Functions of the Structural Domains of Cartilage Superficial Zone Proteoglycan/Proteoglycan 4 (SZP/PRG4)

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CONTENTS

SUMMARY

Proteoglycan 4 (PRG4) is a mucinous proteoglycan with an apparent molecular weight of ~345 kDa. It has been detected in a variety of tissues including cartilage, tendon, bone heart and liver, and is also known as cartilage superficial zone protein (SZP), lubricin, megakaryocyte stimulating factor (MSF) precursor protein and camptodactyly-arthropathy-coxa vara pericarditis (CACP) protein. PRG4 has been shown to reduce friction in joints, and immunological studies have located PRG4 on the surface of articular cartilage, in synovial fluid, on the surface of meniscus and on the surface of mature compressed tendon. PRG4 is involved in the congenital joint pathology known as CACP.

Analyses of the PRG4 sequence reveal a propensity for a diverse number of functions including lubrication, matrix-binding, self-aggregation, cytoprotection and cell proliferation. This study set out to investigate the potential functions of the N- (exons 2-5) and C-terminal (exons 7-12) structural domains of human and bovine PRG4, facilitated by recombinant protein expression. Preliminary results from solid-phase binding assays showed an interaction between the N-terminal domain of PRG4 and plasminogen activator inhibitor-1 (PAI-1), type II collagen and a 70kDa fibronectin fragment. Immunoprecipitaion experiments demonstrated a potential interaction between the PRG4 C-terminal domain and fibronectin fragments. Both the N- and C-terminal contain functional heparin binding sites. The C-terminal domain was shown to interact with superficial zone chondrocytes, an interaction that was perturbed by heparin.

The study also used purified full-length PRG4 from three sources: human recombinant, bovine articular cartilage and bovine tendon. These PRG4 species bound to heparin and displayed similar glycosylation profiles. This study also shows that PRG4 is susceptible to degradation by a number of matrix proteinases including elastase, plasmin, matrix metalloproteinase-7 and cathepsin B.

Collectively, the data presented in this thesis provides novel information concerning the biochemistry, susceptibility to digestion and functional capabilities of PRG4.

LIST OF ABBREVIATIONS

ADAM	A Disintegrin And Metalloproteinase
ADAMTS	A Disintegrin And Metalloproteinase with ThromboSpondin motifs
APMA	aminophenyl mercuric acetate
BCIP	5-bromo-4-chloro-indolyl phosphate
bcPRG4	bovine cartilage proteoglycan 4
BovC	recombinant bovine C-terminal (exons 7-12) PRG4
BovN	recombinant bovine N-terminal (exons 2-5) PRG4
BSA	bovine serum albumin
btPRG4	bovine tendon proteoglycan 4
CHO	Chinese hamster ovary
CS	chondroitin sulphate
DDFT	deep digital flexor tendon
DMEM	Dublecco's modified eagle medium
DS	dermatan sulphate
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N, N, N′, N′-tetraacetic acid
FACIT	fibril associated collagens with an interrupted triple helix
FCS	foetal calf serum
FLAG	flag epitope peptide (amino acid sequence DYKDDDK)
Fn	fibronectin
GAG	glycosaminoglycan
HA	hyaluronic acid
HBS	HEPES-buffered saline
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
hrPRG4	human recombinant proteoglycan 4
HS	heparan sulphate
Ig	immunoglobulin

IL	interleukin
kDa	kilodalton
KS	keratan sulphate
mAb	monoclonal antibody
MMP	matrix metalloproteinase
MSF	megakaryocyte stimulating factor
MT	metallothionein
MW	molecular weight
NBT	nitroblue tetrazoleum
NC	noncollagenous
OA	osteoarthritis
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor 1
PCR	polymerase chain reaction
PG	proteoglycan
PNA	peanut agglutinin
PMSF	phenylmethyl sulfonyl fluoride
PRG4	proteoglycan 4
RA	rheumatoid arthritis
SDS	sodium dodecyl sulphate
SMB	somatomedin B
SZP	superficial zone proteoglycan
TGF-β	transforming growth factor β
TLCK	Nα-p-tosyl-L-lysine chloromethyl ketone hydrochloride
TPCK	N-p-tosyl-L-phenylalanine chloromethyl ketone
TNF-α	tumour necrosis factor α
uPA	urokinase plasminogen activator
uPAR	urokinase receptor
Vn	vitronectin

1. INTRODUCTION

1.1 Articular Cartilage Morphology

Hyaline articular cartilage is a tissue that has an important role in the mechanics of the body. It transmits weight-bearing forces between long bones and facilitates diarthrodial joint articulation. It is an avascular, aneural, hypocellular tissue that is, at skeletal maturity, comprised of 70% water, 20% collagen and 7% proteoglycan (all w/w). It is supported by one type of mesenchymal cell, the chondrocyte. The surface of articular cartilage is resistant to wear, and the hydrated macromolecular mesh of the cartilage matrix aids the dissipation of forces involved in the loading of joints. The surface of articular cartilage also provides a remarkably efficient lubrication mechanism that limits the amount of friction and wear. It is not surrounded by a basement membrane, and has a low level of metabolic activity in comparison with other tissues.

1.1.1 The Chondrocyte

Chondrocytes are usually found as single cells within lacunae, surrounded by an extensive extracellular matrix. They are long-lived cells that do not divide during the course of normal adult life. In mature cartilage, nutrients are obtained by diffusion from the synovial fluid. Chondrocytes maintain the integrity of the cartilage matrix through normal turnover (biosynthesis and degradation) of its constituent macromolecules. To do this they must be able to sense changes in matrix composition, either from matrix degradation or the demands placed on the cartilage surface, and compensate by synthesising the appropriate types and amounts of macromolecules. Chondrocytes from the different zones of articular cartilage differ in size, shape and metabolic activity (Aydelotte *et al.*, 1996).

1.1.2 Articular Cartilage Zones

Mature hyaline articular cartilage is a heterogeneous tissue that can be divided into the four distinct layers or zones illustrated in figure 1.1 – superficial, middle, deep and calcified. There are physiological differences between each zone, however these are not apparent in developing cartilage, where the tissue is unstratified and cells are randomly distributed.

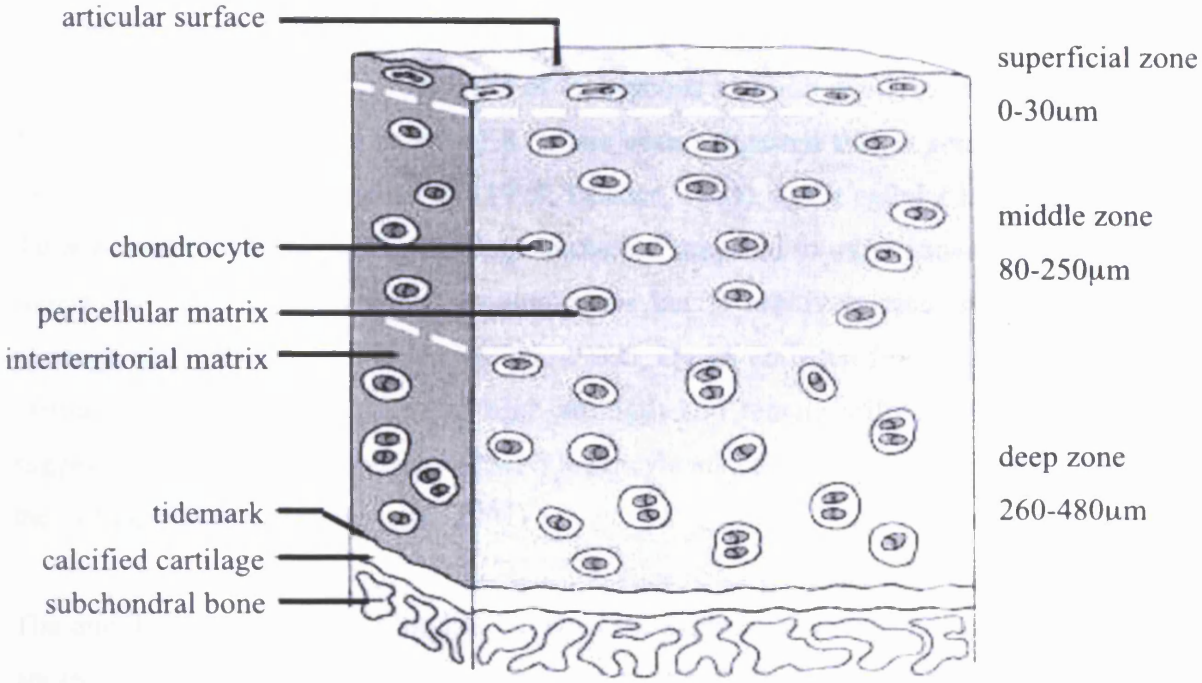


Figure 1.1 – Schematic representation of adult articular cartilage. The diagram illustrates the stratified nature of the tissue and the cellular morphology within each zone. From the website of the UK Centre for Tissue Engineering (adapted from http://www.ukcte.org/images/cartilage.jpg, accessed July 2002).

The superficial zone is nearest to the articular surface of cartilage, and consists of an acellular layer and a deeper, cellular layer. The acellular layer is also known as the *lamina splendens* (MacConaill, 1951) and is a thin layer of amorphous material overlying a sheet of fine fibrils, described in more detail in section 1.8. It has been suggested that in some instances this area contains type I collagen (Eyre *et al.*, 1978; Duance, 1983). In the cellular layer chondrocytes are flattened and lie parallel to the cartilage surface. Compared to other zones within cartilage, the superficial zone contains fewer proteoglycans but is relatively rich in collagen and small proteoglycans (Buckwalter & Hunzicker, 1999). Dense collagen fibrils also lie parallel to the cartilage surface, giving the zone high strength and tensile stiffness. Recent studies have suggested that polymorphonuclear (PMN) leukocyte adhesion is inhibited by macromolecules of the cartilage surface (Mitani *et al.*, 2001).

The middle or intermediate zone lies between the superficial and deep zones. Cells in this zone are more spherical than those of the superficial zone and are randomly organised. Collagen fibrils are thicker than in the superficial zone, though collagen concentration is lower and proteoglycan concentration is higher (Buckwalter & Hunzicker, 1999).

Deep or radial zone cells are aligned in columns of lacunae perpendicular to the cartilage surface. This zone has the thickest collagen fibrils but the lowest concentration of collagen and the highest concentration of proteoglycans. Collagen fibrils from this zone pass into the 'tidemark' – the boundary between uncalcified and calcified cartilage. The zone of calcified cartilage is the borderline between cartilage and the subchondral bone. Chondrocytes within this region are smaller and presumed to be less metabolically active, and have been implicated in the development and progression of osteoarthritis (Oegema & Thompson, 1995).

1.1.3 Matrix Territorial Regions

Within these cartilage zones there are non-uniform regions in the matrix that correspond to the areas surrounding chondrocytes. These are known as matrix territorial regions, of which there are three types: pericellular matrix, territorial matrix and interterritorial matrix. Pericellular matrix surrounds cells, and one or more chondrocytes surrounded by pericellular matrix and an enclosing capsule comprises a structural unit known as a chondron (Poole *et al.*, 1992; Lee *et al.*,

1997). Territorial matrix surrounds the pericellular matrix, and these two regions combined appear to protect chondrocytes from tissue deformation involved in joint loading. The remaining cartilage is termed interterritorial matrix and provides the overall mechanical properties of articular cartilage.

1.2 Articular Cartilage Collagens

Different types of collagen molecules make up a superfamily of matrix proteins that are the most ubiquitous structures in the extracellular spaces of most connective tissues, such as type I collagen in skin, tendon and bone, type II in cartilage and type IV in the basal laminae. They form a 3-dimensional network of fibres into which proteoglycans and other matrix components are trapped. With respect to cartilage, the architecture of these cross-striated fibrils varies to meet specific biomechanical requirements from the surface to the subchondral bone (Bruckner & van der Rest, 1994). All collagens contain a stretch of at least 20 amino acids of the repeat sequence Gly-X-Y where X and Y are often proline and hydroxyproline, respectively. There are 27 distinct types of collagen molecules that have been identified thus far, divided into 3 main groups on the basis of their structure: fibril-forming, network-forming and FACIT (Fibril-Associated Collagens with Interrupted Triple Helices). These groups and their constituent members are shown in table 1.1, along with the collagen molecules that do not fit into the above categories.

The fibril-forming collagens include types I, II, III V and XI, and form organised fibres and fibrils that provide structural support. They are synthesised as large precursors and form 1/4-staggered fibrils that are stabilised through covalent inter- and intra-molecular cross-links. The network-forming family comprises types IV, X and VIII, and form net-like structures by associating at the C-terminus to form dimers and at the N-terminus to form tetramers. The FACIT family include collagen types IX, XII, XIV, XVI and XIX, and also the recently described types XX, XXI and XXII (Koch *et al.*, 2001; Fitzgerald & Bateman, 2001; Koch *et al.*, 2004). They contain two or more short triple-helical domains connected by non triple-helical sequence, and link fibrillar collagens with other matrix macromolecules. These collagens are said to serve as molecular bridges that are important for the organization and stability of extracellular matrices (Shaw & Olsen, 1991).

Table 1.1 – The tissue distribution and collagen group of the 27 known collagens.

Collagen group	Type	Tissue distribution
Fibril forming	I	Skin, bone, tendon, intervertebral disc
	II	Vitreous, cartilage, intervertebral disc
	III	Skin, blood vessels, cartilage, intervertebral disc
	V	Skin, bone, tendon, intervertebral disc
	XI	Skin, bone, tendon, cartilage, intervertebral disc
	XXIV	Developing bone, eye
	XXVII	Cartilage, stomach, lung, gonad, skin
Network forming	IV	Basement membrane, stromal region of cornea
	VIII	Descements' membrane, endothelial cells
	X	Calcifying cartilage
FACITs	IX	Vitreous, cartilage, intervertebral disc
	XII	Skin, cartilage, intervertebral disc
	XIV	Skin, cartilage, intervertebral disc
	XVI	Skin, lung, arterial smooth muscle cells
	XIX	Basement membrane
	XX	Corneal epithelium?
	XXI	Heart, stomach, kidney, skeletal muscle, blood vessels
	XXII	Tissue junctions (e.g. cartilage-synovial fluid, hair follicle-skin, skeletal muscle-heart muscle)
Beaded filaments	VI	Most tissues, including cartilage
Anchoring fibrils for basement membrane	VII	Stroma & basement membrane in skin, cornea
Transmembrane	XIII	Most tissues, including skin and placenta
	XVII	Skin, muscle
	XXIII	Prostate carcinoma
New collagens	XV	Basement membranes in most tissues, placenta, muscle
	XVIII	Most tissues, especially liver
	XXV	Precursor of CLAC (collagen-like Alzheimer amyloid plaque component)
	XXVI	Testis, ovary

Most of the collagen within articular cartilage is of type II (90%), whilst types VI, IX X and XI are present in smaller amounts (Duance *et al.*, 1999). The collagens of articular cartilage are reviewed in (Eyre, 2002).

1.2.1 Type II Collagen

Type II collagen forms fibrillar networks in articular cartilage, and the thickness of these fibrils decreases towards the surface It is a homotrimer of the composition $[\alpha 1(II)]_3$ that is synthesised almost exclusively by chondrocytes (Aydelotte & Kuettner, 1988). The chains are secreted as procollagen molecules with noncollagenous N- and C-terminal segments. The C-terminal propeptides of all the fibril-forming collagens are homologous, and together with the C-telopeptide region contains the information required for chain selection, association and nucleation involved in triple helix formation (Doege *et al.*, 1987). Along with type IX collagen, type II collagen forms the major structural architecture of articular cartilage, which was first illustrated by the arcade model of Benninghoff in 1925.

1.2.2 Type IX Collagen

Type IX collagen belongs to the FACIT group, and is a heterotrimer composed of 3 distinct polypeptide chains, [α1(IX) α2(IX) α3(IX)]. The molecule has three triple-helical domains (COL1 to 3) and four non triple-helical domains (NC1 to NC4) numbered from the C-terminus (Duance *et al.*, 1999). The domains are stabilised by inter-chain disulphide bonds. The NC3 domain of the α2(IX) chain can serve as an attachment point for the glycosaminoglycan chondroitin sulphate. This is an unusual feature, and the degree of glycosylation of collagen IX varies between tissues. Collagen IX is found on the surface of articular cartilage collagen fibrils, covalently bound to type II collagen: cross-linking studies have demonstrated a number of sites where covalent bonds form between type IX collagen and type II collagen, or with other type IX collagen molecules. This interaction can be seen in figure 1.2, along with the interactions of the other major cartilage collagens. Cartilage contains fibrils comprising of collagen types II, IX and XI (Mendler *et al.*, 1989) and the structure is referred to as the collagen II:IX:XI heteropolymer (reviewed in Eyre, 2002).

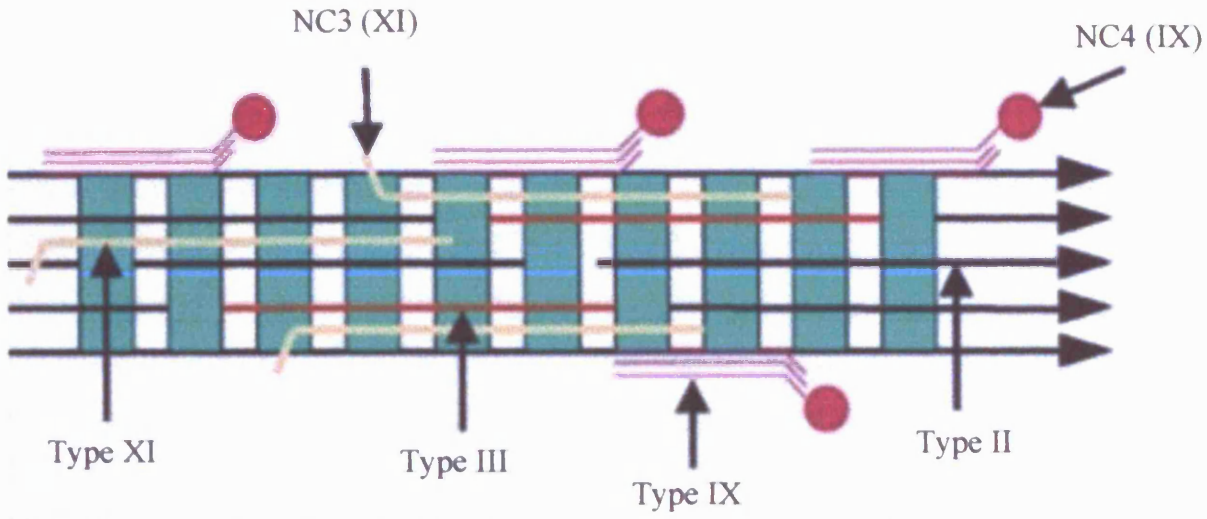


Figure 1.2 – Heterotypic cartilage collagen fibril. Schematic diagram showing the potential interactions between collagen types II, III, IX and XI in articular cartilage. NC – noncollagenous domain. Adapted from (Duance, 2001)

1.2.3 Type XI Collagen

A member of the fibril-forming group of collagens, type XI is found in developing cartilage as a heterotrimer of the composition [α1(XI) α2(XI) α3(XI)]. It has two collagenous (triple-helical) domains – COL1 and 2 - and three noncollagenous (non triple-helical) domains – NC1, 2 and 3. The NC2 domain forms a kink in the molecule between the COL1 and COL2 domains. The molecule is thought to be partially buried within the heterotypic cartilage fibril, and interacts with collagens II and IX as shown in figure 1.2. It also interacts with glycosaminoglycans – including cell-surface species – and has been found bound to or in close proximity to the chondrocyte membrane, implicating the molecule in cell-matrix interactions (Duance *et al.*, 1999). Recent work has demonstrated that collagen XI binds to heparin, heparan sulphate and dermatan sulphate, and that the chondrocyte-type XI collagen interaction is reduced significantly by pre-treatment of cells with heparin or heparanases (Vaughan-Thomas *et al.*, 2001). It is thought that collagen XI may form an interconnecting filamentous network that provides links between as well as within fibrils (Eyre, 2002), consistent with the concept that the molecule restricts the lateral growth of collagen II fibrils (Blaschke *et al.*, 2000).

1.2.4 Other Cartilage Collagens

Type VI collagen is the main constituent of the beaded filaments that are found in most connective tissues, and in cartilage is localised in the pericellular matrix that surrounds cartilage cells, but is also found throughout the matrix (Bruckner & van der Rest, 1994). The meshwork it forms helps to protect the chondrocyte from mechanical stress. Expression of type VI collagen appears to be upregulated in osteoarthritic cartilage (Chang & Poole, 1996), although degradation and/or transformation of collagen VI in osteoarthritic cartilage might cause a significant loss of function of the pericellular microenvironment (Soder *et al.*, 2002).

Type I collagen, a fibril-forming collagen, is abundant in skin, bone and tendon but is only a minor part of articular cartilage. It is present in the superficial zone of avian cartilage, where levels decrease with depth (Eyre *et al.*, 1978). It has also been documented in osteoarthritic cartilage (Aigner *et al.*, 1993). Type III collagen is another fibril-forming collagen and is found in normal and osteoarthritic cartilage co-localised to type II collagen as a minor but regular component (Wu *et al.*, 1996; Young *et al.*, 2000).

In osteoarthritic cartilage, type III collagen is concentrated in the superficial and middle zones (Aigner *et al.*, 1993) and it has been speculated that its synthesis is a damage response akin to the wound-healing role of type III collagen in type I collagen-based tissues (Eyre, 2002). Type V collagen displays homology with type XI, both being fibril-forming collagens, though its exact role in cartilage is unknown at present. Immunolocalisation studies detecting type V collagen in cartilage have shown strong staining in chondrocytes (Furuto *et al.*, 1991). Type X collagen is a network-forming collagen synthesised almost exclusively by hypertrophic chondrocytes, and has been identified in the zone of calcified cartilage surrounding the cells (Gannon *et al.*, 1991). Collagens XII and XIV are FACIT group members and display partial homology to collagen type IX. Both may carry a chondroitin sulphate chain and they are thought to exist in proteoglycan form in bovine articular cartilage. They are not covalently polymerised in the cartilage matrix and are thought to bind physically to fibril surfaces via their COL1/NC1 domains (Watt *et al.*, 1992). Their function is unclear but there may be competition between them and other fibril-binding molecules in cartilage such as decorin, biglycan and fibromodulin.

It should be noted that articular cartilage differs in structure and function to other types of cartilage, for example growth plate and meniscal cartilage. Whilst all cartilage types have a major fibrillar collagen network, the matrix organisation and its interactive properties are greatly influenced by minor components, both collagenous and noncollagenous (Duance *et al.*, 1999). Cartilage collagen breakdown is considered to be a critical and irreversible step in the progression of osteoarthritis (Eyre, 2002).

1.3 Proteoglycans

Proteoglycans (PGs) are made up of glycosaminoglycans (GAGs) and protein, with GAG chains of repeating disaccharides covalently linked to a core protein. The core protein is made on membrane-bound ribosomes, and GAG chains (see section 1.4) are added within the Golgi apparatus. Proteoglycans occur intracellularly (usually in secretory granules), at the cell surface, or in the extracellular matrix (Kjellen & Lindahl, 1991). They are a functionally diverse family of molecules that have the potential to participate in a number of biological functions. These include cell growth, collagen fibrillogenesis, tumour cell invasion and the maturation of specialised tissues including cartilage (Iozzo, 1998). In hyaline cartilage, the large aggregating proteoglycan

aggrecan provides osmotic resistance to resist loading, although many other proteoglycans are present including decorin, biglycan, fibromodulin, lumican epiphycan, perlecan, syndecans and glypican (for a review see Knudson & Knudson, 2001). The protein and carbohydrate domain structures of each individual PG gives it its specific functional properties by means of protein-protein, protein-carbohydrate and protein-cell interactions. A brief description of the structural biology of the major proteoglycans is given below.

1.3.1 Lecticans

Also known as hyalectans or aggregating proteoglycans, the lectican family of PGs share a tridomain structure comprising of an N-terminal domain that binds hyaluronan, a central domain that carries GAG chains and a C-terminal lectin-type domain (Iozzo, 1998). Within these domains there are certain motifs that are common to all lecticans. At present there are four distinct lectican genes – versican, neurocan, brevican and aggrecan.

1.3.1.1 Versican

Versican is the largest member of the lectican family and is a chondroitin sulphate proteoglycan (CSPG) (reviewed in Wight, 2002). Its tissue distribution is wider than that of aggrecan, neurocan or brevican and various forms have been found in fibroblasts, liver, intervertebral disc, skin and cartilage (Dours-Zimmermann & Zimmermann, 1994). Versican is more abundant in the intervertebral disc than in cartilage, suggesting the importance of its function in this tissue (Sztrolovics *et al.*, 2002), though the function of the molecule remains unclear. Versican functions as a structural molecule, creating loose and hydrated matrices during key events in development and disease, interacting either directly with cells or indirectly with molecules that associate with cells to, in part, regulate extracellular matrix assembly and cell adhesion and survival, proliferation, migration and phenotype. As with the other lecticans, versican can be divided into 3 domains – I, II and III – as illustrated in figure 1.3. Briefly, domain I consists of one Ig repeat followed by two link proteins that mediate the binding of hyaluronan. Domain II contains two subdomains, GAGα and GAGβ, that have up to 30 GAG attachment points as well as several potential binding sites for N- or O-linked oligosaccharides. There are four known splice variants of mammalian versican, all involving variations in domain II structure: V0 (GAGα and GAGβ), V1 (GAGβ only), V2 (GAGα only) and V3 (neither GAGα or GAGβ).

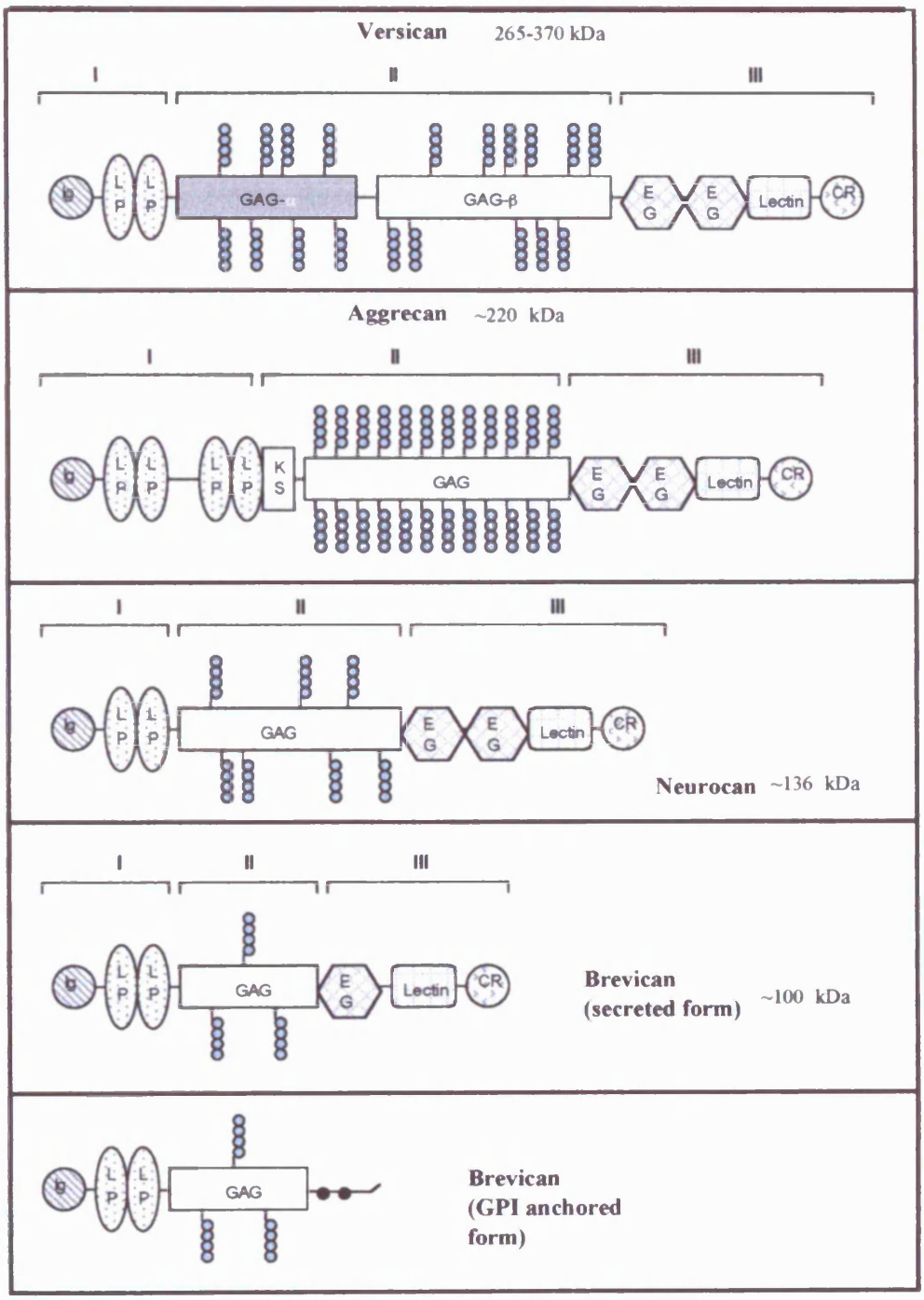


Figure 1.3 – Schematic representation of the domain structures of the four lecticans. Abbreviations: Ig – immunoglobulin-like domain; LP – link protein motif; GAG – glycosaminoglycan domain; EG – epidermal growth factor-like domain; lectin – lectin-like domain; CR – complement-regulatory protein –like domain. Molecular weights refer to unsubstituted protein cores. Adapted from (Iozzo, 1998).

Domain III contains two EGF-like repeats, a C-type lectin domain and a complement regulatory protein segment (Iozzo, 1998). Recent work with recombinant versican has suggested an interaction of cartilage link protein with versican is of a higher affinity than the interaction between link protein and aggrecan (Shi *et al.*, 2004).

1.3.1.2 Neurocan

Neurocan is a CSPG originally cloned from rat brain (Rauch *et al.*, 1992), and is expressed during tissue modelling or remodelling. A schematic diagram of neurocan is shown in figure 1.3. Domain I, the HA binding domain, is similar in structure to that of versican. Domain II contains seven GAG attachment points, and domain III is similar to the lectin-like domains of the other lecticans: between rat neurocan, and human aggrecan and versican, there is a homology of around 60% (Iozzo, 1998).

1.3.1.3 Brevican

Brevican is the most recently discovered lectican and as one can gather from its name it is also the shortest, due to its relatively short GAG attachment region (Jaworski *et al.*, 1994). It exists as a secreted form or as an isoform bound to the plasma membrane via a GPI anchor (Seidenbecher *et al.*, 1995). This isoform contains no EGF-like repeat, lectin domain or complement regulatory protein-like domain (see figure 1.3). These motifs are replaced by a segment of hydrophobic amino acids that resemble the GPI anchor.

1.3.1.4 Aggrecan

Also known as cartilage proteoglycan, aggrecan is the predominating aggregating proteoglycan found in cartilage, and its structure contributes greatly to the biomechanical functionality of the tissue. The GAG binding region has attachment points for up to 150 GAGs – around 50 for keratan sulphate and around 100 for chondroitin sulphate – and this polyanionic region is space-filling and has a high affinity for water. Aggrecan monomers interact non-covalently with HA to form large proteoglycan aggregates (reviewed in Roughley & Lee, 1994), an interaction that is stabilised by link protein (Hardingham, 1979). The structural organisation of aggrecan is similar to that of versican but with a few notable exceptions, as seen in figure 1.3. In domain I, the Ig-like domain and the four tandem repeat modules form two globular domains termed G1 and G2.

The G1 domain contains the Ig repeat and the first two tandem repeats and the G2 domain contains the other two tandem repeats. The interaction between aggrecan and HA is mediated by the G1 domain and is stabilised by a link protein, which is similar in structure to the G1 domain (Perkins *et al.*, 1989). The G1 domain and link protein bind to a decasaccharide segment of HA at intervals of approximately 30nm, and G1 also binds to link protein via its Ig-like repeat. These interactions are shown in figure 1.4, along with the domain structure of the rest of the molecule. The HA backbone of aggrecan-HA polymers can vary in length from several hundred nanometers to over ten thousand nanometres, and may have up to 300 associated aggrecan molecules. The weight of HA-aggrecan complexes has a mass of around 2×10^6 Da.

The G3 domain is similar in structure to domain III of the other lecticans. The region of core protein that links the G1 and G2 domains is known as the interglobular domain (IGD). This contains amino acid sequences that are susceptible to proteolytic attack by matrix proteases. The degradation of aggrecan by these enzymes, some of which are described later, is important both in normal matrix turnover and in matrix degradation and the associated loss of function involved in pathological conditions such as arthritis. The human aggrecan core protein contains a keratan sulphate attachment region and two chondroitin sulphate attachment regions, known as CS-I and CS-II, shown in figure 1.4 (Doege *et al.*, 1991). Aggrecan from rat contains another minor CS attachment region termed CS-III (Doege *et al.*, 1987). Changes in the number, size, sulphation patterns and charge density of the chondroitin sulphate and keratan sulphate regions of aggrecan occur with development and ageing (Hardingham *et al.*, 1994). For example, in ageing cartilage there is an increase in the proportion of keratan sulphate in relation to chondroitin sulphate, and the ratio of 6-sulphated to 4-sulphated chondroitin sulphate increases. Catabolism of aggrecan occurs with the onset of joint diseases such as osteoarthritis (for a review see Caterson *et al.*, 2000).

1.3.2 Small Leucine-Rich Proteoglycans (SLRPs)

Also known as small interstitial proteoglycans, this family of proteoglycans are both small - 70-120 kDa compared to 2.5×10^3 kDa for an aggrecan monomer – and leucine-rich (reviewed in Iozzo, 1999). They contain a central region of leucine-rich repeats (LRR) flanked by two cysteine-rich regions with highly conserved spacing.

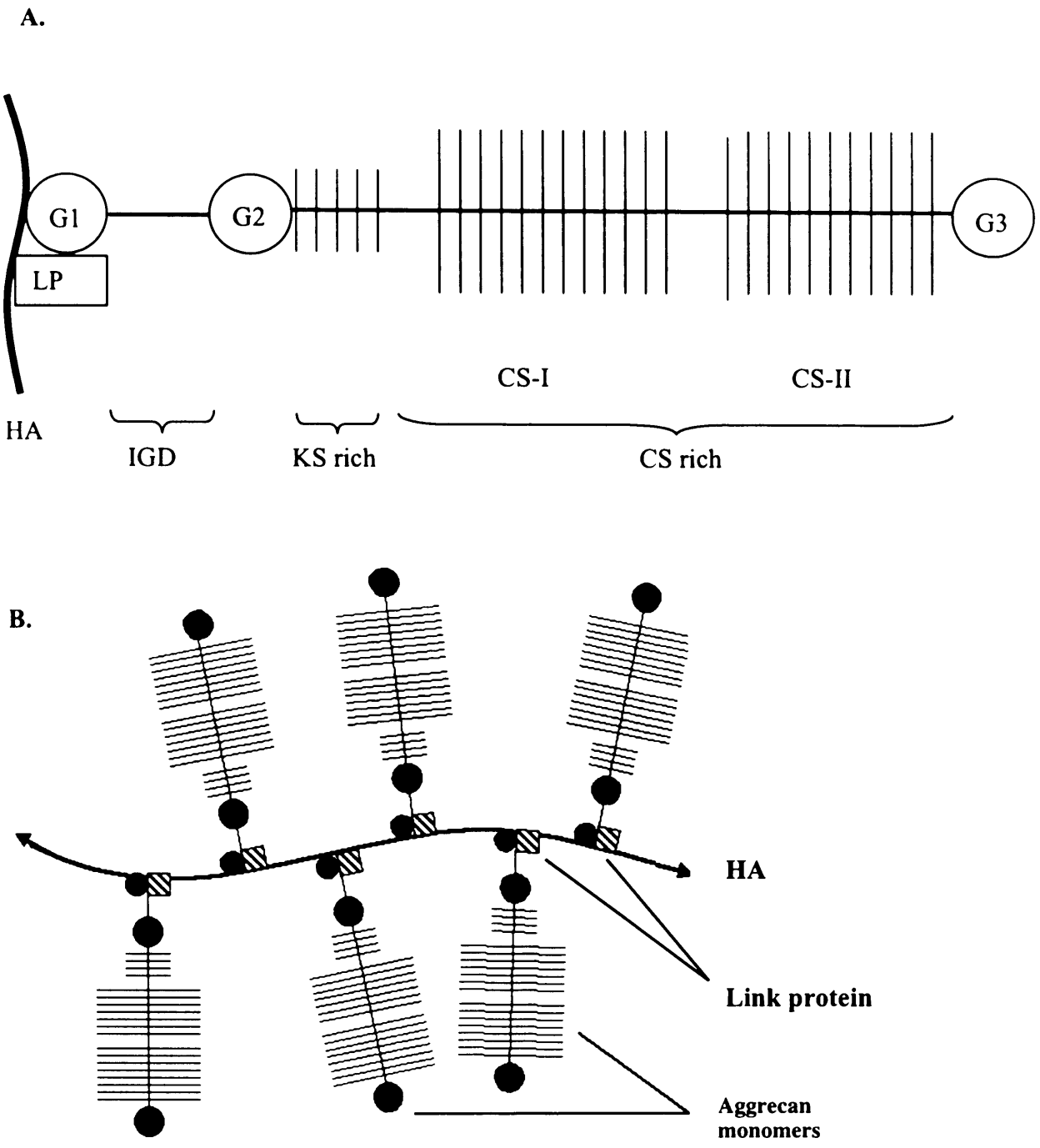


Figure 1.4 – Schematic representation of (A) the interaction between hyaluronan and aggrecan, stabilised by link protein, and (B) the formation of aggrecan aggregates. Abbreviations: G1, G2 and G3 – globular domains of aggrecan; LP – link protein; CS-I, CS-II – chondroitin sulphate I and II domains; IGD – interglobular domain.

SLRPs can be split into three families, classes I, II and III, based on their protein and genomic organisation.

1.3.2.1 Class I SLRPs

This class includes decorin and biglycan. Decorin is the 'prototype' SLRP, and is a tetradomain protein as shown in figure 1.5. Domain I contains a signal peptide and a propeptide, a feature unique to class I SLRPs, both of which are not present in the mature secreted proteoglycan. The propeptide may regulate the attachment of GAG chains – 2 CS or DS chains for biglycan, and one for decorin (Iozzo, 1999). Domain II has a cluster of cysteine residues and contains GAG attachment sites. Domain III is the leucine-rich region, which is common to most SLRPs. Most of the leucine residues are in conserved positions, with the consensus LxxxLxLxxNxLSxL where x is any amino acid, L is leucine, isoleucine or valine, S is serine and N is asparagine. Domain IV contains two cysteine residues separated by 32 amino acids that may aid interaction with collagen. Decorin 'decorates' collagen fibrils and has been found to affect fibrillogenesis: it can regulate the diameter of type I collagen fibrils (Danielson *et al.*, 1997), and it may have a similar role in type II collagen organisation within the cartilage matrix. Collagen malformations exist in *Dcn-/-* mice, reviewed in (Reed & Iozzo, 2002). It has been suggested that the interactions between decorin and other ECM components such as fibronectin and collagen supports formation of a pericellular matrix that stabilises cellular differentiation (Kinsella *et al.*, 2000; Gendelman *et al.*, 2003). Biglycan associates with the cell surface or pericellular matrix of a number of cells, and along with decorin it has been shown to interact with collagen VI (Wiberg *et al.*, 2001). It also binds to the growth factor TGF-β (Hildebrand *et al.*, 1994). Decorin also binds to TGF-β, and prevents it from interacting with its cellular surface receptors, neutralising its effect (Hocking *et al.*, 1998). It is thought that decorin and biglycan may regulate TGF-β activities by sequestering TGF-β in the extracellular matrix.

1.3.2.2 Class II SLRPs

This subfamily includes fibromodulin, lumican, keratocan and osteoadherin, and differ to class I SLRPs in that they are not proteolytically processed after the removal of their signal peptide. They contain keratan sulphate GAG chains, and share a cysteine-rich region that occurs just N-terminal to the 10 LRRs.

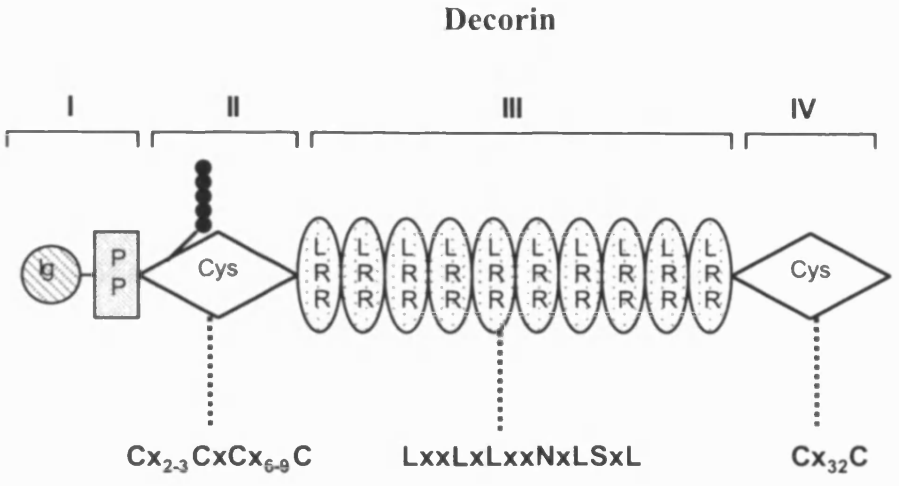


Figure 1.5 – Schematic diagram of the SLRP decorin, illustrating its domain structure. Abbreviations: SP – signal peptide; PP – propeptide; Cys – cysteine-rich region; LRR – leucine-rich repeat. Consensus sequences for the leucine-rich repeats and the cysteine-rich regions are also shown. Adapted from (Iozzo, 1998).

Lumican and fibromodulin inhibit fibrillogenesis, and fibromodulin, like decorin, binds to TGF-β (Hildebrand *et al.*, 1994) and prevents ligand-receptor interactions. Keratocan, like lumican, is a core protein of a major corneal proteoglycan (Corpuz *et al.*, 1996). Osteoadherin binds to hydroxyapatite and also to osteoblasts, and may promote osteocyte attachment (Wendel *et al.*, 1998).

1.3.2.3 Class III SLRPs

This group consists of osteoglycin (PG-LB), epiphycan (mimecan) and opticin (Reardon *et al.*, 2000). They contain 6 LRRs and a unique cysteine-rich consensus sequence. There are two molecules, PRELP (Bengtsson *et al.*, 1995) and chondroadherin, that are members of the leucine-rich repeat (LRR) family of ECM molecules but are not classed as proteoglycans. PRELP interacts with perlecan heparan sulphate chains and collagen, and is thought to anchor basement membranes to the underlying connective tissue (Bengtsson *et al.*, 2002). Chondroadherin has been shown to bind cells (Camper *et al.*, 1997) and type II collagen (Mansson *et al.*, 2001), and may be of functional importance in communications between the cell and its surrounding matrix.

1.3.3 Basement Membrane Proteoglycans

The proteoglycans within the basement membrane bind specifically to its other components. These include type IV collagen, laminin and cellular receptors. They contribute to the formation of a negatively charged barrier that regulates the passage of macromolecules across the basement membrane. At present, four of these proteoglycans have been well characterised: the heparan sulphate proteoglycans (HSPGs) perlecan and agrin, and the chondroitin sulphate proteoglycans (CSPGs) bamacan and leprecan.

Perlecan was the first basement membrane-associated proteoglycan to be characterised (Murdoch *et al.*, 1992). It has a mass of 850 kDa and is a large component of the basement membrane, comparable to laminin at 900 kDa. It is made up of five distinct domains, and the name arises from the appearance of its N-terminal portion: globular domains are interlinked by rod-like segments. Perlecan has particular relevance to cartilage – studies on mutant mice lacking perlecan show severe matrix disorganisation, indicating that perlecan is critical in normal articular cartilage (Arikawa-Hirasawa *et al.*, 1999).

Agrin is a HSPG involved in the development of the neuromuscular junction during embryogenesis, including the aggregation of acetylcholine receptors (Groffen *et al.*, 1998). It is a multidomain protein with four distinct domains, with at least six sites for GAG attachment. Bamacan is structurally dissimilar to other proteoglycan due to the presence of coiled-coil motifs in two of its five domains (Wu & Couchman, 1997). Leprecan is a CSPG that has yet to be fully characterised, though it is possible that both HS and KS chains are attached to the protein core (Wassenhove-McCarthy & McCarthy, 1999).

1.3.4 Cell Surface Proteoglycans

This class of molecules includes the glypicans and syndecans. Glypicans can contain CSPGs, DSPGs or HSPGs whereas syndecans (reviewed in Couchman, 2003) contain HSPGs. Both are involved in cell-matrix and cell-cell interactions, and have been implicated in the cell binding of growth factors. Four isoforms have been cloned in the syndecan family and six in the glypican family. The syndecan core protein contains a single pass transmembrane domain, an extended extracellular domain substituted with HS and a short intracellular domain with roles in signal transduction. Glypicans are attached to the cell surface through a GPI anchor, and have a more globular structure, with HS chains close to the cell surface. Heparan sulphate proteoglycans present on the cell surface may participate in many events during cell adhesion, migration, proliferation and differentiation (reviewed in Tumova *et al.*, 2000). A recent study suggests that the proliferation of chondrocytes effected by fibroblast growth factor-2 (FGF-2) is mediated by syndecan-3 (Kirsch *et al.*, 2002). Syndecan-3 also appears to be expressed by osteoarthritic chondrocytes (Pfander *et al.*, 2001).

1.3.5 Cartilage superficial zone proteoglycan (SZP)

This 345 kDa proteoglycan is synthesised by superficial zone chondrocytes and by some cells of the synovial lining and accumulates on the surface of articular cartilage (Schumacher *et al.*, 1999). It has one CS attachment point, and is also known as lubricin or PRG4. It is the subject of this thesis and is described in more detail in section 1.9.

1.4 Glycosaminoglycans

Glycosaminoglycans (GAGS) are linear polysaccharides made up of repeating unbranched disaccharide units. These units always consist of a hexosamine residue that is invariably sulphated, and a hexuronic acid or galactose residue. The sulphation of GAGs, together with the carbohydrate moiety of hexuronic acid, gives GAG chains a polyanionic charge and therefore a high affinity to water. The functional properties of proteoglycans depend on this feature: in cartilage, aggregated proteoglycans attract water via their hydrophilic GAG side chains and resists its flow out of the cartilage matrix when the tissue is deformed during compression. When the load is removed, GAGs are rehydrated and tissue deformation is reversed.

1.4.1 GAG Species

There are six GAG species: chondroitin sulphate (CS), dermatan sulphate (DS), heparan sulphate (HS), keratan sulphate (KS), heparin (hep) and hyaluronic acid (hyaluronan or HA). HA is not sulphated and is not a constituent of proteoglycans, though it is a major constituent of synovial joint fluid that provides viscosity. Heparin is a more extensively epimerised and sulphated form of HS. HS is found on many cell surfaces and in some matrix proteoglycans. The disaccharide repeat units of the six GAG species present in articular cartilage are shown in figure 1.6. HA, HS and heparin units comprise of N-acetylgalactosamine and uronic acid, KS contains N-acetylglucosamine and galactose, CS and DS contain N-acetylgalactosamine with glucuronic and iduronic acid, respectively. DS is a modified form of CS, formed by the conversion of one or more glucuronic acid residues to iduronic acid by an epimerase (Roughley & Lee, 1994).

Cartilage GAGs display different chain lengths, and with the exception of HA, show varying degrees of sulphation with respect to quantity and position. For example, CS may be sulphated at the 4- or 6-position of the galactosamine residues, sulphation status being loosely dependant on tissue type, age and pathology. CS chains isolated from articular cartilage may vary both in chain length and sulphation pattern, depending on the activities of glycosyl transferases and sulphotransferases that function during synthesis, and the availability of precursors (Roughley & Lee, 1994). The synthesis of sulphated GAGs takes place in the Golgi apparatus, and initiation requires a protein receptor. In contrast, HA is synthesised at the plasma membrane, the growing polysaccharide chain extruding directly into the extracellular space.

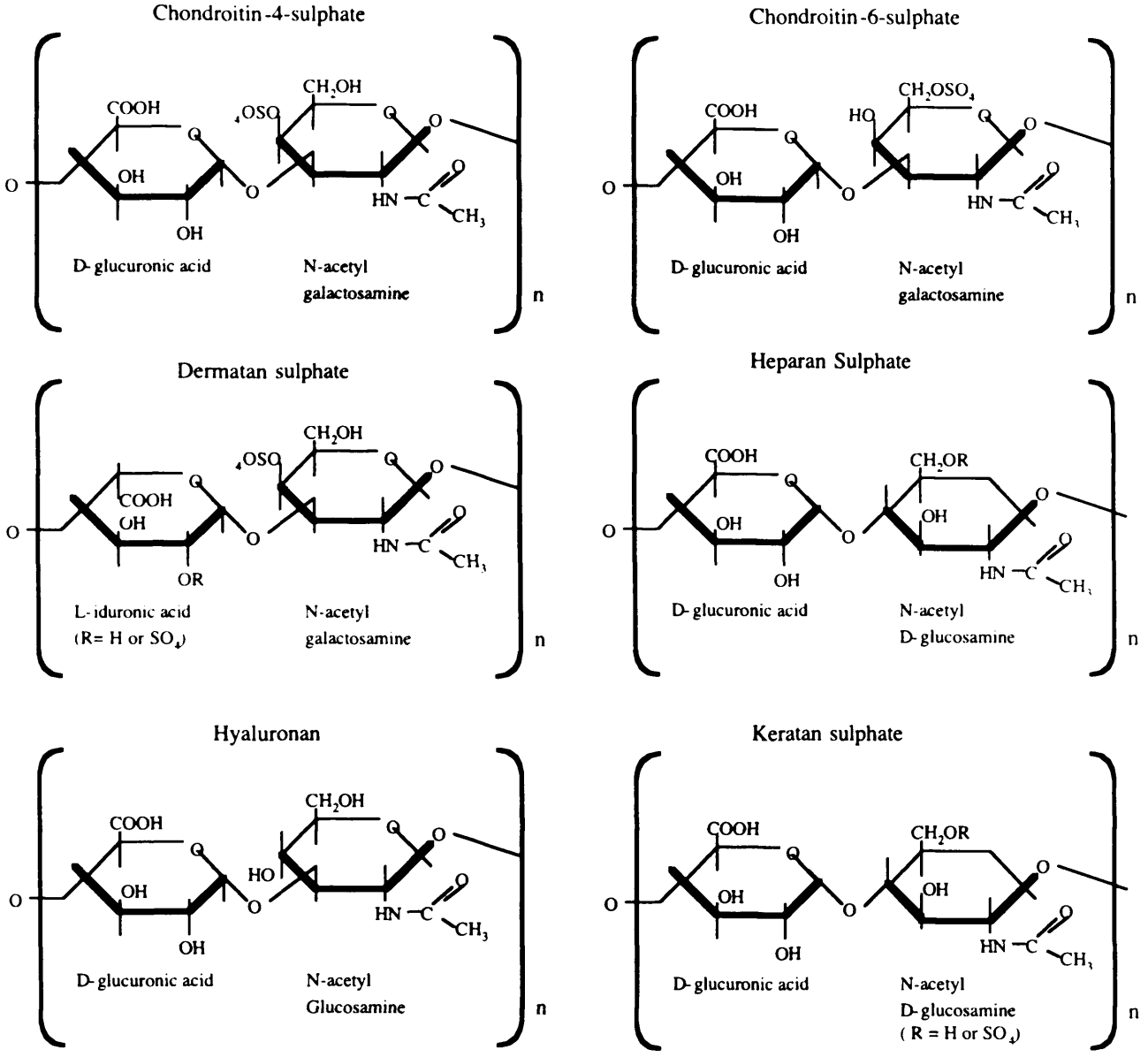


Figure 1.6 – Disaccharide sequences of the six glycosaminoglycan species found in articular cartilage.

1.4.1.1 GAG Attachment

For attachment of GAGs to protein, there are specific amino acid consensus sequences (Esko & Zhang, 1996). The consensus sequences and their method of attachment are shown in figure 1.7. The sequence for CS attachment is a cluster of two or three acidic amino acids followed by Ser-Gly-X-Gly, although there is one exception to this – in type IX collagen, CS is attached through the serine of a Ser-Ala dipeptide. The consensus sequence for the attachment of HS is a Ser-Ala or Ser-Gly dipeptide with nearby clusters of acidic and hydrophobic residues. KS can either be O-linked, as in cartilage, or N-linked as in the cornea. The consensus sequence for N-linked KS attachment is Asn-X-Ser/Thr where X is any amino acid except proline, and KS attaches through asparagines. The sequence for O-linked KS attachment in aggrecan is Glu-Glu/Lys-Pro-Phe-Pro-Ser, with KS being attached through the serine residue (Hering *et al.*, 1997).

1.5 Other Components of Articular Cartilage

An overview of the major macromolecular constituents of articular cartilage is shown in table 1.2. Some of the constituents of the cartilage matrix that are not classed as either collagens or proteoglycans are described briefly in this section.

1.5.1 Fibronectin

Fibronectin is an extracellular matrix glycoprotein that is a soluble constituent of plasma (~300μg/ml) and other body fluids, and it is also part of the insoluble extracellular matrix (reviewed in Pankov & Yamada, 2002). Its increased expression is often associated with tissues undergoing remodelling or repair. Fibronectin is a rod-like molecule that consists of two 250 kDa subunits linked by a disulphide bond at the C-terminus. It has several globular domains that contribute to a number of interactions including binding to collagen, fibrin, heparin, heparan sulphate and cell surface receptors. It has an Arg-Gly-Asp (RGD) tripeptide that mediates the binding of cells to the extracellular matrix via integrins. Fibronectin is a minor constituent of healthy cartilage although elevated levels are present in arthritis. The predominant form of fibronectin in cartilage is the alternatively spliced (V+C)-, which exists as either a homodimer or, unusually, as a monomer. Parts of fibronectin are susceptible to proteolysis, and the resultant catabolites may have the potential to regulate cartilage metabolism (reviewed in Homandberg, 1999).

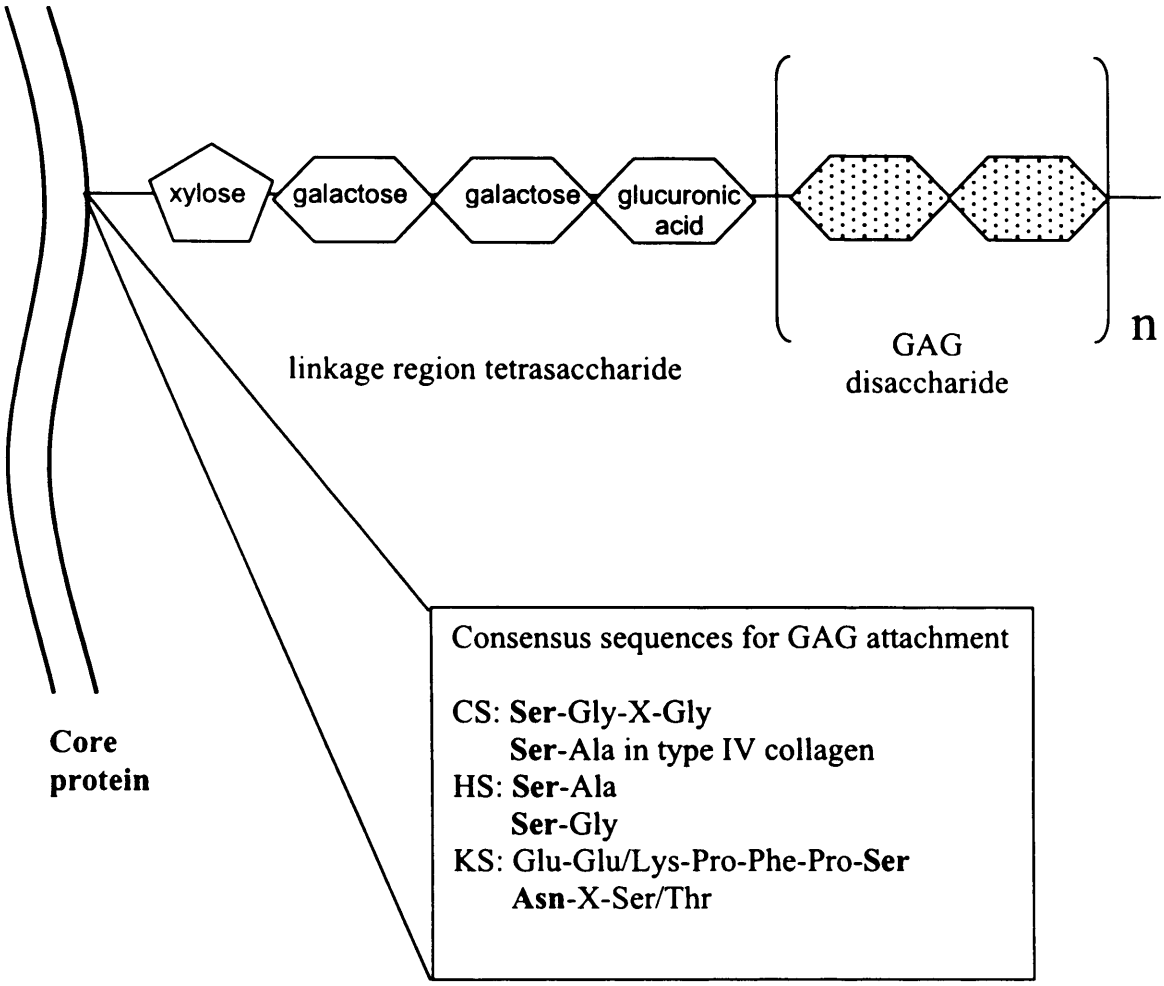


Figure 1.7 –Attachment of common GAGs to a core protein through a linkage tetrasaccharide. Attachment consensus sequences are also shown. Residues in bold indicate amino acids that directly attach GAG. Adapted from (Alberts *et al.*, 1994).

Table 1.2 – Structural matrix molecules of articular cartilage. Adapted from (Poole *et al.*, 2001).

Molecule	Comments	Function
Collagens		
Type II	Principle component of macrofibril	Tensile strength
Type VI	Forms microfibrils in pericellular sites	Unknown
Type IX	Cross-linked to surface of macrofibril	Tensile properties and/or fibril-iterfibrillar connections
Type X	Associated with macrofibril and present in pericellular latticework. Only synthesised by hypertrophic chondrocytes. Only usually present in hypertrophic layer	Unclear but may add structural support
Type XI	Present within and on macrofibrils	Nucleates fibril formation
Types XII and XIV	Each is homotrimeric	Probably part of macrofibril
Proteoglycans		
Aggrecan	Majority proteoglycan by mass. Binds HA through G1 domain. Most concentrated in deep zone.	Compressive stiffness
Leucine-rich core proteins		
Decorin	Has one CS or DS chain near NH_2-terminus. Equimolar to aggrecan. Concentrated at articular surface and in pericellular sites	Regulates formation of macrofibrils
Fibromodulin	May contain as many as 4 KS chains	As for decorin
Lumican	Contains KS in immature form	As for decorin
Biglycan	Has 2 CS or DS chains near NH_2-terminus	Unclear
Perlecan	Located at cell surface	Cell-matrix adhesion
Other molecules		
Cartilage oligomeric protein	Five-armed molecule of thrombospondin family	Binds type II collagen; may be involved in macrofibril assembly
Link protein	Structure homologous to G1 domains of aggrecan/versican	Stabilises binding of aggrecan/versican G1 domains to HA
Matrix-γ-carboxyglutamic acid protein	Pericellular location. Also known as matrix Gla protein	Inhibits calcification
Fibrilin-1	Forms microfibrillar network	Unknown
Hyaluronic acid	Forms macromolecular aggregates with aggrecan/versican. Interacts with collagen	Retention of aggrecan and versican in the matrix
PRG4 (lubricin)	Synthesised by cells of the superficial zone; not retained in matrix	Joint lubrication
Chondroadherin	Leucine-rich protein	Cell-matrix binding
CD44	Cell surface receptor for HA	Cell-matrix binding

It has been reported that up to 50% of fibronectin in arthritic synovial fluids is proteolytically fragmented, with fragments of between 30 and 200 kDa present (Griffiths *et al.*, 1989; Xie *et al.*, 1992). The domain structure of fibronectin and the cleavage point of proteinases is shown in figure 1.8. The relevance of proteolytically fragmented fibronectin to cartilage catabolism was demonstrated *in vivo* by Homandberg *et al.* (1993) who showed that injection of a 29 kDa N-terminal domain fibronectin fragment into rabbit knee joints caused severe depletion of proteoglycan within days. More recently it has been reported that stimulation of cultured chondrocytes and cartilage explants with the 45 kDa N-terminal fragment of fibronectin induces MMP-13 synthesis and aggrecan degradation by aggrecanases (Stanton *et al.*, 2002).

In addition to the presence of proteolytic fragments of fibronectin in joint pathology, novel cartilage-specific splice variants of fibronectin have been identified that may have specific roles in the maintenance of structural integrity or regulation of 'outside-in' signalling in chondrocytes: one fibronectin splice variant was found to be specific to arthritic cartilage (Parker *et al.*, 2002).

Other work has investigated the release of collagen-binding molecules from cartilage explant cultures treated with fibronectin (Johnson *et al.*, 2004). An interesting hypothesis concerning the mechanisms behind these observations is that fibronectin fragments are preferentially active towards chondrocytes because they contain reduced numbers of integrin binding sites (Huhtala *et al.*, 1995). According to the authors, fibronectin appears to be a virtual 'keyboard' of binding sites, upon which various 'chords' of integrins can be played by cells (Peters *et al.*, 2002). Therefore, a catabolic response could be elicited by cells hitting 'bum notes' on a fibronectin fragment 'keyboard' that has a reduced number of integrin-binding sites.

1.5.2 Tenascins

There are five known members of the tenascin multimeric glycoprotein family, as reviewed in (Jones & Jones, 2000): tenascins C, R, W, X and Y, of which tenascin C is the best characterised. Tenascin C has a hexabrachion structure and has been found in cartilage, skin and bone marrow, with several different isoforms variably expressed during embryogenesis, tumourogenesis, angiogenesis and wound healing.

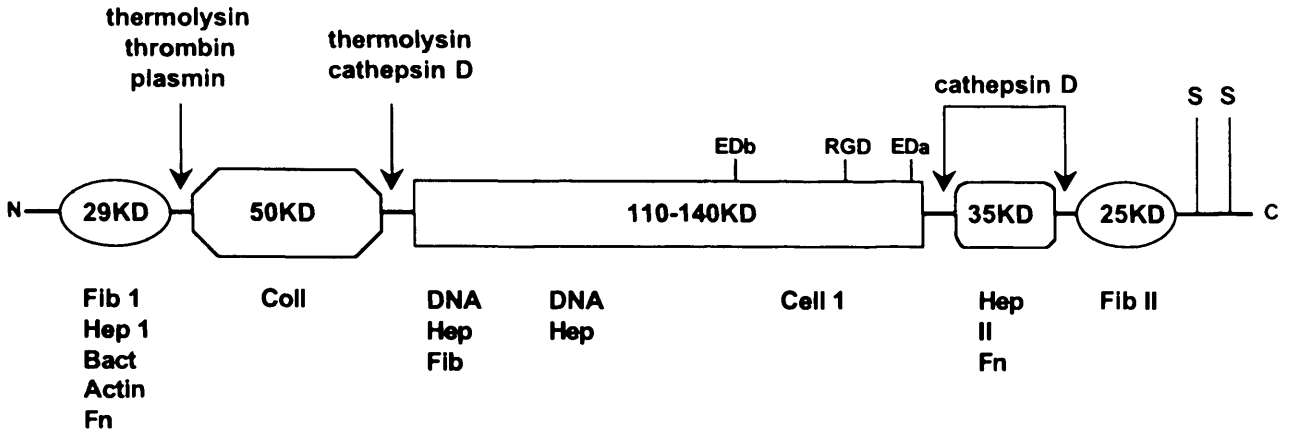


Figure 1.8 – Schematic representation of the domain structure of a fibronectin monomer. Fragments of fibronectin can be isolated by various proteinases, as illustrated above the diagram. The types of ligands each domain can bind are shown below the diagram. Abbreviations: Fib – fibulin; Hep – heparin; Fn – fibronectin; Coll – collagen; Bact – bacterial. Adapted from (Homandberg, 1999).

Tenascins interact with matrix metalloproteinases and can also be cleaved by them. Tenascins C and R both bind to a number of proteins with high affinity including the hyalectan family of proteoglycans: aggrecan-tenascin complexes exist in cartilage, where it is thought to have roles in extracellular matrix assembly and the modulation of interactions. The aggrecan-tenascin C interaction may be affected by alternative splicing of the G3 domain of aggrecan (Day *et al.*, 2004).

1.5.3 Cartilage Intermediate Layer Protein (CILP)

CILP is a 92 kDa single chain glycoprotein. Ten percent of its observed molecular weight is from O-linked oligosaccharides, and there are no N-linked oligosaccharides present (Lorenzo *et al.*, 1998a; Lorenzo *et al.*, 1998b). Its location is restricted to the intermediate or middle zone of cartilage. The function of CILP is not yet known, but it could be involved in the early stages of osteoarthritis and in ageing, as in both processes its expression is upregulated. Recent work suggests that increased CILP expression may be associated with calcium pyrophosphate dihydrate crystal deposition disease (CPPDCD), and that CILP might play a role in promoting CPPD crystal formation (Yamakawa *et al.*, 1999). Of the two isoforms expressed in cartilage, CILP-1 and CILP-2, CILP-1 appears capable of functioning as an insulin-like growth factor-1 (IGF-1) antagonist (Johnson *et al.*, 2003).

1.5.4 Cartilage Oligomeric Matrix Protein (COMP)

COMP is a 524 kDa extracellular, homopentameric glycoprotein belonging to the thrombospondin family (Hedborn *et al.*, 1992). An N-terminal cysteine-rich domain facilitates the assembly of pentamers from monomers, and a globular C-terminal domain may be involved in binding proteins and cells. It is expressed in a variety of tissues including cartilage, tendon, ligament, bone, skeletal muscle and heart. Levels of COMP in serum and synovial fluid have been used as a marker for cartilage degradation (Saxne & Heinegard, 1992). Recent reports have suggested that COMP can interact in a divalent cation-dependent manner with collagens I, II and IX (Rosenberg *et al.*, 1998; Holden *et al.*, 2001; Thur *et al.*, 2001) and also with fibronectin (DiCesare *et al.*, 2002). Mutations in the COMP gene have been implicated in two different inherited skeletal dysplasias: pseudochondroplasia (PSACH) and multiple epiphyseal dysplasia (MED/EDM1), although characterisation of a PSACH-type mutant suggests that the mutation

does not affect COMP-collagen binding (Spitznagel *et al.*, 2004). The *in vivo* function of the protein is not yet fully understood though it may be associated with the elaboration of a weight-bearing chondrocyte matrix.

1.5.5 Secreted Protein Acidic and Rich in Cysteine (SPARC)

SPARC, also known as BM-40, 41K protein and osteonectin, is a 34 kDa glycoprotein made up of three structural domains, one or more of which are implicated in the regulation of cell adhesion, cell proliferation and matrix turnover (Motamed, 1999). It is expressed by a number of cell types, but appears to be upregulated in the superficial and transitional zones of osteoarthritic cartilage. It can bind to collagen & vitronectin, and has also been implicated in the regulation of growth factor activity and lens transparency (reviewed by Brekken & Sage, 2000).

1.5.6 Matrilins

There are currently four identified members of the matrilin family, matrilins 1, 2, 3 and 4. Matrilin-1 was initially identified as an abundant proteoglycan-associated cartilage matrix protein (Paulsson & Henegard, 1981). All matrilins are modular proteins with two von Willebrand factor A (vWFA) domains separated by a varying number of EGF-like domains. It is thought that matrilins connect supramolecular assemblies in cartilage: complexes of matrilin-1 and biglycan or decorin have been shown to connect collagen VI microfibrils to both collagen II and aggrecan (Wiberg *et al.*, 2003). A recent report has suggested that matrilin-3 expression is enhanced in osteoarthritic cartilage and thus may be a cellular response to the modified microenvironment during disease (Pullig *et al.*, 2002).

1.6 Cartilage Turnover

Turnover (synthesis versus degradation) of articular cartilage macromolecules maintains the function of the tissue. Matrix constituents are synthesised by chondrocytes and specifically degraded by a number of matrix proteinases. The same proteinases are also thought to be responsible for the increased catabolism of matrix molecules in degenerative joint diseases. There are four main classes of matrix proteinase, classified according to the amino acid or chemical group at the catalytic centre of the enzyme: aspartate, cysteine, metallo- and serine proteinases.

The main classes of proteases are shown in figure 1.9, along with examples from each family and pH requirement for activity. The cysteine and aspartate proteinases act at low pH levels and are thought to act intracellularly within the lysosomal system. However, these enzymes can also be released extracellularly into small pockets near the cell membrane, where they degrade matrix in a controlled low pH microenvironment. Some enzymes may not catabolise matrix proteins directly but instead are capable of activating other proteinases that degrade the matrix. Membrane-bound proteinases may be involved in cytokine processing, receptor shedding and regulation of cell-cell and cell-matrix interactions.

1.6.1 Acidic Proteinases

Intracellular proteolysis by lysosomal proteinases occurs when cleaved matrix components are phagocytosed, but the relative contributions of intra- versus extra-cellular pathways are not clearly understood. Papain-like lysosomal cysteine proteases known as cathepsins are ubiquitous processive and digestive enzymes. Expression of cathepsins B, H and L is elevated in antigen-induced arthritic cartilage, whilst levels of cathepsins B and D are elevated in osteoarthritic tissues and have been implicated in the degradation of aggrecan core protein (Fosang *et al.*, 1992; Mort *et al.*, 1998; Handley *et al.*,2001). Cathepsin B (reviewed in Mort & Buttle, 1997) may also act as an intracellular activator of other proteinases that then degrade cartilage components. Acid-induced cathepsin K is expressed by phenotypically altered chondrocytes and may cause degradation of the superficial gliding surfaces of cartilage in osteoarthritis (Konttinen *et al.*, 2002). Cathepsin K is capable of degrading aggrecan complexes (Hou *et al.*, 2003).

1.6.2 Matrix metalloproteinases

At present, 23 distinct members of the human MMP family have been identified (for a review see Somerville *et al.*, 2003). They fall into four main groups: collagenases, stromelysins, gelatinases and membrane bound (MT-MMPs), as shown in figure 1.9. All members contain a zinc atom at their catalytic centre and are secreted as inactive pro-MMPs or zymogens that require activation, apart from MT-MMPs, which are active when they reach the plasma membrane. MMP activation can be mediated by other factors including other MMPs (particularly MT-MMPs), plasmin and reactive oxygen species released by inflammatory cells.

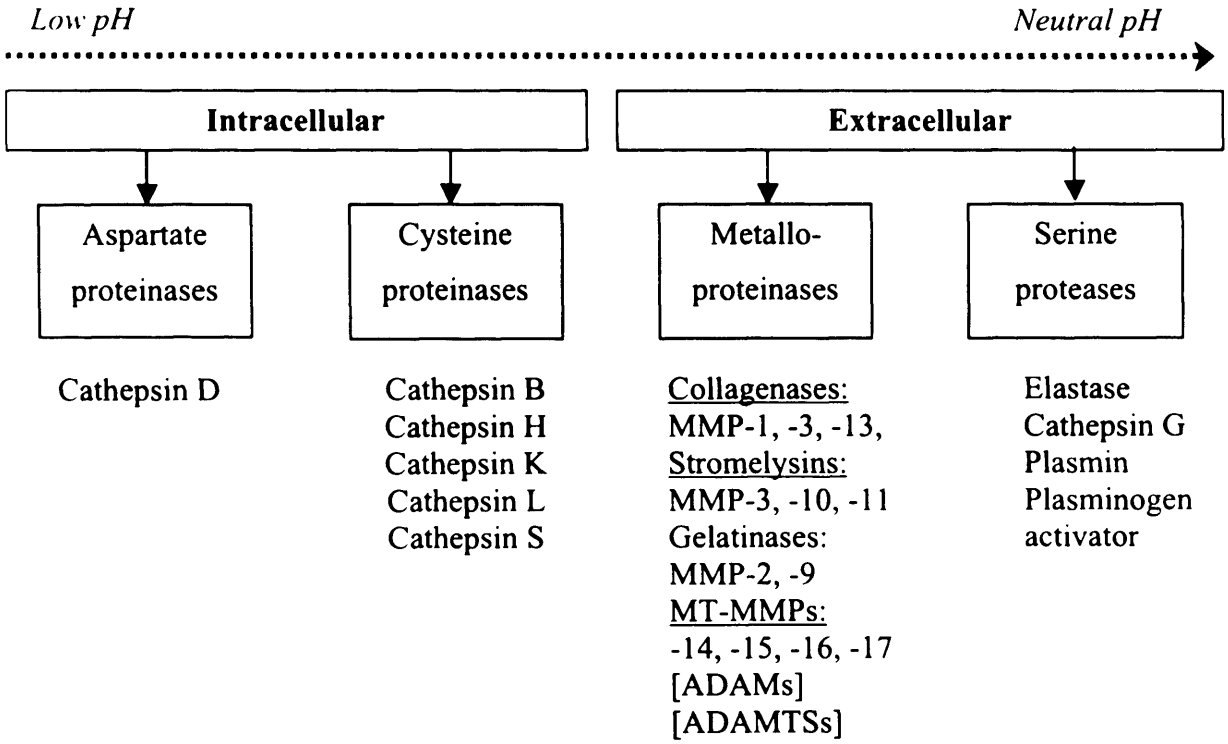


Figure 1.9 – The four main classes of matrix proteinases. Examples are given for each family along with pH range in relation to enzyme activity. Figure adapted from (Cawston & Rowan, 2002).

Most proMMPs have a propeptide domain that contains a unique 'cysteine switch' sequence – PRCG(V/N)PD – in which the cysteine residue ligates the catalytic zinc to maintain the inactive state of the zymogen (Nagase & Woessner, 1999). Invertebrate MMPs are referred to based on their substrate (e.g. collagenase 1) whereas vertebrate MMPs are assigned a number (e.g. MMP-1). All connective tissues contain endogenous Tissue Inhibitors of MetalloProteinases (TIMPS 1-4) that bind to active MMPs. Some MMPs are stored in granules within neutrophils and can be released when these cells are stimulated; others are produced by cells as a result of stimulation by cytokines. A brief description of the main MMP families is given below.

- Stromelysins have a broad substrate specificity that includes proteoglycan, laminin and fibronectin. Stromelysin-1 (MMP-3) is not widely expressed but is induced by IL-1.
- Collagenases cleave fibrillar collagens at a single site to produce quarter and three-quarter length catabolites that are further degraded by gelatinases. They include MMP-1 (interstitial collagenase), MMP-8 (neutrophil collagenase) and MMP-13 (collagenase 3). MMP-13 appears to be important in cartilage as it preferentially cleaves type II collagen.
- Gelatinases MMP-2 and –9 cleave degraded collagens, type IV and V collagen and elastin.
- MT-MMPs are activated by furin in the Golgi vesicles and may activate pro-MMPs.

MMPs play a role in tissue pathologies such as arthritis, cancer and cardiovascular disease. Information regarding MMP structure and function has led to the development of synthetic MMP inhibitors with a view to treating patients with these conditions. However, although this may be beneficial, MMPs also function in vital non-pathological processes such as embryonic development, organ morphogenesis, wound healing, nerve growth and bone remodelling, and the effect of MMP inhibitors on these processes is not clearly understood (reviewed in Nagase & Woessner, 1999).

1.6.3 ADAM/ADAM-TS proteins

1.6.3.1 ADAM proteinases

ADAMs are enzymes with A Disintegrin And Metalloprotoeinase domain, of which over 30 have been found. ADAMs have cell adhesion and protease activities, and act in a highly diverse set of

biological processes, including, neurogenesis, myogenesis, embryonic TGF-alpha release and the inflammatory response, reviewed in (Primakoff & Miles, 2000).

1.6.3.2 ADAM-TS proteinases

ADAMTS stands for A Disintegrin And Metalloproteinase with ThromboSpondin motifs. These include the recently described aggrecanases-1 and –2 (ADAMTS-4 and –5), which can cleave aggrecan, brevican and versican. The importance of aggrecanase-mediated aggrecan degradation in arthritis has been of considerable interest in recent years (for a review, see Arner, 2001). Recombinant ADAMTS-4 and –5 cleave aggrecan at five distinct sites along the core protein, and catabolites generated by cleavage at all five sites have been identified in cartilage explants undergoing matrix degradation. Aggrecanase activity can be modulated by altered expression, activation by cleavage at a furin-sensitive site, binding to aggrecan through the C-terminal thrombospondin motif, activation through post-translational modification of a portion of the C-terminus and inhibition of activity by tissue inhibitor of metalloproteinase-3 (TIMP-3).

1.6.4 Serine proteinases

Endogenous serine proteinases such as elastase and plasmin are found at low levels within cartilage and are thought to be derived from neutrophils. The activation of pro-MMPs may be mediated by plasmin as well as other serine proteinases. Tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) are both found in cartilage and can convert plasminogen to plasmin.

1.6.5 Neutrophil-dependent proteolysis

Neutrophils store large quantities of enzymes such as elastase, cathepsin G, neutrophil collagenase and gelatinase B. In many inflammatory situations there is an influx of neutrophils into the joint, and neutrophil enzymes degrade cartilage if released close to the surface. In most synovial fluids there is an excess of inhibitors such as TIMPS 1 and 2, α2-macroglobulin and α1-antiproteinase which can clear the degradative enzymes from the joint. In septic arthritis this inhibitory capacity may be exceeded and neutrophil enzymes are able to degrade the cartilage surface.

1.7 Arthritis

The Latin word 'arthritis' is derived from Greek: *arthro* (joint) and *itis* (inflammation). There are many different types of arthritis, ranging from mild localised forms such as tendonitis to severe systemic forms such as rheumatoid arthritis. However, most arthritides fall into one of two categories – osteoarthritis (OA) and rheumatoid arthritis (RA). According to the Arthritis Research Campaign (UK) website (www.arc.org.uk, July 2003) one in every five visits to a general practitioner is arthritis related, and more than seven million people have long-term health problems due to arthritis or a related condition. This situation is likely to worsen as the population of the developed world ages due to advances in other areas of medical research, though not only adults are affected - one child in every thousand has arthritis. The unravelling of the events during and leading up to degenerative joint disorders is a matter of considerable scientific and commercial interest. Information regarding non-collagenous cartilage matrix proteins is fragmentary at present, even though this group of molecules has proven to be of great importance in mediating matrix-matrix and cell-matrix interactions in other tissues. The insufficient characterisation of these presumably important components of the cartilage matrix is a considerable handicap in understanding and treating lesions and diseases of cartilage.

1.7.1 Osteoarthritis

OA is a 'wear-and-tear' disease, and its onset is usually related to alteration in the mechanical stresses applied to articular cartilage. The tissue attempts to repair itself to compensate for these changes by altering its biochemical composition, but this attempted remodelling only exacerbates the disability of the cartilage to cope with loading. For example, in the superficial zone there is a loss in proteoglycan that results in a change in the way that the tissue holds water, compromising performance under load. A feature of early OA is the increased hydration of cartilage, possibly due to an alteration in the collagen network (Hamerman, 1989). The matrix exhibits a decrease in keratan sulphate and a switch to chondroitin-4-sulphate from chondroitin-6-sulphate as the major cartilage CSPG (Roughley, 1986). Matrix proteases are activated through cytokines such as IL-1, and these degrade the cartilage matrix leading to joint space narrowing. In later stages there is a change in chondrocyte phenotype and morphology: cells divide in their lacunae and express type X collagen. There is no 'cure' for OA, and commonly used treatments only modify the symptoms of the disease rather than the underlying process. The 'old wives tale' of the use of cod liver oil

for the treatment of OA symptoms has recently been given credence by studies demonstrating that n-3 (but not n-6) fatty acids can inhibit the expression and activity of degradative and inflammatory factors such as aggrecanases, cyclooxygenase-2, IL-1 and TNFα (Curtis *et al.*, 2000; Curtis *et al.*, 2002). Glucosamine sulphate has recently received attention as a disease-modifying agent based upon its role in the metabolic pathway of GAG production and PG assembly. Recent *in vitro* results have shown that glucosamine sulphate can stimulate mRNA and protein levels of aggrecan core protein and inhibit production and enzymatic activity of MMP-3 in chondrocytes from OA articular cartilage (Dodge & Jimenez, 2003). However, the jury is still out in clinical circles as concerns its effectiveness in combating degenerative joint disease when used as a dietary supplement.

1.7.2 Rheumatoid Arthritis

RA is a systemic disease with diverse and fluctuating manifestations, and the mechanisms behind it are not fully understood. Viruses or toxins may trigger an autoimmune response in genetically susceptible individuals. Immune complexes in the synovial joint cause an influx of inflammatory cells such as macrophages and neutrophils into the synovial cavity, and these cells release inflammatory cytokines such as IL-1 and TNF-α, which induce the synthesis, secretion and activation of matrix proteases. A major site of tissue damage resulting from this, the pannus, eventually grows over the cartilage surface, invades the cartilage matrix and destroys it. Pannus is an invasive granulation tissue composed of aggressive macrophage- and fibroblast-like mesenchymal cells (Fassbender & Simmling-Annefield, 1983) that contribute to cartilage destruction by the release of collagenolytic enzymes. Pannus-like tissue also exists in advanced OA cartilage (Shibakawa *et al.*, 2003). RA symptoms can also include general stiffness, inflammation of other tissues such as blood vessels or the lungs, and fatigue. RA has a variable course, with flares and remissions that fluctuate unpredictably, which complicates the task of quantifying the effects of RA therapies (Schiff, 2003).

1.7.3 Surface zone of articular cartilage in arthritis

In OA, the surface of cartilage is altered, such that the normal gliding surface (Mankin grade 0) becomes fibrillated (grade 1). During the progression of the disease, superficial fissures (grade 2) and deep fissures that penetrate to the subchondral bone (grade 3) develop. This implies that the

surface of cartilage is the region to be first affected by OA. Superficial cracks or fissures caused by unusual loading are a feature of post-traumatic osteoarthritis (Kafka, 2002). In late-stage OA pannus tissue can also accumulate on the cartilage surface (Shibakawa *et al.*, 2003). The biochemical and biomechanical features of the surface of cartilage is described in the following section.

1.8 Surface Zone of Articular Cartilage

1.8.1 Introduction - surface of articular cartilage

Articular cartilages serve to absorb and dissipate load, and provides almost frictionless articulation within a diarthrodial joint. The surface zone is important in both these aspects, as its position influences both articulation between cartilage surfaces and the tensile and compressive properties of the tissue as a whole (Guilak *et al.*, 1994). The surface zone of articular cartilage has been studied since the 18th century, when the surgeon and anatomist William Hunter (1718-1783) noted that when 'viewed with a glass,' it appeared to be 'smooth, polished and covered with a membrane' (Hunter, 1743). It is now known that articular cartilage is not covered by a membrane, but by a layer of collagen fibrils that lie tangential to the surface topped with a layer of amorphous, electron-dense material (Ghadially, 1983). It was termed the 'lamina splendens' by MacConaill in 1951 (MacConaill, 1951), who observed a bright line at the surface of cartilage examined by phase contrast microscopy. The term lamina splendens was disputed by Aspden & Hukins in 1979, who predicted that its appearance was an artefact of phase contrast microscopy, a 'halo' effect that is apparent along any incision or scratch made into a section of cartilage (Aspden & Hukins, 1979). Since then, studies have confirmed the presence of a specific lamina splendens by polarising (Dunham *et al.*, 1988), scanning electron (Teshima *et al.*, 1994) and atomic force (Kumar *et al.*, 2001) microscopy. It appears to be comprised of collagen fibrils arranged tangentially to the surface, which is only loosely connected to the underlying fibrous structures (Teshima *et al.*, 1994).

1.8.2 Structure & composition of the surface of articular cartilage

The structure and composition of the articular surface has been the subject of considerable debate in the recent past (Ghadially, 1983; Jeffery *et al.*, 1991; Jurvelin *et al.*, 1996; Kumar *et al.*, 2001). Viewed by scanning electron microscopy, the surface appears as a tangentially arranged layer of

collagen fibrils covered by a single layer of electron dense, amorphous material (Ghadially, 1983). This is shown in figure 1.10. There are irregularities in the surface coat, and these undulations are likely to be a shrinkage artefact of SEM (Jurvelin *et al.*, 1996). This surface coat is proteinic in nature - it can be digested with trypsin and chymopapain but is resistant to degradation by testicular and microbial hyaluronidases, keratanase and chondroitinase ABC (Stanescu, 1985; Kumar *et al.*, 2001).

1.8.2.1 Proteins at the articular surface of cartilage

On the intact articular surface of cartilage type II collagen is masked from recognition by antibodies to collagen. The surface material that protects surface zone type II collagen is thought to be mainly proteinic, non-covalently bound to the underlying intercellular matrix and synthesised by chondrocytes located in the superficial zone (Noyori *et al.*, 1994a; Jasin *et al.*, 1993). It is sensitive to *in vivo* degradation in a rabbit model of acute inflammatory arthritis, and *in vitro* by activated polymorphonuclear leukocytes and neutrophil elastase (Jasin & Taurog, 1991). The major proteins of the articular surface of cartilage are the collagen-binding small proteoglycans fibromodulin and albumin, with fibronectin, decorin and biglycan detected in smaller amounts (Noyori *et al.*, 1998). It has been found that the amount of decorin and biglycan in the superficial zone of intact osteoarthritic cartilage is decreased in comparison to normal cartilage (Poole *et al.*, 1996). Other surface zone-specific molecules include clusterin (Khan *et al.*, 2001), Dell (Pfister *et al.*, 2001) and superficial zone proteoglycan (SZP) (Schumacher *et al.*, 1994), which is described in more detail in section 1.9.

1.8.2.2 Phospholipids at the articular surface

In recent years, many studies have championed the hypothesis that surface-active phospholipid (SAPL) is a vital lubricant on the surface of articular cartilage (Schwarz & Hills, 1996; Hills, 1990; Hills & Monds 1998a). The surface of articular cartilage is hydrophobic, based on the observation that a droplet of water beads up when placed on a rinsed articular surface, and Hills and others propose that SAPL produced by type B synoviocytes and adsorbed to the surface of cartilage via synovial fluid is responsible for this hydrophobicity (reviewed in Hills & Crawford, 2003). Water droplets do not appear to bead to the same degree on osteoarthritic cartilage.

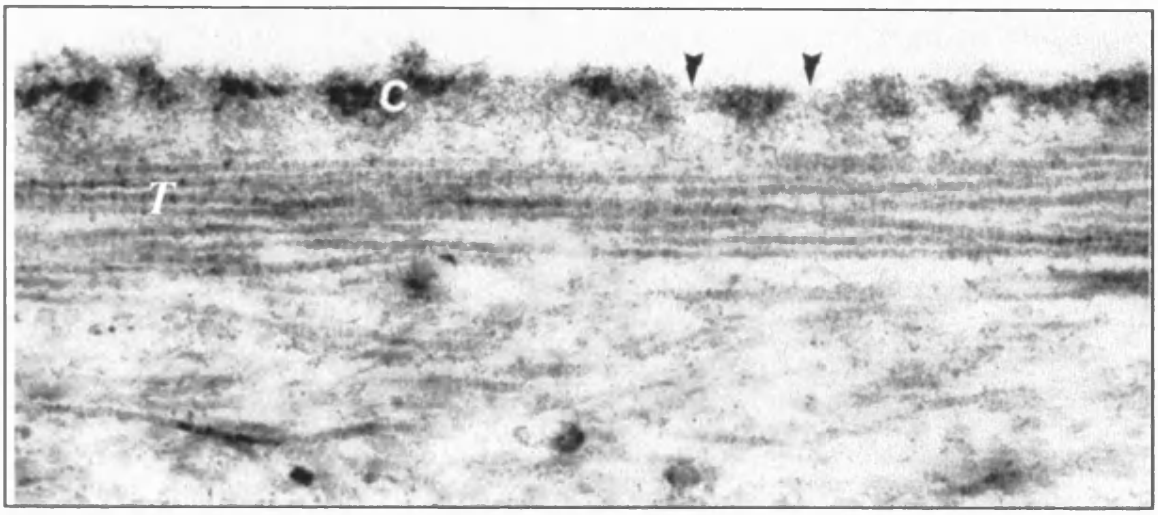


Figure 1.10 – Scanning electron micrograph of a section of rabbit articular cartilage processed attached to bone, showing a fairly straight surface. The surface coat, C, presents as a layer of electron-dense material lying on the surface. T represents the collagen fibrils arranged tangentially to the articular surface. The coat is rarefied or attenuated in places, as shown by arrowheads. From (Ghadially, 1983).

A study to characterise the phospholipid components adsorbed onto the surface of cartilage identified phosphatidylcholine, phosphatidylethanolamine and sphingomyelin as the major components, with oleic acid as the predominating fatty acid observed with all 3 lipid types (Sarma *et al.*, 2001). SAPL has been shown to be deficient on the surface of osteoarthritic hip and knee cartilage (Hills & Monds, 1998b). SAPL may be prone to lysis by phospholipase A_2, a mechanism that may be inhibited by HA present in synovial fluid (Nitzan *et al.*, 2001). It is proposed by Hills and others that the protein lubricin (section 1.9) acts as a carrier for SAPL, and that SAPL is the true lubricant, as reviewed in (Hills, 2002a). This dispute is described in section 1.8.3.2. Microscopical studies have identified globular lipid-like structures in the upper surface layer of hip and knee cartilage (Watanabe *et al.*, 2000).

1.8.3 Function of the surface of articular cartilage

1.8.3.1 Joint lubrication

The surface structure of articular cartilage provides a highly efficient boundary lubrication mechanism with an extremely low coefficient of friction. Boundary lubrication is a term first introduced by Hardy in 1922, when he stated that very thin adsorbed layers were sufficient to cause two glass surfaces to slide over each other with very low friction, and that under such circumstances the lubrication depended wholly upon the chemical constitution of the fluid and not on its viscosity, and that a good lubricant was a fluid which was adsorbed by the solid surfaces (Hardy & Doubleday, 1922). Synovial joints are lubricated and nourished by synovial fluid, and in a healthy individual can operate in a wear resistant, frictionless manner for decades. In the 1960s two conflicting theories for joint lubrication were proposed, taking into account fluid transport across the articular surface. The 'weeping lubrication' model of McCutchen (McCutchen, 1959; McCutchen, 1962) proposed that the synovial film is supplied by the exudation of interstitial fluid from compressed cartilage. Conversely, the 'boosted lubrication' model of Walker *et al.* (1968) assumes that viscous accumulations of synovial fluid in the surface pores of articular cartilage separate the two sliding surfaces. A mechanical, 'biphasic' model of articular cartilage was developed by Mow and co-workers, where the collagen-proteoglycan matrix is modelled as an intrinsically incompressible porous permeable solid matrix, and the interstitial fluid is modelled as an incompressible fluid (Mow *et al.*, 1980). The triphasic model described by Lai *et al.* (1991) is an extension of the biphasic theory, where proteoglycans are

modelled as a negative charge density fixed to the solid matrix and monovalent ions in the interstitial fluid are modelled as additional phases. A series of formulas that relate the biphasic and triphasic models has recently been described by Ateshian and others (Ateshian *et al.*, 2004). Using the development of these models it has been shown that interstitial fluid pressurisation is responsible for increasing the stiffness of cartilage under dynamic loading, which allows it to sustain physiological levels of stress in the range of 2-12 MPa. This interstitial pressurisation is thought to be the primary mechanism imparting low friction and wear to the surface of cartilage (McCutchen, 1962). In 1994, Macirowski and others utilised unique prosthetic instruments to collect *in situ* data regarding cartilage stresses in the human hip joint, the results of which support the 'weeping' mechanism of joint lubrication as proposed by McCutchen (Macirowski *et al.*, 1994). This process has been literally frozen in time by Clark *et al.*, (1999) who have performed experiments involving the plunge-freezing of rabbit joints loaded *in vitro* followed by sectioning and scanning electron microscopy. This reveals a continuous fluid film that separates one cartilage surface from the other, avoiding direct contact.

The study of joint lubrication is a complex area: many different types of lubrication are involved (boundary, fluid film, elastohydrodynamic, boosted, weeping), and it is unclear exactly when each type of lubrication comes into play during the gait cycle. The surface of cartilage is not uniform, and the types of models used to study joint lubrication and the various factors that affect it, such as cartilage on glass bearings or latex on glass bearings, are all subject to certain limitations.

1.8.3.2 Lubricin – a proteoglycan constituent of synovial fluid and the surface of articular cartilage

Lubricin was first described by Swann *et al.* (Radin *et al.*, 1970; Swann & Radin, 1972; Swann *et al.*, 1973) as a 227.5 kDa mucinous glycoprotein constituent of synovial fluid that facilitates boundary lubrication in diarthrodial joints. It has since been established that lubricin is also produced by chondrocytes in the superficial zone of articular cartilage (Schumacher *et al.*, 1994), and immunohistochemical analyses have shown that it accumulates at the articulating surface (Schumacher *et al.*, 1999; Tudor, 2002). Lubricin has been shown to be a product of PRG4/MSF gene expression by synovial fibroblasts (Jay *et al.*, 2000). This molecule is the subject of this

thesis and will be discussed at greater length in section 1.9. Both PRG4 and lubricin contain a central domain of 904 amino acids, 208 of which are threonine in a repeating sequence of KEPAPTT, putative sites for O-linked glycosylation. Bovine lubricin is 50% (w/w) glycosylated with multiple residues of O-linked β (1-3) Gal-GalNAc, which are incompletely capped with NeuAc to an unknown degree (Swann *et al.*, 1981). Recent digestion studies have found that boundary lubrication by lubricin *in vitro* is provided primarily by extensive O-linked β (1-3) Gal-GalNAc (Jay *et al.*, 2001a). This indicates that lubricin, by virtue of its extensive glycosylations, somehow organizes water to support contact loading. The altered frictional characteristics observed in the joints of PRG4 knockout mice seem to confirm that PRG4/lubricin is critical for normal joint lubrication (Jay *et al.*, 2003a). PRG4/lubricin is present in tendon (Rees *et al.*, 2002), and recent preliminary studies have shown that lubricin improves the gliding function of extrasynovial tendon *in vitro* (Kutsumi *et al.*, 2004).

At present, opinions differ as to the true role of lubricin/PRG4 in boundary lubrication. Hills and Mond postulate that lubricin is a carrier for surface-active phospholipid, the true lubricant, based on the loss of synovial fluid lubrication following phospholipase digestion and the apparent hydrophobicity of the articular surface (Hills & Mond, 1998a; Hills, 2002a). This would imply a similar role for a lubricin/surface active phospholipid complex as for phospholipoprotein in alveolar lubrication. Jay and Cha challenged these results by demonstrating trypsin-like activity in the phospholipase used by Hills (Jay & Cha, 1999). Hills countered these suggestions by stating that removal of the putative carrier of surface-active phospholipid, lubricin, would have a similarly detrimental effect on lubrication as would removal of surface-active phospholipid (Hills, 2002b). Jay responded (Jay, 2002) by questioning the purity of lubricin obtained in a previous study supporting the hypothesis that lubricin is a carrier for surface-active phospholipid (Schwarz & Hills, 1998) and drew attention to the results obtained in the study of the removal of synovial fluid lubrication by glycosidases (Jay *et al.*, 2001a). According to Jay, resolution of this issue will depend on the detection of elemental phosphorous in purified lubricin. Another view of the role of lubricin in boundary lubrication is provided by a recent biomechanical study: 'it is likely that μ_{eq} [coefficient of friction at the articular surface] can be further reduced by a lubricant such as lubricin, SZP, or phospholipids..... [but that] the primary mechanism of lubrication is governed by interstitial fluid pressurisation' (Park *et al.*, 2003).

Hyaluronan gives healthy synovial fluid its viscosity and is present at the surface of articular cartilage. However, it is thought that it does not directly contribute to the boundary lubrication mechanism described above (Tadmor *et al.*, 2002). Joint articulation is an inherently cyclic process, and the viscosity of synovial fluid due to hyaluronan could be important in minimising the outflow of the fluid film at the surface during the period of time that a particular area of cartilage is loaded (reviewed in Simkin, 2000). An interaction between lubricin and hyaluronan has been proposed but not conclusively demonstrated (Jay *et al.*, 2003a; Jay *et al.*, 2003b). Viscosupplementation (reviewed in Kirwan, 2001) refers to the intra-articular injection of hyaluronan, and is purported to increase lubrication as well as having an anti-inflammatory effect. However, the large placebo response in viscosupplementation studies makes it hard to assess the clinical efficacy of this technique.

1.8.3.3 Barrier function of the surface of articular cartilage

Recent work has suggested that the mature superficial zone of articular cartilage can act as a barrier against substances that invade from the surface (Takada *et al.*, 1999). However, cartilage is an avascular and alymphatic tissue, and the resident cells depend on diffusion of soluble nutrients from the joint cavity and from subchondral bone. It has been found that the superficial zone of articular cartilage does not constitute a barrier to the transport of macromolecular solutes of up to 440 kDa into the deeper zones (Uesugi & Jasin, 2000).

IgG is found in small amounts in normal cartilage, and has been shown to bind to an unspecified cartilage component in cartilage homogenates (Alomari *et al.*, 1983). It has been proposed that immune complexes found in the superficial zone of articular cartilage in RA (Cooke *et al.*, 1975) and in antigen-induced arthritis are formed because pre-existing antibody normally present in cartilage irreversibly traps antigen within the tissue (Jasin, 1975).

The intact surface of articular cartilage is thought to resist the adhesion of inflammatory cells (Shiozawa *et al.*, 1992; McCurdy *et al.*, 1995; Mitani *et al.*, 2001). Macromolecules normally present on articular cartilage surfaces, such as fibromodulin, decorin and biglycan (Poole *et al.*, 1996) are thought to act as a barrier to polymorphonuclear neutrophil (PMN) adhesion (Mitani *et al.*, 2001). This is in contrast to elastase-treated cartilage explants reconstituted with fibronectin,

which sustain PMN adhesion (Noyori & Jasin, 1994). PMN make up a significant proportion of the synovial fluid cells in rheumatoid arthritis and there is evidence to suggest these cells and their products, particularly proteolytic enzymes, are present at the cartilage-pannus junction and may be responsible for cartilage damage (Jasin & Taurog, 1991; Edwards & Hallett, 1997). Studies concerned with the organisation of the intact surface of adult articular cartilage should yield valuable insights into the pannus-mediated degradation of cartilage in inflammatory arthritis - breakdown of the surface layer of articular cartilage could be a preliminary event leading to the attachment of inflammatory cells and the invasion of pannus. The term pannus applies to macroscopical cloth-like tissue covering articular cartilage in rheumatoid arthritis, but recent studies have shown the presence of a pannus-like tissue on osteoarthritic cartilage (Shibakawa *et al.*, 2003).

1.9 Proteoglycan 4 (PRG4)

A number of surface zone-specific molecules have been found in articular cartilage, and these include Dell (Pfister *et al.*, 2001), clusterin (Khan *et al.*, 2001) and PRG4. PRG4 is a proteoglycan of 1054 amino acids with a molecular weight of around 345 kDa. It has a number of aliases owing to its different routes of discovery (shown in figure 1.11) including megakaryocyte stimulating factor (MSF) precursor protein (Merberg *et al.*, 1993), camptodactyly-coxa-vara-pericarditis (CACP) protein (Marcelino *et al.*, 1999), lubricin (Swann *et al.*, 1985) and cartilage superficial zone proteoglycan (SZP) (Schumacher *et al.*, 1999). PRG4 is the unifying gene name assigned by the Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC). The molecule is not homologous to p53-responsive gene 4, which is also known as PRG4 (Horikoshi *et al.*, 1999).

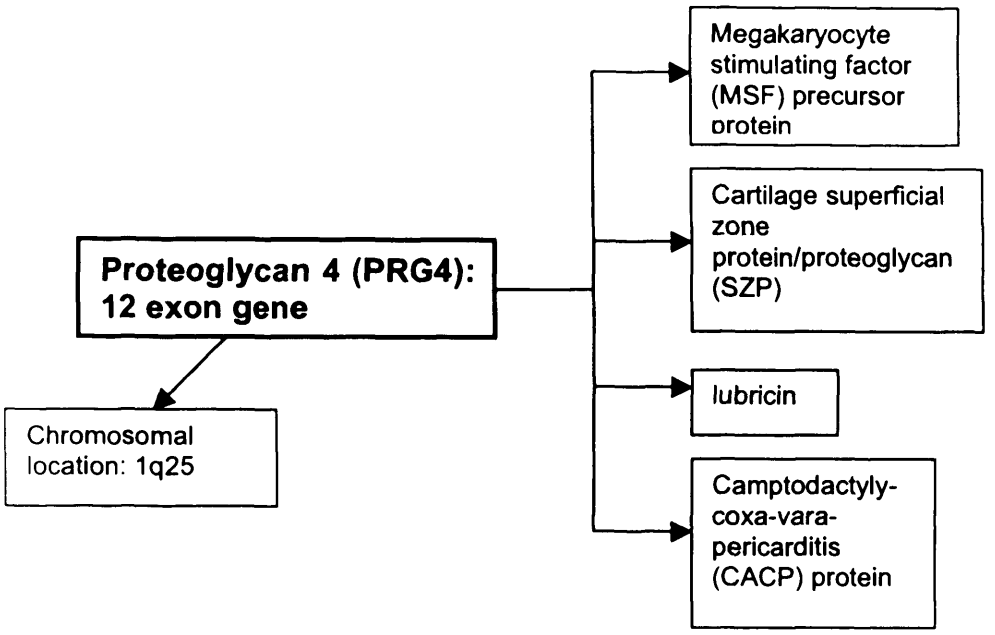


Figure 1.11 – The many monikers of PRG4: clarification of a confusing nomenclatural situation. Different names have been given to products of the PRG4 gene, resulting from different routes of discovery.

1.9.1 Isolation and Location of PRG4

PRG4 was first described in articular cartilage synthesised specifically by chondrocytes of the superficial zone, and also by some of the surface lining cells present in the villi of the synovium of diarthrodial joints (Schumacher *et al.*, 1994; Schumacher *et al.*, 1999). Cartilage PRG4 was first isolated by Schumacher *et al.* (1994) from the culture medium of thin slices of bovine superficial zone articular cartilage. It was not found to be produced in the middle or deep zones. It contains KS and CS chains, but treatment with chondroitinase and keratanase resulted in only a minor shift in electrophoretic mobility, suggesting that there are low levels of KS and CS substitution. It is not retained in the cartilage matrix, and can be detected in synovial fluid. Immunodetection of PRG4 at the surface of cartilage and tendon is more apparent in mature tissues, and this may be due to the maturation of the collagen architecture at the matrix surface, which could form a barrier that entraps PRG4 (Schumacher *et al.*, 1999; Rees *et al.*, 2002).

Studies with monoclonal antibodies raised against bovine PRG4, including 3-A-4 and 6-A-1, confirmed the location of PRG4 at the cartilage surface (Schumacher *et al.*, 1999). Immunodetection studies also revealed that although PRG4 is not incorporated into the matrix *per se*, it does accumulate in the superficial matrix at the interface between the cartilage surface

and the synovial cavity. Along with cartilage intermediate layer protein (CILP), PRG4 appears to be a phenotypic marker, indicative of chondrocytes of a particular morphological zone. Figure 1.12 shows the location of PRG4 in bovine articular cartilage as described in the immunohistochemical studies of Tudor (2002).

PRG4 has also been found in other tissues. Further work with the antibodies described above has located PRG4 as residing in a distinct layer of the surface of mature compressed tendon (Rees *et al.*, 2002). Expression of human and mouse PRG4 mRNA has been found not only in cartilage but also in liver, heart, lung and bone (Ikegawa *et al.*, 2000). Other researchers are involved in quantifying PRG4 concentrations in the serum and synovial fluid of healthy and diseased individuals (Schmid *et al.*, 2001), and developing and utilising cross-species PRG4 antibodies (Su *et al.*, 2001).

1.9.2 Structure and potential functions of PRG4

Monoclonal antibodies were also used by Schumacher *et al.* (Schumacher *et al.*, 1999) to isolate a λgt11 clone producing a fusion protein corresponding to the C-terminal portion of PRG4. The partial cDNA sequence obtained was subjected to a BLAST search and was shown to display a high degree of homology with human megakaryocyte stimulating factor (MSF) precursor mRNA. MSF has been shown to be a complex glycoprotein that displays some homology to vitronectin (Merberg *et al.*, 1993). In work from this laboratory, primers were designed specific to N-terminal regions of bovine PRG4 using the previously published sequence for human MSF precursor. This study further highlighted the homology between human and bovine PRG4 and MSF precursor, and led to the postulation that the two proteins originate from the same 12-exon gene (Flannery *et al.*, 1999).

1.9.2.1 PRG4 domain structure

Based on the partial cDNA sequence for bovine PRG4 and the published sequence for MSF precursor protein, PRG4 is considered to have a number of structural motifs, which in turn indicate that the molecule has the potential for numerous diverse functions (Merberg *et al.*, 1993; Flannery *et al.*, 1999). A schematic representation of the structure of PRG4 is shown in figure 1.13, and a sequence comparison between bovine and human species is shown in figure 1.14.

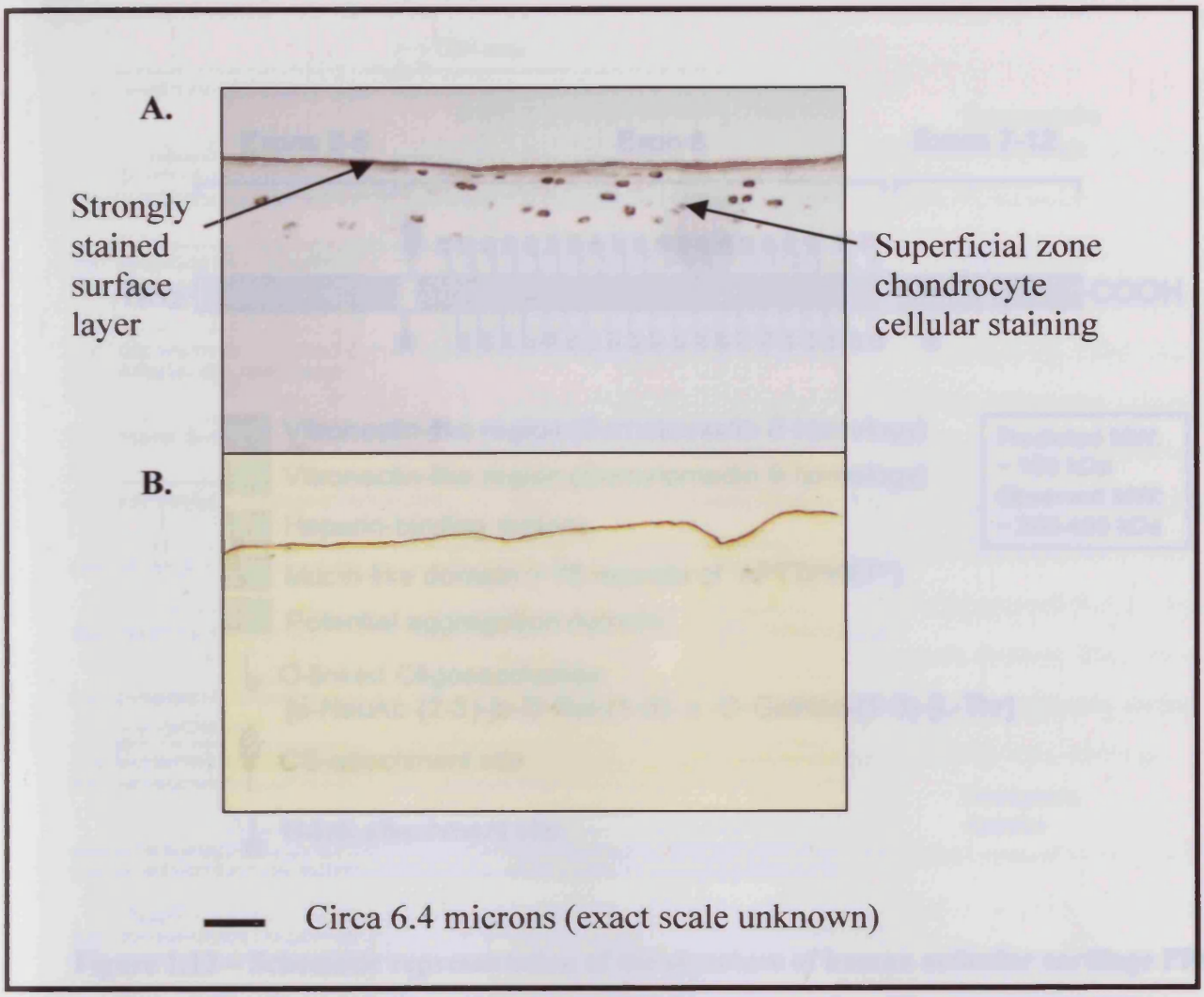


Figure 1.12 – Immunolocalisation of PRG4 in adult bovine articular cartilage using the monoclonal antibody 3-A-4. (A) – Vertical section of cartilage stained with 3-A-4 and viewed by microscopy. (B) – Similar section viewed by microscopy but with no primary antibody. Adapted from (Tudor, 2002).

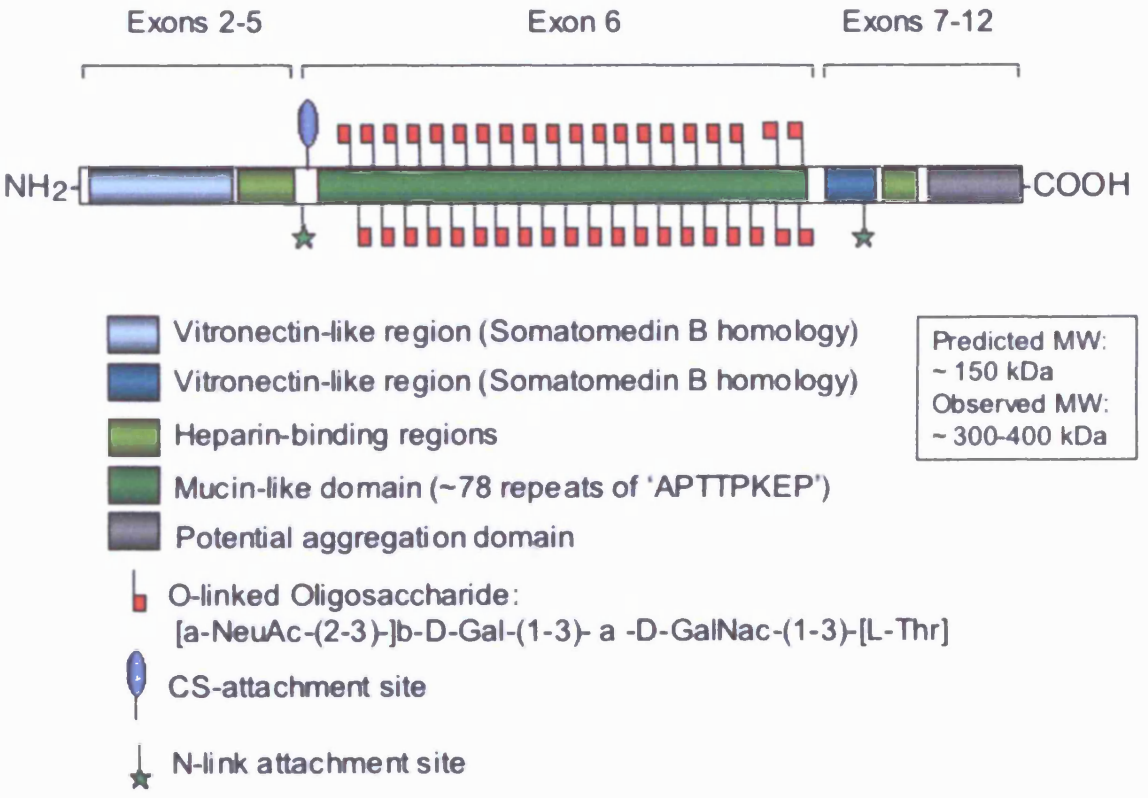


Figure 1.13 – Schematic representation of the structure of human articular cartilage PRG4.

Functional domains are indicated by coloured boxes. Adapted from Flannery *et al.*, 1999.

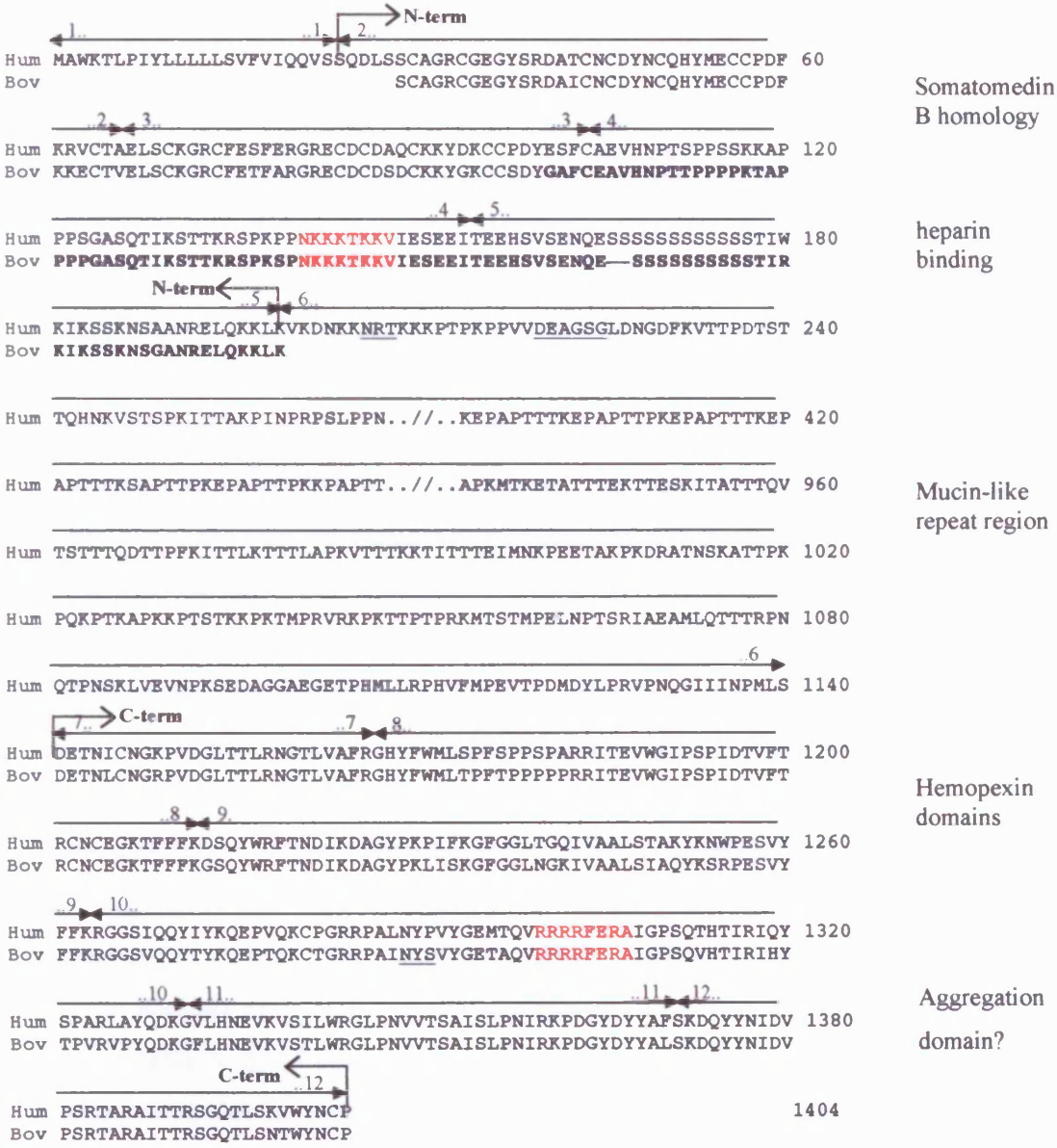


Figure 1.14 – Alignment of amino acid sequences for human and bovine PRG4. Sequences are from human monocytes and bovine articular cartilage chondrocytes (GenBank Accession Nos. NM005807 and AF056218, respectively). The top (numbered) line in each row represents the sequence for human PRG4, with exons and boundaries indicated by lines and arrowheads above the sequence. Bovine PRG4 sequence identified in this study is shown in bold. Consensus sequences for N-glycosylation (**N**XS/T) and chondroitin sulphate substitution (DEAGSG) are underlined. Several of the mucin-like repeats are included though most of the exon 6 sequence has been omitted for clarity. Heparin binding consensus sequences are shown in red. N-terminal and C-terminal domains targeted for cloning and expression in this study are indicated by 'N-term' and 'C-term' bold type. Adapted from Flannery *et al.*, 1999.

At the N- and C-termini there are domains similar to those found in vitronectin, and these may impart cell-proliferative and matrix binding properties. The central part of the molecule contains a 940 amino acid stretch of large and small mucin-like repeats, and this region is likely to be heavily substituted with O-linked oligosaccharides indicating that PRG4 has a lubricating role. Lubricin is a 228 kDa mucinous glycoprotein responsible for the boundary lubrication of synovial joints, and it appears to be the product of PRG4/MSF/SZP gene expression, displaying 100% homology with exons 6 through 9 (Jay 2000; Jay *et al.*, 2001b). The exact relationship between lubricin and PRG4 is still unclear, but based on the assumption that both molecules undergo the same post-translational modifications it is likely that PRG4 functions as a boundary lubricant in the same way that lubricin does. Jay *et al.* (2001b) have suggested that PRG4 and lubricin occupy a new class of biomolecules termed tribonectins, a term derived from tribology and the Latin *nectere*, meaning "to bind or connect". Tribology is the study of sliding surfaces in relative motion and the impact of different factors on the friction between the two surfaces.

Two somatomedin B-type domains at the PRG4 N-terminus could bind plasminogen activator inhibitor-1 and inhibit plasmin generation, based on sequence comparisons with vitronectin (Merberg *et al.*, 1993). The C-terminal portion of PRG4 contains an uneven number of cysteine residues, and this may facilitate self-aggregation, as seen in secretory mucins (Gum, 1995). This self-aggregation could explain the accumulation of PRG4 at the surface of cartilage. Matrix binding could also be explained by the putative heparin-binding domain located within the N-terminal domain of the molecule (Merberg *et al.*, 1993; Flannery *et al.*, 1999).

1.9.3 Megakaryocyte Stimulating Factor (MSF) precursor

PRG4 was first documented with the cloning of the homologous MSF precursor protein and the subsequent determination of its genomic and cDNA sequences led to the prediction of its multidomain structure (Merberg *et al.*, 1993). MSF was first detected in and subsequently purified from the urine of bone marrow patients during the period of acute thrombocytopaenia (Ogata *et al.*, 1990; Turner *et al.*, 1991). Purified urinary MSF has a molecular weight of 28-35 kDa under reducing conditions, and represents an N-terminal portion of the PRG4 protein. In murine bone marrow fibrin clot cultures, urinary MSF stimulates the growth of both pure and mixed megakaryocyte colonies with a specific activity of greater than 5×10^7 units per milligram

of protein. The researchers that isolated and cloned the MSF (PRG4) gene performed sequence analyses and noticed that several regions were very similar to domains of vitronectin (Merberg *et al.*, 1993). Vitronectin is a 75 kDa glycoprotein, and is a member of a group of adhesive plasma proteins which includes fibrinogen, fibronectin and von Williebrand factor (for a review see Schvartz *et al.*, 1999). It is a multifunctional regulator of both complement and coagulation systems, and is also known as serum spreading factor or complement S protein. It is anchored to the extracellular matrix via collagen or proteoglycan binding, and promotes cell attachment, spreading and migration through specific interactions of a single RGD peptide with integrins. Vitronectin can potentially influence extracellular matrix breakdown by virtue of its ability to bind plasminogen activator inhibitor-1 (PAI-1). Some of the structural similarities between vitronectin and MSF/PRG4 are detailed below. These similarities contribute to the theoretical functions of cartilage PRG4.

1.9.3.1 Somatomedin B domains

There are two somatomedin B (SMB) domains in the N-terminal portion of PRG4, encoded for by exons 2 and 3, and these domains are similar to the SMB domain encoded by exon 2 of vitronectin (Merberg *et al.*, 1993). SMB, produced by proteolytic cleavage of vitronectin, circulates in serum and was originally believed to have growth promoting activity, although this was later found to result from contamination by EGF (Heldin *et al.*, 1981). The somatomedin B domain of vitronectin has been implicated in the binding of plasminogen activator inhibitor-1 (PAI-1) (Seiffert & Loskutoff, 1991). PAI-1 is a highly specific serine protease inhibitor, and regulates the activity of plasminogen activators that catalyse the conversion of the zymogen plasminogen into plasmin. Plasmin is a protease of broad specificity, and has the capacity to degrade many extracellular proteins including proteoglycans, either directly or by the activation of procollagenase. PAI-1 in solution is unstable and rapidly decays into an inactive inhibitor, and the binding of PAI-1 to vitronectin appears to stabilise PAI-1 in an active configuration. A number of potential PAI-1 binding sites have been identified within vitronectin, the highest affinity being within the SMB domain (Seiffert & Loskutoff, 1991; Royle *et al.*, 2001). There is also evidence that cells may bind to vitronectin through urokinase receptor (uPAR) mediated attachment to the SMB domain (Okumura *et al.*, 2002). Furthermore, it appears that PAI-1 bound to vitronectin sterically hinders cellular attachment to the SMB domain through uPAR (Deng *et*

al., 2001), and that urokinase plasminogen activator (uPA) can reverse this process by binding to PAI-1 and causing it to be released from vitronectin (Mimuro & Loskutoff, 1989). This would suggest that both the fibrinolytic system and cell adhesion and migration are locally modulated by the relative balance between vitronectin, uPAR and PAI-1. SMB contains 8 cysteine residues that were thought to create 4 disulphide bonds in a linear, uncrossed manner (Kamikubo *et al.*, 2002). However, more recent work investigating the crystal structure of the somatomedin B-PAI-1 complex has revealed the presence of crossed disulphide bonds which constrain the peptide as a novel disulphide bonded knot (Zhou *et al.*, 2003), as illustrated in 1.15B. The conservation of cysteine residues between SMB and PRG4 shown in figure 1.15A would suggest a similar secondary structure for the two SMB domains of PRG4.

Besides vitronectin and PRG4, three other proteins have been found to contain somatomedin B domains: PC-1, a plasma cell membrane glycoprotein (Oda *et al.*, 1991); Tcl-30, a T cell specific protein expressed in immature thymocytes (Baughman *et al.*, 1992) and PP11, a human placental-specific protein (Grundmann *et al.*, 1990). Between these five molecules, eight cysteine residues are conserved in the somatomedin B domains. The presence of somatomedin B domains in the form of MSF/PRG4 that stimulates megakaryocytes implies that this domain has proliferative effects, but this has yet to be shown definitively (Merberg *et al.*, 1993).

1.9.3.2 Heparin binding domains

PRG4 contains 2 'Cardin-Weintraub' heparin binding consensus sequences (Cardin & Weintraub, 1989) in exons 4 and 10, shown in red in figure 1.14. The heparin binding domain of vitronectin has been shown to exhibit conformational flexibility. Vitronectin exists in a native form that does not bind heparin and a conformationally altered, heparin binding form. Both forms bind to the extracellular matrix, however there is evidence to suggest that only the conformationally altered form is cleared from the matrix by receptor-mediated endocytosis for subsequent degradation by lysosomes (Panetti & McKeown-Longo, 1993). Vitronectin contains a cAMP–dependent protein kinase phosphorylation site at the carboxyl terminus of the heparin binding domain, KKKTK, which has shown to be phosphorylated (McGuire *et al.*, 1988). An identical site exists in exon 4 of PRG4, though its phosphorylation status has not been investigated. The heparin binding capability of PRG4 has not been formally documented.

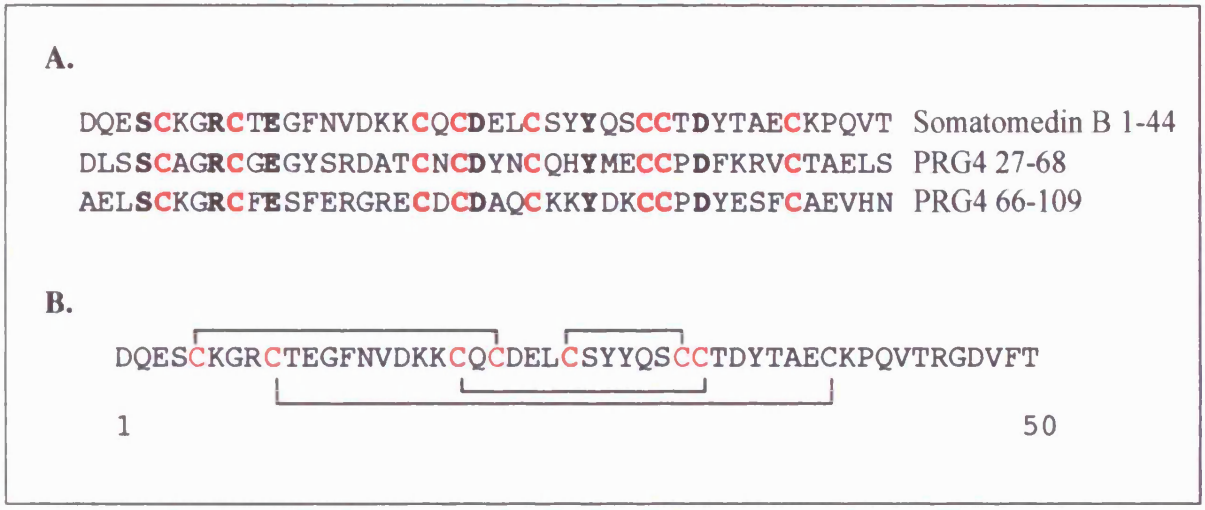


Figure 1.15 – Amino acid sequence comparison between human SMB and human PRG4 and predicted disulphide bonds of human somatomedin B (SMB). Sequence comparisons between somatomedin B and the two somatomedin B-like domains of PRG4 are shown in (A). Conserved residues are highlighted in bold. Disulphide bonds in human somatomedin B were determined by crystallography (Zhou *et al.*, 2003) and are shown in (B).

1.9.3.3 Hemopexin domains

Exons 8 and 9 of PRG4 are homologous to exons 4 and 5 of vitronectin, and are hemopexin-like domains, with central hydrophobic motifs and proximal aromatic residues. Hemopexin is a 60 kDa heme-binding plasma glycoprotein that transports heme to the liver: heme-hemopexin complexes are delivered to hepatocytes by receptor-mediated endocytosis after which hemopexin is recycled to the circulation (reviewed in Delanghe & Langlois, 2001). It is not only the plasma transporter of heme but also acts as a multifunctional agent in processes including iron homeostasis, antioxidant protection and bacteriostatic defence. After haptoglobin, hemopexin forms the second line of defence against haemoglobin-mediated oxidative damage during intravascular haemolysis. The C-terminal hemopexin-like domains of matrix metalloproteinases have specific roles in the binding of collagens and TIMPs (reviewed in Murphy & Knauper, 1997).

1.9.3.4 Other PRG4-vitronectin comparisons

Unlike vitronectin, PRG4 does not contain an RGD tripeptide for integrin-mediated cell binding. It may however share some of the adhesive properties of vitronectin: PRG4 exon 12 contains the pentapeptide IDVPS, a similar sequence to the LDVPS motif found in the CS-1 peptide of a fibronectin splice variant. This motif is responsible for the binding of fibronectin to the VLA-4 integrin receptor. Exon 7 of PRG4 is very similar to the carboxy end of vitronectin exon 3. The high proportion of conserved residues suggests a biological property common to both vitronectin and PRG4, as these sequences appear to be unique (Merberg *et al.*, 1993).

1.9.4 PRG4 exon 6: mucin domain

In its central region of around 940 amino acids, encoded by exon 6, PRG4 contains large and small mucin-like repeat domains composed of the sequences KEPAPTTT/P (76-78 repeats) and XXTTTX (6-8 repeats) (Flannery *et al.*, 1999). The length of these mucin-like domains may vary between species (Ikegawa *et al.*, 2000). The potential of PRG4 to act as a synovial joint lubricant due to its central mucin domain is discussed in section 1.8.3.1.

1.9.4.1 Mucins

Mucins are the major glycoprotein components of mucous, a viscous secretion that protects epithelial cells from infection, dehydration and physical or chemical injury (Perez-Vilar & Hill 1999). Members of the mucin family vary in size but all contain domains rich in proline, threonine and/or serine (PTS regions) that are substituted with O-linked oligosaccharides, to a degree of 40-80% [w/w] in a mature mucin (Van Klinken *et al.*, 1995). These glycosylated PTS regions consist of tandem repeat sequences of serine and/or threonine that contain at least one proline residue per repeat (Gum, 1995). The length of these repeats and their total number vary from mucin to mucin. Many individual mucins also show length polymorphisms resulting from multiple alleles that encode different numbers of tandem repeats (Vinall *et al.*, 1998). The attached oligosaccharides may contain between one and twenty sugar residues, and these may be neutral, sulphated or substituted with sialic acid. The functions of mucins depend on these carbohydrate structures in forming viscous gels by virtue of their filamentous conformation, large volume and charge repulsion in aqueous solution. Because of their large, often negatively charged structure, mucins can act as a barrier protecting the cell, although when other cells have specific receptors for mucins, adhesion can override the barrier function (Van Klinken *et al.*, 1995). They also help to protect against infection by microorganisms that bind to cell surface carbohydrates, and mucin genes appear to be upregulated by substances derived from bacteria, such as lipopolysaccharides (Dohrman *et al.*, 1998). There are two groups of mucin: membrane-bound and secreted. Membrane-bound mucins such as MUC1 form part of the cell surface glycolax and protect against pathogens (Perez-Vilar & Hill, 1999). They can also function as anti-cell adhesion molecules, and their overexpression in cancer may be related to tumour dissemination. Secreted mucins such as MUC2 are generally larger, with cysteine-rich regions flanking the central glycosylated region. These domains have been implicated in the aggregation of MUC2 monomers into large polymers, and are thought to be similar in structure to vWF, an extracellular molecule that is known to multimerise. For example, the mucin PSM is thought to form dimers in the endoplasmic reticulum (ER) through its C-terminal CK domains. These dimers then form multimers in the Golgi complex through their N-terminal D-domains (Perez-Vilar & Hill, 1999). O-glycosylation is thought to occur in the Golgi complex and N-glycosylation occurs in the ER. The role of secretory mucins is to lubricate and protect the tissues they line.

1.9.5 PRG4 in Disease

1.9.5.1 Influence of cytokines and growth factors on PRG4 expression in vitro

PRG4 and its associated functionality may be critical in the onset of joint pathology, as it is situated at the interface between cartilage and synovium. Superficial zone chondrocytes cultured in agarose have been shown to differentially express PRG4 under the influence of cytokines and growth factors. IL-1 appears to inhibit PRG4 biosynthesis, whilst TGF-β and IGF-1 upregulate levels of both PRG4 message and protein (Flannery *et al.*, 1999). IL-1 mimics aspects of inflammation *in vitro*, so it could be postulated that the inhibition of PRG4 expression in inflamed joints would remove its potential protective proclivity at the cartilage surface. In rheumatoid arthritis, this may contribute to the invasion of the articular surface by a proliferating synovial pannus. IL-1 also seems to downregulate PRG4 expression in mature compressed and young tensional tendon (Rees *et al.*, 2002), and recent studies have described the suppressive effect of IL-1 and the stimulatory effect of TGF-β on PRG4 expression by chondrocytes in cartilage explants (Schmidt *et al.*, 2004). However, a conundrum exists: why would the body switch off the expression of a protein with potential protective properties during inflammation? However, although IL-1 appears to downregulate the expression of PRG4 by chondrocytes *in vitro*, recent work by Schumacher and others has described the presence of PRG4 at the surface of osteoarthritic cartilage (Schumacher *et al.*, 2004).

1.9.5.2 Osteoathritis & PRG4

In osteoarthritic cartilage, an alternatively spliced form of PRG4 mRNA exists which lacks exons encoding part of the N-terminal matrix-binding and cell-proliferative domain, including the heparin binding site (Flannery *et al.*, 1999). Expression of this truncated form during osteoarthritis and the possible resultant change in structure and function of PRG4 could contribute to joint pathology. Multiple tissue-specific splice variants of the PRG4 gene have been detected as mRNAs in healthy cartilage, liver, lung, heart and bone (Ikegawa *et al.*, 2000). As well as confirming that PRG4 and splice variants thereof could function in several distinct biological processes, this also suggests that PRG4 splice variants are not necessarily inherent to disease.

1.9.5.3 CACP syndrome

Recently, a study into the autosomal recessive disorder camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome discovered that its cause is a result of mutations in the CACP gene, effectively the PRG4 gene (Marcelino *et al.*, 1999). This disorder affects joints and tendons. In affected individuals, flexion contractions of finger joints (camptodactyly) are usually present at birth, or noticed within the first year of life (Athreya & Schumacher, 1978). Swelling and stiffness of large joints (arthropathy) typically develops between 3 and 8 years of age, and is associated with a non-inflammatory synovial hyperplasia. Some CACP patients also develop a non-inflammatory pericarditis (thickening of the fibrous sac around the heart) in the first or second decades of life). Alternatively, coax-vara deformity (a radiographic change in the angle of the hip joint) may occur (Verma *et al.*, 1995; Bahabri *et al.*, 1998). Eight mutations of the CACP protein (PRG4) have been found, all of them truncated at the C-terminus that contains the hemopexin-like and possible aggregation domains (see figure 1.12). Analysis of the frictional characteristics of PRG4/CACP knockout mice joints using a modified Stanton pendulum technique has confirmed that the CACP phenotype includes a reduction in diarthrodial lubrication (Jay *et al.*, 2003a). This is supported by the observation that synovial fluid from CACP patients displays a reduced boundary lubricating ability (Jay *et al.*, 2003b). Jay and co-workers postulate that a potential interaction between HA with the C-terminal of PRG4 is central to the lubricating function of PRG4, and that this interaction may be interrupted by the C-terminal truncating mutations of CACP syndrome.

1.9.5.4 Tissue engineering and PRG4

Articular cartilage can degenerate over time, and has a minimal capacity for self-repair. Tissue engineering approaches to repair cartilage defects are under active investigation, and recent efforts have focussed on fabricating cartilaginous tissue *in vitro* for transplantation into patient tissue, reviewed in (Risbud & Sittinger, 2002). Most attempts have been based around the seeding of cultured cells into an artificial, degradable scaffold, although new approaches are being developed. For example, the alginate-recovered-chondrocyte (ARC) method retains certain properties of immature cartilage, which has the capacity for repair, by culturing cells in a three-dimensional gel to generate a pericellular matrix. These cells and their newly formed matrix can then be released from the gel and seeded onto cell culture inserts at a high density, which

produces a *de novo* cartilaginous matrix (Masuda *et al.*, 2003). A recent study has utilised this method with the intention of engineering cartilage constructs that include features of normal stratification by culturing subpopulations of chondrocytes from different cartilage zones (Klein *et al.*, 2003). By culturing superficial and middle zone chondrocytes, a cartilage construct has been produced that displays some aspects of normal physiological stratification, including the expression of PRG4 at the superficial surface. This 'biomimetic' approach of engineering cartilage tissue with zonal stratification may be useful within a variety of methods for forming cartilaginous tissue *in vivo*.

1.9.5.5 PRG4 and prosthesis loosening

Approximately 10% of hip endoprostheses loosen after 10 years, caused by either septic or aseptic loosening. A recent study investigating the differences in gene expression in these two pathomechanisms found a strong upregulation (from 20- to 323-fold) of MSF/PRG4 gene expression in all aseptic and some septic cases (Morawietz *et al.*, 2003), leading to the hypothesis that the lubricating property of PRG4 may disturb the tight interaction between bone and prosthesis. Further investigation of this phenomenon is ongoing.

1.9.5.6 Other studies relating to PRG4 in disease

A subtractive suppressive hybridisation study into the differential gene expression in the synovium of rheumatoid and osteoarthritic joints has found PRG4 to be constitutively over-expressed in rheumatoid synovium (Jüsten *et al.*, 2000). Along with the other genes identified, this suggests that different matrix proteins and cell-matrix interactions are relevant for rheumatoid arthritis and osteoarthritis, and that some of these interactions could involve PRG4. Suppression subtractive hybridisation studies into immune-relevant genes affected by hepatic acute phase response in the livers of rainbow trout have documented the upregulation of a vitronectin-like protein very similar to MSF. The tentative conclusion drawn by the authors was that the molecule could boost thrombocyte production, a useful activity during an acute phase response (Bayne *et al.*, 2001). Megakaryocyte stimulating factor (PRG4) has also been identified as a viral-induced gene of Japanese flounder (Aoki *et al.*, 1999).

1.10 Project Aims

PRG4 is a relatively newly identified extracellular matrix component that is secreted by chondrocytes of the superficial zone of articular cartilage and by synoviocytes. Primary sequence information and experimental data from a number of studies indicate that the molecule is a multidomain glycoprotein/proteoglycan that has the potential for a number of functions including cell proliferation and adhesion, cytoprotection, self-aggregation and lubrication These functions may be compromised during arthritic disease. However, at present there is little information regarding the potential biological activities that can be mediated by different domains of PRG4.

The aims of this project were:

- To obtain the non-mucinous N- and C-terminal domains of human and bovine PRG4 by means of recombinant protein expression.
- To investigate the potential multifunctionality of PRG4 by characterising the functions of its different structural domains. This is to be achieved by utilising recombinant proteins in binding/interaction assays with extracellular and cellular components of the synovial joint.
- To determine the susceptibility of PRG4 to degradation by matrix proteinases using recombinant domains and purified full-length PRG4 as substrates in digestion experiments.
- To determine the biological role or roles of PRG4 and its different structural domains in the synovial joint.

2. PURIFICATION AND BIOCHEMICAL CHARACTERISATION OF PRG4

2.1 Introduction

The objective of this section was to develop methods for the purification of PRG4 from the conditioned culture medium of (a) superficial zone chondrocytes isolated from bovine articular cartilage, (b) bovine tendon explant cultures, and (c) a stable, human PRG4-expressing Chinese hamster ovary (CHO) cell line. This material could then be used for biochemical characterisation studies and analyses of PRG4 domain biological function(s). Purified PRG4 could also then be used for *in vitro* proteinase digestion experiments as described in Chapter 5 of this study.

2.2 Materials and Methods

2.2.1 Materials

All chemicals were of analytical grade and were purchased from Sigma-Aldrich (UK) unless indicated otherwise. A detailed list of the materials used in this research project and their suppliers is listed in Appendix A. Tissue culture plastics were purchased from Corning, and all water used was drawn from a MilliQ Plus 185 purification unit (Millipore). Pipette tips and glassware were autoclaved prior to use in tissue culture.

2.2.1.1 Stock solutions

Stock solutions and their components are listed in table 2.1. Non-sterile solutions for tissue culture were autoclaved or filtered before use (0.22μm filters, Millipore). Hydrochloric acid, acetic acid, sodium hydroxide, methanol and ethanol were all purchased from Fisher Scientific, UK. Phosphate buffered saline (PBS) was purchased as tablets from Oxoid, UK (Dublecco A formula.).

2.2.1.2 Origin of PRG4-expressing CHO cell line (CHO-MegK)

The CHO-MegK stable cell line, expressing full-length human PRG4, was a gift from Dr. Lori Fitz of Wyeth Research (formerly Genetics Institute, Cambridge, MA, US). In the generation of this cell line, the plasmid used to transfect CHO DUKX cells utilised the dihydrofolate reductase (DHFR)/methotrexate selection and amplification system (Alt *et al.*, 1978).

Table 2.1 – Stock solutions and their components.

Solution	Components
2X SDS-PAGE sample buffer	0.125M Tris-HCl, pH 6.8, 4% [w/v] SDS, 20% [w/v] glycerol, 0.01% [w/v] bromophenol blue
SDS-PAGE running buffer	25mM Tris-HCl, 192mM glycine, 0.1% [w/v] SDS
Western blot transfer buffer	25mM Tris-HCl, pH 8.1-8.4, 192mM glycine, 20% [v/v] methanol
Tris-buffered saline with azide (TSA)	50mM Tris-HCl, pH 7.4, 0.2M NaCl, 0.02% [w/v] NaN_3
AP buffer for BCIP/NBT substrate	100mM Tris-HCl, pH 9.55, 100mM NaCl, 5mM $MgCl_2$
BCIP (5-bromo-4-chloro-3-indoyl phosphate)	50mg/ml in dimethylamide
NBT (nitro blue tetrazolium)	50mg/ml in 70% [v/v] dimethylformamide
Dissociative buffer for CsCl density gradient centrifugation	8M Guanidine-HCl, pH 6.8, 0.1M sodium acetate, protease inhibitors 0.02M EDTA, 0.2M 6-AHA, 10mM benzamidine and 1mM PMSF
Heparin agarose equilibration buffer	100mM Tris–HCl, pH 7.5, 50mM NaCl.
Heparin agarose elution buffer (stock)	100mM Tris-HCl, pH 7.5, 2M NaCl
PNA lectin agarose equilibration buffer	10mM Tris-HCl, pH 7.8, 0.15M NaCl
PNA lectin agarose elution buffer	10mM Tris-HCl, pH 7.8, 0.15M NaCl, 0.1-1M D-galactose
Tris-buffered saline for HA affinity blotting	20mM Tris-HCl, pH 7.4, 0.5M NaCl
Phosphate buffered saline (PBS)	Dulbecco A 0.16M NaCl, 3mM KCl, 8mM Na_2HPO_4, KH_2PO_4, pH 7.3

Briefly, methotrexate is a folate analogue and binds to and inhibits DHFR, an enzyme that catalyses the regeneration of tetrahydrofolate, a versatile carrier of activated one-carbon units that are needed for the *de novo* synthesis of purines and pyrimidines. CHO DUKX cells are deficient in DHFR, so cells transfected with a vector containing a DHFR gene and a target protein gene will be resistant to methotrexate (Kaufman, 1989). The heat-inactivated FCS used to culture the CHO cells was dialysed against PBS using 12-14,000 molecular weight cut-off dialysis membrane (Spectrapor) to remove nucleosides. For expansion of cell culture, cells were grown in medium containing 0.5mM methotrexate. Methotrexate was purchased from Sigma as (+)-Amethopterin. A stock solution was prepared by dissolving 1g methotrexate in 20ml of 0.1M NaOH, followed by the addition of 10µl volumes of 10M NaOH until the solution became clear. This yielded a stock with a methotrexate concentration of approximately 0.1M, and molarity was calculated by reading absorbance at 370nm, based on the assumption that a 1M solution has an absorbency value of 8100. Methotrexate stock solutions were wrapped in foil and stored at -20°C.

2.2.1.3 Tissue culture

All cell culture plates and 0.2µm filter units were from Corning Costar and Millipore respectively. Bovine metacarpophalangeal joints from animals aged 2-3 years were obtained from a local abattoir. Pronase (*Streptomyces griseus*) was from Boehringer-Mannheim (Roche

Diagnostics). Type 2 collagenase (*Clostridium histolyticum*) was from Worthington Biochemical Corporation, New Jersey, USA. Dulbecco's' Modified Eagle Medium (DMEM), MEMα medium, chemically defined (CD) CHO medium, L-Glutamine, penicillin, streptomycin, gentamicin, trypsin and foetal calf serum were obtained from Invitrogen. All media, serum, antibiotics and other supplements were filtered before use. Low melting temperature agarose (SeaPlaque GTG) was obtained from FlowGen, Staffordshire, UK. Formulations of the culture media used are detailed in table 2.2.

Table 2.2 – Formulation of cell and tissue culture media

Function of culture medium	**Culture medium**	**Supplements**
Pronase digestion of bovine cartilage	DMEM (Dublecco's modified eagle medium)	10% [v/v] FCS, 50μg/ml gentamicin, 0.1% [v/v] pronase
Collagenase digestion of bovine cartilage	DMEM	10% [v/v] FCS, 50μg/ml gentamicin, 0.04% [v/v] type II collagenase
Agarose culture of bovine chondrocytes	DMEM	5% [v/v] FCS, 50μg/ml gentamicin, 25μg/ml phosphotin C
Bovine tendon explant preculture	DMEM	10% [v/v] FCS, 50μg/ml gentamicin
Bovine tendon explant culture	DMEM	50μg/ml gentamicin
CHO cell expansion	MEMα	10% [v/v] heat inactivated FCS (dialysed against PBS), 1mM L-glutamine, 100U/ml penicillin G, 100μg/ml streptomycin, 0.5mM methotrexate
CHO cell expression	CD (chemically defined) CHO medium	1mM L-glutamine, 100U/ml penicillin G, 100μg/ml streptomycin

2.2.1.4 Protein purification

Heparin-agarose and PNA lectin-agarose was obtained from Sigma-Aldrich Co. The matrix in each case was cross-linked 4% beaded agarose.

2.2.1.5 SDS-PAGE

Pre-cast gradient SDS-PAGE gels (4-12%) and gel running apparatus were from Invitrogen. Slab gels were poured using acrylagel and bisacrylagel from National Diagnostics. Molecular weight standards were from BioRad. Protran BA 83 pure nitrocellulose (0.2 μM) designed for high binding capacity and low background noise was from Schleicher and Schuell. Transfer tanks and power packs for the electro-elution of SDS-PAGE gels onto nitrocellulose were from BioRad.

2.2.1.6 Proteinase inhibitors

Proteinase inhibitors were added to the conditioned culture medium of CHO cells, tendon explants and chondrocytes, and also to purification products, but not if the products were to be used in *in vitro* proteinase digestion experiments (see chapter 5). The inhibitors used are listed in table 2.3, along with their concentration and specificity.

Table 2.3 – Proteinase inhibitors used in this study

Inhibitor	Concentration	Specificity of inhibitor
Na_2EDTA	10mM	Metalloproteinases
Benzamidine HCl	5mM	Thrombin and trypsin
Phenylmethyl sulphonyl fluoride (PMSF)	0.5mM	Serine proteases, papain
ε-Aminocaproic acid (6-AHA)	0.1M	Fibrinolysin, chymotrypsin

2.2.1.7 Western blotting

The origin and specificities of the monoclonal and polyclonal antibodies used for detection of PRG4 are described in table 2.4. All were used at a dilution of 1:1000. For use with monoclonal antibodies, the anti-mouse IgG (H+L) alkaline phosphatase secondary antibody (affinity purified goat anti-mouse) was purchased from Promega, UK. A similar anti-rabbit secondary antibody from Promega was used in conjunction with the polyclonal antibodies. For all blots the alkaline phosphatase substrate was 0.33mg/ml nitro blue tetrazoleum (NBT) and 0.165mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) from Promega, made up in the AP substrate buffer as detailed in table 2.1.

2.2.1.8 Affinity blotting

For detection of the carbohydrate structures on PRG4, a DIG Glycan Differentiation kit was used according to the manufacturers instructions (Boehringer Mannheim). The lectins used and their specificities for different carbohydrate structures are listed in table 2.5. For HA affinity blotting, biotinylated HA and avidin-AP conjugate were purchased from Sigma.

2.2.2 Isolation and agarose culture of bovine superficial zone chondrocytes

The superficial layer of bovine articular cartilage was aseptically harvested from the metacarpophalangeal joints of 2-3 year old cows as previously described (Tudor, 2002). For the isolation of superficial zone chondrocytes, tissue slices were washed in DMEM and subjected to

digestion with 0.1% pronase [w/v] in DMEM containing 10% [v/v] FCS for 1-2 hours. After this time the pronase solution was removed and tissue was digested with 0.04% collagenase [w/v] in DMEM containing 10% [v/v] FCS overnight (14-16 hours). Isolated chondrocytes were then washed out of collagenase and into DMEM by centrifugation (1800rpm for 8 minutes) and aspiration. All DMEM used contained 50μg/ml gentamicin. Agarose culture plates were established by preparing a 2% [w/v] solution of SeaPlaque GTG agarose in DMEM. 60mm plates were coated with 2 ml of 1% agarose and placed at 4°C for 15 minutes to set, followed by 15 minutes at 37°C. Chondrocytes were resuspended at a concentration of 12 x 10^6 cells/ml. This suspension was diluted with an equal volume of 2% agarose, and 1ml of the resultant 1% [w/v] agarose cell suspension was laid over the agarose plug (6 x 10^6 cells/plate). Plates were placed at 4°C for 15 minutes to set. Experimental medium was then added to the plates which were maintained at 37°C in 5% CO_2. The medium consisted of DMEM, 5% [v/v] FCS and 25μg/ml phosphotin C. Phosphotin C is an ascorbate analogue, and ascorbate functions as a cofactor in the proline hydroxylation of collagen. Culture media was harvested and replaced every 4-6 days. The conditioned media of agarose cultures was centrifuged at 3200 x g for 30 minutes at 4°C to remove agarose, and was passed through a 0.22μm filter after the optional addition of proteinase inhibitors (table 2.3) and stored at -20°C prior to processing.

2.2.3 Explant culture of bovine deep digital flexor tendon

Bovine deep digital flexor tendon (DDFT) explant cultures were established by cutting diced tendon pieces (10-50mg) from 2-3 year old bovine DDFT and pre-culturing them at 1 explant/ml in 100mm dishes with DMEM supplemented with 10% [v/v] FCS, at 37°C, 5% CO_2 for two days (Rees *et al.*, 2002). At this time explants were washed 3 times in DMEM (without FCS) and placed in serum free DMEM. Conditioned media was harvested every 3 days, and was filtered after the optional addition of proteinase inhibitors (table 2.3) and stored at -20°C until use.

2.2.4 CHO cell culture

2.2.4.1 Initiation of cell culture and preparation of frozen stocks

For the preparation of frozen CHO cell stocks, confluent plates were washed with PBS, removed from the plastic by trypsinization and re-suspended at a density of 2 x 10^6 cells/ml in MEMα medium containing 10% [v/v] heat inactivated FCS and 10% [v/v] DMSO. Cells were placed in

Nalgene cryovials (1ml cell suspension/vial) and frozen to -80°C in Styrofoam containers. Stocks were transferred to liquid nitrogen for long term storage. For the initiation of cell culture from frozen stocks, a single vial was thawed by warming in a 37°C water bath and added to 9ml of CHO expansion medium (table 2.2) in a 100mM plate. Cells were allowed to attach for 24 hours before the aspiration of the DMSO-containing medium and the addition of fresh expansion medium.

2.2.4.2 Expansion of cultures and collection of conditioned medium

The human PRG4-expressing cell line CHO-MegK (Wyeth) was grown in MEMα with 10% [v/v] heat-inactivated FCS (dialysed against PBS), 1mM L-Glutamine, 100U/ml penicillin G, 100μg/ml streptomycin and 0.5mM methotrexate (see section 2.2.1.2 for more information on the use of methotrexate). Cells were grown on 100mm plates with 10ml of media at 37°C in 5% CO_2 until visibly confluent and were split following trypsinisation. Cells were pelleted by centrifugation at 450 x g for 5 minutes. Following three washes with PBS, cells were then plated in serum free CD CHO medium (with glutamine and antibiotics) and conditioned media was harvested at 48 hours with replacement and again at 96 hours. Conditioned media was stored at -20°C either with or without protease inhibitors (table 2.3) depending on the different experimental applications used – if PRG4 was to be used in proteinase digestion experiments, enzyme inhibitors were omitted.

2.2.5 Heparin-agarose affinity chromatography

In general, a 0.5-2.0ml bed volume of heparin-agarose was used in a 10ml plastic gravity flow chromatography column (BioRad), which was equilibrated with 10 column volumes of heparin-agarose equilibration buffer (100mM Tris-HCl, pH 7.5, 50mM NaCl) and pre-eluted with 10 column volumes of 2M NaCl in 100mM Tris-HCl, pH 7.5 before re-equilibration. Heparin-agarose was then mixed with the sample in a 50ml tube (Corning) and agitated at room temperature for 30 minutes. The affinity gel was re-packed by passing the agarose-sample suspension over a 10ml BioRad column, and unbound proteins were removed with 20 column volumes of equilibration buffer. Heparin-binding proteins were eluted with 0.1-2M NaCl in equilibration buffer in stepwise aliquots of 1 column volume unless otherwise stated. After

elution, columns were re-equilibrated and stored at 4°C in heparin-agarose equilibration buffer supplemented with 0.02% [w/v] sodium azide.

2.2.6 PNA lectin-agarose affinity chromatography

Samples were applied to a PNA lectin-agarose column with a bed volume of 1ml in a 10ml BioRad chromatography column. Before use the agarose was equilibrated in 10 column volumes of PNA agarose equilibration buffer (10mM Tris-HCl, pH 7.8, 0.15M NaCl), pre-eluted with 10 column volumes of 1M D-galactose in equilibration buffer and re-equilibrated. PNA-agarose was then mixed with the sample and agitated at room temperature for 30 minutes. The suspension of sample and agarose was then passed over the BioRad column to collect the agarose. Following a wash step of 20 column volumes of equilibration buffer to remove unbound proteins, PNA lectin-bound glycoproteins were eluted with aliquots of 0.1-1M D-galactose (Lotan *et al.*, 1977) in equilibration buffer. After elution, columns were re-equilibrated and stored at 4°C in PNA-agarose equilibration buffer supplemented with 0.02% [w/v] sodium azide.

2.2.7 CsCl density gradient centrifugation

Briefly, an equal volume of CsCl dissociative buffer was added (table 2.1), and this solution was made up to a density of 1.47-1.48g/ml by addition of solid CsCl. The sample was then centrifuged at 100,000 x g for 40 hours at 10°C in a Beckman L-60 ultracentrifuge. The gradient was fractionated into five fractions (see figure 2.1) and the PRG4-containing D5 fraction (density of approximately 1.3g/ml) was dialysed exhaustively against MilliQ water before freezing.

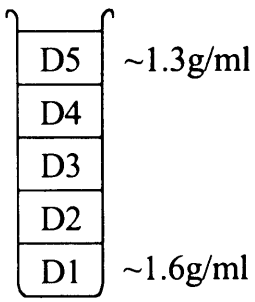


Figure 2.1 – Fractionation of a sample into 5 x 5ml fractions after CsCl density gradient centrifugation under dissociative conditions, with an overall starting density of 1.47-1.48g/ml.

2.2.8 Procedures for the purification of PRG4

Full-length PRG4 was purified from a number of sources. The protein concentration of eluents was calculated by reading absorbancy values at 280nm in an Ultrospec 2000 spectrophotometer (Pharmacia).

2.2.8.1 Purification of human recombinant PRG4 from conditioned media of the CHO-MegK cell line

Conditioned CHO cell media was centrifuged at 600 x g for 10 minutes and supernatant was passed through a 0.22μM filter to remove cellular debris. Heparin affinity chromatography was performed as described in section 2.2.5 (40ml conditioned media/1ml heparin agarose). Heparin column eluents were further purified by PNA lectin agarose affinity chromatography (section 2.2.6). Fractions were separated by SDS-PAGE and analysed by Western blotting (section 2.2.9), lectin affinity blotting (section 2.2.10), HA affinity blotting (section 2.2.11) and silver staining (section 2.2.12).

2.2.8.2 Purification of bovine cartilage PRG4 from the conditioned media of superficial zone chondrocyte agarose cultures

The conditioned medium from bovine superficial zone chondrocyte agarose cultures was collected and centrifuged to remove agarose (section 2.2.4.1), and CHAPS was added to a final concentration of 0.5% [w/v]. Bovine cartilage PRG4 was partially purified from conditioned medium by heparin agarose affinity chromatography (section 2.2.5), PNA lectin affinity chromatography (section 2.2.6) and dissociative density gradient centrifugation (section 2.2.7). These fractions were then used in proteinase digests and Western/affinity blotting experiments (sections 2.2.9-11).

2.2.8.3 Purification of bovine tendon PRG4

Tendon PRG4 was partially purified by directly applying the conditioned media of bovine DDFT explant cultures to heparin agarose. Prior to this, media was passed through 0.22μM filters to remove debris. A 1ml bed volume of heparin beaded agarose was pre-eluted and added to 40ml of conditioned media, and mixed at room temperature for 30 minutes before the elution of heparin bound proteins with a NaCl gradient (section 2.2.5).

2.2.9 *SDS-PAGE and Western blotting*

For SDS-PAGE, the Laemmli buffer system was used (Laemmli, 1970), and experimental samples were diluted (1:1) with 2X SDS-PAGE sample buffer (table 2.1). β-mercaptoethanol was added to 10% [v/v] if samples were to be reduced. Samples were routinely run under reducing conditions. Samples were boiled for 3-5 minutes prior to loading, and were separated on either 4-12% pre-cast gradient gels or slab gels of 10% or 15% depending on target protein size, using vertical Novex gel apparatus (Invitrogen). The SDS-PAGE running buffer used is listed in table 2.1, and gels were run at 120-150V for 1.5 hours or until the bromophenol blue in the SDS-PAGE sample buffer reached the heel of the gel. Samples were electrophoretically transferred onto nitrocellulose membrane using transfer buffer (table 2.1) formulated with a Towbin recipe (Towbin *et al.*, 1979), in BioRad apparatus for 1 hour at 100 volts. For Western blotting, transfer membranes were blocked for 1 hour at room temperature or overnight at 4°C in 5% [w/v] BSA in TSA (see table 2.1). Membranes were probed with antibodies (see table 2.4) in 1% [w/v] BSA in TSA for a minimum of 1 hour at the dilutions listed in table 2.4. Primary antibody was removed with 3 x 10 minute washes in TSA before incubation with an anti-mouse (dilution of 1:7500) or anti-rabbit (dilution of 1:3000) secondary antibody diluted in 1% [w/v] BSA/TSA for one hour at room temperature. After 3 x 10 minute washes in TSA, the immunoblots were incubated with alkaline phosphatase substrate (section 2.2.1.7) for 5-60 minutes at room temperature to achieve optimum colour development. Membranes were washed with MilliQ water. If a comparison between Western blots was required, all incubation times were kept consistent.

Table 2.4 – PRG4-specific antibodies

Antibody name	Origin and PRG4 specificity	Source	Dilution
6-A-1	Monoclonal against purified bovine PRG4	Prof. B. Caterson (Schumacher *et al.*, 1999)	1:1000 (ascites) 1:100 (media)
3-A-4	Monoclonal against purified bovine PRG4 Epitope disrupted by reduction	Prof. B. Caterson (Schumacher *et al.*, 1999)	1:1000 (ascites) 1:100 (media)
5-B-6	Monoclonal against purified bovine PRG4	Prof. B. Caterson	1:1000 (ascites) 1:100 (media)
5-D-1	Monoclonal against purified bovine PRG4	Prof. B. Caterson	1:1000 (ascites) 1:100 (media)
06A10	Polyclonal – purified human recombinant (Gln^{1}-Leu^{924})	Wyeth Research	1:1000
378	Polyclonal – human exon 3 peptide (Gly^{72}-Asp^{85})	Wyeth Research	1:1000
01B-10	Polyclonal – human exon 12 peptide (Gln^{1374}-Ala^{1387})	Wyeth Research	1:1000

2.2.10 Lectin affinity blotting

A DIG glycan differentiation kit (Roche) was used according to the manufacturers instructions. The specificities of the lectins in the kit are listed in table 2.5. Blots were developed with NBT/BCIP in AP substrate buffer (section 2.2.1.7).

Table 2.5 – Lectins of the DIG Glycan Differentiation kit (Roche) and their specificity. Lectins that recognise PRG4 are indicated with an asterix.

Lectin name	Specificity
Peanut agglutinin (PNA)*	Core disaccharide galactose β(1-3) N-acetylgalactosamine. Identifies O-linked oligosaccharides
Maackia amurensis agglutinin (MAA)*	Sialic acid linked α(2-6) to galactose. Identifies complex, sialylated chains
Galanthus nivalis agglutinin (GNA)	Terminal mannose α(1-3), α(1-6) or α(1-2) linked to mannose. Identifies high mannose N-glycan chains
Sambucus nigra agglutinin (SNA)	Sialic acid linked α(2-6) to galactose. Identifies complex, sialylated N-glycan chains
Datura stamonium agglutinin (DSA)	Galactose linked β(1-4) to N-acetyl glucosamine.

2.2.11 Affinity blotting with biotinylated hyaluronan

Preparations of purified or partially purified PRG4 were separated by SDS-PAGE under reducing and non-reducing conditions and transferred to nitrocellulose. As a positive control for HA binding, 5μg of a preparation of link protein was used that runs at 45 kDa and binds HA under non-reducing conditions (bovine nasal cartilage A1D4 fraction, prepared by Dr. C. R. Flannery). Membranes were blocked for 16 hours at 4°C in TBS (20mM Tris-HCl, pH 7.4, 0.5M NaCl) with 0.1% [v/v] Tween-20, without rocking. Membranes were subjected to 3 x 10 minute washes with TBS/0.05% [v/v] Tween-20 prior to incubation with biotinylated HA at a concentration of 5μg/ml in TBS/0.05% Tween-20 for 1 hour at room temperature with rocking. Biotinylated HA was removed with 6 x 5 minute washes with TBS/0.05% Tween-20 prior to the addition of 0.5μg/ml avidin-alkaline phosphatase (in TBS/0.05% Tween 20), which was incubated for 1 hour at room temperature. Membranes were washed six times as previously described before incubation with NBT/BCIP substrate (section 2.2.1.7) as for Western blotting experiments.

2.2.12 Silver staining

For detection of total protein, SDS-PAGE gels were silver stained using a method adapted from (Morrisey, 1981). Briefly, gels were pre-fixed for 1.5-16 hours in 30% [v/v] ethanol and 10%

[v/v] acetic acid. After 2 x 10 minute washes in 10% [v/v] ethanol and 3 x 10 minute washes in MilliQ H_2O, gels were placed in 5μg/ml DTT for 30 minutes then 0.1% [w/v] silver nitrate for 30 minutes. Gels were developed with 3% [w/v] sodium carbonate containing 0.05% [v/v] formaldehyde solution. A small amount of this solution was added and incubated with the gel, and was removed when it became cloudy before adding fresh solution for full development. The developing solution was neutralised with 5ml of 5M citric acid for 10 minutes. Following neutralisation, gels were washed with repeated changes of H_2O.

2.3 Results

2.3.1 Purification of human recombinant PRG4 from the culture medium of a PRG4-secreting CHO cell line

2.3.1.1 Purification of human recombinant PRG4 by heparin-agarose affinity chromatography

Previous reports have indicated that PRG4 may be capable of binding to heparin through an arginine/lysine-rich sequence present in exon 4 (Merberg *et al.*, 1993). Therefore, it was decided to investigate this potential interaction with respect to the affinity purification of human recombinant PRG4 from the conditioned media of the CHO-MegK polyclonal cell line (obtained as a gift from Wyeth, MA), which expresses and secretes full-length human PRG4. The results of utilising this procedure are shown in figure 2.2, which shows the results of applying 40mls of CHO serum free conditioned medium to a 1ml heparin agarose column. Heparin-binding proteins were sequentially eluted with 2ml volumes of 0.1-1.0M NaCl in 0.1M increments. Proteins were visualised by silver staining (figure 2.2A) and Western blotting with the polyclonal antibody 06A10 (figure 2.2B). The majority of the PRG4 present in 40mls of conditioned CHO medium was bound by 1ml of heparin-agarose, as assessed by comparing the staining seen with the starting material (lane 1) and the flowthrough (lane 2), and PRG4 was eluted at a salt concentration of between 0.2M and 0.4M NaCl (lanes 4-6). The Western blot with antibody 06A10 seen in figure 2.2B verifies that the eluted protein present in lanes 4-6 running at >250 kDa is human PRG4. Other proteins eluted from the column may be contaminants or catabolites of full-length PRG4 lacking the 06A10 epitope.

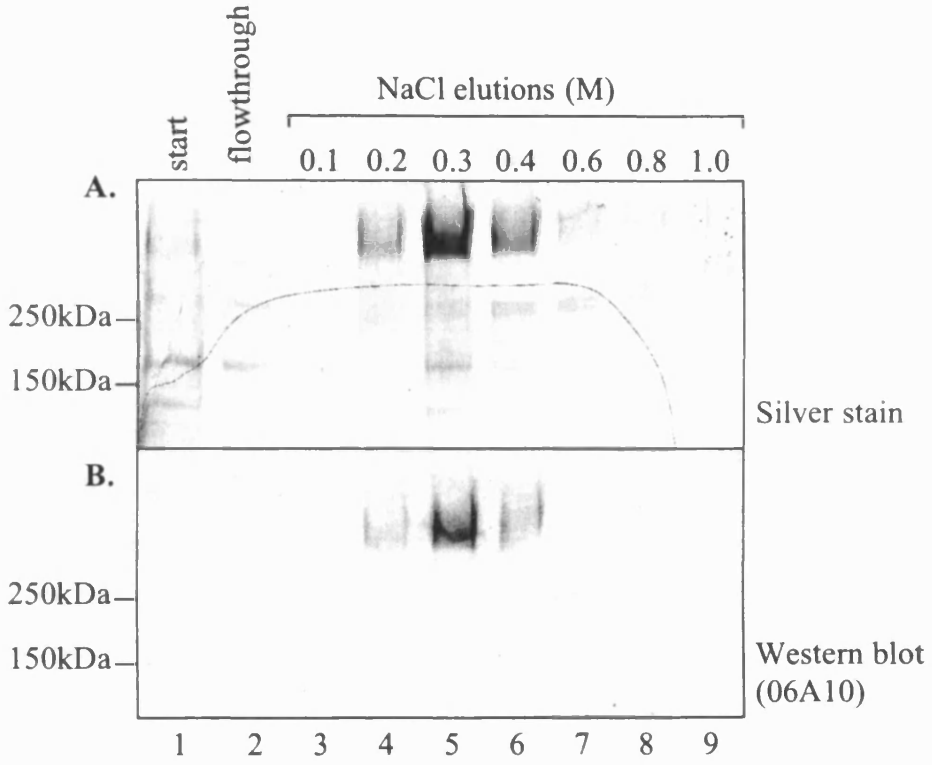


Figure 2.2 – Purification of human recombinant PRG4 (hrPRG4) by heparin-agarose affinity chromatography. Starting material was 40mls of conditioned culture medium from the CHO-MK cell line, which was applied to heparin-agarose (1ml bed volume). Bound proteins were eluted from the column using a stepwise NaCl gradient (lanes 2-9, 2mls per fraction) Fractions were separated by SDS-PAGE and hrPRG4 was visualised by (A) silver staining and (B) Western blot probed with polyclonal antibody 06A10. Migration of molecular weight standards (kDa) is shown to the left.

2.3.1.2 Purification of human recombinant PRG4 by PNA lectin-agarose affinity chromatography

The O-linked oligosaccharide substitution of PRG4 from bovine cartilage has previously been characterised by affinity blotting using a panel of lectins, with positive reactions occurring with the lectins PNA and MAA (Tudor, 2002). The lectin PNA recognises the core disaccharide galactose β(1-3) N-acetylgalactosamine (Carter & Sharon, 1977) and the lectin MAA recognises sialic acid linked α(2-6) to galactose (Wang & Cummings, 1988). These glycosylation features of the molecule were used in the further purification of heparin-purified human recombinant PRG4. Although studies with a MAA-agarose affinity column were unsuccessful (data not shown), purification of PRG4 was successful with PNA-agarose affinity chromatography. This was achieved by adding samples obtained during heparin-agarose affinity chromatography to a 1ml PNA-agarose column and eluting bound protein with 1ml aliquots of D-galactose (Lotan *et al.*, 1977) in a stepwise manner, using a concentration range of 0.1-0.7M. Figure 2.3 shows fractions from this purification after separation by SDS-PAGE, visualised by silver stain. Not all PRG4 from the heparin-agarose column eluent was bound by the column, as observed by comparing the start and flowthrough fractions (lanes 1 & 2). The inability of the flow through material to bind to the PNA-agarose column when passed over the column for a second time (data not shown) suggests that the PRG4 sample may contain differentially glycosylated sub-populations. PRG4 bound to the column was eluted with 0.2-0.3M D-galactose in two apparently homogenous fractions (lane 4 & 5). Therefore, this data describes a two-step procedure for the purification of human recombinant PRG4 from the culture medium of a PRG4-expressing CHO cell line. Protein concentrations of purified preparations were calculated by reading absorbance at 280nm, and purified protein was used in Western and affinity blotting experiments as shown later in this chapter, and also in proteinase digestion experiments described in Chapter 5.

2.3.2 Partial purification of bovine articular cartilage PRG4 from the culture medium of superficial zone chondrocytes

2.3.2.1 Purification of bovine cartilage PRG4 by heparin-agarose affinity chromatography

Following the success of the heparin- and PNA-agarose affinity chromatography methods used for the purification of hrPRG4, the potential of these methods for purifying native bovine PRG4 was investigated.

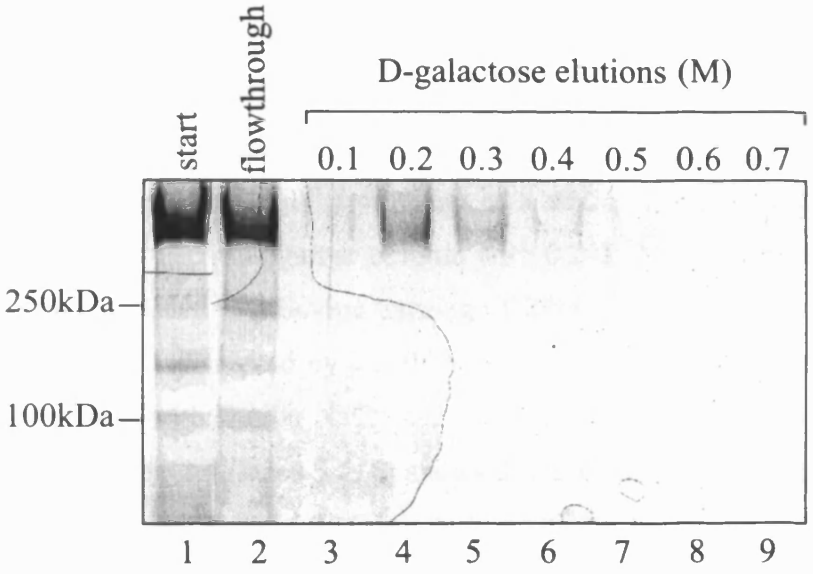


Figure 2.3 – Purification of human recombinant PRG4 (hrPRG4) by PNA lectin-agarose affinity chromatography. Starting material was 6mls of heparin-agarose affinity column eluent (start, see figure 2.2), which was applied to PNA lectin-agarose (1ml bed volume). HrPRG4 was eluted from the column with a stepwise gradient of D-galactose (lanes 2-9), with elution volumes of 1 column volume (1ml). Fractions were separated by SDS-PAGE (30µl/lane) and total protein was visualised by silver staining. Migration of molecular weight standards is shown to the left.

Bovine articular cartilage PRG4 (bcPRG4) obtained from the conditioned medium of agarose cultures of superficial zone chondrocytes was concentrated and partially purified using heparin-agarose affinity chromatography. Figure 2.4 shows the result of applying 40mls of conditioned medium to 0.5ml of heparin-agarose, eluting proteins with sequential 5ml volumes of 0.1-1.0M NaCl. A Western blot of the starting material (conditioned medium) with the antibody 6-A-1 is shown in lane 1 to confirm the presence of bovine PRG4 at ~345 kDa. Eluted proteins were detected using silver staining, and PRG4 is apparent as a strongly stained band of around 345 kDa and was eluted from the heparin-agarose column with 0.2-0.3M NaCl (figure 2.4, lanes 5 & 6). It can therefore be concluded that bovine cartilage PRG4 is capable of binding to heparin-agarose, an interaction that is disrupted by 0.2-0.3M NaCl. Human recombinant PRG4 is also eluted from heparin-agarose with similar NaCl concentrations as shown in figure 2.3. However, the silver stain shown in figure 2.4 (lanes 5 & 6) shows that the eluents are heterogeneous, which would suggest that endogenous bovine PRG4 is only partially purified from the conditioned media of superficial zone chondrocyte agarose cultures by a heparin-agarose affinity chromatography step.

2.3.2.2 Purification of bovine articular cartilage PRG4 heparin-agarose column eluents by CsCl density gradient centrifugation

Further purification of bovine cartilage PRG4 from heparin-agarose column eluents was performed using dissociative CsCl density gradient centrifugation (section 2.2.7), a process that has previously been used in the purification of bovine PRG4 (Tudor, 2002). The results of this procedure can be seen in figure 2.5, which shows a silver stain analysis of the 5 fractions. PRG4 (~345 kDa) appeared in the D5 fraction (figure 2.5, lane 4), but only in a slightly purer mixture than the starting material figure 2.5, lane 1). A contaminant of around 275 kDa can be clearly seen in the D5 fraction (lane 6). This may well be fibronectin, a component of FCS that has a heparin-binding domain. A Western blot of the D5 fraction with mAb 6-A-1 is shown in figure 2.5, lane 7, confirming the presence of PRG4 at ~345 kDa.

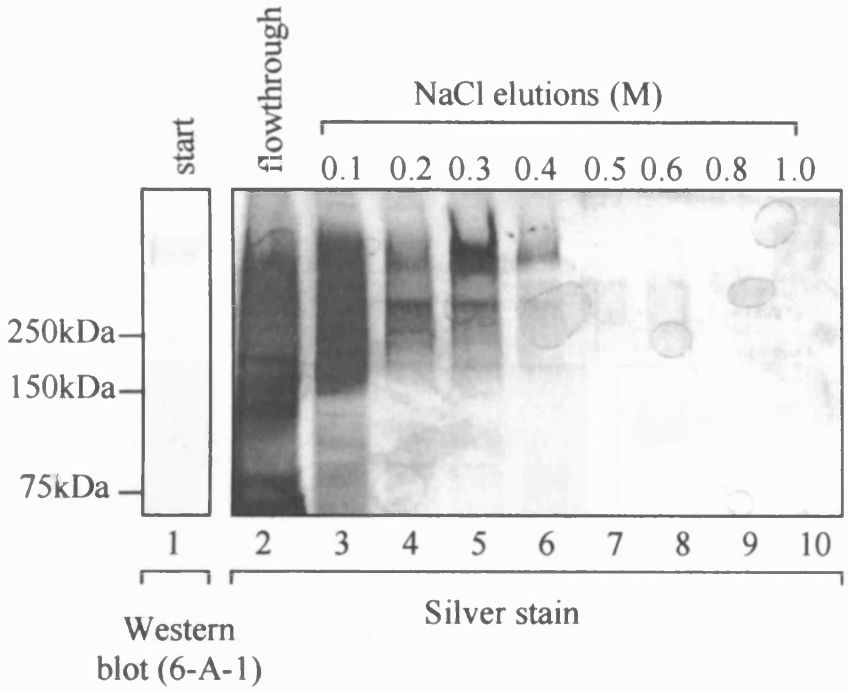


Figure 2.4 – Purification of bovine articular cartilage PRG4 (bcPRG4) by heparin-agarose affinity chromatography. Starting material was 40mls of conditioned culture medium from superficial zone chondrocyte agarose cultures Figure 2.4 – Analysis of fractions (30µl/lane) Conditioned medium from bovine superficial zone chondrocyte agarose cultures (40ml) was applied to 0.5ml heparin-agarose. Bound proteins were eluted using a stepwise NaCl gradient (lanes 3-10, 5ml per fraction) and fractions were separated by SDS-PAGE under reducing conditions (30µl/lane). The starting material is shown as a Western blot probed with mAb antibody 6-A-1 (lane 1), heparin-agarose column eluents were silver stained. Migration of molecular weight standards (kDa) is shown to the left.

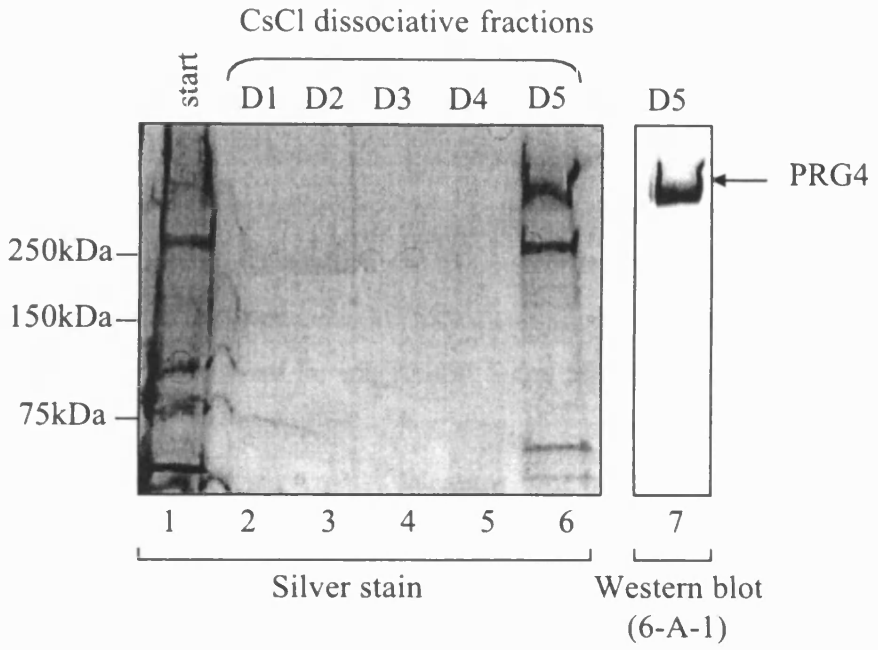


Figure 2.5 – Purification of bovine articular cartilage PRG4 by heparin-agarose affinity chromatography followed by CsCl density gradient centrifugation. Heparin column eluent (5ml, lane 1) was subjected to cesium chloride density gradient centrifugation under dissociative conditions (total volume 32.4ml). Following fractionation of the sample into 5 parts (D1-D5), fractions were separated by SDS-PAGE (25µl/lane) under reducing conditions and visualised by silver stain. A Western blot of the D5 fraction using mAb 6-A-1 is shown on the far right (lane 7), and an arrow marks the position of PRG4. Migration of molecular weight standards is shown to the left.

2.3.2.3 Purification of bovine articular cartilage PRG4 by PNA lectin affinity chromatography

Heparin column eluents were applied to a PNA lectin-agarose column in a similar manner to the purification method described for human recombinant PRG4 (section 2.3.1.1). Similarly to human recombinant PRG4, bovine cartilage PRG4 bound to the column and was eluted with ~0.2M D-galactose, as seen in an analysis of purification fractions by SDS-PAGE followed by silver staining (figure 2.6A). However, the fraction is heterogeneous, and the same ~275 kDa contaminant is present in PRG4 purified by PNA lectin-agarose affinity chromatography as that seen with PRG4 purified by CsCl density gradient centrifugation. The contaminant of around 75kDa is removed by PNA lectin-agarose affinity chromatography (figure 2.6A & B). The PNA affinity purification step proved to be inefficient – the binding capacity of the PNA-agarose seemed to be low, based on the observation that a considerable amount of PRG4 was present in flowthrough material (data not shown). A Western blot of the heparin affinity/CsCl density gradient centrifugation-purified fraction is shown in figure 2.6C, probed with mAb 6-A-1 to verify the presence of bovine PRG4. For the later applications in this study (Western and affinity blot analyses, *in vitro* proteinase digests), bovine articular cartilage PRG4 was partially purified by heparin agarose affinity chromatography followed by density gradient centrifugation. The purity could be improved by utilising other previously reported PRG4 purification methods such as ion exchange chromatography (Tudor, 2002), anti-fibronectin immunoaffinity chromatography (Jay *et al.*, 2000) and size exclusion chromatography. Size exclusion column chromatography and immunoaffinity chromatography (using mAbs 6-A-1 and 3-A-4) were utilised in an attempt to further purify bovine cartilage PRG4, but the experimental approaches proved to be unsuccessful (data not shown).

2.3.3 Partial purification of bovine tendon PRG4

Recently, PRG4 has been immunolocated on the surface of bovine compressed deep digital flexor tendon, and is also found to be expressed by tenocytes at the mRNA level (Rees *et al.*, 2002). Bovine tendon PRG4 was partially purified from the conditioned medium of bovine deep digital flexor tendon (DDFT) explant cultures using heparin-agarose affinity chromatography.

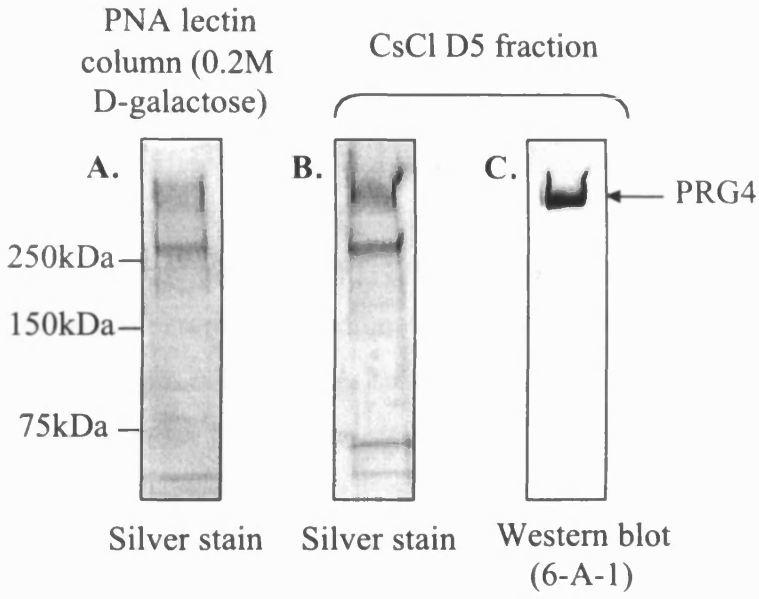


Figure 2.6 – Purification of bovine articular cartilage PRG4 by heparin-agarose affinity chromatography followed by PNA lectin-agarose affinity chromatography. Conditioned medium from superficial zone chondrocyte agarose cultures was subjected to (A) heparin and PNA affinity chromatography, and (B) heparin affinity chromatography and CsCl density gradient centrifugation. Fractions were separated by SDS-PAGE (30μl/lane) and were visualised by silver stain. Material shown in (B) is also shown as a Western blot in (C), probed with monoclonal antibody 6-A-1 to show the presence of bovine PRG4. Migration of molecular weight standards is shown to the left.

Figure 2.7 shows fractions from this purification after separation by SDS-PAGE, which were visualised by (A) a silver stain and (B) Western blot probed with monoclonal antibody (mAb) 3-A-4. PRG4 eluted from the column at a salt concentration of between 0.2 and 0.6M NaCl (figure 2.7, lanes 2-6), and was identified by mAb 3-A-4 as a protein running at around 345 kDa. This demonstrates that bovine tendon PRG4 exhibits the same heparin-binding characteristics as human recombinant and bovine cartilage PRG4. Purification of tendon PRG4 from this source was facilitated by the use of serum free medium within the culture system, and the conditioned media of bovine DDFT explant cultures may be a useful source of tendon PRG4 for functional analyses when coupled with other purification strategies.

2.3.4 Characterisation of anti-PRG4 antibodies using human recombinant, bovine cartilage and bovine tendon PRG4

Purified human recombinant PRG4 (hrPRG4) and partially purified bovine articular cartilage PRG4 (bcPRG4) and bovine tendon PRG4 (btPRG4) were used in Western blotting analyses to determine the cross-species reactivity of a number of antibodies to epitopes present in human and bovine PRG4. HrPRG4 was purified to homogeneity by heparin-agarose and PNA lectin-agarose affinity chromatography (section 2.3.1), bcPRG4 was partially purified by heparin affinity chromatography and CsCl density gradient centrifugation (section 2.3.2) and btPRG4 was partially purified by heparin-agarose affinity chromatography (section 2.3.3).

Figure 2.8 shows Western blots of hrPRG4, bcPRG4 and btPRG4 probed with polyclonal antibodies raised against purified human recombinant PRG4 (Gln^{1}-Leu^{924}, 06A10) and a synthetic peptide from exon 3 of human PRG4 (Gly^{72}-Asp^{85}, Ex3). Both antibodies recognise epitopes of hrPRG4, but showed no cross-species reactivity with PRG4 from bovine tendon and articular cartilage.

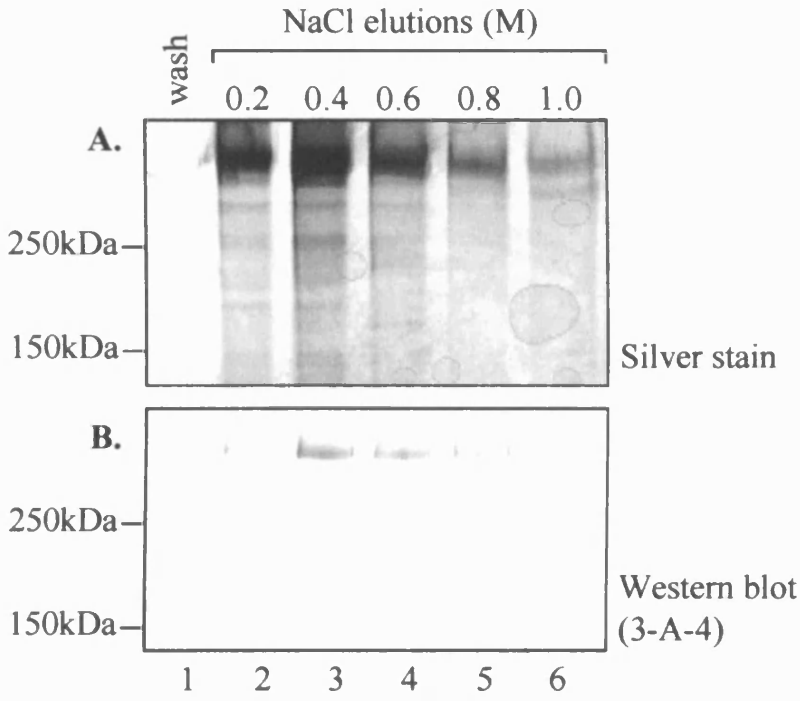


Figure 2.7 – Heparin-agarose affinity purification of bovine tendon PRG4. The starting material was 80mls of conditioned culture medium from bovine DDFT explant cultures, which was applied to heparin-agarose (1ml bed volume). Bound proteins were eluted from the column with a stepwise NaCl gradient (lanes 2-6, 1ml per fraction) Eluent fractions were separated by SDS-PAGE (30µl/lane) under non-reducing conditions and were visualised by (A) silver stain showing total protein and (B) Western blot probed with mouse anti-bovine mAb 3-A-4, which recognises an non-reduced epitope of native bovine PRG4 (Schumacher, *et al.*, 1999). Migration of molecular weight standards (kDa) is shown to the left.

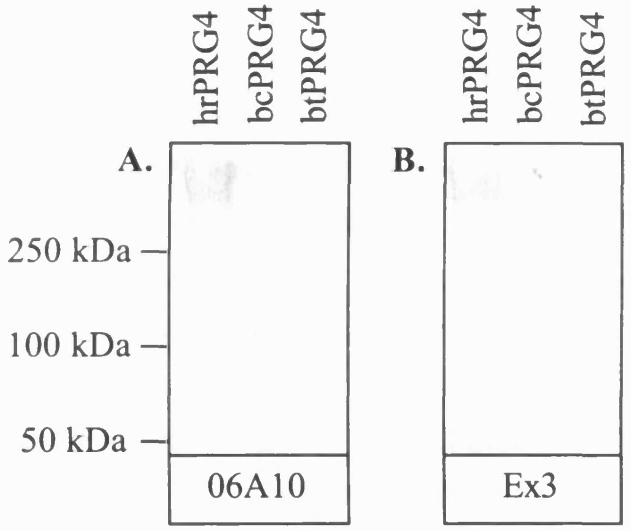


Figure 2.8 – Western blot analysis of purified human recombinant PRG4 (hrPRG4), partially purified bovine cartilage (bcPRG4) and tendon PRG4 (btPRG4) with polyclonal antibodies raised against hrPRG4. Proteins were run under reducing conditions at a concentration of 5μg total protein/lane. Western blots were probed with (A) 06A10, a polyclonal antibody raised against purified human recombinant PRG4 and (B) anti-exon 3 (Ex3), a polyclonal antibody raised against a synthetic peptide in exon 3 of human PRG4. The migration of molecular weight standards is shown to the left.

Figure 2.9 shows Western blot analysis of hrPRG4, bcPRG4 and btPRG4 (5μg total protein/lane), using a panel of monoclonal antibodies (mAbs) raised against purified bovine PRG4: 6-A-1, 3-A-4, 5-B-6 and 5-D-1. Antibodies 6-A-1 and 3-A-4 have been used to immunolocalise PRG4 in bovine articular cartilage (Flannery *et al.*, 1999; Schumacher *et al.*, 1999; Tudor, 2002), tendon (Rees *et al.*, 2002), synovial fluid (Schumacher *et al.*, 1999; Tudor, 2002) and meniscus (Schumacher *et al.*, 2003). For Western blotting analyses the samples were separated under reducing (figure 2.9A) and non-reducing (figure 2.9B) conditions, as previous studies have indicated that the 3-A-4 epitope is lost under reducing conditions whilst the 6-A-1 epitope is better exposed under reducing conditions (Tudor, 2002; Schumacher *et al.*, 1999). As expected, bcPRG4 was immunostained with 6-A-1 under reducing conditions (figure 2.9A) and 3-A-4 under native conditions (figure 2.9B). There appeared to be weak staining of bcPRG4 by mAbs 5-B-6 and 5-D-1 under non-reducing conditions (figure 2.9b) but not under reducing conditions. A btPRG4 band of ~345 kDa, representing full-length tendon PRG4, was apparent with 3-A-4 under non-reducing conditions (figure 2.9b). Interestingly, 5-B-6 immunostained a number of btPRG4 bands under reducing and non-reducing conditions that could represent degradation fragments. These fragments are not recognised by the other antibodies, and surprisingly, 6-A-1 does not recognise btPRG4 under reducing or non-reducing conditions. Disappointingly, there appeared to be no strong staining of hrPRG4 by any of the mAbs to bovine PRG4, suggesting that these antibodies may not cross-react between bovine and human PRG4.

2.3.5 Characterisation of carbohydrate moieties on recombinant human and native bovine PRG4 by lectin affinity blotting

Lectins are proteins that recognise and bind to different carbohydrate moieties, and this feature allows the carbohydrate moieties of glycoproteins to be characterised using lectin affinity blot analysis (reviewed in Vijayan & Chandra, 1999). The procedure in this study involved immobilising the target glycoprotein onto a nitrocellulose membrane after separation by SDS-PAGE followed by affinity blotting with lectins conjugated with the steroid hapten digoxigenin. This enables a secondary immunological detection of the digoxigenin hapten on the bound lectins. Previous work has demonstrated the reactivity of bovine PRG4 with the lectins peanut agglutinin (PNA) and Maackia amurensis agglutinin (MAA) (Tudor, 2002).

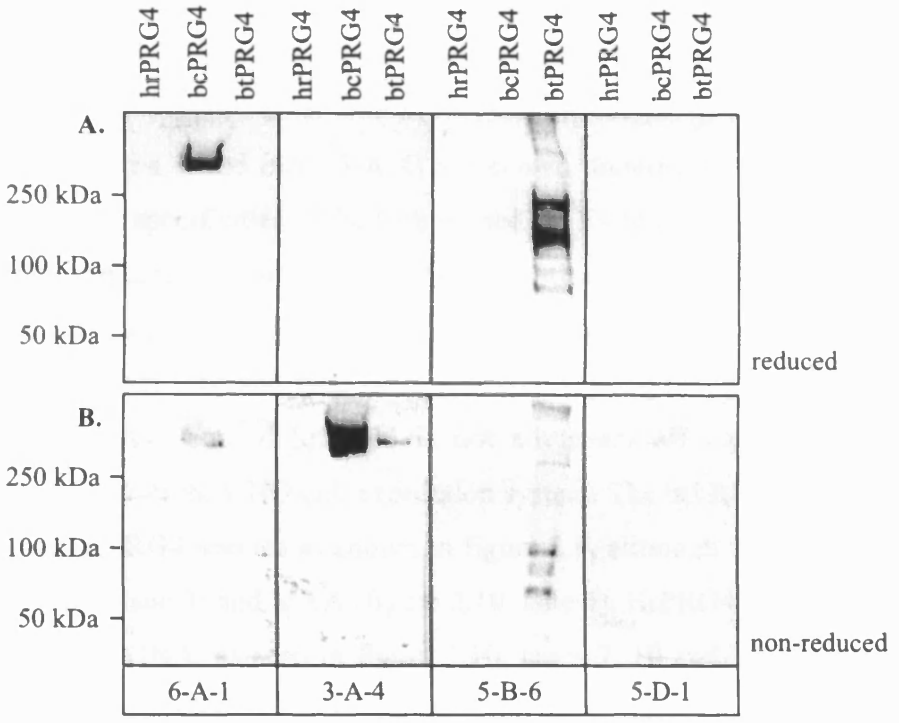


Figure 2.9 – Western blot analysis of purified human recombinant PRG4 (hrPRG4), partially purified bovine articular cartilage PRG4 (bcPRG4) and partially purified bovine tendon PRG4 (btPRG4) with monoclonal antibodies raised to bcPRG4. Proteins were run under reducing (A) and native (B) conditions at a concentration of 5µg total protein/lane. Western blots were probed with mAbs 6-A-1, 3-A-4, 5-B-6 and 5-D-1 which were raised against purified PRG4 from bovine cartilage. (Schumacher *et al.*, 1999). Migration of molecular weight standards (kDa) is shown to the left.

PNA recognises the core disaccharide galactose linked β(1-3) to N-acetylgalactosamine; MAA identifies sialic acid linked α(2-6) to galactose. Lectin affinity blotting experiments with hrPRG4, bcPRG4 and btPRG4 are shown in figure 2.10, along with a control blot performed without primary lectin (no 1°). Western blots with polyclonal antibodies (pAbs) specific to human and bovine PRG4 (anti exon 3 and mAb 3-A-4) are shown underneath to illustrate the migration position of PRG4. The specificities of the lectins used are listed in table 2.5, and all proteins were separated under reducing conditions with the exception of the 3-A-4 Western blot. These experiments demonstrated that the human and bovine PRG4 molecules both react with PNA and MAA (figure 2.10, lanes 1-6) and thus share a similar glycosylation pattern. This pattern also shows that the glycosylation of hrPRG4 is not adversely affected by the different post-translational machinery in the CHO-cell expression system. The btPRG4 preparation contained a number of degraded PRG4 species as shown in figure 2.9, although there was positive staining for PNA (figure 2.10, lane 3) and MAA (figure 2.10, lane 6). HrPRG4 was not recognised by the lectins DSA, SNA or GNA, as seen in figure 2.10, lanes 7, 10 and 13, respectively. DSA and SNA recognise some species in bcPRG4 and btPRG4, but it must be borne in mind that these are partially purified preparations, and there is little, if any, staining at the migration position of PRG4 as assessed by Western blot, 345 kDa. These bands may therefore represent contaminants in the protein preparations used.

2.3.6 Analysis of PRG4-hyaluronan interactions by affinity blotting

Previous work has proposed a physical interaction between a synovial lubricating factor (PSLF), thought to be part of the lubricin/PRG4 gene, and HA (hyaluronic acid), and that this interaction contributes to the lubricating function of lubricin/PRG4 (Jay *et al.*, 1992). Exons 8-9 of PRG4 contain haemopexin-like domains similar to vitronectin, and haemopexin is a serum haeme-scavenging protein that has been shown to interact with HA (Hrkal *et al.*, 1996). At present there have been no biochemical studies demonstrating the potential interaction between PRG4 and HA. This study investigated this potential interaction by means of affinity blotting experiments using the purified PRG4 molecules described earlier in this chapter along with biotinylated HA, and the results can be seen in figure 2.11.

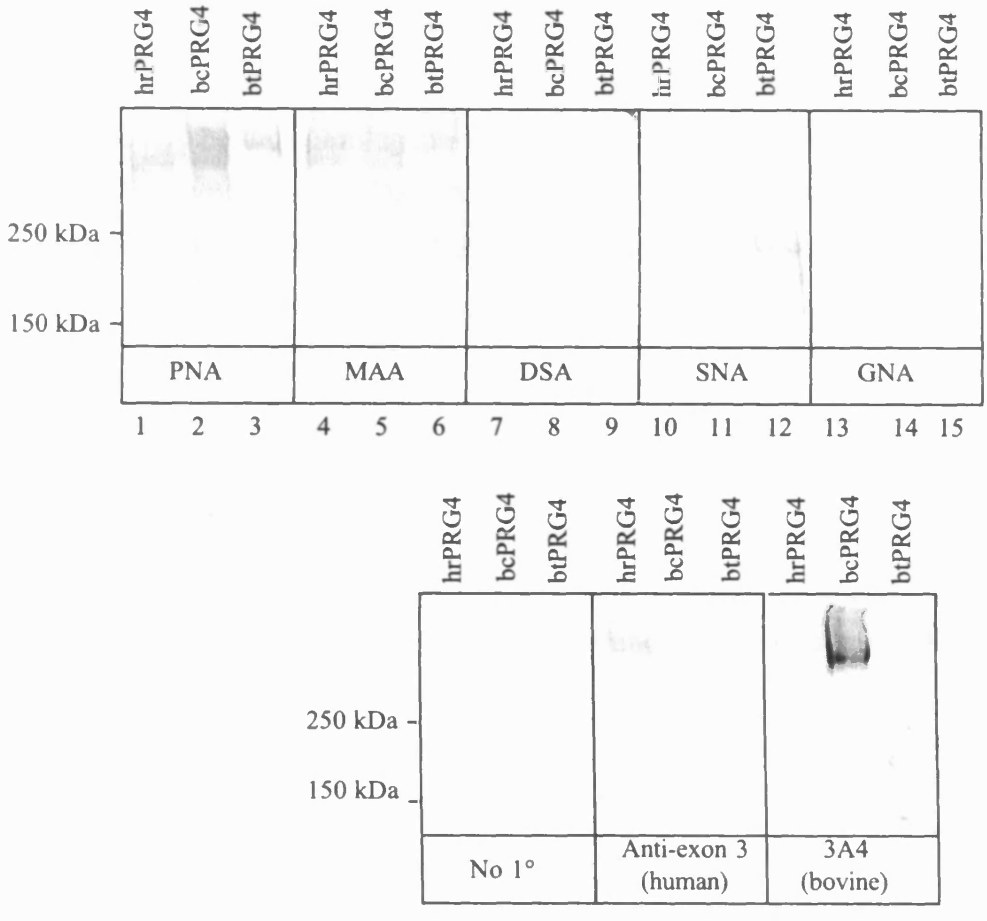


Figure 2.10 – Lectin affinity blotting analysis of purified human recombinant PRG4 (hrPRG4), partially purified PRG4 from bovine cartilage (lane 2) and partially purified PRG4 from bovine tendon (btPRG4). Proteins were run on SDS-PAGE gels under reducing conditions at a concentration of 5μg total protein/lane. Membranes were probed with the lectins PNA, MAA, DSA, SNA and GNA, the specificities of which are listed in table 2.5. The positive controls for human recombinant and bovine cartilage PRG4 are Western blots with anti-Exon 3 (reduced) and 3-A-4 (non-reduced) antibodies, respectively. A negative control was performed in the absence of primary lectin (no 1°). Migration of molecular weight standards (kDa) is shown to the left.

PRG4 was separated under reducing (figure 2.11A) and non-reducing (figure 2.11B) conditions and transferred to nitrocellulose, which was then incubated with biotinylated HA. A positive control for the procedure was performed using 5μg of a bovine nasal cartilage A1D4 preparation, which contains the HA-binding cartilage molecule link protein. Lane 6 of figure 2.11A shows that HA binds to link protein (~40 kDa) under non-reducing conditions, but in this procedure there was no visible interaction between HA and hrPRG4 or bcPRG4 (lanes 1& 2, 4& 5).

2.4 Discussion

The results presented in this chapter demonstrate that human recombinant, bovine articular cartilage and bovine tendon PRG4 all bind to heparin, and that this is a feature that can be used to purify the molecule from conditioned culture media by means of heparin-agarose affinity chromatography. PRG4 contains 2 Cardin-Weintraub heparin-binding consensus sequences (Cardin & Weintraub, 1989), as highlighted in the amino acid sequence of PRG4 shown in chapter 1, figure 1.14. These results suggest that one or both of these sites is functionally active in PRG4. This chapter also details the use of PNA lectin-agarose affinity chromatography in the purification of human recombinant and bovine cartilage PRG4, a technique that has also been used in the purification of the homologous molecule lubricin from human synovial fluid (Jay *et al.*, 2000). In other studies, bovine PRG4 has been shown to be recognised by the lectins PNA and MAA (Tudor, 2002), and PNA affinity chromatography has been shown in this study to be a useful technique for the purification of PRG4, although MAA affinity chromatography proved to be unsuccessful. This chapter details a two-step protocol for the purification of human recombinant PRG4 from the culture media of a stable, PRG4-expressing CHO cell line, which involved heparin-agarose affinity chromatography (figure 2.2) followed by PNA lectin-agarose affinity chromatography (figure 2.3). Using these methods, full-length human recombinant PRG4 of around 345 kDa can be purified to homogeneity, as assessed by SDS-PAGE and silver staining, and such a pure fraction was used in later chapters of this study and could conceivably be used in further applications such as binding assays and cell biology experiments.

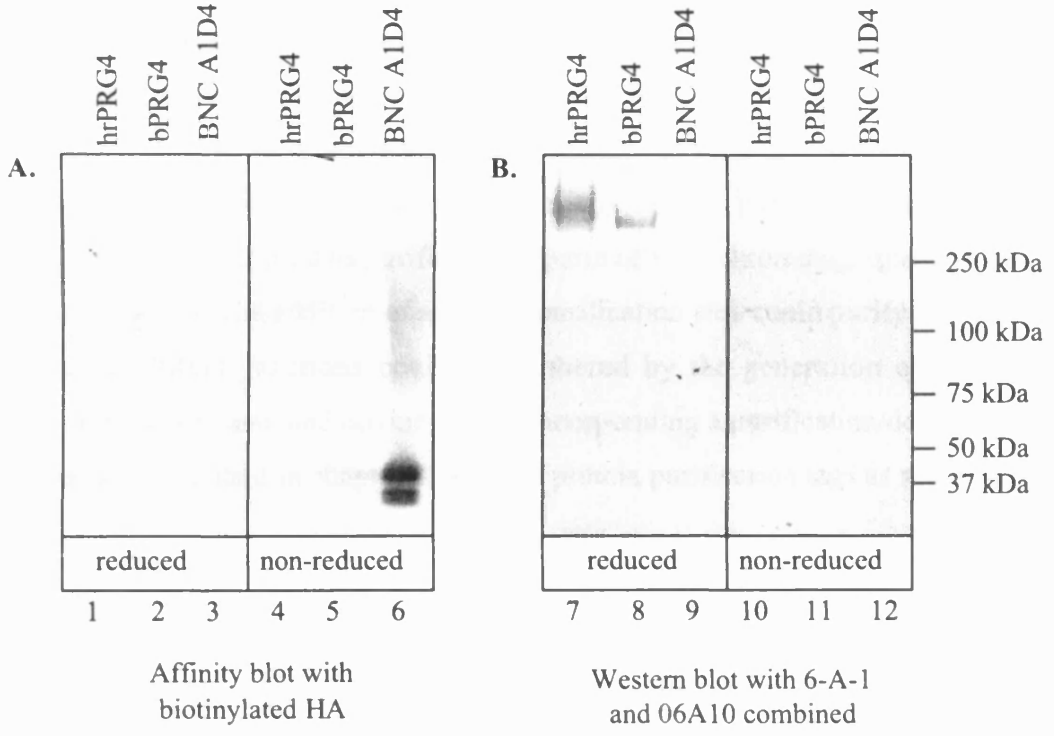


Figure 2.11 – HA affinity blotting experiments with purified human PRG4 and partially purified bovine cartilage PRG4. PRG4 preparations were separated under reducing (lanes 1-3) and non-reducing (lanes 4-6) conditions (5μg total protein/lane). Membranes were probed with biotinylated hyaluronan (HA), shown in (A). A bovine nasal cartilage A1D4 prep containing link protein is used as a positive control (5μg, lanes 3 and 6). A Western blot probed with antibodies to human and bovine PRG4 is shown in (B). Migration of size standards is shown to the right.

Bovine articular cartilage PRG4 was partially purified from the conditioned medium of superficial zone chondrocyte agarose cultures using heparin-agarose affinity chromatography (figure 2.4) and CsCl density gradient centrifugation (figure 2.5). PNA lectin-agarose affinity chromatography was also used, but despite being successful the step was inefficient and did not produce a homogenous PRG4 preparation (figure 2.6). For the purpose of further experiments, bovine cartilage PRG4 was used as purified by heparin affinity chromatography and CsCl density gradient centrifugation. The addition of a further purification step could purify bovine PRG4, and understanding of PRG4 functions could be furthered by the generation of stable cell lines expressing full-length human and bovine PRG4, incorporating a purification/detection tag such as the FLAG epitope described in chapter 3, or other protein purification tags as reviewed in (Terpe, 2003).

Immunohistochemical analyses have demonstrated the presence of PRG4 on the surface of mature compressed bovine tendon (Rees *et al.*, 2002). In this study, bovine PRG4 was partially purified from the conditioned medium of deep digital flexor tendon explant cultures by heparin-agarose affinity chromatography, and PRG4 was detected with mAb 3-A-4 (figure 2.7). Bovine tendon PRG4 runs at around 340 kDa on a reduced SDS-PAGE gel, similar to human recombinant and bovine articular cartilage PRG4. This culture system and initial chromatography step could be useful in the large-scale purification of tendon PRG4. The culture system is (a) less costly and time-consuming than the isolation of chondrocytes or tenocytes, as there is no need for pronase and collagenase, and (b) lends itself better to purification, as no calf serum is present in conditioned medium of the explant cultures. Tendon PRG4 could be used towards the determination of the differences, or lack thereof, between PRG4 molecules present in different anatomical sites within the joint, which would contribute to a better characterisation of the overall PRG4 phenotype.

Affinity blotting with biotinylated hyaluronan failed to reveal an interaction between hyaluronan and PRG4. However, this does not necessarily disprove the hypothesis that an interaction exists between PRG4 and HA (Jay *et al.*, 2003a & b), as there are a number of methods for studying protein-HA interactions. These include column chromatography (Heinegard & Hascall, 1979), affinity chromatography, co-precipitation with cetylpyridinium chloride (Acharya *et al.*, 1998;

Watanabe *et al.*, 1997), ELISA with lipid-conjugated HA, (Watanabe *et al.*, 1997), gel electrophoresis with agarose incorporating HA (Hrkal *et al.*, 1996) and BIAcore analyses (Watanabe *et al.*, 1997). Jay and others have suggested that the potential HA-PRG4 interaction may be interrupted by the C-terminal truncating mutations that are characteristic of CACP syndrome, the pathology associated with PRG4, and that this may explain why synovial fluid from patients with CACP syndrome displays a decrease in boundary lubricating ability (Jay *et al.*, 2003b). The potential for HA interactions with recombinant PRG4 domains is discussed later in this thesis.

Western blot analyses of human and bovine PRG4 show that the 06A10 and anti-Exon 3 polyclonal antibodies to human PRG4 do not recognise bovine PRG4 (figure 2.8), and that monoclonal antibodies to bovine PRG4 do not detect human PRG4 (figure 2.9). Interestingly, mAb 5-B-6 appeared to react with degradation products of bovine tendon PRG4 (figure 2.9). The cross-species reactivity of these antibodies could be further tested by Western blotting experiments with PRG4 from other species. Lectin affinity blotting experiments show that both human and bovine PRG4 are recognised by the lectins PNA and MAA, demonstrating a similarity in terms of post-translational modifications with respect to N- and O-linked oligosaccharides. The contribution of carbohydrates to the lubricating ability of PRG4 at the cartilage surface has been demonstrated by Jay *et al.* (2001a), who have determined that boundary lubrication by PRG4 is mediated by O-linked β(1-3) Gal-GalNAc oligosaccharides. MAA recognises complex, sialylated chains (Wang & Cummings, 1988), and the importance of these terminal sialic acids in lubrication could be investigated further using sialyltransferase and their inhibitors. Sialyltransferases are a family of enzymes that catalyse the transfer of sialic acids onto terminal positions of complex carbohydrates from glycoproteins and glycolipids. PNA recognises galactose linked β(1-3) to N-acetylgalactosamine, a chain that is elongated by β(1-3) galactosyltransferase. Inhibitors of sialyltransferases (Kleineidam *et al.*, 1997) and β(1-3) galactosyltransferase (Delannoy *et al.*, 1996) could be applied to specifically inhibit the glycosylation of PRG4, and the implications could be studied by lubrication analyses.

2.5 Chapter Summary

- Human recombinant PRG4, bovine articular cartilage PRG4 and bovine tendon PRG4 all bind to heparin-agarose and were eluted with 0.2-0.4M NaCl.
- Human recombinant and bovine cartilage PRG4 both react with the lectin PNA as assessed by affinity blotting. This feature was used in the lectin affinity purification of PRG4, which eluted from a PNA-agarose column with 0.2M D-galactose.
- Human recombinant PRG4 was purified to apparent homogeneity from the conditioned medium of a stable, PRG4-expressing cell line by a two-step process: heparin-agarose affinity chromatography followed by PNA lectin-agarose affinity chromatography.
- Bovine tendon PRG4 was partially purified from the conditioned media of deep digital flexor tendon explant cultures, which may be a good source of tendon PRG4 for functional analyses
- The PRG4-specific antibodies available did not display any cross-reactivity between human and bovine species
- Bovine cartilage and human recombinant PRG4 are both recognised by the lectins PNA and MAA, indicating that the two species of PRG4 incorporate similar post-translational modifications.

3. CLONING, EXPRESSION AND PURIFICATION OF PRG4 STRUCTURAL DOMAINS

3.1 Introduction

Sequence analyses of PRG4 have indicated that it is a multifunctional domain protein that has the potential for a number of physiological functions including cell proliferation, cytoprotection, lubrication and self-aggregation/matrix binding (Flannery *et al.*, 1999). The N- and C-terminal domains of PRG4, which lie either side of the central mucin domain, contain a number of protein motifs relevant to functionality. To assess these potential functions the N- and C-terminal domains of bovine and human PRG4 were cloned for expression in a *Drosophila* Schneider-2 insect cell expression system, and this chapter describes the production of these PRG4 domains as recombinant proteins. The domains that were targeted for PCR cloning are detailed in figure 3.1. The N-terminal sequence is 525 base pairs in length, encoding for a protein with a calculated mass of 20 kDa, whilst the C-terminal sequence is 792 base pairs long representing a protein with a calculated molecular weight of 30 kDa (Stothard, 2000). A cleavable FLAG epitope (reviewed in Einhauer & Jungbauer, 2001) was inserted N-terminal to the PRG4 domains for the detection and purification of the secreted proteins. The expression and purification of these polypeptides facilitated studies involving the characterisation of the biological activity of these PRG4 domains, based on their ability to interact with other matrix molecules, using binding assays such as ELISA and BIACore, and also by utilising the recombinant PRG4 domains in cell biology systems.

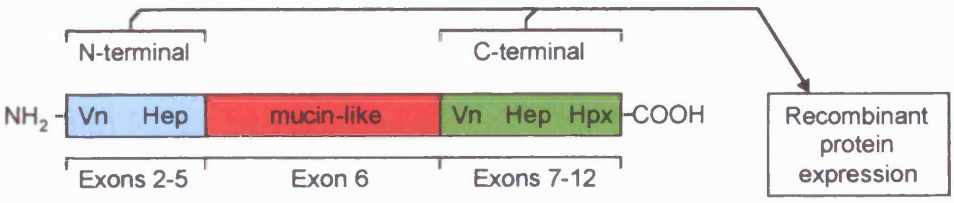


Figure 3.1 – Schematic diagram of PRG4 illustrating the domains targetted for PCR cloning and recombinant protein expression. Vn – vitronectin-like; Hep – heparin-binding site; Hpx – hemopexin-like.

3.2 Materials and Methods

3.2.1 Materials

All chemicals were of an analytical grade and were purchased from Sigma-Aldrich unless indicated otherwise. A detailed list of the materials used in this research project and their suppliers is listed in Appendix A. Tissue culture plastics were purchased from Corning.

3.2.1.1 Stock solutions

Stock solutions and their components are listed in table 3.1. Solutions for use in tissue culture were autoclaved or filtered before use (0.22μm filters, Millipore).

Table 3.1 – Stock solutions and their components.

Solution	Components
2X HEPES buffered saline (HBS)	50mM HEPES, pH 7.1, 1.5mM $NaHPO_4$, 280mM NaCl
0.5X Tris-borate-EDTA (TBE)	From a 5X solution (0.445M Tris-borate, pH 8.3, 10mM EDTA) made with Milli-Q H_2O and a powdered stock (Sigma T3913)
Phosphate buffered saline (PBS)	Dulbecco A: 0.16M NaCl, 3mM KCl, 8mM Na_2HPO_4, 1mM KH_2PO_4, pH 7.3
Tris-buffered saline (TBS)	50mM Tris-HCl, pH 7.4, 0.15M NaCl
100 mM $CuSO_4$	100mM in H_2O, passed through 0.22μM filter

3.2.1.2 Culture media

Schneider's Drosophila medium, Drosophila-SFM (serum free medium), heat-inactivated FCS, and L-glutamine were all purchased from Invitrogen. Culture medium was formulated as listed later in table 3.10, and was passed through 0.22μm filters (Millipore, UK) before use.

LB plates (100mm) for the growth of transformed *E. Coli* were prepared with LB agar (Sigma) containing 50μg/ml ampicillin. For blue-white selection, 40μl of 40mg/ml X-gal solution was spread onto plates 1 hour before the addition of *E. coli*. For the expansion of *E. Coli,* LB broth (Sigma) was supplemented with 100μg/ml ampicillin.

3.2.1.3 Antibiotics

Penicillin-Streptomycin-Glutamine solution (100X) was purchased from Invitrogen and comprised of 10,000 units/ml penicillin G, 10mg/ml streptomycin sulphate and 29.2 mg/ml L-Glutamine in a 8.85% saline and 10mM citrate buffer to maintain penicillin potency. L-

Glutamine (100X) and Hygromycin B solution (50mg/ml in PBS) were purchased from Invitrogen.

3.2.1.4 Host strains/cell lines/plasmid vectors

The competent cells used for transformation were from the TOP10 *E. Coli* strain from Invitrogen. *Drosophila* S2 cells were purchased from Invitrogen. Information concerning the pMT-BiP, pCoHYGRO (Invitrogen) and pTAg (R & D Systems) plasmid vectors used can be found in appendix B. The CHO-MegN141 cell line was a gift from Wyeth Research.

3.2.1.5 Molecular biology reagents

RT-PCR was carried out using an RNA PCR core kit from Applied Biosystems. The kit consists of GeneAmp 10X PCR buffer II (100mM Tris-HCl, pH 8.3, 500mM KCl), $MgCl_2$ (25mM), dNTPs (dATP, dCTP, dGTP, dTTP); all at 10mM, RNase inhibitor, MuLV (murine leukaemia virus) reverse transcriptase and oligo dT. *Taq* DNA polymerase from *Thermus aquaticus* strain YT1 was purchased from Promega.

3.2.2 General molecular biology methods

3.2.2.1 Preparation of glassware & plastics

All glassware and media used for culture of *E. Coli* was autoclaved before use. Pre-sterilised filter pipette tips were used for PCR and also for tissue culture after opening within a sterile environment. Non-sterile pipette tips without filters were autoclaved before use in tissue culture. Glassware and Eppendorf™ tubes were autoclaved prior to use in DNA manipulation and tissue culture.

3.2.2.2 Transformation of competent E. coli

A 20µl aliquot of TOP10 *E. coli* cells was used for each transformation. Plasmid ligation mixture (1µl) was added and mixed with a pipette tip, and the mixture was incubated on ice for 30 minutes. The mixture was then transferred to a 42°C water bath for 30 seconds before the addition of 225µl SOC medium, followed by incubation at 37°C for 1 hour with shaking. This culture was then spread on LB agar plates (50-100µl/plate) and incubated overnight at 37°C.

3.2.2.3 Agarose gel electrophoresis of DNA

Agarose gels were prepared using SeaKem agarose (Cambrex) and 0.5X TBE buffer (table 3.1). For DNA fragments over 1000bp, 1% [w/v] gels were used, for DNA fragments under 1000bp, 2% [w/v] gels were used. Ethidium bromide was included in the gels at a concentration of 1μg/ml: 3μl of a 10mg/ml stock solution was added for a 30ml gel. 6X running buffer (Cambio) was added to DNA samples before loading on to agarose gels. Low (50bp-1000bp) and high molecular weight (500bp-10kb) DNA size standards were supplied by Cambio, UK. Electrophoresis was carried out in horizontal gel apparatus (BioRad, UK) at a constant voltage of 100 volts for approximately 1 hour in 0.5X TBE (table 3.1). Gels were visualised under ultraviolet light, and image capture was performed using a gel documentation system (BioRad) linked to an Apple G3 computer.

3.2.2.4 Extraction of DNA from agarose gel

DNA bands of interest within ethidium bromide-containing gels were viewed with a UV transilluminator and excised with the aid of scalpel blades. DNA was purified from the excised agarose bands using the QIAquick gel extraction kit (Qiagen) according to the manufacturers instructions. The resultant DNA was analysed to assess purity and concentration by measuring absorbancies at 260nm and 280nm using a spectrophotometer (UltroSpec 2000, Pharmacia).

3.2.2.5 Small-scale preparation of plasmid DNA

Transformed *E. coli* colonies were picked from inoculated LB agar/ampicillin plates with a clean 10μl pipette tip and grown overnight in 3ml of ampicillin-containing LB broth (section 3.2.1.2) at 37°C with shaking. Plasmid DNA was purified using the Wizard®*Plus* SV Miniprep DNA Purification System from Promega according to the manufacturers instructions. DNA concentration was calculated by reading absorbance values at 260/280nm.

3.2.2.6 Large-scale preparation of plasmid DNA for transfection

For transfections, plasmid DNA was purified from a 100ml LB/ampicillin broth culture grown overnight at 37°C in 500ml conical flasks with vigorous shaking (300rpm). Plasmid DNA was purified using an EndoFree plasmid midiprep kit (Qiagen) according to the manufacturers instructions in order to remove endotoxin, or lipopolysaccharide (LPS). A single *E. Coli* cell

contains about 2 million LPS molecules (Rietschel & Brade, 1992; Raetz, 1990), and during lysis of bacterial cells for plasmid DNA purifications, endotoxin molecules are released from the outer membrane into the lysate. Endotoxin or LPS can reduce transfection efficiency in eukaryotic cell lines (Weber *et al.*, 1995). Levels of endotoxin in plasmid preparations depend on the type of purification method employed (Cotten *et al.*, 1994), and the EndoFree purification kit removes most endotoxin, and yields around 400µg of plasmid from a 100ml *E. Coli* culture. DNA concentration was calculated by reading absorbance values at 260/280nm.

3.2.2.7 Preparation of frozen plasmid stocks

Ampicillin-containing LB broth cultures (3ml) of transformed colonies picked from agar plates were grown overnight at 37°C with continuous shaking (225rpm). For the preparation of glycerol stocks for long-term storage of plasmids, 0.5ml of broth culture was mixed with 0.5ml of sterile 50% [v/v] glycerol in a Nalgene cryovial. Stocks were stored at -80°C.

3.2.2.8 Restriction enzyme digestion

Restriction endonuclease digestions were performed according to the manufacturers instructions, in the buffers recommended by the suppliers (Promega) for 2 hours at 37°C, in the absence of acetylated BSA. For a reaction volume of 20µl, 1µg of DNA was used. Following digestion, 6X running buffer (Cambio) was added and products were separated on 1% (vector DNA) or 2% (PCR product DNA) agarose gels containing 0.5µg/ml ethidium bromide (section 3.2.2.3). Bands of interest were excised and purified using the QIAquick gel purification system (section 3.2.2.4).

3.2.3 RNA isolation

3.2.3.1 Cell sources

(a) Bovine chondrocytes

For the amplification of bovine cDNA, total RNA was isolated from bovine superficial zone chondrocyte monolayer cultures. Chondrocytes were isolated as described in section 2.2.2. Cells were then cultured in monolayer at an initial seeding density of 0.8 x 10^6 cells/ml in DMEM supplemented with 5% [v/v] FCS and 50µg/ml gentamicin. Upon confluency, cells were washed with PBS prior to the addition of 3ml Tri-Reagent (Sigma) per 60mm dish. The Tri-Reagent was

allowed to sit for 15 minutes before removal into 1.5ml microcentrifuge tubes containing chloroform (0.2ml/1ml Tri-Reagent).

(b) Human chondrocytes

For the PCR amplification of human cDNAs, total RNA originating from explant cultures of human osteoarthritic cartilage was used (gift from Dr. C. R. Flannery).

(c) Drosophila S2 cells

To investigate the expression of PRG4 domains by stable S2 cell lines, 12 x 10^6 cells from confluent cultures were washed twice with PBS before the extraction of total RNA with 1ml Tri-Reagent.

3.2.3.2 Procedure for RNA isolation

The Tri-Reagent/chloroform samples were vortexed and left to stand at room temperature for 15 minutes, then centrifuged at 16,000 x g for 15 minutes. The upper aqueous phase was carefully removed and mixed with an equal volume of 70% [v/v] ethanol. Total RNA was isolated using RNeasy mini-columns and reagents (Qiagen) according to the manufacturers protocol. This procedure is based on the binding properties of a silica gel-based membrane with microspin technology. RNA contained in the sample binds to the membrane allowing contaminants to be washed away. To remove contaminating DNA, DNAse I solution (80µl, Qiagen) was incubated with the membrane in between two wash steps. RNA was eluted from the membrane with sterile water, and amount and purity was assessed by measuring absorbance values at 260 and 280nm using a spectrophotometer (UltroSpec 2000, Pharmacia).

3.2.4 Reverse transcription of RNA and PCR amplification of cDNA

Reverse transcription-polymerase chain reaction (RT-PCR) is a two-step process comprised of the reverse transcription of RNA into cDNA, followed by the amplification of cDNA by PCR with oligonucleotide primers specific to the required target sequence. For the first step, cDNA was synthesised by reverse transcription by using 50-100ng of RNA in a reaction with a final volume of 20µl, as detailed in table 3.2. This reaction was mixed by vortexing and then incubated at room temperature for 10 minutes. The mixture was then subjected to the following cycle: 42°C for 30 minutes, 99°C for 5 minutes with a final soak of 4°C.

Table 3.2 – Reverse transcription reaction mixture

Component	Volume
10X PCR buffer II	2.0µl
25mM $MgCl_2$	1.6µl
2.5mM dNTPs	1.6µl
RNase inhibitor	1.0µl
MuLV reverse transcriptase	1.0µl
Oligo dT	1.0µl
Total RNA sample (50-100ng)	Xµl
Water	To 20µl

3.2.5 PCR reactions

3.2.5.1 Oligonucleotide primers

For the construction of oligonucleotides to amplify PRG4 cDNA sequence(s), a number of primer design criteria were considered. These included the following:

(i) 'standard' PCR primers were 20-25 nucleotides long;

(ii) melting temperatures were within the range of 50-65°C;

(iii) the GC content was generally kept to 40-60%; and

(iv) self-complementarity was avoided to minimise primer secondary structure and primer-dimer formation.

In addition, 'expression primers' were designed to generate PCR products that could be inserted into an expression vector. These primers were designed to contain the following features:

(i) primers, and therefore PCR products, contained a 5′ NcoI cleavage site and a 3′ XhoI cleavage site so that digested PCR products can be inserted into the polylinker region of a cleaved pMT-BiP vector (appendix B);

(ii) 5′ and 3′ restriction sites to include an appropriate overhang in order to ensure efficient restriction enzyme digestion of the amplified cDNA ends (technical information obtained from Promega 2002 catalogue);

(iii) forward primers contained a FLAG® protein purification/detection epitope, encoding for the amino acid sequence DYKDDDDK;

(iv) forward primers were designed so that the PRG4 protein-encoding sequence of the PCR product would be in frame with the FLAG epitope, and that after restriction digestion the PCR product could be ligated into the cleaved expression vector pMT-

BiP, with PRG4 sequence in frame with the BiP secretion signal present in the vector (for details see appendix B);

(v) reverse primers were to contain a stop codon (AMB) in frame with the PRG4-encoding PCR product.

For the amplification of specific portions of human and bovine PRG4, primers were designed using sequences that are available in Genbank, and their Accession numbers are listed in table 3.3. Primer sequences and relevant features are listed in table 3.4. Primers were designed using the Oligo 4.0s program for Macintosh (MBI Inc.) or the MacDNAsis system (Hitachi Software, Japan).

3.2.5.2 Protocol for PCR reactions

PCR reaction mixtures were prepared as detailed in table 3.5. Reactions were performed in a Technegene thermal cycler (Techne, UK). Cycling parameters were as follows: following an initial denaturation step of 60 seconds at 95°C, amplification consisted of 35 cycles of 30 seconds at 95°C, 45 seconds at the annealing temperature of the primer set (52°C for the primers listed in table 3.4), 45 seconds at 72°C followed by a final extension step of 5 minutes at 72°C. Reaction mixtures were then separated by agarose gel electrophoresis as detailed in section 3.2.2.3.

Table 3.3 – Sequences used in the design of PRG4-specific primers

Target	Genbank Accession No.	Sequence	Reference
Human PRG4, exons 2-5	U70136	Homo sapiens megakaryocyte stimulating factor	(Turner *et al.*, 1991)
Bovine PRG4, exons 2-5	U70136	Homo sapiens megakaryocyte stimulating factor	(Turner *et al.*, 1991)
Human PRG4, exons 7-12	U70136	Homo sapiens megakaryocyte stimulating factor	(Turner *et al.*, 1991)
Bovine PRG4, exons 7-12	AF056218	Bos taurus superficial zone protein	(Flannery *et al.*, 1999)

Table 3.4 – Oligonucleotide primers used in the RT-PCR amplification of human and bovine N- and C-terminal domains of PRG4.

Primer pair	Specificity	Sequence	Expected size of product
HC1 HC2	Human PRG4 Exons 7-12 (C-terminal)	5'-GATGAGACCAATATATGCAAT 5'-CTAAGGACAGTTGTACCAGAC	792bp
BC1 BC2	Bovine PRG4 Exons 7-12 (C-terminal)	5'-GATGAAACTAATCTATGCAACG 5'-CTAAGGACAGTTGTACCAGGTA	792bp
N1 N2	Human & Bovine PRG4 Exons 2-5 (N-terminal)	5"-CAAGATTTATCAAGCTGTGCA 5"-CTATTTGAGTTTCTTCTGTAA	525bp
HC1a HC2a	Human PRG4 Exons 7-12 (C-terminal) w/ restriction sites & FLAG	5'- ACATGCCATGGGACTACAAGGACGACGA TGACAAGGATGAGACCAATATATGCAAT 5'-CCGCTCGGAGCTAAGGACAGTTGTACCAGAC	815bp
BC1a BC2a	Bovine PRG4 Exons 7-12 (C-terminal) w/ restriction sites & FLAG	5'- ACATGCCATGGGACTACAAGGACGACGA TGACAAGGATGAAACTAATCTATGCAAC	815bp
N1a N2a	Human & Bovine PRG4 Exons 2-5 (N-terminal) w/ restriction sites & FLAG	5'- ACATGCCATGGGACTACAAGGACGACGA TGACAAGCAAGATTTATCAAGCTGGGCA 5'- CCGCTCGAGCTATTTGAGTTTCTTCTGTAA	548bp

Table 3.5 – PCR reaction mixture

Component	Volume
10X PCR buffer II	2µl
25mM $MgCl_2$	1.2µl
2.5mM dNTPs	1.6µl
Primers (stock = 10µM)	0.4µl of each
Taq polymerase	0.12µl
RT sample	1µl
Water	To 20 µl

3.2.5.3 Cloning of PRG4 cDNA fragments into pTAg TA cloning vector

PRG4 domain cDNAs were amplified by PCR with the primers detailed in table 3.4. If a strong single band was apparent, the PCR reaction mixture was included in a ligation mixture as detailed in table 3.6. This mixture was incubated overnight at 14°C and transformed into *E coli* as described in section 3.2.2.2. Transformants were grown on LB agar with X-Gal (5-bromo-4-

chloro-3-indolyl-ß-D-galactoside) for blue-white selection of transformed colonies (section 3.2.1.2).

Table 3.6 – Ligation reaction mixture for plasmid vector pTAg

Component	Volume
10X ligation buffer (Promega)	0.5µl
T4 DNA Ligase	0.5µl
20-50ng cleaved pMT vector	Xµl
Cleaved PCR product	Xµl
H_2O	To 5µl

3.2.5.4 Cloning of PRG4 cDNA fragments into pMT expression vector

DNA fragments were generated by PCR using primers containing sequences designed to incorporate a 5′ NcoI site and a 3′ XhoI site into the product (table 3.4). The forward primer was designed so that a FLAG detection/purification epitope was incorporated into the product. The PCR products and the pMT-BiP vector (see appendix B) were both cleaved with the aforementioned restriction endonucleases (section 3.2.2.8) and subjected to agarose gel electrophoresis (section 3.2.2.3). The desired products were excised from the ethidium bromide-stained gel with the aid of a UV transilluminator and gel-purified (section 3.2.2.4). The purified PCR products were then cloned into the cleaved pMT-BiP vector. This was performed by preparing a 5µl ligation reaction containing the components listed in table 3.7.

Table 3.7 – Ligation reaction mixture for plasmid vector pMT-BiP

Component	Volume
10X ligation buffer (Promega)	0.5µl
T4 DNA Ligase	0.5µl
20-50ng cleaved pMT vector	Xµl
Cleaved PCR product	Xµl
H_2O	To 5µl

This mixture was then incubated overnight at 14°C and transformed into the TOP10 *E. Coli* strain (Invitrogen) as in section 3.2.2.2. Transformants were grown on LB agar (section 3.2.1.2).

3.2.5.5 Screening of colonies

Analysis of transformants was carried out by preparing plasmid miniprep DNA of colonies apparent on LB agar plates (see section 3.2.2.5). To confirm the insertion of PCR products, plasmid preparations were analysed by restriction digestion with NcoI/XhoI, PCR followed by gel electrophoresis, and automated DNA sequencing (section 3.2.5.6).

3.2.5.6 Automated DNA sequencing

Plasmid preparations were sequenced using the primers relevant to the plasmid vectors used, as listed in below in table 3.8.

Table 3.8 – Sequencing primers for plasmid vectors

Vector	Primer	Sequence
pMT-BiP	MT forward	5'-CATCTCAGTGCAACTAAA
pMT-BiP	BGH reverse	5'-TAGAAGGCACAGTCGAGG
pTAg	M13 (+)	5'-GTAAAACGACGGCCAGT
pTAg	M13 (-)	5'-AACAGCTATGACCAT

Sequencing reactions were prepared using the ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA) as detailed in table 3.9. BigDye™ contains dNTPs and the four ddNTPs with their associated differing fluorescence labels. The reaction mixture was then subjected to a thermal cycling program using a Technegene cycling machine (Techne). The parameters used were as follows: an initial denaturation step of 95°C for 30 seconds was followed by 26 cycles of 96°C for 20 seconds, 50°C for 10 seconds and 60°C for 4 minutes, with a final hold at 4°C.

Table 3.9– Sequencing reaction mixture

Component	Volume
BigDye solution	1µl
ABI Better Buffer	5µl
Sequencing primer (2µM)	1.5µl
DNA	200-400ng
H_2O	To 15µl

The products of sequencing reactions were purified after cycling. 3M Sodium acetate, pH 3.1 (2µl), H_2O (3µl) and ethanol (40µl) were added to the reaction mixture, which was vortexed and

allowed to stand at room temperature for 20 minutes. The mixture was centrifuged for 20 minutes at 16,000 rpm on a benchtop centrifuge and the supernatant was removed, and the DNA pellet was washed with 250µl of 70% [v/v] ethanol and centrifuged for 10 minutes. The supernatant was removed and the pellet was allowed to dry at 90°C and stored at -20°C before sequencing on an ABI Prism 310 automated sequencer, or transfer to the Molecular Biology Support Unit, Cardiff University, to be sequenced on an ABI Prism 3100 automated sequencer.

3.2.6 Cloning of PRG4 domains

3.2.6.1 Overview

The overall aim of this technique was to obtain the cDNAs for human and bovine C-terminal and N-terminal domains of PRG4 in order to produce them in a protein expression system. The position of the N- and C-terminal domains targeted for PCR cloning are indicated in figure 2.1. The system used in this instance utilises the pMT-BiP-V5 plasmid vector from Invitrogen. This vector gives inducible, secreted expression of protein encoded by insert DNA in *Drosophila* S2 cells.

3.2.6.2 Primers for PCR amplification of PRG4 domains

PRG4-specific primers without 'expression primer' features (HC1/HC2, BC1/BC2, N1/N2; see table 3.4) were used to amplify the desired PRG4 sequence using cDNA from human and bovine chondrocytes. The products from these reactions were then cloned into a TA cloning vector (pTAg, section 3.2.5.3). Plasmid preparations of transformed colonies were then used as templates for PCR using the FLAG/restriction site 'expression' primers, alongside RT samples from human or bovine chondrocytes. The 'expression' primer sets HC1a/HC2a, BC1a/BC2a and N1a/N2a (see table 3.4) were all designed with the appropriate restriction sites at the 5' end to enable cloning of the resultant PRG4 cDNA products into a digested pMT-BiP vector, in frame with the vector BiP secretion signal sequence. Sets HC1a/HC2a and BC1a/BC2a are specific to human and bovine PRG4 C-terminal regions, respectively. The primer pair N1a/N2a was designed using the human PRG4 N-terminal sequence but was also functional with bovine samples. Forward primers were designed with an enterokinase-cleavable FLAG identification/purification epitope (amino acid sequence DYKDDDDK) 5` to the start of the PRG4 sequence. Reverse primers included a stop codon (AMB). The restriction sites used were

NcoI for the forward primers and XhoI for the reverse primers, and the sites were designed so that the start codon, FLAG epitope and PRG4 sequence would be in frame with the BiP secretion signal of the expression vector.

3.2.6.3 Expression constructs

Before ligation into the expression vector, PRG4 domain PCR products were double-digested with NcoI and XhoI (section 3.2.2.8) and gel purified (section 3.2.2.4). Digested PCR products were ligated into the pMT-BiP expression vector (section 3.2.5.4) following digestion of the expression vector (pMT-BiP-V5) with NcoI and XhoI, and gel purification of the linearised vector. Plasmid preparations (section 3.2.2.5) of all constructs were sequenced (section 3.2.5.6) to check for in-frame insertion of PRG4 cDNA and lack of PCR-introduced mutants. Verified expression constructs and the selection vector pCoHYGRO (see appendix B) were prepared to a high degree of purity with an endotoxin-removal kit (see section 3.2.2.6) prior to transfection into *Drosophila* S2 cells (section 3.2.8.1).

3.2.7 Drosophila S2 cell culture

3.2.7.1 Drosophila S2 cell line

The pMT-BiP expression vector (appendix B) is designed for use with Schneider-2 (S2) cells, a cell line derived from a primary culture of late stage (20-24 hours old) *Drosophila melanogaster* embryos (Schneider, 1972).

3.2.7.2 Culture media

The culture media used in conjunction with the *Drosophila* S2 cell line is listed in table 3.10.

Table 3.10 – Culture media used with *Drosophila* S2 cells

Media	Components
DES complete medium	Schneider's Drosophila Medium with 10% heat-inactivated FCS, 50μg/ml penicillin G, 50μg/ml streptomycin & 2mM L-glutamine
DES selection medium	Schneider's Drosophila Medium with 10% heat-inactivated FCS, 50μg/ml penicillin G, 50μg/ml streptomycin, 2mM L-glutamine and 300μg/ml hygromycin B
DES expression medium	Drosophila-SFM with 50μg/ml penicillin G, 50μg/ml streptomycin, 2mM L-glutamine and 300μg/ml hygromycin B
DES freezing medium	45% [v/v] fresh DES complete medium, 45% [v/v] conditioned DES complete medium, 10% [v/v] DMSO

3.2.7.3 Culture and maintenance of S2 cells

DES complete medium (see table 3.10) was used for the initiation and expansion of the *Drosophila* S2 cell line. S2 cells grow at room temperature as a loose, semi-adherent monolayer and have a doubling time of approximately 24 hours. Cells were cultured in $25cm^2$ (3-5ml culture volume) or $75cm^2$ (10-15ml culture volume) tissue culture flasks with vented lids (Corning), and were incubated at 26°C without CO_2. Cells were passaged when cell density reached between 8 and 20 x 10^6 cells/ml and were split using dilution factors of 1:2 to 1:5. S2 cells do not require trypsinisation; they can be detached by washing the surface of the flask with conditioned medium with a pipette during passage. Conditioned media was always carried over; for example a 1:3 dilution of a confluent 15ml culture would involve transferring 5ml of the culture to 10ml of fresh medium in a new flask. For expansion purposes cells were subcultured in serum-containing medium to a final density of 2 - 4 x 10^6 cells/ml. S2 cells were never seeded below a density of 0.5 x 10^6 cells/ml, as these cells do not grow well at low density.

3.2.7.4 Initiation of S2 cell culture from frozen stocks/preparation of frozen S2 cell stocks

For the initiation of cell culture from frozen stocks, frozen aliquots were thawed quickly at 37°C and added to $25cm^2$ flasks containing 5ml of complete medium. Following a 45-minute incubation, cells were centrifuged at 1000 x g for 4 minutes and the DMSO-containing freezing medium was aspirated. Cells were re-suspended in 5ml of complete medium and grown to confluency (8-20 x 10^6 cells/ml) before dilution. For storage, 11 x 10^6 cells from confluent (10-20 x 10^6 cells/ml) cultures in serum-containing media were frozen to -80°C in 1ml freezing medium (see table 3.10) in Nalgene cryovials. Styrofoam containers were used to ensure a slow freezing process, and vials were transferred to liquid nitrogen for long-term storage.

3.2.8 Generation of PRG4 domain-expressing Drosophila S2 cell lines

3.2.8.1 Transfection of cultured S2 cells

The aim of this technique was to generate stable *Drosophila* S2 cell lines that secrete target proteins (PRG4 domains) into the culture media upon induction with $CuSO_4$. S2 cells were co-transfected with pMT-BiP PRG4 domain-containing expression constructs (section 3.2.6.3) and a hygromycin selection vector (pCoHYGRO, appendix B) using a calcium phosphate transfection kit (Invitrogen). A 19:1 [w/w] ratio of expression vector to selection vector was used, and

expression or selection vectors were omitted where appropriate as negative controls for selection and expression experiments. Briefly, the solution described in table 3.11 was added dropwise to 300µl of HBS (see table 3.1) with continuous mixing.

Table 3.11 - Calcium phosphate transfection solutions for *Drosophila* S2 cells

Component	Volume
2M $CaCl_2$	36µl
19µg pMT expression construct	Xµl
1µg pCoHYGRO selection vector	Xµl
H_2O	to 300µl

The resulting solution was incubated for 45 minutes at room temperature, mixed and added dropwise to S2 cells. For transfections, 3 x 10^6 cells were grown in 25cm^2 flasks for 16 hours in 3ml of complete medium before the addition of transfection mixture. Cells were incubated for 24 hours before centrifugation and removal of the transfection mixture from the cell pellet by aspiration. Cells were resuspended in 3ml of complete medium and allowed to recover for 48 hours

3.2.8.2 Selection of stable S2 cell lines

Following the recovery of cells after transfection in complete medium (48 hours), cells were pelleted by centrifugation and re-plated in 3ml of selection medium (complete medium with 300µg/ml hygromycin B, table 3.10) into the same vessel. Selection medium was replaced every 4 days and hygromycin-resistant cells started to expand 2 to 3 weeks post-transfection. This was assessed by applying selection medium to untransfected S2 cells. Hygromycin B is an aminocyclitol antibiotic that strongly inhibits protein synthesis through an action on mRNA translation (Cabanas *et al.*, 1978). The hygromycin-resistance vector (pCoHYGRO) used in these experiments contains a gene encoding for hygromycin B phosphotransferase (Hph). This enzyme catalyses the phosphorylation of the 4-hydroxyl group on the cyclitol ring of hygromycin, producing 7'-O-phosphoryl-hygromycin B which lacks biological activity (Pardo *et al.*, 1985). Hygromycin-resistant S2 cells were split 1:2 upon reaching confluency (6-20 x 10^6 cells/ml), and frozen stocks were prepared after multiple passes and expansion of the stable cell line in hygromycin-containing complete medium. Cell viability was assessed visually or by trypan blue assay: 0.1ml of cell suspension (1 x 10^6 cells/ml, no serum) was mixed with 0.1ml 0.4% [w/v]

trypan blue solution. Trypan blue is excluded from viable cells, and cells were counted and assessed with a haemocytometer.

3.2.8.3 Expression and detection of recombinant protein from stable cell lines

For the expression and purification of recombinant proteins, Stable S2 cell lines were transferred to DES serum free expression medium (see table 3.10) and incubated at 26°C. Expression of recombinant proteins was induced by the supplementation of $CuSO_4$ to a final concentration of 500μM, inducing expression from the metallothionein (MT) promoter of the transfected pMT-BiP expression vector (Bunch *et al.*, 1988). S2 cells transfected with the pCoHYGRO vector only were used as a negative control. For the harvest of conditioned medium, stable cell lines were plated at a density of 4-10 x 10^6 cells/ml in 15ml serum free medium in 75cm^2 flasks with vented lids. Media was harvested every 3-5 days by centrifugation (1000 x g for 4 minutes), or when plates reached confluency. S2 cells are viable in serum free medium up to a density of 30 x 10^6 cells/ml. After harvest of conditioned medium cells were re-plated at a density of 4-10 x 10^6 cells/ml in fresh DES expression medium. 30μl aliquots of conditioned medium were analysed by SDS-PAGE using 15% [w/v] Tris-Glycine slab gels (acrylamide and bis-acrylamide from National Diagnostics). The SDS-PAGE methods used are detailed in chapter 2, section 2.2.9. Recombinant proteins were detected using silver staining for total protein (chapter 2, section 2.2.12) and Western blottting (as described in section 2.2.9) with a monoclonal antibody specific to the FLAG detection/purification epitope at the N-terminus of recombinant proteins, anti FLAG-M2 AP-conjugate (Sigma). This was used at a concentration of 1:1000 in 1% [w/v] BSA diluted in TSA. A FLAG fusion protein, rAgg1 (recombinant aggrecan-1), was used as a positive control for blotting and was supplied by Dr. Clare Hughes (Hughes *et al.*, 1997).

3.2.9 Purification of recombinant PRG4 domains from the conditioned medium of stable S2 cell lines

Conditioned media was passed through 0.22μm filters and dialysed against TBS (table 3.1) with repeated changes before immunoaffinity chromatography using an anti-FLAG M2 affinity gel (Sigma). For each purification, FLAG affinity gel (1ml) was equilibrated with 10ml TBS (see table 3.1) prior to pre-elution with 5ml glycine-HCl (pH 3.5) and re-equilibration in TBS. The FLAG affinity gel was then mixed with TBS and added to 40ml of conditioned medium and

incubated at 4°C for 1 hour on an orbital mixer. The immunoaffinity resin was collected by passing the mixture over an empty 10ml chromatography column (BioRad) that had previously been washed in TBS. Vessels containing the mixture of conditioned medium and affinity gel were washed with TBS to remove the remaining affinity gel, and this TBS was passed over the column. The resin bed was washed with 20ml TBS to remove unbound proteins, and the efficiency of washing was determined by reading absorbance values at 280nm. Bound protein was eluted with 5 x 1ml aliquots of glycine-HCl (pH 3.5), into vials containing 25µl of 1.5M Tris-HCl (pH 8.8). The concentration of eluents was calculated by reading absorbance values at 280nm, and protein species were separated by SDS-PAGE and analysed by Western blot and silver stain. FLAG M2 agarose was washed with 20ml TBS before washing in 10 column volumes of storage buffer (50% [v/v] glycerol with TBS containing 0.02% sodium azide). 5ml of storage buffer was added to the column, which was stored at 4°C without draining.

3.2.10 CHO-MegN141 cell line

The CHO-MegN141 cell line, supplied by Wyeth, was generated to express human PRG4 from the initial Gln^{1} residue to Asn^{141} as a secreted protein from a stably transfected Chinese hamster ovary (CHO) cell line. Cells were cultured and conditioned serum free media was collected as described in chapter 2, section 2.2.4. Conditioned medium was concentrated using a Centriplus centrifugal filter device (Millipore) with a YM10 cut-off membrane to retain macromolecules of over 10 kDa. 30 mls of media was passed over the filter by adding 10 ml at a time and spinning for 1.5 hours at 3000 x g at 4°C. This process was repeated until all the media was passed over the column. The proteins in concentrated media were separated by SDS-PAGE as described in chapter 2, section 2.2.9 and subjected to a Western blot probed with a polyclonal antibody to exon 3 of human PRG4.

3.3 Results

3.3.1 Amplification of cDNAs for human and bovine N- and C-terminal PRG4

Previous studies have deduced the complete cDNA sequence (GenBank Accession No. U70136) encoding for human MSF precursor (Merberg *et al.*, 1993), which is homologous to PRG4. The partial cDNA sequence (GenBank Accession No. AF056218) encoding the C-terminal 401 amino

acids of bovine articular cartilage PRG4 (Flannery *et al.*, 1999). In this study the human MSF precursor sequence was used to design primers N1 and N2, specific for exons 2 to 5 of MSF precursor/PRG4 (amino acids Gln^{25}-Lys^{199}). Exon 1 was not included, as this is the secretion signal for PRG4, and the expression system used in this study has an expression vector with a built-in BiP secretion signal (reviewed in Gething, 1999). PCRs performed using cDNA from bovine chondrocyte monolayer and human cartilage explant cultures generated products of the expected size of 525bp (figure 3.2, lanes 1 and 3, respectively).

The human MSF precursor sequence was also used to design primers HC1 and HC2, specific to exons 7-12 (amino acids Asp^{1441}–Pro^{1404}) of human MSF precursor/PRG4, and PCR with human cartilage cDNA generated a product of the expected size of 792bp (figure 3.2, lane 4). Previously identified cDNA sequence encoding the C-terminal portion of bovine articular cartilage PRG4 was used to design primers BC1 and BC2, specific for exons 7-12 of bovine PRG4. RT-PCR amplification using bovine articular cartilage chondrocyte cDNA as a template produced a fragment with the expected size of 792bp (figure 3.2, lane 2).

3.3.2 Sequencing of cDNAs for PRG4 N-terminal and C-terminal structural domains

The cDNAs encoding human and bovine N-terminal (exons 2-5) and C-terminal (exons 7-12) PRG4 were ligated into the TA-cloning vector pTAg and sequenced using the M13 forward and reverse primers listed in table 3.8. The sequences obtained were subjected to BLAST searches (Altschul *et al.*, 1997) and their homology with existing MSF precursor/PRG4 cDNA sequences was confirmed. Sequence data was deposited in GenBank as detailed in table 3.12. Novel bovine cartilage PRG4 N-terminal cDNA sequence (^{29}Ser-^{191}Glu) was deposited to Genbank under the Accession No. AY397646, and the position of this sequence is shown in figure 3.3. The bovine amino acid sequence displays a homology of 86% with the human sequence. Other sequences were almost completely homologous to already published PRG4 sequence data.

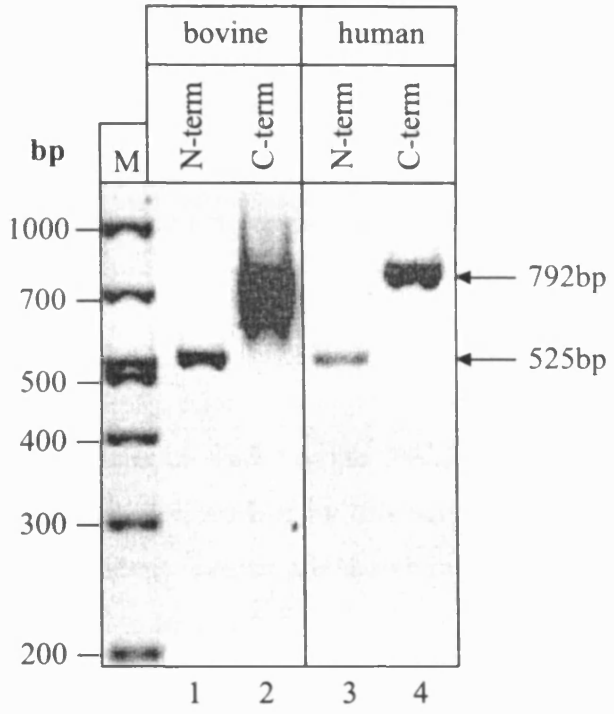


Figure 3.2 – RT-PCR analyses of bovine and human chondrocyte cDNA using primers specific for N-terminal (exons 2-5) and C-terminal (exons 7-12) PRG4. The primer pair N1/N2, designed using human N-terminal PRG4 sequence, was used in lanes 1 and 3 to amplify human and bovine PRG4 DNA, respectively. Primer pairs BC1/BC2 and HC1/HC2, designed using bovine and human C-terminal PRG4 sequence, were used in lanes 2 and 4, respectively. The migration positions of PRG4 N-terminal and C-terminal cDNAs are indicated by arrows on the right. Migration of size standards is shown on the left.

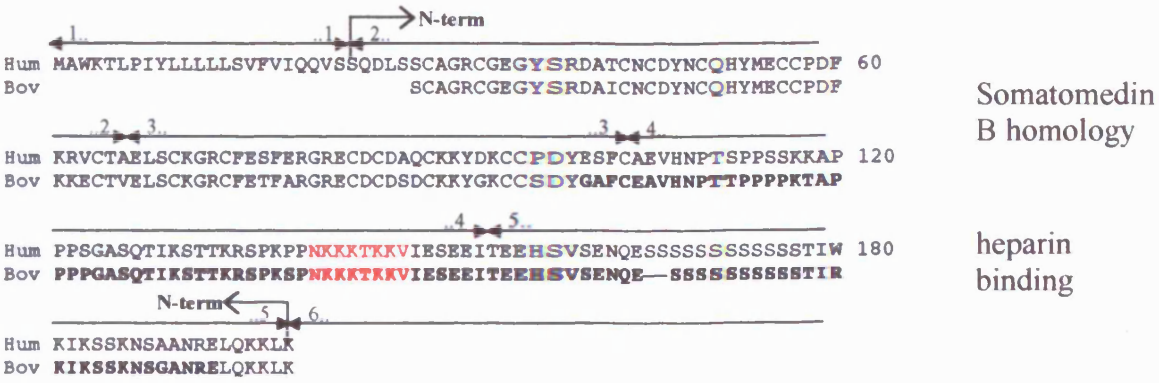


Figure 3.3 – Alignment of human and bovine PRG4 N-terminal domain amino acid sequences. Bovine PRG4 sequence identified by this study (Genbank Acc. No. AY397646) is highlighted in bold. Heparin-binding regions are shown in red. Sources of the other human and bovine PRG4 sequence are listed in table 3.3.

Table 3.12 – PRG4 sequence data obtained during this study, along with their GenBank Accession numbers.

Species and PRG4 domain	PRG4 region	GenBank Acc. No.
Bovine N-terminal (exons 2-5)	^{29}Ser-^{191}Glu	AY397646
Human N-terminal (exons 2-5)	^{29}Ser-^{193}Glu	AY653037
Bovine C-terminal (exons 7-12)	^{1146}Gly-^{1395}Ser	AY536119
Human C-terminal (exons 7-12)	^{1148}Gly-^{1397}Ser	AY653038

3.3.3 Generation of expression constructs for human and bovine PRG4 domains

The expression system chosen for the purpose of producing recombinant PRG4 domains was the *Drosophila* Expression System from Invitrogen, which utilises a cell line derived from *Drosophila melanogaster* Schneider 2 (S2) cells and a simple plasmid vector. There are a number of vectors available for use with this system, for this application the pMT-BiP-V5/His plasmid was used. This vector allows inducible expression from the metallothionein (MT) promoter, and inserts can be cloned in-frame with an N-terminal *Drosophila* secretion signal sequence (BiP) which causes recombinant proteins to be secreted into the culture medium of transfected S2 cells. The expression vector also encodes a C-terminal peptide containing the V5 epitope for antibody detection and a polyhistidine (6xHis) tag for purification, but in this study these features were not used, and a stop codon was included in the reverse expression primers. Instead, an 8 amino acid FLAG® epitope (AspTyrLysAspAspAspAspLys) was included in forward expression primers to facilitate the production of PRG4 domains as N-terminal FLAG fusion proteins, which can be detected and purified using anti-FLAG antibodies. The FLAG epitope was specifically designed for immunoaffinity chromatography and contains an enterokinase cleavage site (reviewed in Einhauer & Jungbauer, 2001). 'Expression' primer sets were designed in order to manipulate PRG4 domains into this system. These comprised of the regular primers as used in figure 3.2, with added features including a FLAG epitope sequence in the forward primer and a stop codon in frame with PRG4-coding sequence in the reverse primer. The expression primers also included NcoI (forward) and XhoI (reverse) restriction sites to enable the insertion of restriction enzyme-digested PCR products into the pMT-BiP vector. PRG4 cDNA sequence was checked for NcoI and XhoI sites using MacDNAsis software (Hitachi). Figure 3.4 shows the results of PCR using human and bovine chondrocyte cDNA and pTAg cloning vectors with PRG4 inserts as template material. PRG4 primer pairs containing only PRG4 sequence (1), and modified 'expression' primer pairs (2).

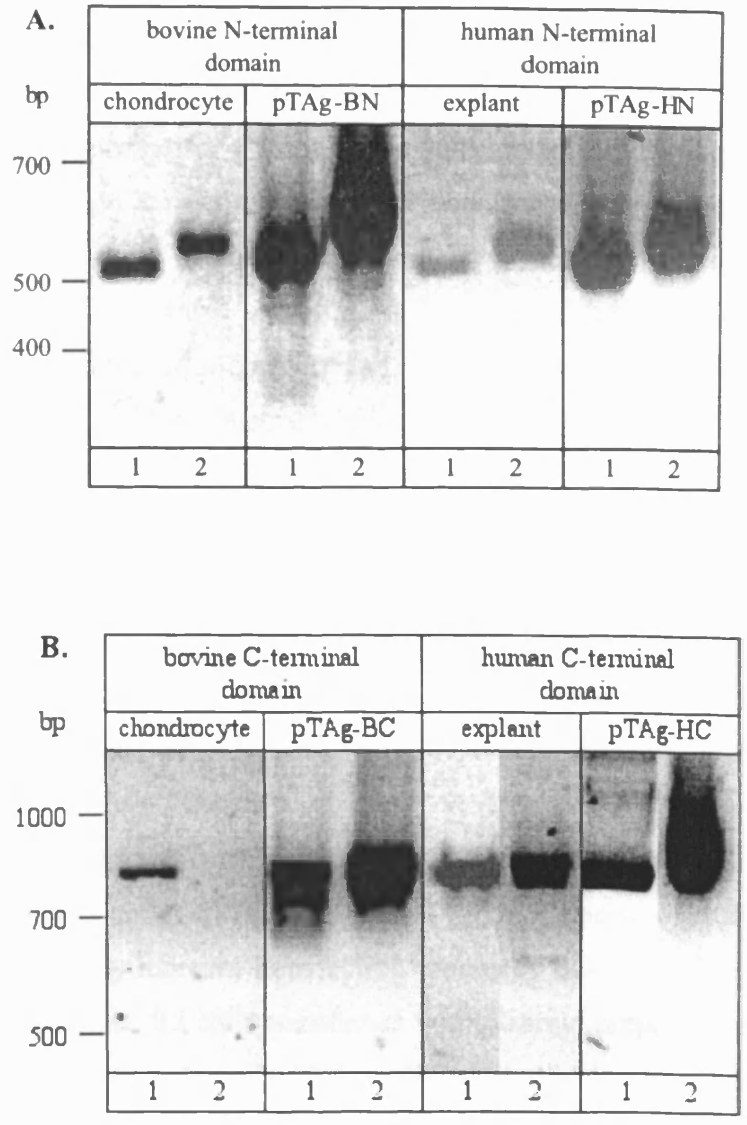


Figure 3.4 – PCR analyses of bovine chondrocyte and human cartilage explant cDNA (RT samples) and TA-cloned pTAg-PRG4 domain constructs for the bovine and human N- and C-terminal domains of PRG4. In each case conventional (A) and 'expression' (B) primer sets were used. Expression primers incorporated the modifications necessary to ligate the PCR products into the pMT-BiP expression vector in order to express FLAG-tagged recombinant PRG4. The migration of molecular size standards is shown on the left.

As shown in figure 3.4, in most cases PCR products of the correct size were generated with expression primers when using bovine or human cDNA samples as a template, but in the case of bovine C-terminal PRG4 a fragment was only generated when using the pTAg vector with inserted bovine PRG4 sequence as a template (figure 3.4, lower left panel). The cDNA products resulting from PCRs with the 'expression' primer sets were subsequently gel purified, digested with NcoI and XhoI, gel purified for a second time and ligated into the expression vector pMT-BiP. To enable this ligation, the expression vector was digested with NcoI and XhoI and gel purified. Plasmid preparations of transformed colonies were sequenced with the supplied sequencing primers MT-F (forward) and BGH-R (reverse) to verify that the inserted PRG4-expressing domains contained no mutations and were in frame with the BiP secretion signal included in the vector. Expression constructs are shown in figure 3.5 after digestion with either one or both of the restriction endonucleases used to cut the polylinker region of the expression vector prior to insertion of the PRG4 cDNA, with unmanipulated pMT-BiP vector as a control (lanes 9 & 10). In the first lane in each case the vector is digested with XhoI alone yielding a linearised product of around 3.6kb, whereas in the second lane the construct has been digested with NcoI and XhoI, and the PRG4 cDNAs of 562 (N-terminal, lanes 2 & 4) and 829bp (C-terminal, lanes 6 & 8) are apparent.

After the expansion of expression construct LB broth cultures, plasmids were purified using a kit designed for the removal of endotoxin (EndoFree, QIAgen). Removal of endotoxin from plasmid preparations can dramatically increase transfection efficiency in many cell lines. This effect was apparent in these experiments: S2 cells transfected with plasmid preparations without endotoxin removal steps were not viable under selection conditions, indicating poor transfection efficiency (data not shown). The expression constructs generated by this study and their associated PRG4 domains are listed in table 3.13.

Table 3.13 – List of PRG4 expression constructs

Construct name	PRG4 domain
pMT-BN	Bovine N-terminal (exons 2-5)
pMT-HN	Human N-terminal (exons 2-5)
pMT-BC	Bovine C-terminal (exons 7-12)
pMT-HC	Human C-terminal (exons 7-12)

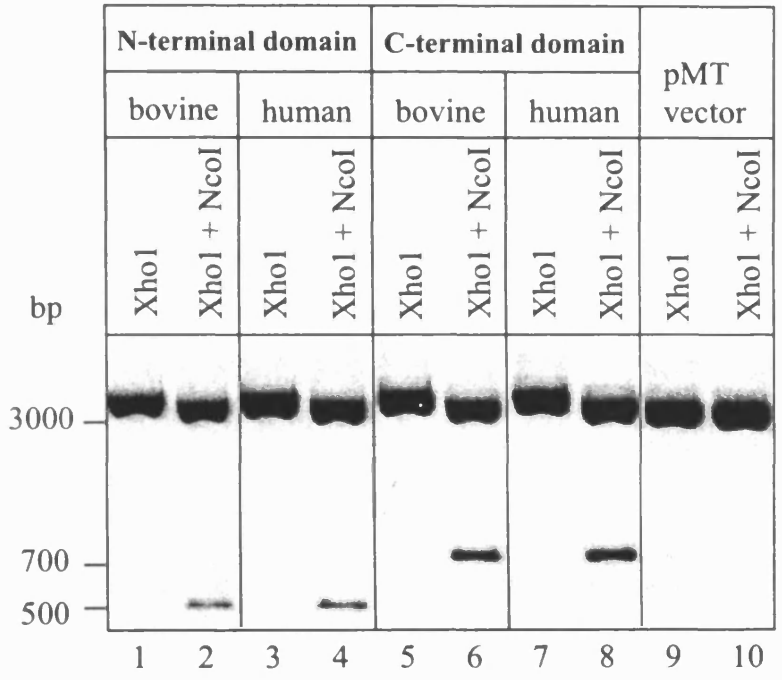


Figure 3.5 – Restriction digestion analysis of bovine and human N- and C-terminal domain PRG4 expression constructs. One or both of the endonucleases NcoI and XhoI was used, which were the endonucleases used to insert the PRG4 PCR product into the pMT expression vector (3.6 kb). Linearised vector can be seen after digestion with XhoI. Excised PRG4 domains (562bp for N-terminal domain, 815bp for C-terminal domain) can be seen after digestion with XhoI and NcoI. Migration of size standards is shown to the left.

3.3.4 Generation of stable, PRG4 domain-expressing S2 cell lines

To facilitate the large-scale production of recombinant proteins from stable S2 cell lines, *Drosophila* S2 cells were transfected with PRG4 domain expression constructs as listed in table 3.12 along with the selection vector pCoHYGRO (see appendix A). Following the seeding of transfected cells into selection medium containing hygromycin B, resistant cells started to grow out after 2-3 weeks. Selection was monitored by culturing untransfected S2 cells in selection medium and monitoring cell death visually and by trypan blue assay. Stocks of stable cell lines were prepared for long-term storage before expansion into serum free medium for analysis of recombinant protein expression. Hygromycin-resistant cell lines were seeded into serum free medium at a density of 8 x 10^6 cells/ml and induced with 500μM $CuSO_4$, and conditioned medium was harvested after 5 days.

Figure 3.6 shows a silver stain and a Western blot of conditioned medium (30μl) probed with anti-FLAG M2 mAb after separation under native (3.6A) and reduced (3.6B) conditions. Production of FLAG-tagged recombinant PRG4 domains was seen in 3 cell lines: S2-HN (human N-terminal PRG4, exons 2-5), S2-BN (bovine N-terminal PRG4, exons 2-5) and S2-BC (bovine C-terminal PRG4, exons 7-12). A stable S2-HC line was generated (human C-terminal PRG4, exons 7-12), but under the conditions used did not appear to express any detectable recombinant human C-terminal PRG4 protein. An S2 cell line transfected with only the selection vector pCoHYGRO was generated as a negative control and did not secrete any FLAG-tagged protein (data not shown). Table 3.14 details the nomenclature of all S2 cell lines generated by this study and their expressed proteins.

Table 3.14 – Nomenclature of PRG4 domain-expression stable *Drosophila* S2 cell lines

Cell line	Expression construct	PRG4 domain	Expressed protein
S2-BN	pMT-BN	Bovine N-terminal (exons 2-5)	BovN-PRG4
S2-HN	pMT-HN	Human N-terminal (exons 2-5)	HumN-PRG4
S2-BC	pMT-BC	Bovine C-terminal (exons 7-12)	BovC-PRG4
S2-HC	pMT-HC	Human C-terminal (exons 7-12)	-
S2-Hyg	-	-	-

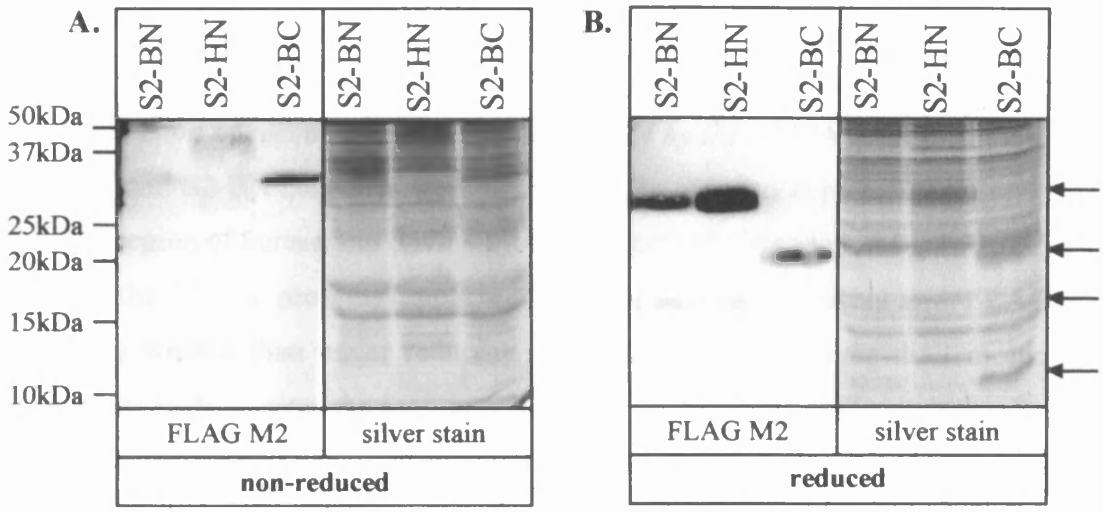


Figure 3.6 – Western blot and silver stain analysis of serum free conditioned media (30µl) from the S2-HN, S2-BN and S2-BC cell lines. Cell lines were seeded into serum free conditioned medium at a density of 6 x 10^6 cells/ml and induced for 72 hours in the presence of 500µM $CuSO_4$. Proteins were separated under native (A) and reducing (B) conditions. The Western blot was probed with anti FLAG M2 mAb. Migration of size standards is shown to the left. The arrows to the right indicate the migration positions of recombinant PRG4 proteins.

3.3.5 Production of recombinant N-terminal PRG4 by the S2-HN and S2-BN cell lines

The S2-HN and S2-BN cell lines were established in order to express recombinant N-terminal (exons 2-5) region of human and bovine PRG4, respectively. As seen in figure 3.6A, under native conditions the FLAG proteins appeared as high MW aggregates, and FLAG staining is considerably weaker than under reducing conditions (3.6B), making their molecular weights more difficult to determine. As seen in figure 3.6B, SDS-PAGE under reducing conditions and Western blotting enabled the visualisation of two FLAG-positive proteins with molecular weights of 27 and 16 kDa in the conditioned medium of the S2-HN and S2-BN cell lines after induction with 500μM $CuSO_4$. Both the S2-BN and the S2-HN cell lines appeared to express more of the 27 kDa species, based on the strength of FLAG-immunopositive staining. The predicted molecular weights of HumN and BovN were calculated using a web-based protein sequence analysis program (Stothard, 2000), and were found to be 20.92 kDa and 20.5 kDa, respectively. These are based on sequences of 187 (HumN) and 185 (BovN) amino acids including the 8 residue FLAG tag and the 4 residues (RSPW) of the expression vector polylinker region that is between the vector BiP signal sequence and the inserted PCR product. However, the cell lines S2-BN and S2-HN each appeared to secrete two FLAG-immunopositive proteins that migrated with apparent molecular weights of 27 and 16 kDa under reducing conditions (figure 3.6B). This discrepancy in predicted and apparent molecular weight(s) of recombinant proteins may be due to post-translational modifications, degradation within the culture system or anomalous migration in SDS-PAGE. The observation that both human and bovine recombinant proteins run at similar molecular weights would indicate that post-translational modifications for recombinant human and bovine N-terminal (exons 2-5) PRG4 are similar in this expression system. Discrepancies between the predicted and apparent molecular weights of recombinant PRG4 N-terminal proteins and the presence of two protein species is observed elsewhere. The CHO-MegN141 cell line (Wyeth) was generated to express human PRG4 from the initial ^{1}Gln residue to ^{141}Asn, a 117-residue protein with a predicted molecular weight of 12.96 kDa (Stothard, 2000). In figure 3.7, a Western blot of concentrated conditioned medium from this cell line (under reducing conditions) was probed with a polyclonal antibody raised to a peptide from exon 3 of human PRG4, and the antibody detected two protein species of ~21 and 18 kDa.

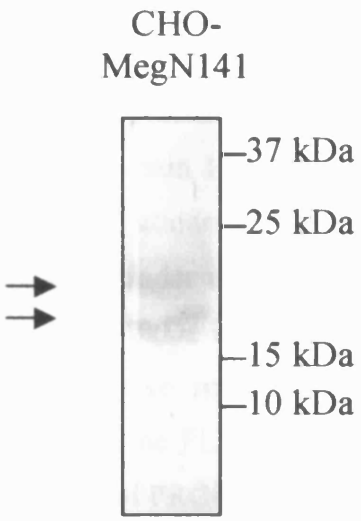


Figure 3.7 – Western blot analysis of concentrated conditioned medium from the CHO-MegN141 cell line (Wyeth). This cell line secretes human PRG4 N-terminal domain Gln^{1} to Asn^{141}). 30ml of conditioned medium was concentrated to 66µl, a concentration factor of 454. 20µl of the concentrated medium was run on a 15% (w/v) SDS-PAGE gel under reducing conditions, and a Western blot was probed with a polyclonal antibody to exon 3 of human PRG4. This Western blot illustrates the atypical migration of N-terminal recombinant PRG4 molecule. Migration of molecular size standards is shown to the right.

3.3.6 Production of recombinant C-terminal PRG4 by the S2-BC cell line

Figure 3.6 also shows a 30µl aliquot of conditioned medium from the cell line S2-BC after separation by SDS-PAGE and detection by silver stain and Western blotting with anti-FLAG M2 antibody. The S2-BC cell line was expected to secrete recombinant bovine C-terminal PRG4 from the start of exon 7 to the end of exon 12 (BovC). The predicted molecular weight of the entire BovC protein is 31.77 kDa (277 amino acid residues, including FLAG and 4 residues of vector polylinker) (Stothard, 2000). Under non-reducing conditions (figure 3.6A), a FLAG-immunopositive protein species of ~30kDa is apparent. However, under reducing conditions (figure 3.6B) it was apparent that two recombinant proteins are produced, the FLAG-immunopositive BovC1 (20 kDa) and the FLAG-immunonegative species BovC2 (12 kDa). If both proteins represent the C-terminal of PRG4 it suggests that a splice mechanism or proteolysis is causing BovC to be visible as two separate molecules under reducing conditions. BovC1 and BovC2 appeared to be associating under native conditions resulting in the appearance of a FLAG-immunopositive protein species of ~30 kDa, the approximate sum of the apparent molecular weights of BovC1 (20 kDa) and BovC2 (12 kDa).

3.3.7 Effect of $CuSO_4$ concentration on the secretion of recombinant proteins

To assess the levels of protein secretion over time, and the effect of differing concentrations of the inducing agent $CuSO_4$ on the levels of protein secretion, cell lines were seeded into serum free medium at a density of 6×10^6 cells/ml and induced with $CuSO_4$ concentrations in the range of 0-1000µM. Aliquots of conditioned media (30µl) were removed at 24, 48 and 72 hour intervals. Proteins were visualised by means of SDS-PAGE under reducing conditions using 15% gels (30µl media/lane) and Western blotting with anti-FLAG M2 monoclonal antibody, and in the case of the S2-BC cell line, silver staining. The effect of increasing $CuSO_4$ concentrations on the production of FLAG-tagged protein by the three S2 cell lines is seen in figures 3.8 (S2-HN line), 3.9 (S2-BN line), and 3.10 (S2-BC line). These results show that optimal secretion of recombinant proteins occurred with a $CuSO_4$ concentration of between 500-1000µM, based on intensity of staining. Levels of protein increased significantly over a 72 hour culture period. Both species of BovC protein were apparent (figure 3.10), and increased with equal intensity of staining in the 72 hour culture period. However, only the 27 kDa species was detected in the culture medium of the S2-HN and S2-BN cell lines (figures 3.8 and 3.9).

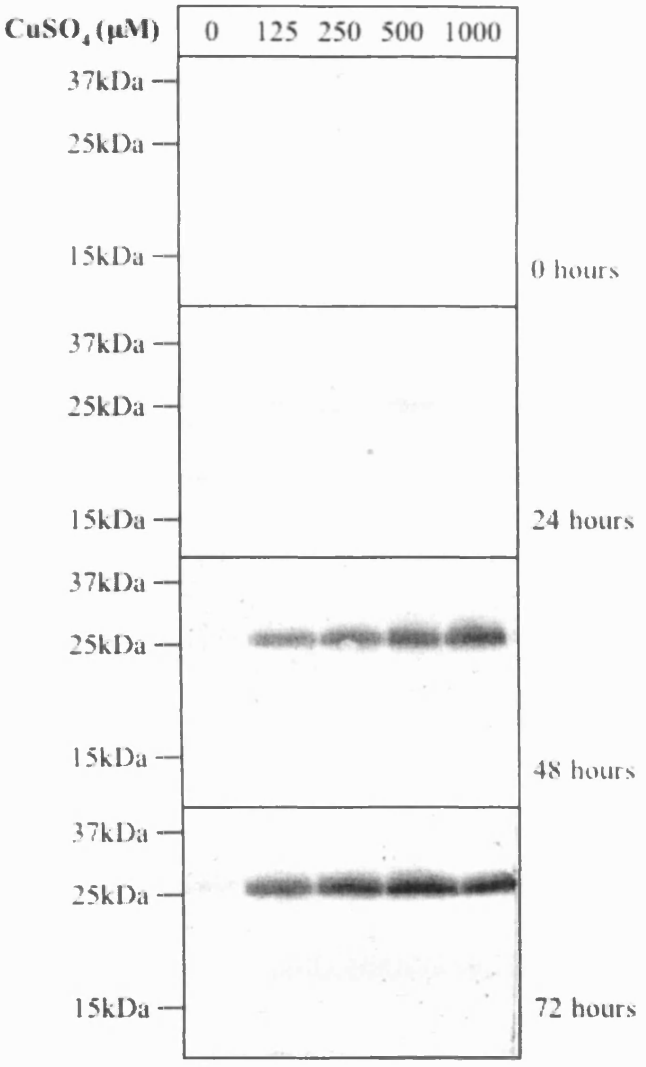


Figure 3.8 – Western blot analysis of recombinant human N-terminal (exons 2-5) PRG4 domain expressed by the stable *Drosophila* S2 cell line S2-HN. Stably transfected cells were seeded at a density of 6 x 10^6 cells/ml and treated with different concentrations of inducing agent ($CuSO_4$). Recombinant protein levels in 30μl of conditioned media was analysed over a 72 hour period by SDS-PAGE (reducing conditions) and Western blot probed with an anti-FLAG M2 antibody. Migration of standards is shown to the right.

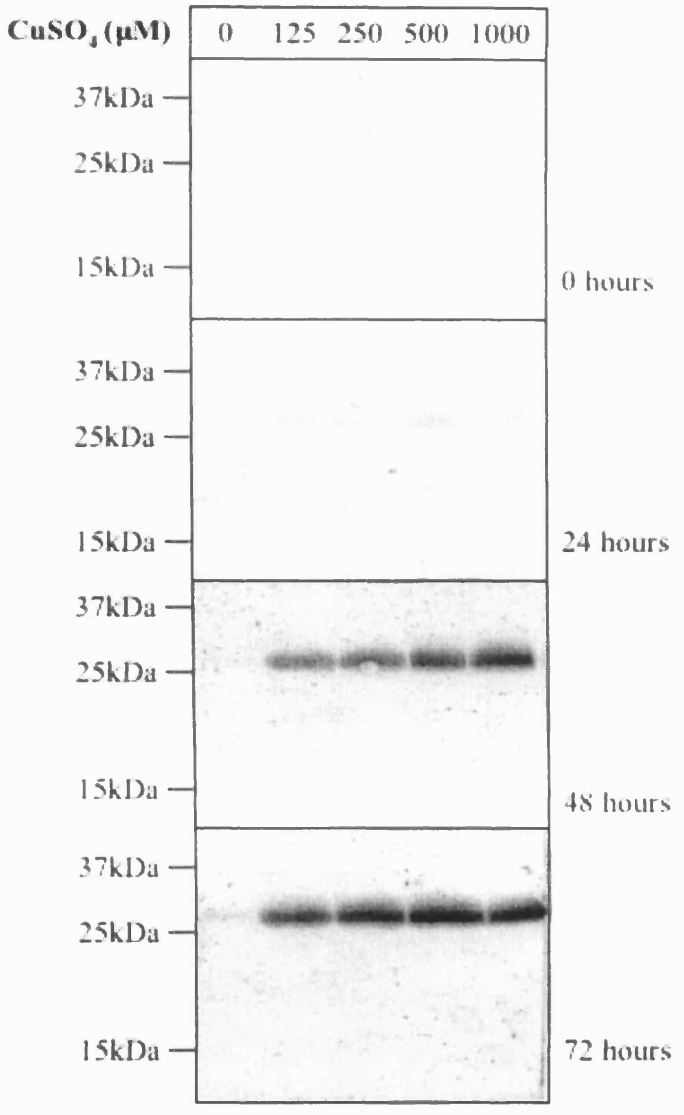


Figure 3.9 – Western blot analysis of recombinant bovine N-terminal (exons 2-5) PRG4 domain expressed by the stable *Drosophila* S2 cell line S2-BN. Stably transfected cells were seeded at a density of 6 x 10^6 cells/ml and treated with different concentrations of inducing agent ($CuSO_4$). Recombinant protein levels in 30μl of conditioned media was analysed over a 72 hour period by SDS-PAGE (reducing conditions) and Western blot probed with an anti-FLAG M2 antibody. Migration of standards is shown to the right.

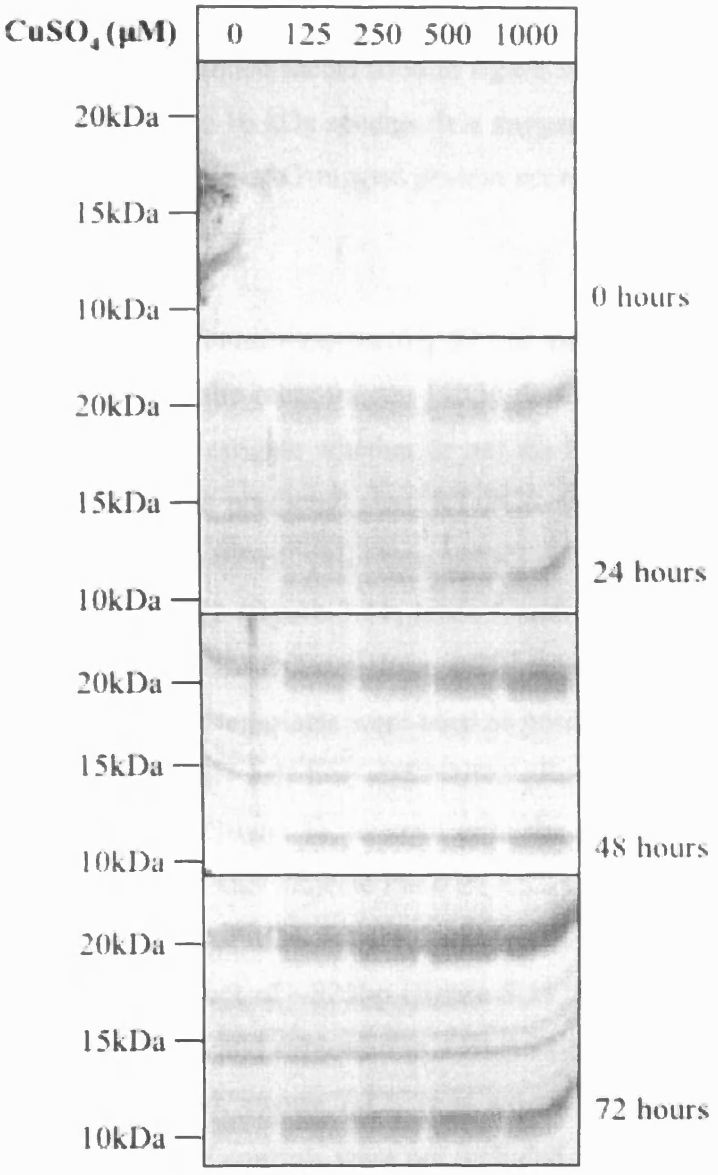


Figure 3.10 – Analysis of recombinant bovine C-terminal (exons 7-12) PRG4 domain expressed by the stable *Drosophila* S2 cell line S2-BC. Stably transfectd cells were seeded at a density of 6 x 10^6 cells/ml and treated with different concentrations of inducing agent ($CuSO_4$). Recombinant protein levels in 30μl of conditioned media was analysed over a 72 hour period by SDS-PAGE (reducing conditions) and silver staining (30μl medium/lane). Migration of standards is shown to the right.

This may be due to the initial cell density being lower than for the blot shown in figure 3.6B, in which both the 27 and 16 kDa species were detected. Conditioned medium was also harvested sooner (72 hours) than for the conditioned media used in figure 3.6 (5 days), which could further account for the non-appearance of the 16 kDa species. It is suggested that the 16 kDa protein is a minor degradation production of the FLAG-tagged protein secreted by the S2-HN and S2-BN cell lines.

3.3.8 RT-PCR analyses of PRG4 domain-expressing S2 cell lines

The observed molecular weights of the recombinant PRG4 domains differed greatly from their predicted molecular weights. To investigate whether or not the PRG4-expressing stable S2 cell lines were expressing mRNA of the predicted size, RT-PCR analyses were performed. Total RNA was extracted from S2-HN and S2-BC cell lines, and RT-PCR experiments were conducted (figure 3.11) with the primers N1/N2 (figure 3.11, lanes 1 and 3) for the S2-HN cell line and BC1/BC2. (lanes 2 and 4) for the S2-BC cell line. PCR experiments using the expression constructs pMT-HN and pMT-BC as templates were used as positive controls (lanes 3 & 4). It is evident that RT-PCR with the S2-HN cell line and N1/N2 primers generates a product of the expected size of 525bp (figure 3.11, lane 1), as seen with the positive control (lane 3). This product was subsequently sequenced and subjected to a BLAST search, and was found to encode the N-terminal (exons 2-5) of human PRG4. RT-PCR experiments with the S2-BC cell line and BC1/BC2 primers generates a product of ~825bp (figure 3.11, lane 2), similar to the positive control. This product was sequenced and subjected to a BLAST search, whereupon it was found to encode bovine C-terminal PRG4, exons 7-12. It is worth noting that as these were not quantitative experiments, as GAPDH controls were not included. Also, these experiments do not provide any data that would detect the expression of splice variants in the stable cell lines, which could also explain the secretion of two proteins by each cell line where only one is expected. Splicing of the transfected PRG4 domain-expressing segment could be investigated by designing a series of primer pairs spanning each region.

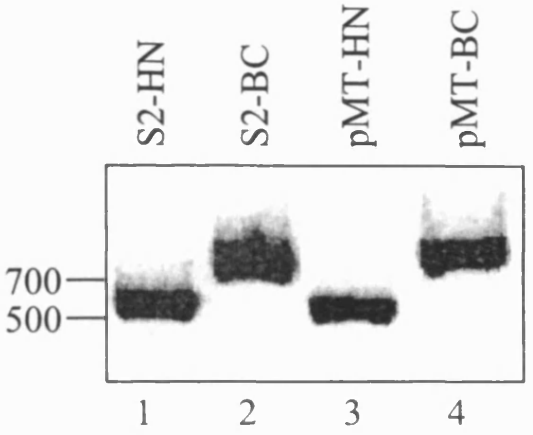


Figure 3.11 – RT-PCR analyses of RNA from stable *Drosophila* S2 cell lines expressing recombinant human N-terminal PRG4 (lane 1) and bovine C-terminal PRG4 (lane 2). For positive controls, PCRs were performed using pMT expression constructs as templates (lanes 3 and 4). The primer pairs used were N1/N2 (lanes 1 and 3) and BC1/BC2 (lanes 2 and 4). Migration of size standards is shown on the left.

3.3.9 Purification of recombinant bovine and human N-terminal and bovine C-terminal PRG4 by FLAG M2 immunoaffinity chromatography

The recombinant PRG4 proteins (HumN, BovN and BovC) produced in the S2 expression system all have an N-terminal FLAG epitope (DYKDDDDK). As demonstrated in the expression experiments, the FLAG epitope of these recombinant proteins can be detected on a Western blot by a commercial anti-FLAG M2 monoclonal antibody (Sigma), and this interaction was utilised to purify recombinant proteins from the conditioned media of induced cell lines using anti-FLAG M2 mAb covalently linked to an agarose support (Sigma), a technique first described by Brizzard *et al.* (1994). Yields of recombinant proteins were in the region of 2-5 mg/L for each of the S2 cell lines.

3.3.9.1 Purification of N-terminal PRG4 by FLAG immunoaffinity chromatography

Figure 3.12 shows the results of passing 40ml of conditioned serum free media from induced S2-BN cells over a FLAG-M2 agarose column and eluting bound protein under acidic conditions. Two BovN protein species of 27 and 16 kDa (BovN1 and BovN2) were purified to homogeneity as seen by silver staining (figure 3.12, eluent fractions 3-6). Both species were detected on a Western blot probed with anti-FLAG M2 mAb (figure 3.12, Western blot). Table 3.14 describes the protein concentrations of each eluent fraction and the amount loaded on to the gel. A similar result to the purification of bovine N-terminal PRG4 was obtained for the purification of human N-terminal PRG4 from the conditioned medium of the S2-HN cell line (data not shown).

3.3.9.2 Purification of C-terminal PRG4 by FLAG immunoaffinity chromatography

FLAG M2 immunoaffinity chromatography was also used to purify recombinant bovine C-terminal PRG4 (BovC) from the conditioned medium of the S2-BC cell line (figure 3.13). Two BovC protein species were purified with apparent molecular weights of ~20 and 12 kDa, visualised using silver staining after separation by SDS-PAGE under reducing conditions (figure 3.13, eluents 3-6). Table 3.15 describes the protein concentrations of each eluent fraction and the amount loaded on to the gel. Immunoblotting with FLAG M2 shows that only the 20 kDa species contains a FLAG epitope, and both the 20 kDa species and the FLAG-immunonegative 12 kDa species are purified by this process, which suggests that association between the two species may be occurring under non-reducing conditions.

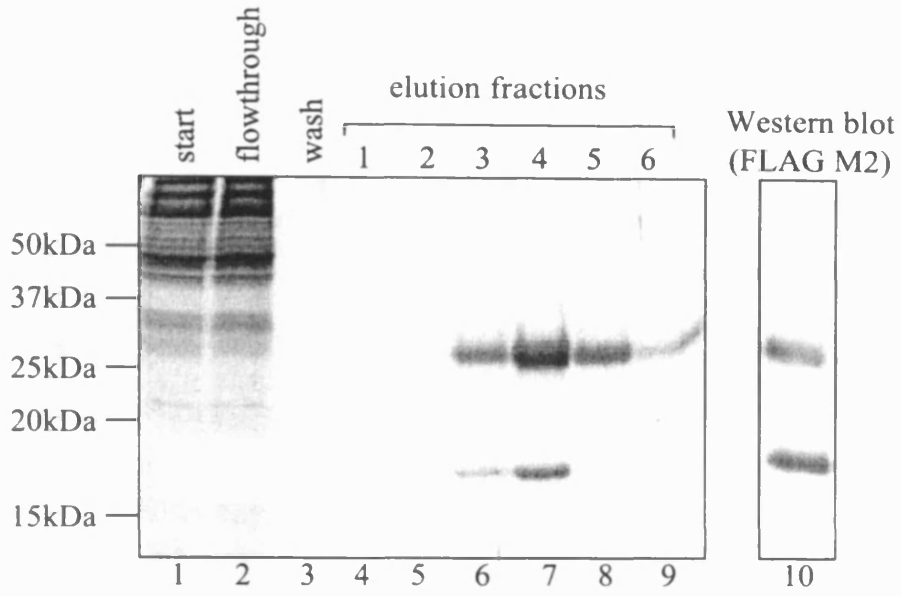


Figure 3.12 – FLAG M2 immunoaffinity chromatography purification of recombinant bovine N-terminal PRG4 domain (exons 2-5) from the conditioned medium of S2-BN cells. Conditioned medium from the *Drosophila* S2-BN cell line (40ml) was passed over a 1ml bed volume of anti-FLAG M2 agarose, and bound protein was eluted with 6 x 1ml aliquots of glycine-HCl, pH 3.5. Fractions were separated by SDS-PAGE under reducing conditions (30µl/lane) and visualised by silver staining (lanes 1-9). A Western blot of elution fraction 4 using anti-FLAG M2 mAb is shown to the right (lane 10), and migration of molecular size standards is shown to the left.

Table 3.15 – Calculation of the concentration of BovN FLAG column eluent fractions and protein loaded in figure 3.12, as assessed by absorbancy measured

Eluent	Concentration (mg/ml, A280)	Amount loaded (µg)
1	0.002	0.06
2	0.011	0.33
3	0.075	2.25
4	0.372	11.16
5	0.148	4.44
6	0.034	1.02

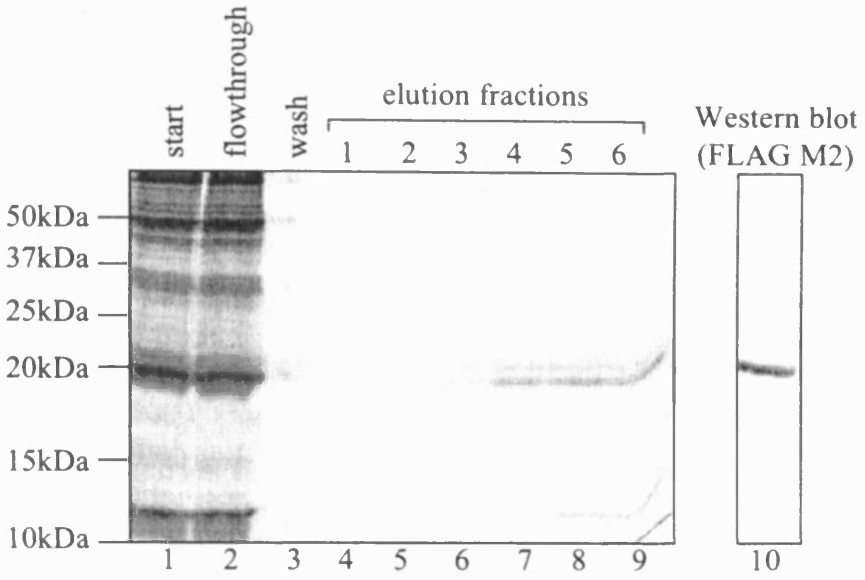


Figure 3.13 – FLAG M2 immunoaffinity chromatography purification of recombinant bovine C-terminal PRG4 domain (exons 7-12) from the conditioned medium of S2-BC cells. Conditioned medium from the *Drosophila* S2-BC cell line (40ml) was passed over a 1ml bed volume of anti-FLAG M2 agarose, and bound protein was eluted with 6 x 1ml aliquots of glycine-HCl, pH 3.5. Fractions were separated by SDS-PAGE under reducing conditions (30µl/lane) and visualised by silver staining (lanes 1-9). A Western blot of elution fraction 4 using anti-FLAG M2 mAb is shown to the right (lane 10), and migration of molecular size standards is shown to the left.

Table 3.16 – Calculation of the concentration of BovC FLAG column eluent fractions and protein loaded in figure 3.12, as assessed by absorbancy

Eluent	Concentration (mg/ml, A280)	Amount loaded (µg)
1	0	0
2	0.004	0.12
3	0.017	0.51
4	0.015	0.45
5	0.019	0.57
6	0.016	0.48

This is supported by the observation that when separated by SDS-PAGE, the BovC protein runs as a single band under non-reducing conditions (figure 3.6A) and 2 proteins under reducing conditions (figure 3.6B).

3.4 Discussion

In its alternate guises as cartilage superficial zone proteoglycan, lubricin, megakaryocyte stimulating factor and CACP protein, PRG4 has been studied using a number of different experimental approaches. These include the study of its homologies to other proteins (Merberg *et al.*, 1993; Ikegawa *et al.*, 2000; Flannery *et al.*, 1999), its ability to lubricate (Swann *et al.*, 1985; Jay *et al.*, 2001a), its expression in a number of healthy and diseased tissues including cartilage and tendon (Ikegawa *et al.*, 2000; Tudor, 2002; Rees *et al.*, 2002) and its alteration during inherited CACP syndrome (Marcelino *et al.*, 1999). However, to date there have been no investigations into the predicted functions of the N- and C-terminal domains of the molecule. PRG4 is made up of 12 exons, where exons 2-5 and 7-12 flank a central mucin domain, exon 6. The aim of this work was to produce recombinant PRG4 non-mucin domains and use them to investigate the functions of individual domains of PRG4 by using binding assays directed towards ECM molecules, and by conducting cell biology experiments. A recent example of the strategy of utilising a recombinant segment of an extracellular matrix protein in functional analyses is the N-terminal heparin-binding domain of thrombospondin-1, which was found to constitute an adhesive support for human umbilical vein endothelial cells (HUVECs), possibly mediated by syndecan-4 (Outeiro-Bernstein *et al.*, 2002). This chapter describes the successful production of stable *Drosophila* S2 cell lines that secrete human and bovine N-terminal (HumN & BovN) and bovine C-terminal (BovC) PRG4 domains as N-terminal FLAG fusion proteins (S2 cells also were transfected with an expression construct to produce the human recombinant C-terminal domain of PRG4, although no protein was detected in the conditioned medium of induced cells. The reason for the absence of recombinant protein secretion is unclear. Production and secretion was induced with $CuSO_4$, which stimulates expression of recombinant proteins from the metallothionein promoter region in the expression construct. The presence of an 8 amino acid FLAG tag (DYKDDDDK) at the N-terminus of these recombinant PRG4 proteins greatly facilitated their purification from crude insect cell culture medium. A wide variety of affinity tags are available to facilitate the detection and purification of recombinant proteins,

reviewed in (Terpe, 2003), but the FLAG-tag was used in this instance. The FLAG epitope of the tagged proteins was used for immunoaffinity purification using a commercially available anti-FLAG monoclonal antibody (M2) covalently attached to an agarose chromatography matrix. In addition, an anti-FLAG antibody conjugated to alkaline phosphatase has been used to immunolocate recombinant proteins in Western blotting experiments.

With each stable cell line, two recombinant proteins were identified by SDS-PAGE under reducing conditions and were subsequently purified by FLAG immunoaffinity chromatography, not the expected single protein. The presence of two proteins may be due to splice variants or partial degradation within the expression system. Degradation during purification may be a factor, but two species are usually apparent in freshly isolated conditioned media (figure 3.6). For the PRG4 N-terminal domain-expressing lines S2-HN and S2-BN, 'N1' and 'N2' FLAG-immunopositive proteins were purified, running on SDS-PAGE gels under reducing conditions at 27 and 16 kDa respectively. For the bovine PRG4 C-terminal domain-expressing cell line, S2-BC, two proteins BovC1 and BovC2 were purified by FLAG affinity chromatography that ran on reduced SDS-PAGE gels at 20 and 12 kDa, respectively. Only BovC1 is FLAG-immunopositive, which would indicate that there is association between the 2 species under non-reducing conditions in order for them to be co-purified by FLAG chromatography. This is supported by the observation that under non-reducing conditions, BovC is apparent as a single, FLAG-positive band of around 32 kDa.

Recombinant protein expression in a *Drosophila* S2 cell system has a number of advantages, and a variety of proteins have been expressed using *Drosophila* S2 cells including recombinant secreted, membrane and cytosolic proteins (Benting *et al.*, 2000), erythropoietin (Lee *et al.*, 2000), hepatitis B virus surface antigen (Deml *et al.*, 1999) and human glucagon receptor (Tota *et al.*, 1995). Cells are easily maintained at room temperature, do not require CO_2, have a doubling time of around 24 hours and do not require trypsinisation during passaging as they are loosely adherent and may grow in suspension. When grown in suspension to a high density, cell numbers can reach 30 x 10^7 cells/ml, which is 10-fold higher than other insect cell lines such as *Sf9* (Perret *et al.*, 2003), and there is growing interest in using insect cell culture for the industrial production of recombinant proteins (Ikonomou *et al.*, 2003). Cells can be maintained in the presence of the

inducing agent $CuSO_4$ for up to 4 months (Johansen *et al.*, 1989). Insect cells perform comparable post-and co-translational modifications compared to mammalian cells except for N-glycosylation: *Drosophila* N-linked glycosylation is less complex in that it is not trimmed and sialylated (Jarvis & Finn, 1995). Post-translational modifications of recombinant proteins, including glycosylation, may affect functionality. In some cases, the utilisation of commonly-used mammalian cell lines for recombinant protein expression, such as the CHO (Chinese hamster ovary, *Cricetulus griseus*) or COS (African green monkey kidney, *Cercopithecus aethiops*) lines, may be more appropriate for functional assays of mammalian recombinant proteins. The characterisation of the purified recombinant proteins described in this chapter is further described in chapter 4, and their utilisation in proteinase digestion experiments and functional analyses is described in chapters 5 and 6, respectively.

3.5 Chapter Summary

- The N-terminal (exons 2-5) and C-terminal (exons 7-12) cDNAs of human and bovine PRG4 were successfully amplified by PCR and ligated into a TA cloning vector. cDNAs were sequenced and a number of sequences were deposited in Genbank.
- Expression constructs were manufactured in order to produce human and bovine N- and C-terminal PRG4 domains in *Drosophila* S2 cells as fusion proteins containing an N-terminal FLAG purification/detection epitope.
- Stable cell lines were generated for the expression of human and bovine N-terminal PRG4 (termed HumN and BovN) and bovine C-terminal PRG4 (BovC)
- Recombinant proteins were purified to homogeneity by FLAG immunoaffinity chromatography. Their predicted molecular weights, apparent molecular weights and FLAG immunoreactivity are listed in table 3.17.

Table 3.17 – Recombinant PRG4 domains produced by this study

Cell line	Species	Exons	Predicted molecular weight	Apparent molecular weights (reduced, 15% SDS-PAGE)	N-terminal FLAG epitope (assessed by Western blot)
S2-HN	Human	2-5	20.9 kDa	27 kDa 16 kDa	+ +
S2-BN	Bovine	2-5	20.5 kDa	27 kDa 16 kDa	+ +
S2-BC	Bovine	7-12	31.7 kDa	20 kDa 12 kDa	+ -

4. CHARACTERISATION OF RECOMBINANT PRG4 DOMAINS

4.1 Introduction

Chapter 3 describes the cloning, production and purification of recombinant PRG4 domains. However, these proteins did not correspond to their predicted molecular weights, and two recombinant protein species were secreted by each stable *Drosophila* S2 cell line, rather than a single species. Before embarking on further studies with these proteins, such as their ability to interact with other matrix molecules in binding assays and their activity in cell biology systems (chapter 6), it was important to further characterise these recombinant proteins. This was achieved using several approaches: (i) by studying the interactions of recombinant PRG4 domains with a panel of antibodies raised against PRG4; (ii) N-terminal amino acid sequencing; (iii) study of the heparin-binding capability of the molecules; and (iv) by studying post-translational modifications by means of lectin affinity blotting and enzymatic deglycosylation.

4.2 Materials and Methods

4.2.1 Materials

All chemicals were of an analytical grade and were purchased from Sigma-Aldrich unless otherwise indicated. A detailed list of the materials used in this research project and their suppliers is listed in Appendix A. Sequencing-grade PVDF transfer membrane was purchased from Millipore. The enzymatic deglycosylation (EDEGLY) kit was purchased from Sigma. Recombinant enterokinase was from Calbiochem. Other materials are listed earlier in this thesis, and section references are provided in the relevant methods sections within this chapter.

4.2.2 Purification of recombinant PRG4 domains by heparin-agarose affinity chromatography

The recombinant PRG4 domains purified by FLAG immunoaffinity chromatography (chapter 3, section 3.3.8) were subjected to heparin-agarose affinity chromatography as described in chapter 2, section 2.2.5. In addition, heparin-binding proteins were also concentrated from the conditioned media of the stable cell lines S2-HN, S2-BN and S2-BC by heparin-agarose affinity chromatography. Fractions were separated by SDS-PAGE under reducing conditions (chapter 2,

section 2.2.9) using 15% slab gels and proteins were visualised by silver staining for total protein (chapter 2, section 2.2.12) and Western blot probed with an anti-FLAG M2 AP-conjugate mAb (chapter 3, section 3.2.8.3) to detect recombinant FLAG-tagged PRG4 domains.

4.2.3 Protein sequencing

Proteins were concentrated from the conditioned media of stable *Drosophila* S2 cell lines S2-HN, S2-BN and S2-BC (described in chapter 3) by heparin affinity chromatography as described in chapter 2, section 2.2.5. Fractions were separated by SDS-PAGE under reducing conditions using 15% slab gels, and proteins were then transferred to sequencing-grade polyvinylidene fluoride (PVDF) transfer membrane (Immobilon-P^{SQ}, Millipore) as described in chapter 2, section 2.2.9. Before use, the PVDF membrane was soaked in methanol for 1-3 seconds, immersed in water for 2 minutes and equilibrated in transfer buffer (chapter 2, table 2.1) for 3 minutes. Membranes were stained for total protein with Coomassie Blue R250 (1g/L in 50% [v/v] methanol, 40% [v/v] water and 10% [v/v] acetic acid). Membranes were washed 3 times in water before the addition of Coomassie solution for 1 minute with gentle agitation. Membranes were de-stained with a 50% [v/v] methanol solution followed by 10% [v/v] methanol solution followed by water. The membranes were dried, scanned and sent to an off-campus protein sequencing facility (Dr. W.J. Manby, Department of Biochemistry, Bristol University), with instructions on a duplicate printout of the scanned membrane highlighting the bands to be sequenced.

4.2.4 Western blot analysis of recombinant PRG4 domains

Purified PRG4 domains were subjected to SDS-PAGE using 15% [w/v] slab gels (5μg/lane), under reduced and non-reduced conditions (section 2.2.9). Protein concentrations were calculated by taking absorbance readings at 280nm in an Ultrospec 2000 spectrophotometer (Pharmacia). Proteins were transferred to nitrocellulose as described in chapter 2, section 2.2.9 before Western blotting with a panel of PRG4-specific antibodies (chapter 2, table 2.4) as well as an anti-FLAG M2 monoclonal antibody (chapter 3, section 3.2.8.3).

4.2.5 Identification of the carbohydrate side chains of recombinant PRG4 domains

Characterisation of the carbohydrate moieties of recombinant PRG4 domains was performed using lectin affinity blotting. PRG4 domains purified by FLAG M2 immunoaffinity

chromatography and heparin-agarose affinity chromatography were run on 15% SDS-PAGE gels at 5μg/lane under reducing conditions followed by transfer to nitrocellulose. Lectin affinity blotting was performed using the DIG Glycan Detection system (Roche), previously used to characterise the carbohydrate moieties of full-length PRG4 from a number of sources in chapter 2 of this study. Kit components and methods are listed in chapter 2, section 2.2.10.

4.2.6 Deglycosylation of recombinant PRG4 domains

Deglycosylation of recombinant proteins was performed using the EDEGLY protein deglycosylation kit from Sigma-Aldrich. The enzymes contained in this kit and their specificities, sources and molecular weights are listed in table 4.1.

Table 4.1 – Specificity of enzymes contained within the EDEGLY kit

Enzyme	Source	MW	Specificity
PNGase F	from *Chryseobacterium meningosepticum*	36 kDa	Asparagine-linked complex, hybrid or high mannose oligosaccarides unless α(1-3) core fucosylated
α-2(3,6,8,9) Neuraminidase	recombinant from *Arthrobacter ureafaciens*	60 kDa and 69 kDa	All non-reducing terminal branched and unbranched sialic acids.
O-Glycosidase	recombinant from *Streptococcus pneumonia*	180 kDa	Serine or threonine-linked unsubstituted Gal-β(1-3)-GalNAc-α-
β(1-4)-Galactosidase	recombinant from *Streptococcus pneumonia*	350 kDa	Releases only β(1-4)-linked, non-reducing terminal galactose
β-N-Acetylglucosaminidase	recombinant from *Streptococcus pneumonia*	140 kDa	All non-reducing terminal β-linked N-acetylglucosamine residues

Three reactions were performed for each of the recombinant proteins comprising of (a) PNGase F alone, (b) a combination of O-glycosidase, α-2(3,6,8,9) Neuraminidase, β(1-4)-Galactosidase and β-N-Acetylglucosaminidase and (c) a negative control. For each reaction 20μg of FLAG-purified recombinant PRG4 domain was lyophilised and resuspended in 30μl H_2O before the addition of the supplied 5X reaction buffer (10μl) and denaturation solution (2.5μl). The mixture was heated at 100°C for 5 minutes and allowed to cool to room temperature before addition of Triton X-100 (2.5μl) and 1μl each of the relevant enzymes. Reactions were made up to 50μl with MilliQ H_2O and incubated at 37°C for 3 hours before the addition of 40μl 2X SDS-PAGE sample buffer and 10μl β-mercaptoethanol. Reactions were separated on two SDS-PAGE gels (10μg protein per gel) and visualised by silver stain (section 2.2.12) and Western blot with FLAG M2 monoclonal antibody (section 3.2.8.3).

4.3 Results

4.3.1 Characterisation of the heparin-binding properties of recombinant PRG4 domains by heparin-agarose affinity chromatography

Heparin-binding site sequences are found in a number of matrix proteins, and are characterised by an overall positive charge. The consensus sequences BB*X*B, *X*BB*X*B*X* and *X*BBB*XX*B*X* (where B designates a basic amino acid and *X* designates any other residues) have been identified, and are known as 'Cardin-Weintraub' sequences (Cardin & Weintraub, 1989). In exons 4 and 10 of human PRG4 there are two sequences that match the eight-residue consensus sequence as illustrated in figure 4.1. It has already been demonstrated in Chapter 2 of this study that full-length human recombinant PRG4, bovine tendon PRG4 and bovine articular cartilage PRG4 bind to heparin as assessed by heparin-agarose affinity chromatography, so the potential heparin-binding activity of recombinant N-terminal and C-terminal PRG4 domains was investigated on the basis that both the N-terminal (exons 2-5) and C-terminal (exons 7-12) domains of PRG4 contain a heparin-binding consensus sequence (figure 4.1).

Cardin-Weintraub heparin-binding consensus sequences:	XBBBXXBX XBBXBX
PRG4 exon 4:	Human ^{134}KRSPKPPNKKKTKKV Bovine ^{134}KRSPKSPNKKKTKKV
PRG4 exon 10:	Human ^{1298}QVRRRRFERAIG Bovine ^{1298}QVRRRRFERAIG

Figure 4.1 – Predicted heparin-binding consensus sequences in PRG4. For the consensus sequence, B represents a basic amino acid and X denotes any amino acid (Cardin & Weintraub, 1989). Basic amino acid residues relevant to the heparin-binding consensus sequence are highlighted in red.

4.3.1.1Characterisation of the heparin-binding properties of human recombinant PRG4 N-terminal domain (HumN) by heparin-agarose affinity chromatography

Heparin-agarose affinity chromatography was performed using human N-terminal PRG4 (HumN) purified by FLAG M2 immunoaffinity chromatography as starting material (5ml of ~0.2mg/ml in heparin-agarose equilibration buffer). As previously described in chapter 3, FLAG

M2 immunoaffinity chromatography yields two FLAG M2-immunopositive molecular species of 27 and 16 kDa, HumN1 and HumN2. These two bands can be seen in lane 1 of figure 4.2, which shows the separation of HumN after heparin-agarose affinity chromatography by SDS-PAGE, visualised by Western blot probed with anti-FLAG M2 mAb (figure 4.2A) and silver stain for total protein (figure 4.2B). This data clearly shows the binding of the 27 kDa form of HumN PRG4 to heparin, with the 16 kDa isoform remaining in the flowthrough fraction of the column (figure 4.2, lane 2). The 27 kDa HumN isoform was eluted from the heparin-agarose column at a salt concentration of 0.4-0.6M (figure 4.2, lanes 6 & 7). This data suggests that HumN1 contains an active heparin-binding site, likely to be the putative heparin-binding site within exon 4 illustrated in figure 4.1, and that HumN2 is likely to represent a C-terminally truncated form of HumN that does not contain the heparin-binding consensus sequence in exon 4.This prediction of C-terminal truncation in HumN2 is supported by the observation that it is immunopositive for the anti-FLAG M2 antibody: it contains the N-terminal FLAG purification/detection epitope. These results therefore demonstrate that heparin-agarose affinity chromatography can separate the two recombinant HumN PRG4 species.

Similar data was obtained when FLAG-purified recombinant bovine N-terminal PRG4 (BovN) was applied to a heparin-agarose affinity chromatography column. BovN1 (27 kDa) was seen to bind to heparin-agarose and was eluted with a salt concentration of 0.4-0.6M NaCl, whilst BovN2 did not bind to heparin-agarose and was apparent in the flowthrough fraction (data not shown).

4.3.1.2 Characterisation of the heparin-binding properties of bovine recombinant PRG4 N-terminal domain (BovN) species by heparin-agarose affinity chromatography

The heparin-binding activity of bovine recombinant N-terminal PRG4 domain (BovN) was investigated by applying the conditioned media of induced S2-BN cells (chapter 3, section 3.3.4) to a heparin-agarose affinity chromatography column. Conditioned media from the S2-Hyg cell line (transfected with only the pCoHYGRO selection vector) was also passed over a heparin-agarose affinity column as a negative control, and fractions from these purifications can be seen in figure 4.3 as analysed by Western blot probed with anti-FLAG M2 mAb.

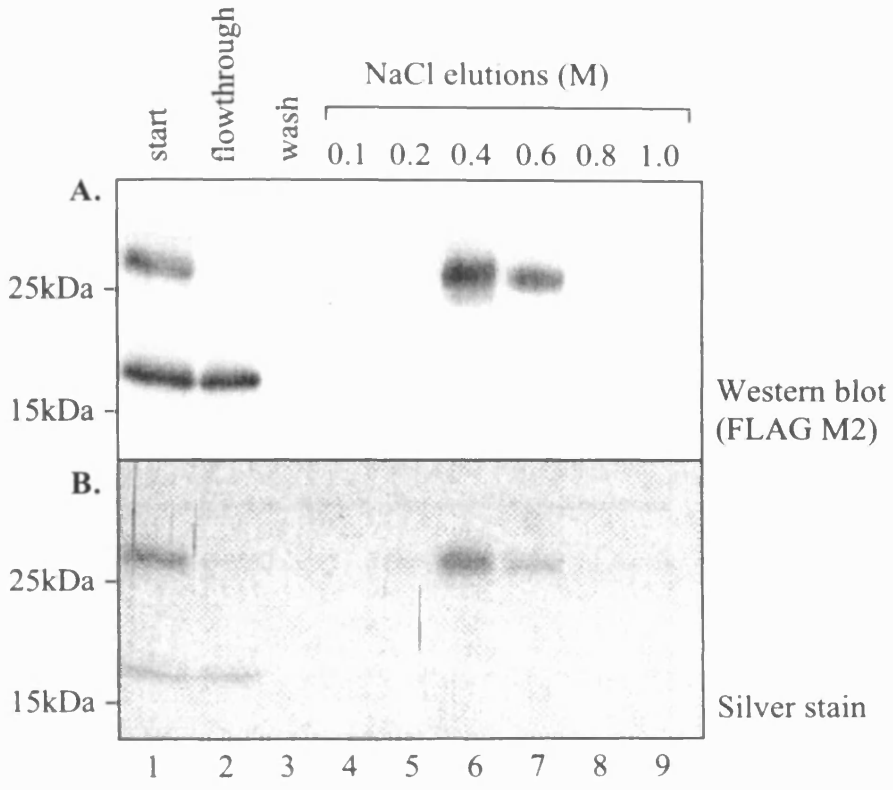


Figure 4.2 – Heparin-agarose affinity chromatography of purified recombinant human N-terminal PRG4 (exons 2-5). FLAG-purified recombinant protein was applied to a 1ml heparin-agarose column and bound protein was eluted with a stepwise gradient of NaCl in 1ml fractions. Fractions were separated by SDS-PAGE (30µl/lane, reducing conditions) and visualised by (A) Western blot probed with anti-FLAG M2 mAb and (B) silver stain showing total protein. Migration of molecular weight standards (kDa) is shown to the left.

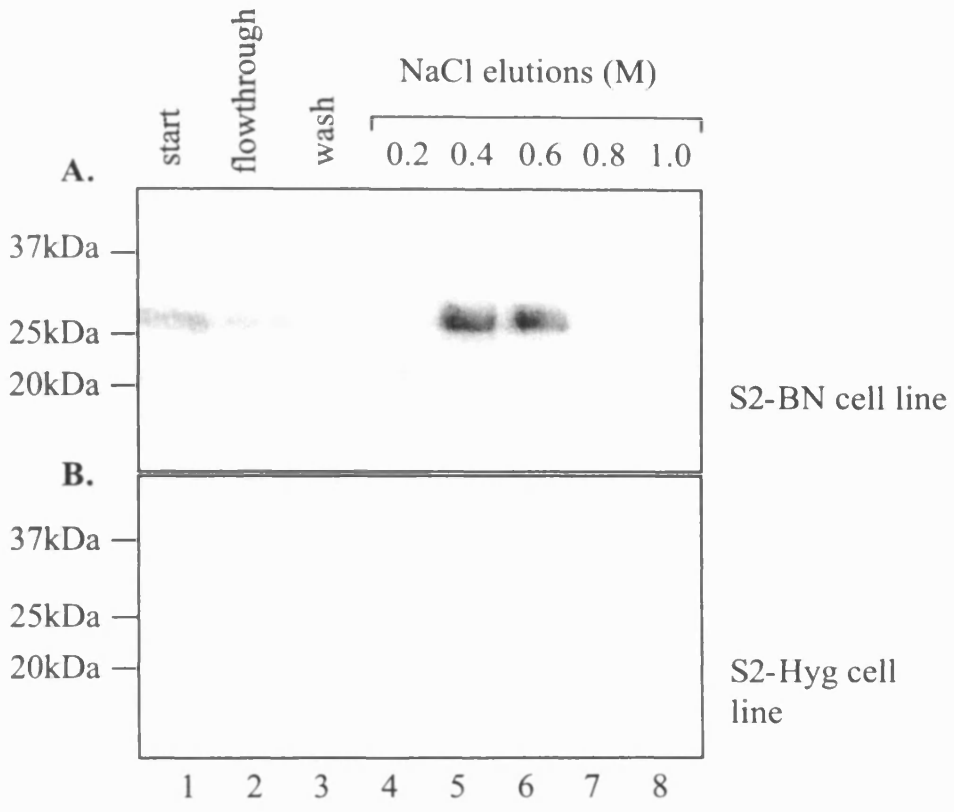


Figure 4.3 – Heparin-agarose affinity chromatography purification of recombinant bovine N-terminal PRG4 (exons 2-5). Conditioned medium (15ml) from the S2-BN (A) and S2-Hyg (B) lines was applied to a 1ml heparin-agarose column and bound protein was eluted sequentially with increasing concentrations of NaCl in 1ml fractions. Fractions were separated by SDS-PAGE (30µl/lane, reducing conditions) and visualised by Western blot probed with anti-FLAG M2 mAb. The S2-Hyg cell line was transfected with the selection vector pCoHYGRO only. Migration of molecular weight standards (kDa) is shown to the left.

The 27 kDa BovN species (BovN1) bound to heparin-agarose and was eluted with 0.4-0.6M NaCl (figure 4.3A, lanes 5 & 6). Therefore this data shows that BovN and HumN (figure 4.2) bind heparin with a similar affinity. As stated previously, the 16 kDa BovN species does not bind to heparin (data not shown), similar to the 16 kDa HumN species (figure 4.2), but it should be pointed out that in this instance the 16 kDa BovN species is not visible in conditioned media or the flowthrough fraction (figure 4.3, lanes 1 & 2). The likely reason for this is that the S2-BN and S2-HN cell lines tended to secrete more detectable 27 kDa FLAG fusion protein compared to 16 kDa FLAG protein, as discussed in chapter 3. As expected, the conditioned medium of the S2-Hyg cell line does not contain any FLAG-tagged protein (figure 4.3B, lane 1), and no FLAG-tagged proteins are purified by heparin-agarose affinity chromatography (figure 4.3B, lanes 3-8).

4.3.1.3 Characterisation of the heparin-binding properties of bovine recombinant PRG4 C-terminal domain (BovC) by heparin-agarose affinity chromatography

Heparin-agarose affinity chromatography was also performed using recombinant bovine C-terminal (exons 7-12) PRG4 (BovC) initially purified from the conditioned medium of the S2-BC cell line by FLAG immunoaffinity chromatography. Under reducing conditions 2 protein species of ~20 kDa (BovC1) and 12 kDa (BovC2) were apparent using SDS-PAGE (chapter 3, figure 3.11). Figure 4.4 shows a silver stain (A) and Western blot (B) of BovC eluted from a heparin-agarose affinity chromatography column. Both species appeared to bind to heparin as demonstrated by the analysis of the start and flowthrough fractions (figure 4.4, lanes 1 & 2). BovC protein was eluted from the column with a NaCl concentration of 0.4- 0.8M (figure 4.4, lanes 6-8). However, that is not to say that both species are capable of binding to heparin: BovC1 is FLAG-immunopositive and BovC2 is not, yet both are purified by FLAG affinity chromatography, no doubt due to an interaction between the two species under non-reducing conditions. It is likely that this interaction may cause a non-heparin-binding BovC species to be co-purified with a heparin-binding BovC species.

4.3.2 N-terminal amino acid sequencing of recombinant PRG4 domains

The aim of this work was to determine the N-terminal amino acid sequence for the bovine C-terminal PRG4 protein species BovC2 (12 kDa). The other recombinant PRG4 proteins produced by this work - HumN1, HumN2, BovN1, BovN2 and BovC1 – are stained with an anti-FLAG

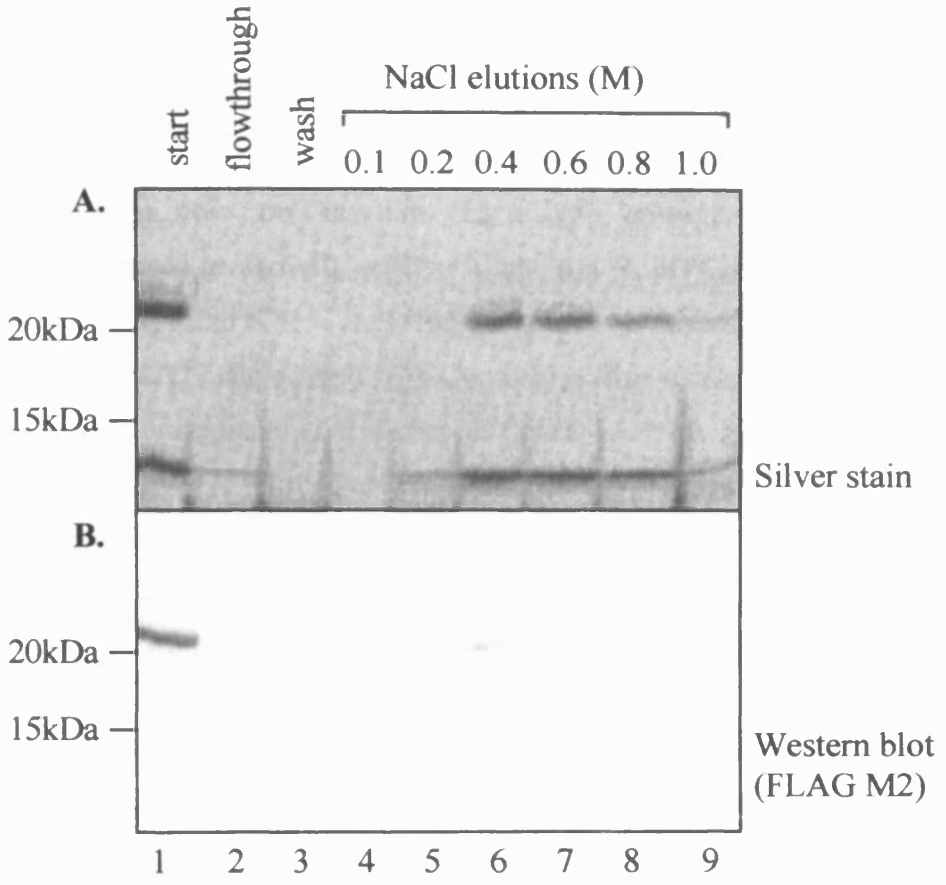


Figure 4.4 – Heparin-agarose affinity chromatography of purified recombinant bovine C-terminal PRG4 (exons 7-12). FLAG-purified recombinant protein was applied to a 1ml heparin-agarose column and bound protein was eluted sequentially with a stepwise gradient of NaCl in 1ml fractions. Fractions were separated by SDS-PAGE (30µl/lane, reducing conditions) and visualised by (A) silver stain showing total protein and (B) Western blot probed with anti-FLAG M2 mAb. Migration of molecular weight standards (kDa) is shown to the left.

M2 monoclonal antibody, which indicates that the FLAG tag is present at the amino terminus of the protein. In contrast, BovC2 is FLAG immunonegative, strongly suggesting that its N-terminal amino acid sequence does not contain the FLAG epitope. Heparin-agarose affinity chromatography was used to partially purify HumN and BovC from the conditioned media of stably transfected S2-HN and S2-BC cell lines, respectively. Proteins were separated by SDS-PAGE, transferred to PVDF and stained with Coomassie Blue to visualise total protein. Bands of interest were excised for sequencing as shown in figure 4.5. This sequencing was performed by an off-campus facility (Bristol University). The sequences obtained for the FLAG immunopositive protein species HumN1 and BovC1 (figure 4.5A & B) contained 4 amino acids originating from the expression vector and the FLAG epitope, as expected. The sequence obtained for BovC2 (figure 4.5C) relates to a sequence that resides in exon 10 of PRG4: ^{1307}AIGPSQVHT. The predicted molecular weight of BovC from ^{1307}AIGPSQVHT to the end of exon 12 is 11.16 kDa, and the predicted molecular weight of BovC from the start of exon 7 to ^{1307}AIGPSQVHT is 20.56 kDa. These molecular weights approximate to the observed molecular weights of the BovC1 and BovC2 proteins as seen on reduced SDS-PAGE gels. Collectively, these results indicate that BovC is a polypeptide of approximately 30 kDa that has been cleaved at the RRRFER^{1306} - ^{1307}AIGPSQ site.

4.3.3 Characterisation of recombinant PRG4 domains by Western blotting with a panel of PRG4-specific antibodies

The recombinant PRG4 domains produced in this study were used in Western blotting experiments with a panel of antibodies designed to recognise PRG4 (chapter 2, table 2.4) These included polyclonal antibodies raised against synthetic peptides from human PRG4 (anti-exon 3, anti-exon 12) (Lori Fitz, Wyeth, Cambridge MA), and monoclonal antibodies raised against purified bovine superficial zone chondrocyte PRG4 (6-A-1, 3-A-4) (Schumacher *et al.*, 1999). This was carried out in order to confirm the presence of PRG4 epitopes within the recombinant PRG4 domains and to characterise the recombinant protein species produced by the stable S2 cell lines. Work of this nature would also help to partially map the PRG4 recognition epitopes for the antibodies 3-A-4 and 6-A-1, and would help determine the extent of cross-species reactivity displayed by each of these antibodies.

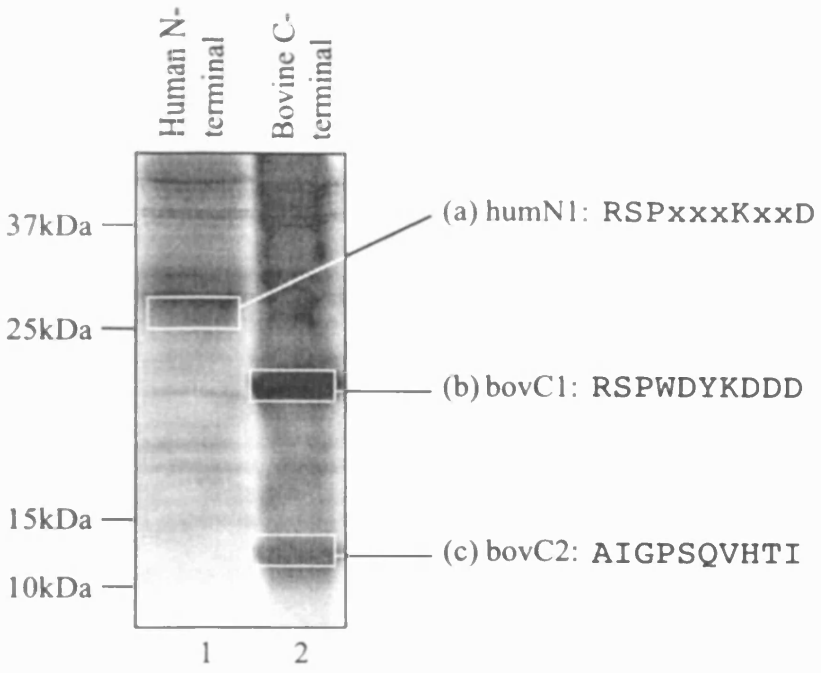


Figure 4.5 – Amino terminal sequence analysis of recombinant human N-terminal PRG4 (lane 1) and recombinant bovine C-terminal PRG4 (lane 2) proteins expressed by stable *Drosophila* S2 cell lines. Conditioned media (30ml) was concentrated by heparin-agarose affinity chromatography (1ml bed volume), separated by SDS-PAGE, transferred to a PVDF membrane and stained with Coomassie blue. Bands of interest were selected based on prior Western blotting experiments, excised, and sequenced by a core facility at Bristol University. The sequences obtained are shown on the right (one-letter amino acid code). Migration of molecular size standards is shown to the left.

4.3.3.1 Immunodetection of recombinant PRG4 domains using polyclonal antibodies to human PRG4

Recombinant PRG4 domains BovN1, HumN1 and BovC were purified by FLAG immunoaffinity chromatography and heparin-agarose affinity chromatography and subjected to SDS-PAGE (5μg/lane) under reducing and non-reducing conditions. Western blots probed with anti-exon 3 and anti-exon 12 polyclonal antibodies raised against human PRG4 synthetic peptides (Wyeth, see table 2.4 for details) are shown in figure 4.6. A silver stain and Western blot probed with anti-FLAG M2 mAb are shown as a reference (lanes 1-6). It is apparent from the silver stains and FLAG M2 immunoblots of the recombinant proteins that they run very differently under native (figure 4.6A) and reducing conditions (figure 4.6B). BovN1 and HumN1 both run at ~27 kDa under reducing conditions, whereas under non-reducing conditions BovN appears as a single band of ~50 kDa and HumN1 runs as 2 bands of ~37 and 55 kDa (figure 4.6, lanes 1 & 2). Under reducing conditions, two species of BovC are apparent at ~20 (BovC1) and 12 (BovC2) kDa, whereas under non-reducing conditions 1 band of ~32 kDa is visible, which indicates that the two molecular species are disulphide-bonded with one another (figure 4.6, lane 3). This proposed interaction is supported by the observation that both species of BovC are purified by FLAG immunoaffinity chromatography even though only the 20 kDa BovC1 species is FLAG immunopositive (chapter 3, figure 3.12). The polyclonal antibody to exon 3 of human PRG4 recognised HumN1 in reduced and non-reduced forms (figure 4.6, lane 8), although staining was enhanced in reducing conditions. There was a weak interaction of the anti-exon 3 antibody with reduced BovN1 (figure 4.6, lane 7). This antibody was raised against a synthetic peptide from exon 3 of human PRG4, ^{72}GRCFE**SF**ERGRECD. The corresponding bovine PRG4 amino acid sequence is ^{72}GRCFE**TFA**RGRECD, and the difference of 2 amino acids between the 2 species (highlighted in bold) appears to be sufficient to significantly reduce the binding of the antibody to this epitope. The difference between ^{79}E in human PRG4 and ^{79}A in bovine PRG4 could be particularly relevant: glutamic acid possesses an acidic side chain whereas the side chain of alanine is nonpolar. The anti-exon 3 antibody also fails to react with full-length bovine articular cartilage or tendon PRG4, as shown in chapter 2, figure 2.9. HumN1 shows greater immunoreactivity with the anti-exon 3 antibody than HumN2, demonstrated by the Western blot shown in figure 4.7. This suggests that HumN2 contains a truncated anti-exon 3 epitope, and that the site of C-terminal truncation of the HumN molecule occurs near the anti-exon 3 epitope site.

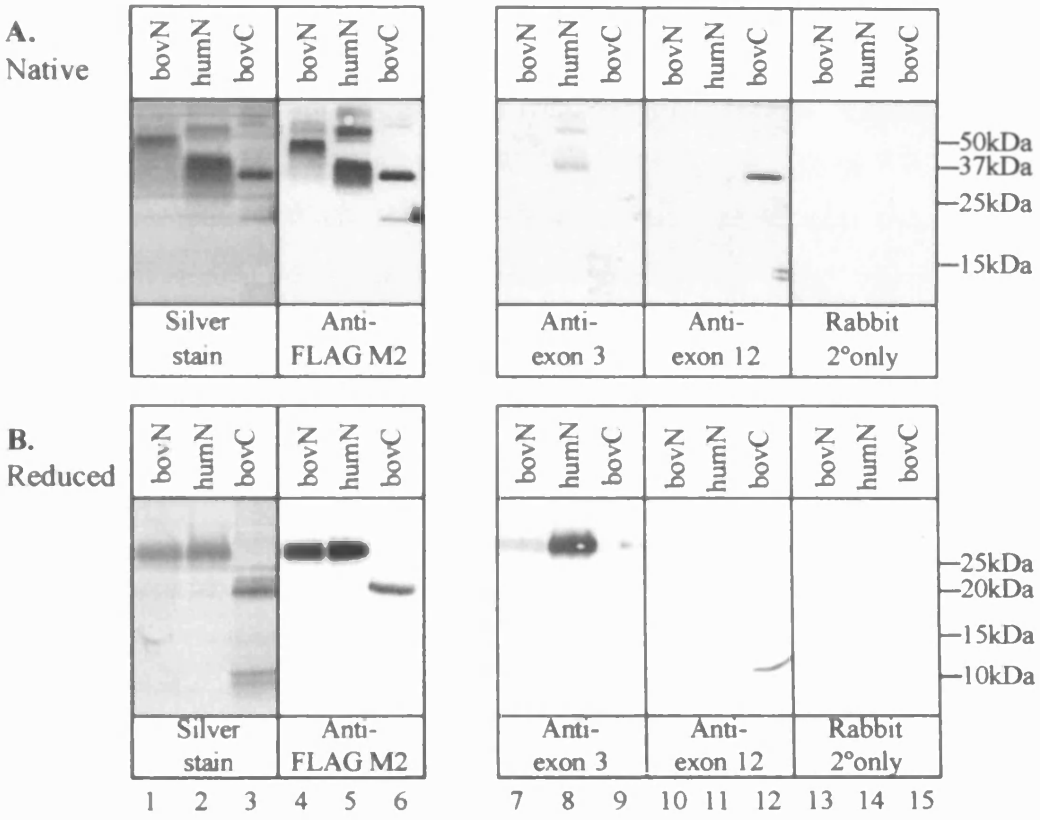


Figure 4.6 – Immunoblotting experiments using bovine and human N-terminal (exons 2-5) PRG4 (BovN & HumN) purified by FLAG and heparin affinity chromatography, and bovine C-terminal (exons 7-12) PRG4 (BovC) purified by FLAG affinity chromatography. All samples were run in (A) native and (B) reduced states, at 5μg total protein/lane. Silver stain and Western blot probed with anti-FLAG M2-AP conjugate mAb are shown alongside blots probed with polyclonal antibodies to human PRG4, and an anti-rabbit secondary antibody control is shown in the far right panel. Migration of molecular size standards is shown to the left.

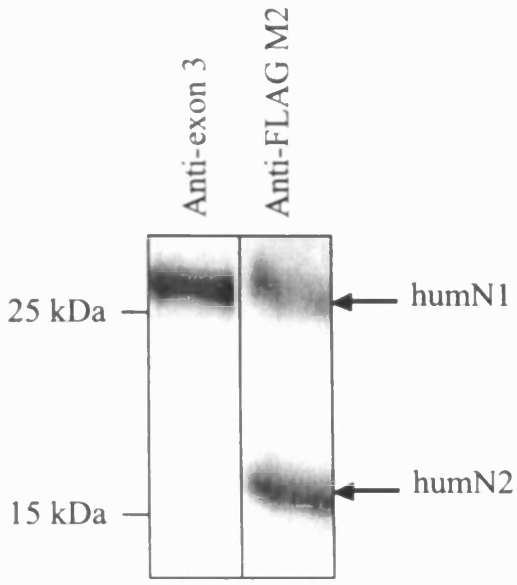


Figure 4.7 – Western blot analysis of humN1 and humN2 PRG4 protein species. Proteins were purified by FLAG immunoaffinity chromatography and separated by SDS-PAGE under reducing conditions (5μg total protein/lane). A Western blot was probed with a polyclonal antibody to an epitope within exon 3 of human PRG4 and FLAG M2 monoclonal antibody. Migration of standards is shown on the left.

As expected, the anti-exon 12 antibody, raised against a peptide sequence in the C-terminal domain of human PRG4, did not react with BovN or HumN (figure 4.6, lanes 10 & 11). The antibody did recognise a single non-reduced BovC band of 32 kDa and also reacted with the BovC2 species under reducing conditions (figure 4.6, lane 12), but not the FLAG immunopositive BovC1 20 kDa protein species. This supports the conclusion made earlier after the amino-terminal sequencing of BovC2, that BovC1 (20 kDa) represents bovine PRG4 from the start of exon 7 to R^{1306}, and that BovC2 (12 kDa) represents bovine PRG4 starting at A^{1307} in exon 10 to the end of exon 12. The anti-exon 12 antibody was raised against a synthetic peptide from human PRG4, ^{1373}QYYNIDVPSR, and there is no difference in amino acid sequence between human and bovine PRG4 within this peptide. This would explain why an antibody raised against a human PRG4 peptide would recognise the recombinant bovine protein.

4.3.3.2 Immunodetection of recombinant PRG4 domains using monoclonal antibodies raised against purified bovine articular cartilage PRG4

In a similar fashion to figure 4.6, figure 4.8 shows the results of immunoblotting experiments using bovine and human N-terminal (exons 2-5) PRG4 (BovN1 & HumN1) purified by FLAG immunoaffinity chromatography and heparin-agarose affinity chromatography, which purifies the higher molecular weight species only (see figure 4.2). Bovine C-terminal (exons 7-12) PRG4 (BovC) purified by FLAG immunoaffinity chromatography was also used in these analyses. After separation under native (figure 4.8A) and reducing (figure 4.8B) conditions, proteins were immunoblotted using two monoclonal antibodies raised against purified bovine PRG4, 3-A-4 and 6-A-1 (Schumacher *et al.*, 1999). A silver stain and Western blot probed with anti-FLAG M2 mAb are shown as a reference (figure 4.8, lanes 1-6). Non-reduced forms of BovN1 and HumN1 show weak immunopositive staining with 6-A-1 and 3-A-4 (figure 4.8A). Figure 4.8B shows that under non-reducing conditions, HumN1 and BovN1 are weakly stained by the antibodies 6-A-1 and 3-A-4. There was no staining of BovC protein with any of the mAbs used. This result was unexpected as 6-A-1 was thought to recognise a C-terminal bovine PRG4 epitope based on the isolation of a λgt11 clone with 6-A-1 (Schumacher *et al.*, 1999). As seen in figure 4.8A, lane 10, 3-A-4 recognises non-reduced BovN1, but not HumN1. This is in keeping with results shown earlier in this study (chapter 2, figure 2.9) and results shown by others (Tudor, 2002), which show that 3-A-4 recognises a non-reduced epitope on non-reduced full-length bovine PRG4

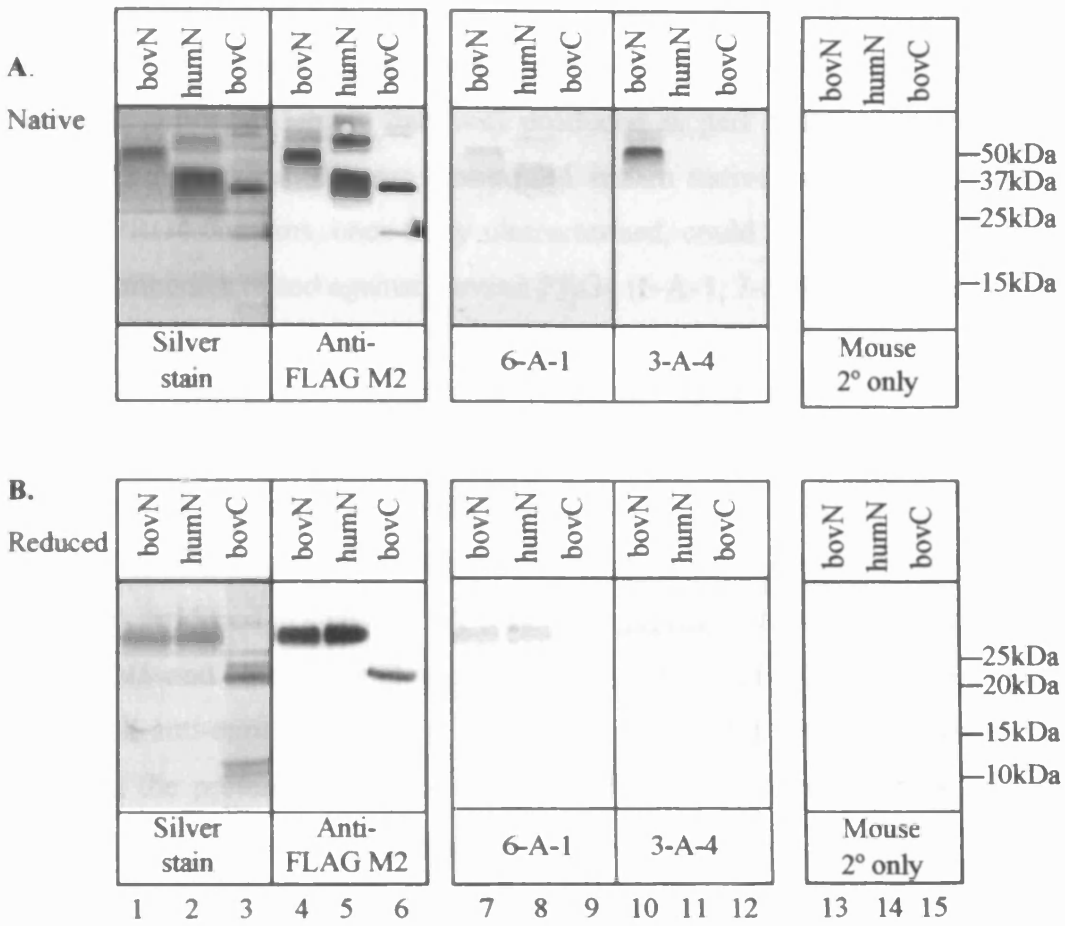


Figure 4.8 – Immunoblotting experiments using bovine and human N-terminal (exons 2-5) PRG4 (BovN & HumN) purified by FLAG and heparin affinity chromatography, and bovine C-terminal (exons 7-12) PRG4 (BovC) purified by FLAG affinity chromatography. All samples were run in (A) native and (B) reduced states, at 5μg total protein/lane. A silver stain and Western blot probed with anti-FLAG M2-AP conjugate mAb are shown alongside blots probed with monoclonal antibodies raised against purified bovine PRG4, and an anti-mouse secondary antibody control is shown in the far right panel. Migration of molecular size standards is shown to the left.

isolated from cartilage and tendon, but not full-length human PRG4. This work, therefore, maps the 3-A-4 epitope to between exons 2 and 5 of bovine PRG4. This also indicates that the recombinant N-terminal domain that was produced as part of this study has similar structural features to the N-terminal domain contained within native bovine full-length PRG4. These recombinant PRG4 domains, once fully characterised, could be used to further epitope–map the monoclonal antibodies raised against bovine PRG4 (6-A-1, 3-A-4, 5-D-1 and 5-B-6).

4.3.4 Predicted structure of recombinant PRG4 structural domains

4.3.4.1 Predicted structure of recombinant PRG4 N-terminal (exons 2-5) domain species

The results from Western blotting, protein sequencing and affinity chromatography experiments presented in this chapter enable the prediction of structural features for the different recombinant PRG4 proteins produced in chapter 3. A schematic representation of the human N-terminal PRG4 species HumN1 and HumN2 is shown in figure 4.9. As HumN1 is immunopositive for anti-FLAG M2 and anti-exon 3 antibodies (figure 4.6), and also binds to heparin (figure 4.2), it is predicted that the protein represents PRG4 exons 2-5. As HumN2 does not bind to heparin-agarose (figure 4.2) and is immunopositive for anti-FLAG and weakly immunopositive for anti-exon 3 (figures 4.2 and 4.7), it is predicted that N2 comprises exon 2 and exon 3 up to the amino acid sequence used in the manufacture of the anti-exon 3 antibody. Similar structures could be proposed for the BovN1 and BovN2 species, based on similarities with the HumN1 and HumN2 species in terms of molecular weight, heparin-binding characteristics and FLAG M2 mAb immunopositivity.

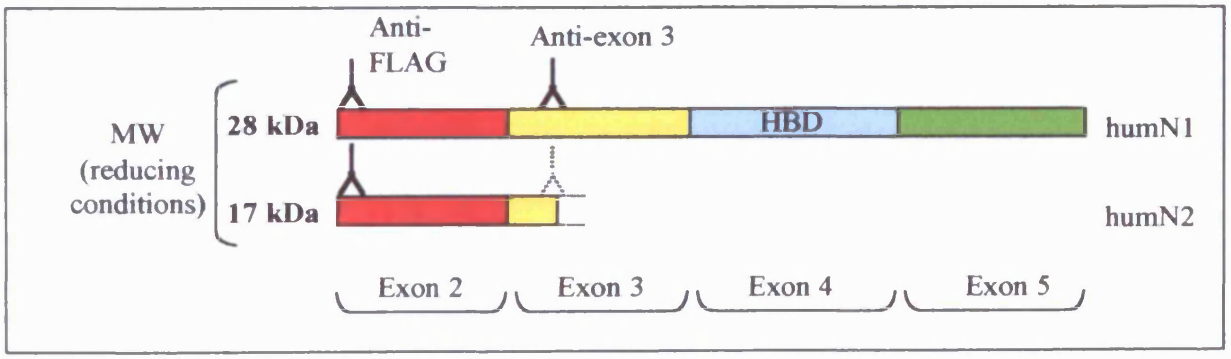


Figure 4.9 – Schematic representation of HumN1 and HumN2 recombinant PRG4 protein species based on Western blotting experiments and heparin-agarose affinity chromatography. HBD = heparin-binding domain.

4.3.4.2 Predicted structure of recombinant PRG4 C-terminal (exons 7-12) domain species

The predicted structure of the human C-terminal PRG4 species, BovC1 and BovC2, is shown in figure 4.10. BovC1 is immunonegative for a polyclonal antibody raised against a peptide from exon 12 whereas BovC2 is immuopositive (figure 4.6). Therefore it can be concluded that BovC2 contains PRG4 exon 12 and BovC1 does not. At the N-terminus of the 2 species, BovC1 has the FLAG epitope and BovC2 has an internal sequence from exon 10 of PRG4 (figure 4.5). From these observations it can be predicted that the sum of BovC1 and BovC2 equate to exons 7-12 of bovine PRG4. It can also be predicted that BovC2 contains a free cysteine residue and BovC1 contains four cysteine residues. Therefore, it is likely that disulphide bonding between the two species must take place in order for them to be co-purified by FLAG immunoaffinity chromatography. The separation of the two BovC species could be a useful tool in further characterisation experiments. However, preliminary reduction/alkylation experiments based on a method used by Calabro *et al.* (1992) proved to be unsuccessful, as under native conditions, BovC1 and BovC2 did not run as two bands on SDS-PAGE gels after this process.

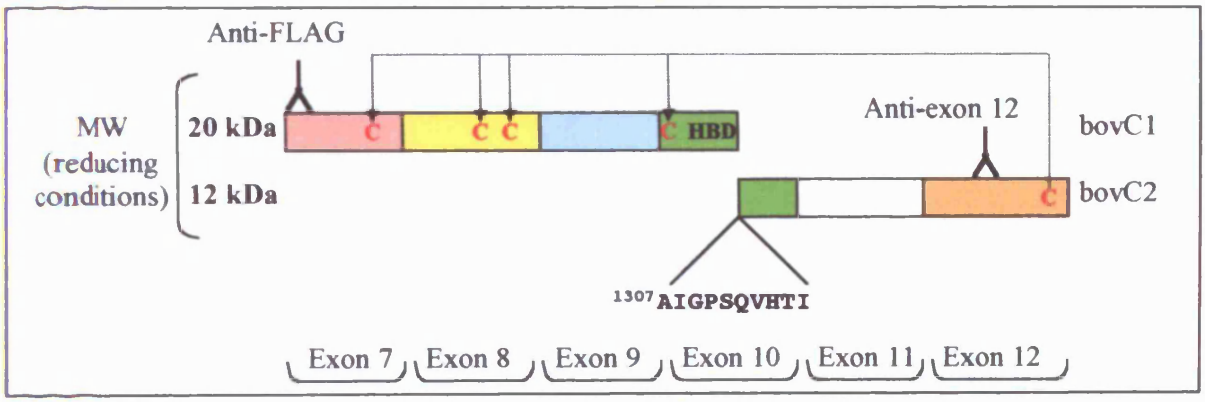


Figure 4.10 – Schematic representation of BovC1 and BovC2 recombinant PRG4 proteins based on Western blotting experiments and N-terminal amino acid sequencing. Arrows indicate potential disulphide bonding. HBD = heparin-binding domain. C = cysteine residue.

4.3.5 Identification of carbohydrate side chains on recombinant PRG4 domains

Glycosylation of recombinant proteins can cause altered migration in SDS-PAGE that is not in concordance with the predicted molecular weight of the protein core. The glycosylation of purified recombinant PRG4 domains was investigated by separation using SDS-PAGE under

reducing conditions followed by lectin affinity blotting experiments performed using methods described in Chapter 2 of this study (section 2.2.10). The specificities of the lectins used are described in table 2.5.

Figure 4.11 shows the results of lectin affinity blotting experiments with HumN1, BovN1 and BovC recombinant proteins. HumN1 and BovN1 show positive staining for the lectin PNA, which recognises the core disaccharide galactose β(1-3) N-acetylgalactosamine (figure 4.11, lanes 1 & 2). Bovine and human full-length PRG4 are also positive for PNA staining as shown in chapter 2, figure 2.10, and also for MAA which specifically binds to sialic acid linked α(2-6) to galactose. As shown in figure 4.11 (lanes 4 & 5), HumN and BovN display a weak affinity with MAA, along with DSA (lanes 7 & 8), which recognises galactose linked β(1-4) to N-acetyl glucosamine, and SNA (lanes 10 & 11), which recognises sialic acid linked α(2-6) to galctose. Full-length human or bovine PRG4 does not appear to be recognised by DSA, SNA or GNA (chapter 2, figure 2.10). BovC1 (though not BovC2) is recognised by GNA (figure 4.11, lane 15), which is specific to terminal mannose α(1-3), α(1-6) or α(1-2) linked to mannose, identifying high mannose N-glycan chains.

4.3.6 Deglycosylation of recombinant proteins

Carbohydrates in the form of asparagine-linked (N-linked) or serine/threonine-linked (O-linked) oligosaccharides are post-translational modifications that are present in many eukaryotic proteins, and often introduce heterogeneity in the mass and charge of glycoproteins. The recombinant PRG4 proteins investigated by this study are predicted to have a number of O- and N-linked glycosylation sites, as illustrated in tables 4.2 and 4.3, respectively. Potential glycosylation sites were predicted by inputting the PRG4 core protein amino acid sequence into the NetOGlyc and NetNglyc online glycosylation prediction engines.

HumN and BovN are predicted to have 28 and 24 O-glycosylation sites, respectively, whilst BovC is predicted to have 2 N-linked and 2 O-linked glycosylation sites. Human and bovine N-terminal PRG4 has 1 consensus sequence for N-linked glycosylation (NXS/T), but as the middle residue is proline the acceptor capability of the site is markedly reduced (Ronin *et al.*, 1981).

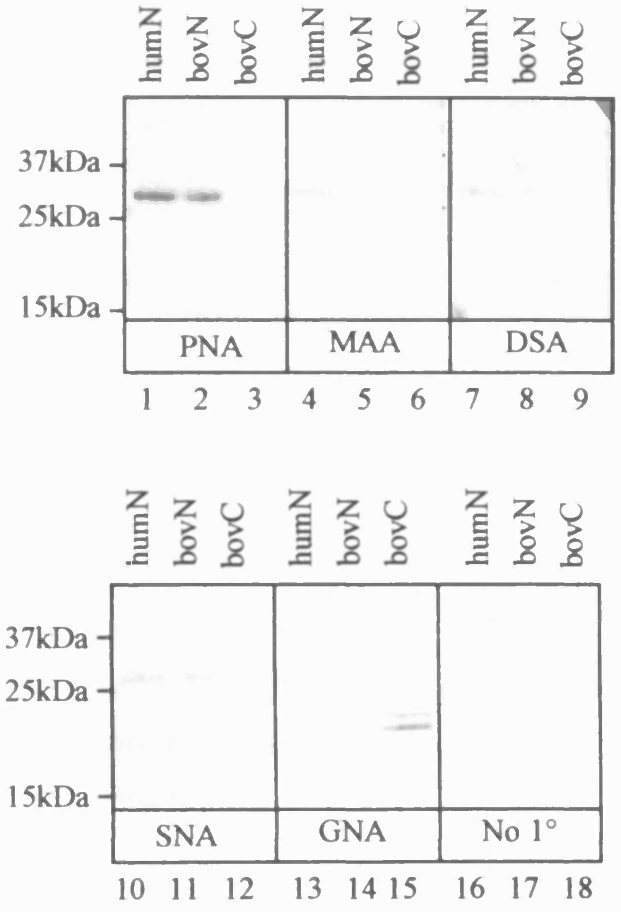


Figure 4.11 – Lectin affinity blot analysis of recombinant human and bovine N-terminal PRG4 (lanes 1 and 2, respectively) and recombinant bovine C-terminal PRG4 (lane 3). All proteins were loaded at 5μg/lane, and under reducing conditions. Migration of molecular weight standards (kDa) is shown to the left.

Table 4.2 – Predicted mucin-type O-linked glycosylation sites in recombinant PRG4 domains, as predicted by the NetOGlyc 3.0 server, URL http://www.cbs.dtu.dk/services/NetOGlyc/ (Hansen *et al.*, 1998). Predicted O-glycosylation sites are shown in red.

Protein	No. of predicted sites	Amino acid sequence
Human recombinant PRG4 N-terminal domain (exons 2-5)	28	[25]QDLSSCAGRCGEGYSRDATCNCDYNCQHYMECCPDFKRVCTAELS CKGRCFESFERGRECDCDAQCKKYDKCCPDYESFCAEVHNPTSPPS SKKAPPPSGASQTIKSTTKRSPKPPNKKKTKKVIESEEITEEHSVS ENQESSSSSSSSSSSTIWKIKSSKNSAANRELQKKLK
Bovine recombinant PRG4 N-terminal domain (exons 2-5)	24	[25]QDLSSCAGRCGEGYSRDAICNCDYNCQHYMECCPDFKKECTVELS CKGRCFETFARGRECDCDSDCKKYGKCCSDYGAFCEAVHNPTTPPP PKTAPPPPGASQTIKSTTKRSPKSPNKKKTKKVIESEEITEEHSVS ENQESSSSSSSSSTIRKIKSSKNSGANRELQKKLK
Bovine recombinant PRG4 C-terminal domain (exons 7-12)	2	[1141]DETNLCNGRPVDGLTTLRNGTLVAFRGHYFWMLTPFTPPPPPRR ITEVWGIPSPIDTVFTRCNCEGKTFFFKGSQYWRFTNDIKDAGYPK LISKGFGGLNGKIVAALSIAQYKSRPESVYFFKRGGSVQQYTYKQE PTQKCTGRRPAINYSVYGETAQVRRRRFERAIGPSQVHTIRIHYTP VRVPYQDKGFLHNEVKVSTLWRGLPNVVTSAISLPNIRKPDGYDYY ALSKDQYYNIDVPSRTARAITTRSGQTLSNTWYNCP

Table 4.3 – Predicted N-linked glycosylation sites in recombinant PRG4 domains (Asn-Xaa-Ser/Thr). Sites were found using the NetNGlyc 1.0 server, URL (http://www.cbs.dtu.dk/services/NetNGlyc/). Predicted N-glycosylation sites are shown in red, consensus sequence residues are shown in blue.

Protein	No. of predicted sites	Sequence(s)
Human recombinant PRG4 N-terminal domain (exons 2-5)	1*	[25]QDLSSCAGRCGEGYSRDATCNCDYNCQHYMECCPDFKRVCTAELS CKGRCFESFERGRECDCDAQCKKYDKCCPDYESFCAEVHNPTSPPSSKK APPPSGASQTIKSTTKRSPKPPNKKKTKKVIESEEITEEHSVSENQESS SSSSSSSSSTIWKIKSSKNSAANRELQKKLK
Bovine recombinant PRG4 N-terminal domain (exons 2-5)	1*	[25]QDLSSCAGRCGEGYSRDAICNCDYNCQHYMECCPDFKKECTVELS CKGRCFETFARGRECDCDSDCKKYGKCCSDYGAFCEAVHNPTTPPPPKT APPPPGASQTIKSTTKRSPKSPNKKKTKKVIESEEITEEHSVSENQESS SSSSSSSSTIRKIKSSKNSGANRELQKKLK
Bovine recombinant PRG4 C-terminal domain (exons 7-12)	2	[1141]DETNLCNGRPVDGLTTLRNGTLVAFRGHYFWMLTPFTPPPPPRRITE VWGIPSPIDTVFTRCNCEGKTFFFKGSQYWRFTNDIKDAGYPKLISKGF GGLNGKIVAALSIAQYKSRPESVYFFKRGGSVQQYTYKQEPTQKCTGRR PAINYSVYGETAQVRRRRFERAIGPSQVHTIRIHYTPVRVPYQDKGFLH NEVKVSTLWRGLPNVVTSAISLPNIRKPDGYDYYALSKDQYYNIDVPSR TARAITTRSGQTLSNTWYNCP

*Denotes Asn-Pro-Ser/Thr sequence, which is less likely to be glycosylated

These potential glycosylation sites within recombinant proteins were investigated by enzymatic deglycosylation, the extent of which was determined by analysing mobility shift on SDS-PAGE gels. Proteins were detected after deglycosylation by silver stain and Western blot using anti-FLAG M2 mAb, and the results can be seen in figure 4.12. A mixture of O-glycosidases (see table 4.1) appeared to reduce the apparent MW of HumN1 and BovN1 proteins by ~2 to 3 kDa (figure 4.12, lanes 3 & 9), and had no effect on the migration of BovC protein (lanes 15 & 18). N-deglycosylation with PNGaseF had no effect on N-terminal PRG4 species, corresponding to the observation that there are no consensus sequences for N-glycosylation within the N-terminal domain (table 4.3). PNGaseF digestion reduced the molecular weight of the 20 kDa BovC1 species by around 2 kDa (figure 4.12, lanes 14 & 17), confirming the activity of the two predicted N-glycosylation sites (table 4.3) as acceptors. The 12 kDa BovC2 protein was unaffected by digestion with PNGase F (lanes 14 & 17), in keeping with the prediction that no N-glycosylation consensus sequences are present after the N-terminal BovC2 sequence ^{1307}AIGPSQVHT (table 4.4), determined by N-terminal amino acid sequencing (figure 4.5). The presence of N-glycans in BovC is supported by the positive staining with the lectin GNA, which identifies N-linked glycans (figure 4.11).

4.4 Discussion

Before embarking on further studies with the recombinant PRG4 domains described in Chapter 3 of this study, it was desirable to characterise the recombinant proteins. The experiments described in this chapter have partially characterised the recombinant PRG4 domains by Western blotting, affinity chromatography and N-terminal amino acid sequence analysis.

HumN1 and BovN1 proteins (27 kDa) bind to heparin-agarose, whereas HumN2 and BovN2 (16 kDa) do not. This would indicate that HumN1 and BovN1 proteins contain a functional heparin-binding site, probably conferred by the consensus sequence within exon 4 of PRG4, whereas HumN2 and BovN2 proteins do not. This in turn led to the prediction that N1 represents PRG4 N-terminal exons 2-5 and N2 represents PRG4 exons 2-4, truncated before the heparin-binding sequence.

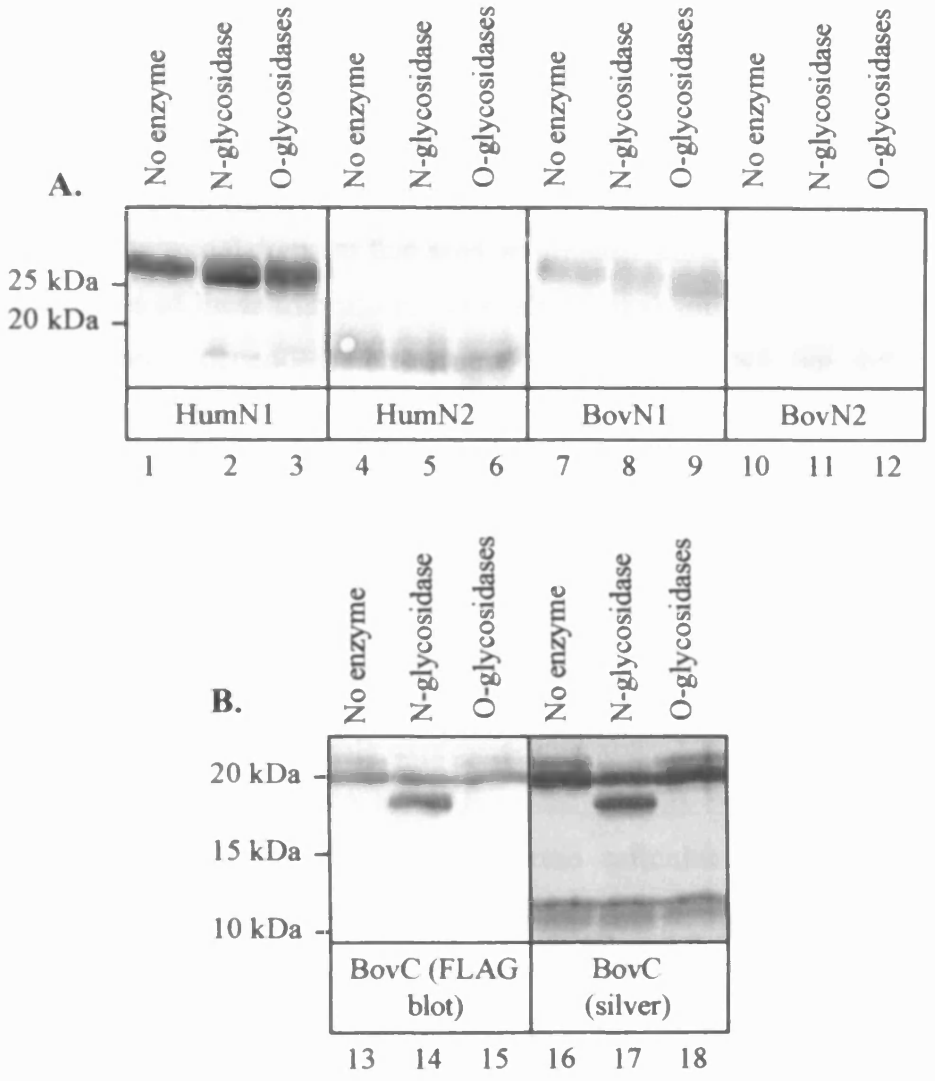


Figure 4.12 – Enzymatic deglycosylation of recombinant PRG4 domains followed by analyses of mobility shift on SDS-PAGE gels. (A) human and bovine N-terminal species detected by Western blot with anti FLAG M2 mAb. (B) bovine C-terminal species detected by Western blot with anti FLAG M2 mAb and silver stain. Migration of molecular size standards is shown to the left.

This work would benefit from a more detailed characterisation of the recombinant proteins, such as by the calculation of the mass of tryptic peptides by Matrix Assisted Laser Desorption Ionisation–Time of Flight (MALDI-TOF) mass spectrometry. BovN2 and HumN2 represent recombinant PRG4 N-terminal domains that are C-terminally truncated and other such techniques could reveal the nature of these truncations. The monoclonal antibody to bovine PRG4, 3-A-4, recognises a conformation-dependant non-reduced epitope on BovN1 (but not HumN1). This is similar to results obtained in immunoblotting experiments with native full-length PRG4, where 3-A-4 recognises a non-reduced conformation-dependent epitope on PRG4 isolated from bovine articular cartilage and synovial fluid (Tudor, 2002), but not full-length human recombinant PRG4 (chapter 2). This work therefore maps the specificity of 3-A-4 to exons 2-5 of bovine PRG4. The presence of this domain in bovine synovial fluid adds to results presented in two papers by Jay and others, which document the presence of PRG4 containing only exons 6-9 in human synovial fluid (Jay *et al.*, 2000), and the variable expression of exons 2, 4 and 5 by synovial fibroblasts and articular chondrocytes at an mRNA level (Jay *et al.*, 2001b). Previously, Tudor (2002) found that chondrocytes from the deep zone of bovine articular cartilage were positive in immunohistochemical experiments with 6-A-1, but not 3-A-4, and that C-terminal but not N-terminal PRG4 mRNA was expressed by deep zone chondrocytes. This led to the hypothesis that 6-A-1 recognises a C-terminal PRG4 epitope and 3-A-4 recognises an N-terminal epitope, the second part of which has been supported by work presented in this study. The physiological function of different PRG4 isoforms in synovial fluid, articular cartilage matrix and chondrocytes from different territorial regions has yet to be resolved, and 3-A-4 could be used to determine the nature of different PRG4 populations once its epitope has been fully mapped.

For the bovine PRG4 C-terminal domain-expressing cell line, S2-BC, two proteins BovC1 and BovC2 were purified by FLAG affinity chromatography, running on reduced SDS-PAGE gels at 20 and 12 kDa, respectively. Results presented in this chapter show that the N-terminal amino acid sequence of BovC2 is ^{1307}AIGPSQVHT, which is located within exon 10 of PRG4. BovC2 is immunopositive for an antibody raised against a peptide from exon 12 of PRG4 whereas BovC1 is immunonegative, which has led to the predicted structure of BovC1 and BovC2 as illustrated in figure 4.10. From these data it is predicted that BovC1 and BovC2 represent products from the cleavage of BovC (exons 7-12) PRG4 at the site RRRFERA^{1306} – ^{1307}AIGPSQ.

The recombinant PRG4 domain proteins HumN1, BovN1 and BovC all appear to be able to bind heparin. Taken together, these results suggest that the heparin-binding consensus sequences in exons 4 and 10 of PRG4 are functionally active in these proteins. This was also the case with the full-length molecule: in chapter 2 of this study it has been shown that human recombinant, bovine articular cartilage and bovine tendon PRG4 bind to heparin-agarose in affinity chromatography experiments. This would indicate that PRG4 has two functionally active heparin-binding sites. It has been suggested that the ability of PRG4 to bind glycosaminoglycan chains on both sides of the central lubricating domain would serve to stabilise lubricating activity on cartilage covered with the lamina splendens (Jay *et al.*, 2001a).

The results presented in this chapter demonstrate a degree of similarity in glycosylation patterns between the recombinant PRG4 domains and full-length PRG4, based on results obtained in lectin affinity blotting experiments with the recombinant domains. Full-length PRG4 is recognised by the lectins PNA and MAA, which also recognise human and recombinant N-terminal PRG4 domains (Tudor, 2002). The bovine C-terminal recombinant PRG4 domain is positive for binding with GNA, and the N-terminal domains are also weakly stained with DSA and SNA, but this data differs with results seen with the full-length native molecule. This could be due in part to the slightly different glycosylation patterns in *Drosophila* cells as compared with mammalian cells. The sialylation of glycoproteins produced by insect cells has been a subject of debate, and it has been concluded that sialylation in insect cells is probably a highly specialised function that occurs rarely (reviewed in Marchal *et al.*, 2001). The recognition of BovN and HumN proteins by MAA and SNA in this study would indicate the presence of sialic acid on these recombinant proteins. However, more extensive controls, such as the use of competing monosaccharides and/or endoglycosidase and neuraminidase treatment, are necessary to verify the carbohydrate-binding specificity of the lectin affinity blot results.

Enzymatic deglycosylation of the recombinant proteins has confirmed the presence of N-glycosylation sites within the C-terminal domain of PRG4 (exons 7-12) and O-glycosylation sites within the N-terminal domain of PRG4 (exons 2-5). Other researchers have utilised fluorophore-assisted carbohydrate electrophoresis (FACE) to discover a small amount of asialo-, digalactosylated bi-antennary, bisecting GlcNAc, tri-mannosyl core fucosylated N-link on human

PRG4 isolated from synovial fluid (Jay & Cha, 2002). This bi-antennary structure on other glycoproteins has been associated with protein folding (reviewed by Yamaguchi, 2002), and the data presented in this chapter details N-glycosylation as being present in the C-terminal domain of PRG4. Interestingly, the study that characterised the N-linked glycans of human PRG4 by FACE did not find N-linked oligosaccharides on bovine PRG4 (Jay & Cha, 2002), which is contrary to the results documented in this study. The glycosylation of recombinant PRG4 domains could be further characterised by deglycosylation with TFMS (trifluoromethanesulphonic acid, reviewed by Edge, 2003). The glycans present could be further characterised by deglycosylation with specific glycosidases (as performed in this study) followed by FACE.

4.5 Chapter Summary

- Affinity chromatography experiments with recombinant PRG4 domains have shown that there are heparin-binding sites in the N- (exons 2-5) and C- (exons 7-12) terminal domains of PRG4.
- N-terminal amino acid sequencing and Western blot analyses of the two BovC protein species has revealed that the BovC2 species starts with internal PRG4 sequence from exon 10. BovC2 is also immunopositive for an antibody to PRG4 exon 12 whereas BovC1 is immunonegative, suggesting that the BovC1 species represents bovine PRG4 exons 7-9 and part of exon 10, and that BovC2 represents the remainder of exon 10 to the end of exon 12.
- Western blot analysis of the recombinant N-terminal PRG4 domains have shown that monoclonal antibody 3-A-4 recognises a non-reduced conformation-dependent epitope on bovine (but not human) PRG4, exons 2-5. This is a similar antibody recognition pattern as seen with native bovine articular cartilage PRG4, and maps the specificity of antibody 3-A-4 to the N-terminal region of PRG4.
- Lectin affinity blotting and enzymatic deglycosylation of recombinant PRG4 domains have partially characterised the glycosylation patterns of the recombinant proteins.
- The experiments described in this chapter have facilitated the characterisation of the recombinant PRG4 protein species.

5. PROTEINASE DIGESTION OF PRG4

5.1 Introduction

This chapter presents results from *in vitro* digestions of full-length PRG4 described in Chapter 2 and the purified recombinant PRG4 structural domains described in Chapters 3 and 4. The rationale for these digestions can be described under the following three sub-headings.

(a) Generation of distinct structural PRG4 domains.

The initial aim of the work presented in this chapter was to digest purified full-length human and bovine PRG4 with a view to obtaining distinct structural domains of PRG4 that could be used for biochemical analyses and cell biology experiments. This would enable investigations into the link between the structural features of PRG4 and its proposed independent physiological functions. The generation of the central mucin-like region encoded for by exon 6 was of particular interest, as the recombinant protein expression work described earlier in this thesis was limited to the non-mucin N- and C-terminal domains. The derivation of other PRG4 domains from the full-length molecule by enzymatic digestion was a secondary aim of this work.

(b) Identification of proteinases capable of degrading full-length PRG4.

Previous work has identified lower molecular weight forms of PRG4 in cartilage and synovial fluid (Schumacher *et al.*, 1999; Tudor, 2002), and the occurrence of a number of different forms of PRG4 within the synovial joint has been documented (Jay *et al.*, 2001b). Along with alternative splicing, proteinase digestion may contribute to the different forms of PRG4 found within the synovial joint. Different forms of PRG4 may possess different structural features, and this may affect the functionality of the molecule. In addition to this, it is interesting to consider that degradation of PRG4 by proteinases present within an inflamed synovial joint could affect the functional integrity of the molecule. The susceptibility of full-length PRG4 to digestion within the synovial joint was investigated by performing *in vitro* proteinase digests of purified preparations of full-length PRG4 (chapter 2) with a panel of physiologically relevant proteinases.

(c) Identification of proteinases capable of degrading recombinant PRG4 domains.

The specificity of the digestions of full-length PRG4 observed in part (b) was investigated by performing *in vitro* proteinase digests with preparations of recombinant PRG4 N- and C-terminal domains (chapter 3) and PRG4-degrading proteinases.

5.2 Materials and Methods

All chemicals were of an analytical grade and were purchased from Sigma-Aldrich unless indicated otherwise. A detailed list of the materials used in this research project and their suppliers is listed in Appendix A.

5.2.1 Materials

5.2.1.1 Source of PRG4 (full-length and domains)

The purification of human recombinant full-length PRG4 from the culture medium of a PRG4-expressing CHO cell line is described in chapter 2, section 2.3.1. The partial purification of bovine cartilage PRG4 from the culture medium of superficial zone chondrocytes cultured in agarose is described in chapter 2, section 2.3.2. The cloning, expression and purification of bovine and human N-terminal (exons 2-5) and bovine C-terminal (exons 7-12) PRG4 domains is described in chapter 3. Protein concentrations were calculated by reading absorbency values at 280nm.

5.2.1.2 Enzymes and reaction buffers

The enzymes used are listed in table 5.1 (over page), along with their sources, EC number and digestion buffers used in each case. Digestion buffers were formulated with the advice of enzyme suppliers, with the exception of the cathepsin digestion buffer, which is a formulation, previously used by Fosang *et al.* (1992).

5.2.2 Enzyme concentrations

The enzyme concentrations used for preliminary experiments using an excess of enzyme are listed in table 5.2 (over page). In experiments with different amounts of enzymes, enzyme concentrations are described within the relevant figures.

5.2.3 Preliminary digestions of full-length PRG4

Human recombinant full-length PRG4 was purified from the conditioned medium of a stable, PRG4-expressing polyclonal CHO cell line as described in chapter 2. Initial digestions were carried out with an excess of each enzyme. For each separate digestion, 40μg of PRG4 was added to a 1.5ml screw-cap tube. Appropriate amounts of MilliQ water, 10X digestion buffer (table 5.1)

and enzyme (table 5.2) was added to total a reaction of 100µl. For control digestions the constituents of the reaction were the same, and included 10X MMP reaction buffer, but did not contain enzyme.

Table 5.1 – Enzymes and digestion buffers used during *in vitro* digestions of PRG4

Enzyme	Source	EC number	Digestion buffer
Cathepsin B, from bovine spleen	Sigma	3.4.22.1	0.25M Na-Acetate, pH 5.5, 0.2M NaCl, 1mM EDTA, 10mM DTT
Cathepsin D, from bovine spleen	Sigma	3.4.23.5	0.25M Na-Acetate, pH 5.5, 0.2M NaCl, 1mM EDTA, 10mM DTT
MMP-1, human recombinant	Gift from Paul Cannon (Roche)	3.4.24.7	0.1M Tris-HCl, pH 7.5, 50mM NaCl, 1mM $CaCl_2$, 1mM APMA
MMP-3, human recombinant	Gift from Paul Cannon (Roche)	3.4.24.17	0.1M Tris-HCl, pH 7.5, 50mM NaCl, 1mM $CaCl_2$, 1mM APMA
MMP-7, human recombinant	Gift from Paul Cannon (Roche)	3.4.24.23	0.1M Tris-HCl, pH 7.5, 50mM NaCl, 1mM $CaCl_2$, 1mM APMA
Trypsin, from bovine pancreas, TPCK-treated	Sigma	3.4.21.4	0.1M Na-Acetate, pH 7.3, 0.1M Tris
Chymotrypsin, from bovine pancreas, TLCK-treated	Sigma	3.4.21.1	10mM Tris-HCl, pH 7.8, 10mM $CaCl_2$
Plasmin, from human plasma	Sigma	3.4.21.7	20mM Tris-HCl, pH 7.4, 0.15M NaCl
ADAMTS-4, human recombinant	Gift from Dr. C. R. Flannery (Wyeth, MA)	3.4.24.82	0.1M Tris-HCl, pH 7.4, 50mM NaCl, 10mM $CaCl_2$, 10mM $MgCl_2$
ADAMTS-5, human recombinant	Gift from Dr. C. R. Flannery (Wyeth, MA)	3.4.24.-	0.1M Tris-HCl, pH 7.4, 50mM NaCl, 10mM $CaCl_2$, 10mM $MgCl_2$
Calpain II, from porcine kidney	Calbiochem	3.4.22.17	0.11M imidazole, pH 7.5, 1mM EGTA, 5mM β-mercaptoethanol
Elastase, from human leukocytes	Sigma	3.4.21.37	0.1M Tris-HCl, pH 8.8, 0.1M $CaCl_2$

A 50mM stock solution of APMA (p-aminophenyl mercuric acetate) was made up in 50mM Tris-HCl, pH 7.5, 1mM $CaCl_2$, 0.05% Triton X-100.

Table 5.2 – Enzyme concentrations used in preliminary digestions of full-length PRG4. These concentrations equate to a large excess of enzyme based on information from the suppliers.

Enzyme	Concentration
Elastase	0.2U/ml
Calpain	1µg/ml
Cathepsin B	0.5U/ml
Cathepsin D	0.5U/ml
Plasmin	0.2U/ml
ADAMTS-4	9.3µg/ml
MMP-1	5µg/ml
MMP-2	5µg/ml
MMP-3	10µg/ml
MMP-7	10µg/ml

Reactions were incubated at 37°C for 16 hours followed by dialysis against water and lyophilisation. Reaction mixtures were reconstituted in 90µl 1X SDS-PAGE sample buffer (chapter 2, table 2.1) and 10µl β-mercaptoethanol and separated on two 4-12% SDS-PAGE gradient gels (50µl/lane).

5.2.4 Titrated digests of full-length PRG4 and domains of PRG4

Human recombinant, bovine cartilage, recombinant human N-terminal (exons 2-5) and bovine C-terminal (exons 7-12) PRG4 were purified and characterised as described in chapters 2 and 3, respectively. For digests with different amounts of enzyme, the type and amount of PRG4, along with the enzyme used and its concentration, are listed within the relevant figures. For all reactions, a total volume of 50µl was adjusted with the appropriate 10X reaction buffer specific to the enzyme used (table 5.1). Control digestions were performed without enzyme. Reactions were incubated for 2 hours at 37°C before the addition of 40µl 2X SDS-PAGE sample buffer (chapter 2, table 2.1) and 10µl β-mercaptoethanol. Proteins were then separated by SDS-PAGE (50µl/lane) prior to detection.

5.2.5 Analysis of digestion products

Digestion products were separated by SDS-PAGE under reducing conditions as described in chapter 2, section 2.2.9. Digestions of full-length PRG4 (~340 kDa) were separated on 4-12% gradient gels and digestions of recombinant PRG4 domains (~10-30 kDa) were separated on 15% slab gels. Detecting PRG4 with a number of methods assessed the extent of proteolytic degradation. Total protein was detected by silver stain as described in chapter 2, section 2.2.12. For the immunolocation of PRG4, gels were electro-eluted on to nitrocellulose and Western blotting was performed using a variety of antibodies, the specificities of which are detailed in chapter 2, table 2.4. The central mucin domain of PRG4 was detected using lectin affinity blotting with the lectin MAA (chapter 2, table 2.5). The Western blotting procedures used are described in chapter 2, section 2.2.9 and the lectin affinity blotting procedures used are described in chapter 2, section 2.2.10.

5.3 Results

The *in vitro* proteinase digests of PRG4 documented in this chapter were either preliminary digests with a fixed amount of enzyme, or digests with the inclusion of different amounts of enzymes. The amount of substrate was constant within each set of digests.

5.3.1 Generation of PRG4 mucin-like domain by proteolytic digestion of full-length PRG4

As stated in the introduction to this chapter, the cloning and expression of PRG4 domains in this study was limited to the non-mucin, vitronectin-like N-terminal (exons 2-5) and C-terminal (exons 7-12) domains, therefore it was of interest to obtain the mucin-like central domain of PRG4 (exon 6). This would enable studies of its physiological function. The strategy to facilitate this was to derive the central mucin-like domain from purified full-length human recombinant and bovine native PRG4 by means of proteolytic digestion. To facilitate the removal of the non-mucin domains from purified human recombinant PRG4 (hrPRG4) for future functional studies, digestion studies were performed using trypsin and chymotrypsin. Figure 5.1 shows the residues within the human PRG4 sequence that would be affected by trypsin or chymotrypsin digestion, highlighted in red and blue, respectively. In the case of trypsin, it was predicted that lysine and leucine residues within the central mucin domain of hrPRG4 would be protected from limited trypsin digestion by virtue of the steric hindrance provided by its extensive oligosaccharide substitutions, a phenomenon observed in other glycoproteins such as ribonuclease (Bernard *et al.*, 1983). As regards chymotrypsin digestion of PRG4, the central mucin domain does not contain aromatic amino acids, so theoretically the region would be left intact (Jay *et al.*, 2001b).

5.3.1.1 Digestion of human recombinant full-length PRG4 with trypsin

For these *in vitro* digestion experiments, the substrate was hrPRG4 purified by heparin agarose and PNA lectin affinity chromatography (see chapter 2). The hrPRG4 substrate was incubated with TPCK-treated trypsin, and the first of these experiments is shown in figure 5.2, where different amounts of trypsin were used to digest 10μg of hrPRG4 in a 50μl reaction with a 2 hour incubation. A no enzyme control is shown in lane 1, and all reactions were visualised by SDS-PAGE followed by silver stain (figure 5.2A) or MAA lectin affinity blotting (figure 5.2B). HrPRG4 was completely digested with trypsin concentrations of 20 and 8 μg/ml (figure 5.2, lanes 2 & 3), as there were no protein species apparent with a silver stain or a lectin affinity blot.

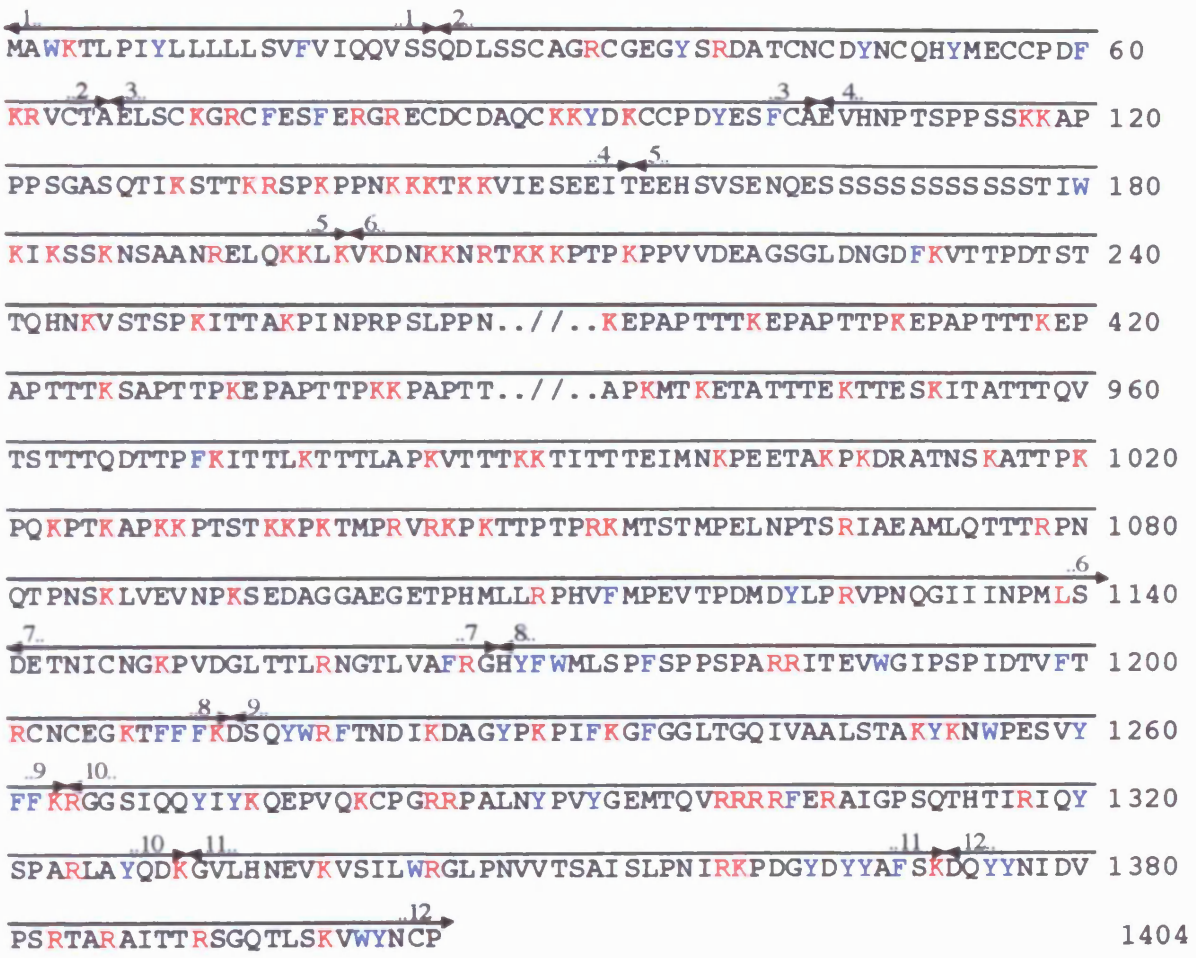


Figure 5.1 – Predicted trypsin and chymotrypsin cleavage sites within human PRG4. Trypsin theoretically cleaves C-terminal to lysine and arginine residues (shown in red); chymotrypsin theoretically cleaves after phenylalanine, tryptophan or tyrosine residues (shown in blue. Exon boundaries are shown above the amino acid sequence. Most of the exon 6 mucin-like repeat sequence has been omitted for clarity. Adapted from Flannery *et al.*, 1999.

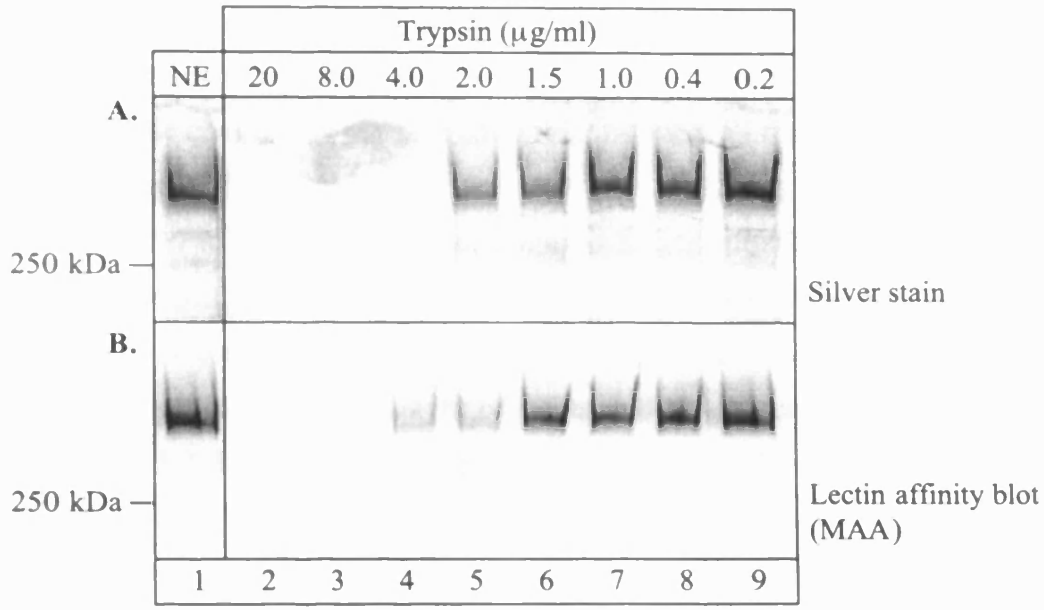


Figure 5.2 – Digestion of human recombinant PRG4 with decreasing concentrations of trypsin. Reaction mixtures of 50μl containing 10μg PRG4 were separated by SDS-PAGE under reducing conditions (25μl/lane, 5μg PRG4/lane) and were visualised by (A) silver stain and (B) lectin affinity blot with the lectin MAA. A control reaction showing PRG4 with no enzyme (NE) is shown in lane 1. Migration positions of molecular mass standards are shown to the left of the panel.

Decreasing trypsin concentrations resulted in a decrease in the extent of hrPRG4 digestion (figure 5.2, lanes 4-9), as the observed silver stain and lectin affinity blot intensity increases. However, the hypothesis that exon 6 of PRG4 is protected from tryptic digestion by its extensive oligosaccharide side chains was not proved in this instance, as there was a lack of any reduced molecular weight hrPRG4 catabolites that were stained by MAA. The presence of any reduced molecular weight hrPRG4 catabolites with oligosaccharide substitutions would signify that the domains either side of the mucin domain had been digested, and these catabolites could then be assessed further by Western blot to examine look for the presence or absence of these non-mucin domains. The overall loss in the strength of silver and MAA staining of the trypsin digestion products in figure 5.2, lanes 4 & 5, suggests that digestion of the mucin domain has also taken place, and that this domain was not protected from proteolytic digestion. These data show, therefore, that under the conditions used, trypsin digestion would be an inefficient way to derive the central mucin domain of hrPRG4 from the full-length molecule.

5.3.1.2 Digestion of human recombinant full-length PRG4 with chymotrypsin.

As stated in the introduction to this section, the central mucin domain of PRG4 does not contain any aromatic amino acid residues and should therefore be insusceptible to chymotrypsin digestion. Figure 5.3 shows the separation by SDS-PAGE of reaction mixtures containing hrPRG4 and different concentrations of chymotrypsin. Again, reactions were incubated at 37°C for 2 hours, and a no enzyme (NE) control is shown in figure 5.3, lane 1. Proteins in the reaction mixture were detected by silver stain (figure 5.3A) and an immunoblot with antibodies to exon 3 and exon 12 of human PRG4 (figure 5.3B). At chymotrypsin concentrations of 20μg/ml and 10μg/ml (figure 5.3, lanes 2 & 3), a reduced molecular weight protein species was generated that was visible by silver stain (figure 5.3A) but was not positive for anti- exon 3 or exon 12 antibody staining (figure 5.3B, lanes 2 & 3). This indicates that either the anti-exon 3 and 12 epitopes were removed by digestion as predicted, or that the protein concentration was too low for antibody staining. However, a reduction in the silver staining intensity of the reduced molecular weight PRG4 species indicates that digestion of the molecule as a whole also took place, and that the mucin domain was not protected from digestion. A chymotrypsin concentration of 10μg/ml equates to an enzyme:substrate ratio of 1:10 in this case.

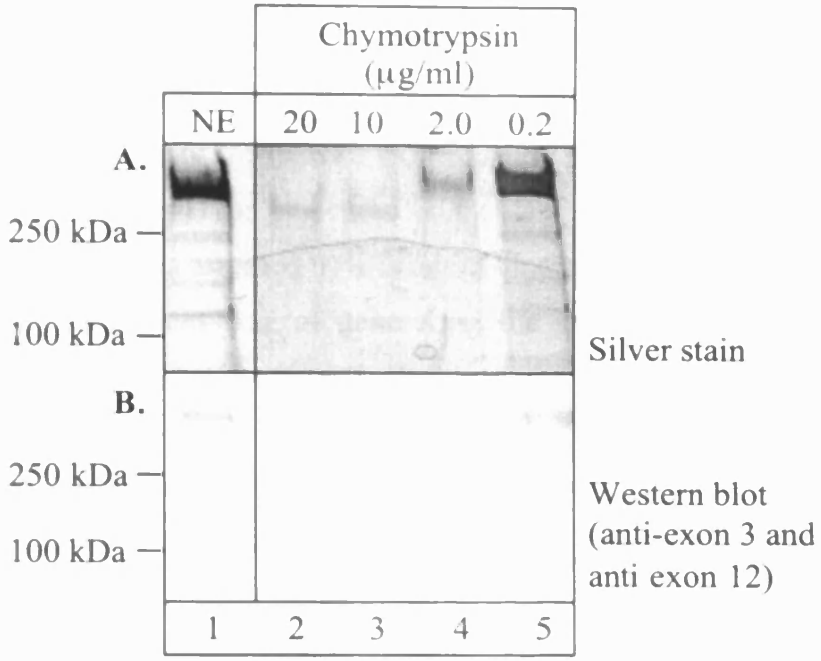


Figure 5.3 – Digestion of human recombinant PRG4 with decreasing concentrations of chymotrypsin. Reaction mixtures of 50µl containing 5µg PRG4 were separated by SDS-PAGE under reducing conditions (25µl/lane, 2.55µg PRG4/lane) and were visualised by (A) silver stain and (B) Western blot with the anti-exon 3 and anti-exon 12 polyclonal antibodies. A control reaction with no enzyme (NE) is shown in lane 1. Migration positions of molecular mass standards are shown to the left of the panel.

Digestion of PRG4 as a whole also occurred at a chymotrypsin concentration of 2μg/ml as seen in figure 5.3, lane 2. In this case there was no shift in molecular weight compared with the no enzyme control (lane 1), but there was a reduction in silver staining intensity, which is likely to signify non-specific digestion of the entire PRG4 molecule rather than specific digestion of the non-mucin domains. Therefore it can be concluded that chymotrypsin digestion removes the non-mucin domains from PRG4, as assessed by a Western blot with anti- exon 3 & 12 antibodies, but that it would be an inefficient way of generating the mucin domain of PRG4 for further experiments due to the losses that occurred under the conditions used.

5.3.1.3 Digestion of native bovine full-length PRG4 with chymotrypsin

Chymotrypsin digestions were also performed using as a substrate bovine articular cartilage PRG4 purified from the conditioned media of superficial zone chondrocyte agarose cultures by heparin agarose affinity chromatography (see chapter 2). Figure 5.4 shows the analysis of reaction mixtures containing bovine articular cartilage PRG4 and different concentrations of chymotrypsin where reactions were incubated at 37°C for 2 hours, and a no enzyme (NE) control is shown in figure 5.4, lane 1. Proteins in the reaction mixture were separated by SDS-PAGE and detected by silver stain (figure 5.4A) and a Western blot with the monoclonal antibody 6-A-1 (figure 5.4B). At chymotrypsin concentrations of 20μg/ml and 10μg/ml, a reduced molecular weight catabolite was apparent by silver stain (figure 5.4A, lanes 2 & 3), although intensity of staining was greatly reduced compared to the no enzyme control, signifying that as in the chymotrypsin experiments with hrPRG4, digestion of the molecule as a whole took place.

These catabolites were negative for staining with the antibody 6-A-1, which has been purported to have a recognition epitope in the non-mucin C-terminal domain of bovine PRG4 (Schumacher et al., 1999). However, this may be due to low overall protein concentration. Another catabolite was observed after digestion with a chymotrypsin concentration of 2.0μg/ml (figure 5.4A, lane 4) that was of a higher molecular weight and silver stain intensity than the catabolites seen in lanes 2 & 3. This catabolite was also negative for 6-A-1, and this is less likely to be due to low protein concentration than for the catabolites seen in lanes 2 & 3, which suggests that the C-terminal domain of bovine PRG4 has been digested.

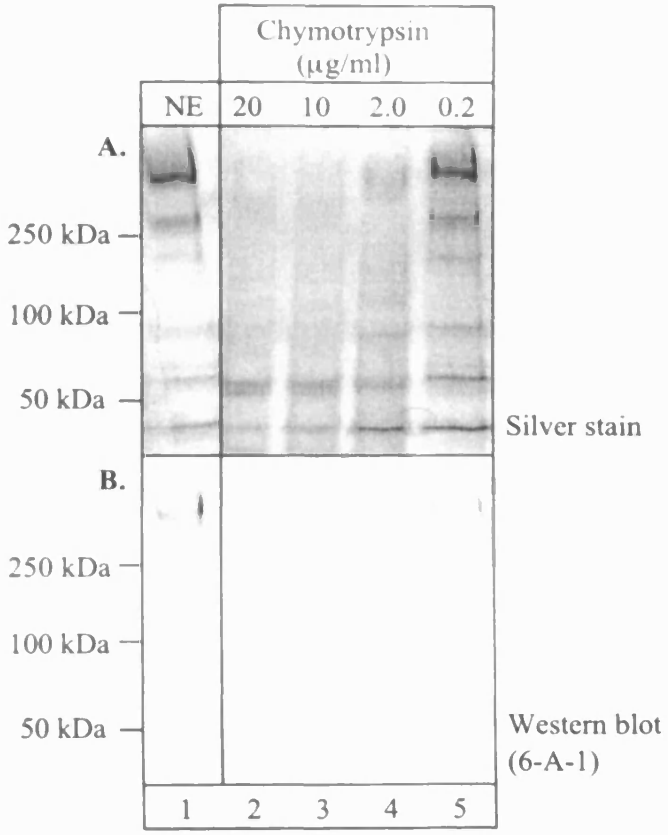


Figure 5.4 – Digestion of bovine articular cartilage PRG4 with decreasing concentrations of chymotrypsin. Reaction mixtures of 50μl containing 2.5μg PRG4 were separated by SDS-PAGE under reducing conditions (25μl/lane, 1.25μg PRG4/lane) and were visualised by (A) silver stain and (B) Western blot with the monoclonal antibody 6-A-1. A control reaction with no enzyme (NE) is shown in lane 1. Migration positions of molecular mass standards are shown to the left of the panel.

Therefore, similarly to the chymotrypsin digestion experiments with human recombinant full-length PRG4, it can be concluded that digestion of the non-mucin domains of bovine PRG4 is effected by chymotrypsin, but that digestion of the molecule as a whole also takes place, making chymotrypsin digestion an inefficient method for the production of the central mucin domain of bovine articular cartilage PRG4.

5.3.2 Identification of physiologically relevant proteinases capable of degrading full-length PRG4

The susceptibility of full-length human recombinant and bovine articular cartilage PRG4 to digestion within the synovial joint was investigated by performing *in vitro* digests of purified preparations of full-length PRG4 (chapter 2) with a panel of physiologically relevant proteinases. These digests were conducted overnight, and the enzyme concentrations used was predicted to be concentrations that would facilitate complete digestion of a substrate based on information from the individual enzyme suppliers.

5.3.2.1 Identification of proteinases capable of degrading human recombinant full-length PRG4

A number of preliminary digestions of full-length human recombinant PRG4 (hrPRG4) were conducted, using a panel of proteinases of potential physiological relevance. These results are summarised in table 5.3.

Table 5.3 – Results of preliminary hrPRG4 digestion experiments, where 40μg hrPRG4 was included in a 100μl reaction with enzymes at the concentrations shown. The table indicates if hrPRG4 was digested (+) or undigested (-).

Enzyme	Concentration	HrPRG4 digestion
Cathepsin B	0.5U/ml	+
Cathepsin D	0.5U/ml	+
Elastase	0.2U/ml	+
MMP-7	10μg/ml	+
Plasmin	0.2U/ml	+
ADAMTS-4	9.3μg/ml	-
ADAMTS-5	18μg/ml	-
Calpain	1μg/ml	-
MMP-1	5μg/ml	-
MMP-2	5μg/ml	-
MMP-3	10μg/ml	-

For these experiments, 40μg of purified hrPRG4 was incubated in a 100μl reaction with a fixed concentration of each enzyme at 37°C for 16 hours. Reaction mixtures were separated by electrophoresis on SDS-PAGE gradient gels under reducing conditions, and catabolites were identified by silver stain, Western blot with the antibody 06A10, or affinity blot with the lectin MAA, which recognises the oligosaccharide side chains substituted on full-length PRG4 (chapter 2, figure 2.10).

As listed in table 5.3, digestion of the hrPRG4 substrate was observed with cathepsin B, cathepsin D, elastase, MMP-7 and plasmin, and SDS-PAGE analyses of these reactions are shown in figures 5.5 and 5.6. No digestion of hrPRG4 was observed with ADAMTS-4, ADAMTS-5, calpain, MMP-1, MMP-2 or MMP-3, and these data are not shown. With these enzymes, the electrophoretic mobility and the antibody staining intensity of PRG4 was not discernibly altered.

Figure 5.5 shows SDS-PAGE analyses of hrPRG4 after digestion by cathepsin B (figure 5.5B) and cathepsin D (figure 5.5C). A no enzyme control reaction is shown in figure 5.5A, and PRG4 was identified in each case by silver stain and Western blot with the antibody 06A10, a polyclonal antibody raised against purified human PRG4. Silver stain analysis of an enzyme only reaction is shown in each panel in order to evaluate the contribution of the enzyme preparations to the overall protein staining pattern. Complete digestion of the hrPRG4 substrate was observed with cathepsin B (figure 5.5B), as no protein was apparent with either silver stain or Western blot. Digestion of hrPRG4 by cathepsin D was less comprehensive (figure 5.5C). No shift in electrophoretic mobility was observed by silver staining, although intensity was reduced. Antibody binding was considerably reduced, suggesting an alteration in the 06A10 antibody recognition epitope.

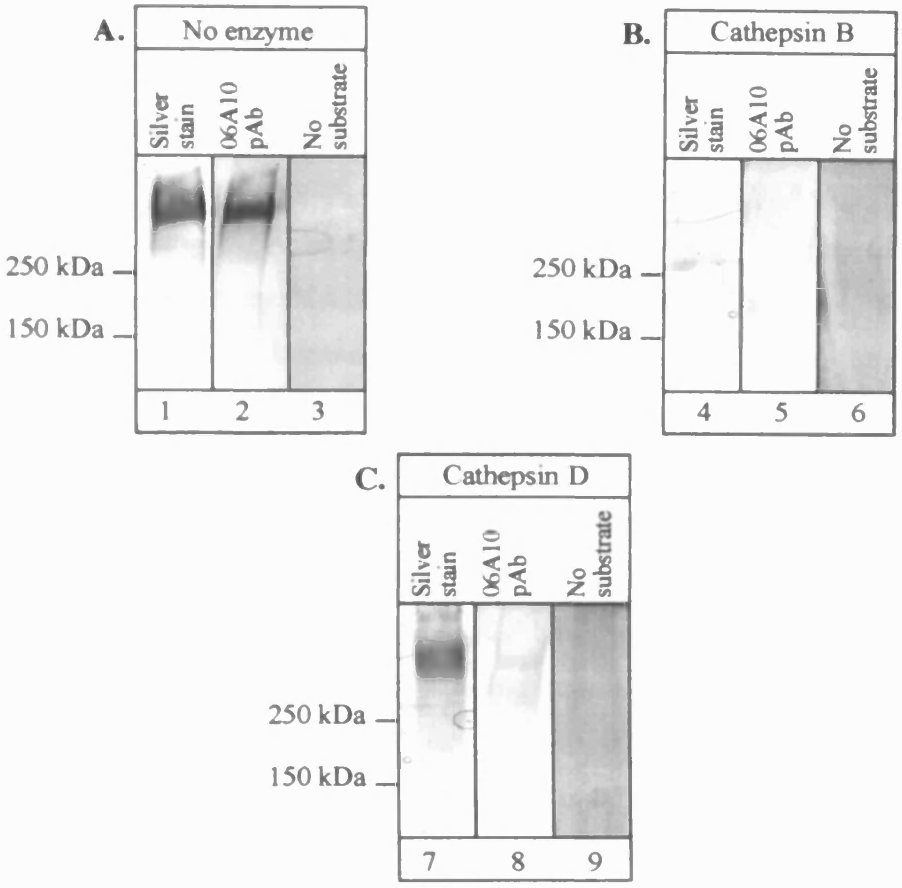


Figure 5.5 – Analysis of human recombinant PRG4 proteinase digestion reactions including (A) no enzyme, (B) cathepsin B or (C) cathepsin D. Reactions (40μg/100μl reaction) were subjected to SDS-PAGE under reducing conditions (20μg/lane) and were visualised by silver stain and Western blot with the polyclonal antibody 06A10. The enzyme concentrations used are listed in table 5.2, and no substrate (enzyme only) control reactions are shown for each enzyme after silver staining. The migration positions of molecular mass standards are shown to the left of each panel.

Figure 5.6 shows SDS-PAGE analyses of digestion reactions including hrPRG4 substrate and elastase (figure 5.6B) MMP-7 (figure 5.6C) or plasmin (figure 5.6D). Again, a no enzyme control reaction is shown in figure 5.6A, and PRG4 was identified in each case by silver stain and either Western blot with the antibody 06A10 or a lectin affinity blot using MAA. Silver stain analyses of reactions performed in the absence of hrPRG4 substrate are shown to the right of each panel. Complete digestion of hrPRG4 was observed with elastase (figure 5.6B). Digestion of hrPRG4 with MMP-7 (figure 5.6C) generated a catabolite of ~280 kDa that was visible by silver staining. No intact hrPRG4 remained in this reaction, a loss in 06A10 antibody binding was observed. Plasmin digestion (figure 5.6D) generated a hrPRG4 catabolite with a slightly reduced molecular weight, although much of the PRG4 was digested based on the reduction in silver stain intensity and the large reduction in 06A10 antibody staining intensity.

In the preliminary experiments shown in figures 5.5 and 5.6, cathepsin B and plasmin appeared to digest hrPRG4. Further digestions with these enzymes were conducted where hrPRG4 was incubated with decreasing concentrations of proteinase, and a shorter incubation period of 2 hours was used. Reactions of 50µl were separated by SDS-PAGE under reducing conditions and were analysed by silver stain and Western blot with antibodies specific to the non-mucin domains of PRG4, the polyclonal antibodies anti-exon 3 and anti-exon 12 (Wyeth). The results can be seen in figure 5.7, where 5.7A is a silver stain and 5.7B is a Western blot. A control reaction that contained no enzyme is shown in lane 1.

Digestion with a cathepsin B concentration of 0.2U/ml (figure 5.7, lane 2) resulted in a PRG4 catabolite of reduced molecular weight (~300 kDa) that was apparent by silver stain. Interestingly, this catabolite was not visible with antibodies directed towards sequences in exon 3 and exon 12 (figure 5.7B, lane 2), and two lower molecular weight catabolites (~25 kDa) are immunopositive for these antibodies. This suggests that limited cathepsin B digestion removes non-mucin domains from hrPRG4. Digestion with higher concentrations of plasmin (figure 5.7, lanes 5-9) resulted in a reduction in silver staining intensity with a slight reduction in electrophoretic mobility, although the relatively large mass of hrPRG4 makes it difficult to assess small changes in electrophoretic mobility on a gradient polyacrylamide gel.

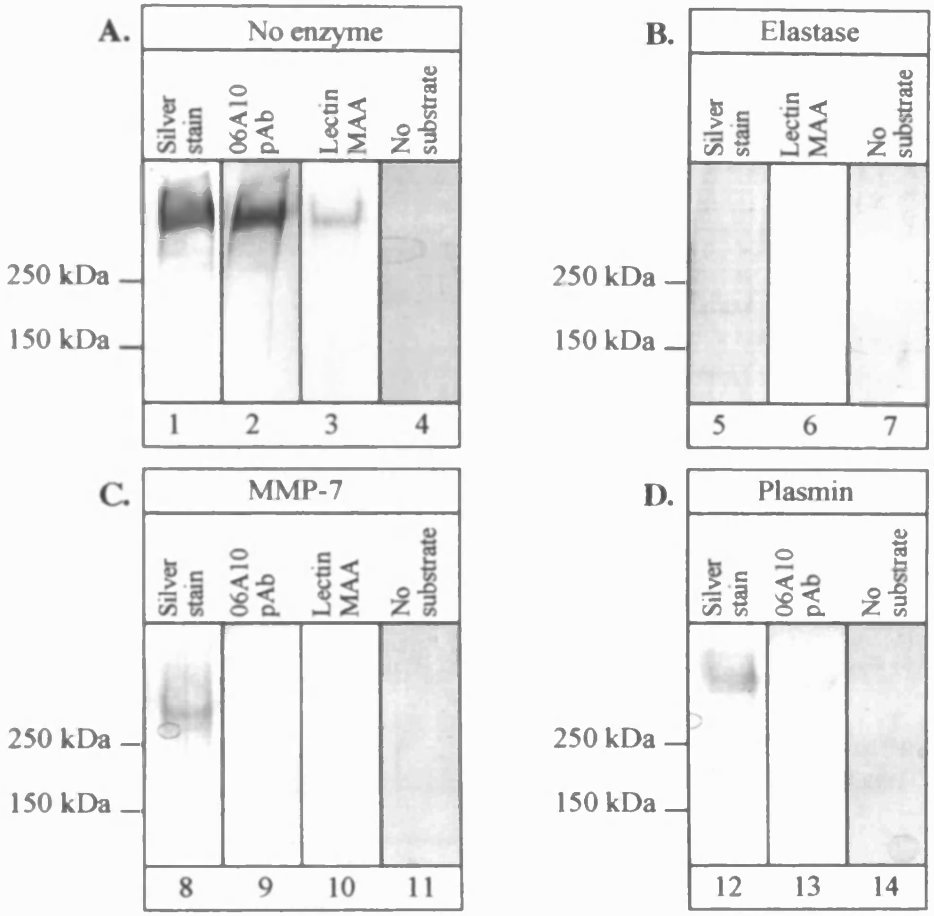


Figure 5.6 – Analysis of human recombinant PRG4 proteinase digestion reactions including (A) no enzyme, (B) elastase, (C) MMP-7 or (D) plasmin. Reactions (40µg/reaction) were subjected to SDS-PAGE under reducing conditions (20µg/lane) and were visualised by silver stain. PRG4 was identified either by MAA lectin affinity blot or a Western blot with the polyclonal antibody 06A10. The enzyme concentrations used are listed in table 5.2, and no substrate (enzyme only) control reactions are shown for each enzyme after silver staining. The control reaction is duplicated from figure 5.5. The migration positions of molecular mass standards are shown to the left of each panel.

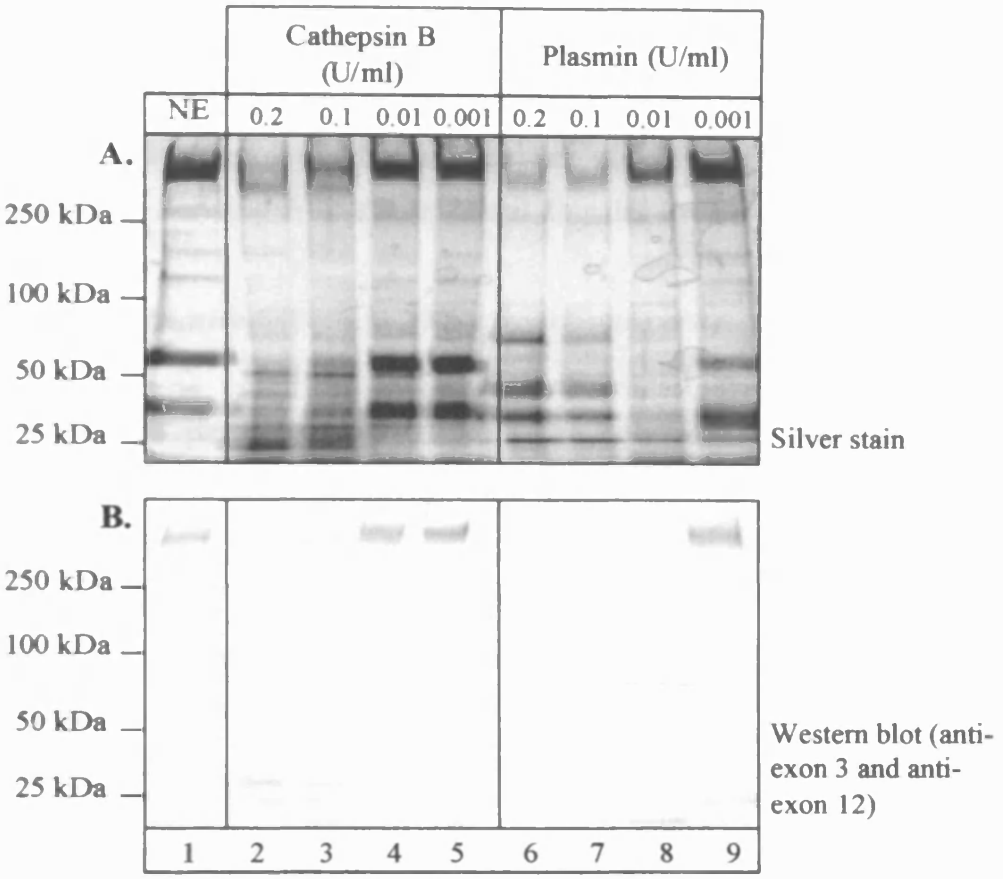


Figure 5.7 – Analysis of human recombinant PRG4 proteinase digestion reactions with decreasing concentrations of cathepsin B or plasmin. Reactions (5µg/50µl reaction) were incubated for 2 hours and subjected to SDS-PAGE under reducing conditions (2.5µg/lane). Total protein was visualised by silver stain (A). PRG4 was identified by Western blotting with polyclonal antibodies to sequences within exon 3 and exon 12 of human PRG4. A no enzyme (NE) control reaction is shown on the left. The migration positions of molecular mass standards are shown to the left.

Interestingly, a loss of staining in the Western blot with antibodies to exon 3 and exon 12 signifies that the remaining PRG4 species does not contain these domains. Catabolites containing these epitopes can be seen towards the bottom of the gel, running at around 20 kDa. Taken together, these results suggest that limited digestion of hrPRG4 by cathepsin B or plasmin may be a useful method for deriving different structural domains from a full-length PRG4 molecule.

5.3.2.2 Digestion of native full-length PRG4 from bovine articular cartilage with cathepsin B and plasmin

Bovine PRG4 was partially purified from the conditioned medium of superficial zone chondrocyte agarose cultures as described in chapter 2. This material was then subjected to digestion experiments with decreasing amounts of cathepsin B and plasmin, with an incubation time of 2 hours at 37°C. The reaction mixtures were separated as before and can be seen in figure 5.8, visualised by silver stain (5.8A) and Western blot with the monoclonal antibody 6-A-1 (5.8B). A negative control is shown to the left (lane 1). At a cathepsin B concentration of 0.2U/ml (figure 5.8, lane 2), there was a shift in the electrophoretic mobility of PRG4 to around 300 kDa and a concomitant loss of 6-A-1 immunoreactivity. However, two 6-A-1 positive catabolites of around 100 kDa and 60 kDa were generated. Amino acid sequencing of these catabolites could be useful in the epitope-mapping of 6-A-1. At lower cathepsin B concentrations (figure 5.8, lanes 3-5) digestion of bovPRG4 was less comprehensive, and the 60 kDa catabolite predominates over the 100 kDa species (figure 5.8B, lanes 3-5).

Digestion of bovPRG4 with a plasmin concentration of 0.2U/ml (figure 5.8, lane 2) resulted in a reduced molecular weight catabolite of ~300 kDa that displays a reduction in silver staining intensity. This catabolite is not positive for 6-A-1 (figure 5.8B). This could be due to the amount of protein being too small, or the lack of 6-A-1 epitope within the catabolite. Interestingly, a catabolite of ~60 kDa is generated that was detected by 6-A-1 (lanes 3 & 4), though this too is degraded by plasmin a 0.2U/ml (lane 4). In general, digestion of bovPRG4 with decreasing concentrations of cathepsin B and plasmin (figure 5.8) resulted in a similar pattern to that seen when hrPRG4 was digested by cathepsin B and plasmin (figure 5.7).

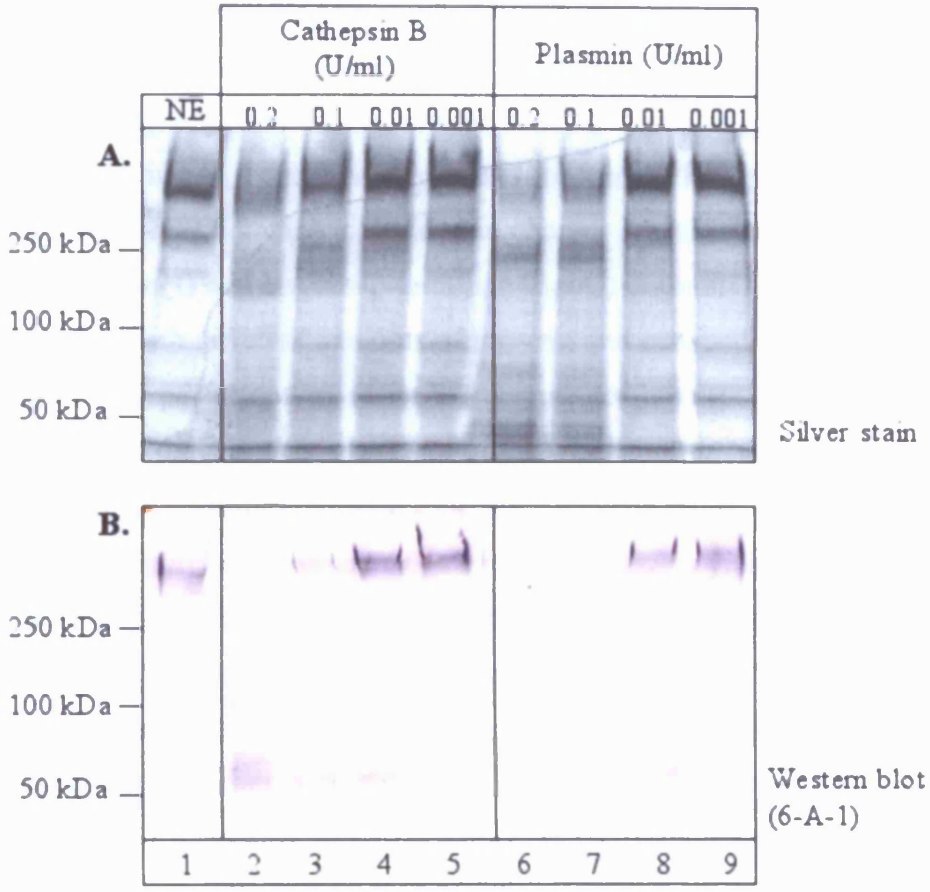


Figure 5.8 – Analysis of bovine articular cartilage PRG4 proteinase digestion reactions with decreasing concentrations of cathepsin B or plasmin. Reactions (2.5µg total protein/50µl reaction) were incubated for 2 hours and subjected to SDS-PAGE under reducing conditions (1.25µg/lane). Total protein was visualised by silver stain (A). PRG4 was identified by Western blotting with the monoclonal antibody 6-A-1. A no enzyme (NE) control reaction is shown on the left. The migration positions of molecular mass standards are shown to the left.

5.3.3 *In vitro* proteinase digestion of recombinant PRG4 structural domains

So far this chapter has documented the *in vitro* digestion of full-length PRG4 by a number of proteinases, and the occurrence of lower molecular weight PRG4 catabolites as a result of these digestions. The action of these proteinases on PRG4 was investigated further by utilising recombinant PRG4 structural domains in similar *in vitro* digestion experiments. The cloning, expression and purification of recombinant human N-terminal PRG4 (exons 2-5) and bovine C-terminal PRG4 (exons 7-12) domains is detailed in chapter 3 of this study. Purified recombinant human N-terminal (HumN) and bovine C-terminal (BovC) domains were utilised in proteinase digestion experiments with different amounts of enzyme and constant amounts of PRG4 domain substrate. Digests were performed in a similar manner to the experiments with hrPRG4 and bovPRG4 described earlier in this chapter, with an incubation time of 2 hours at 37°C. Recombinant human N-terminal domain (HumN) was purified by FLAG immnoaffinity chromatography and heparin-agarose affinity chromatography to yield one protein species that runs at ~27 kDa on an SDS-PAGE gel under reducing conditions. It should be borne in mind that the FLAG-purified recombinant C-terminal domain of bovine PRG4 (BovC) preparation described in chapters 3 and 4 contains two protein species. There is a species of around 20 kDa, BovC1, which is negative for staining with an antibody to exon 12 of human PRG4, and a 12 kDa species, BovC2, which is immunopositive for anti-exon 12. It was concluded (see chapter 3) that BovC1 contains exons 7, 8, 9 and part of exon 10, whereas BovC2 contains the remainder of exon 10 plus exons 11 and 12.

5.3.3.1 Digestion of recombinant PRG4 domains with leukocyte elastase

Aliquots of human recombinant N-terminal PRG4 domain (HumN) and bovine recombinant C-terminal PRG4 domain (BovC) were digested with decreasing amounts of elastase (2.0 – 0.2U/ml) at 37°C for 2 hours. Similar to all the results described in this section, the reaction mixtures were separated by SDS-PAGE and were visualised by silver stain and Western blot with an anti-exon 3 antibody for HumN, or an anti-exon 12 polyclonal antibody for BovC (B). There appeared to be no apparent digestion of HumN with different amounts of elastase under the conditions used, as both silver stain and Western blot (anti-exon 3) intensities were not reduced in comparison with an undigested control (data not shown). It may be that the digestion of PRG4 domains by elastase requires a longer incubation period than other proteinases used in this study.

As seen in figure 5.9, digestion of BovC with higher amounts of elastase reduced the intensity of the 20 kDa BovC1 band in comparison to undigested BovC1 (figure 5.9, lanes 1-3) as viewed by silver staining, and resulted in a BovC2 catabolite of around 10 kDa (lanes 8 & 9) This demonstrates the sensitivity of the C-terminal of PRG4 to digestion by elastase.

5.3.3.2 Digestion of recombinant PRG4 domains with cathepsin B

Figure 5.10 shows the digestion of human recombinant N-terminal PRG4 domain (HumN) with decreasing concentrations of cathepsin B. At a cathepsin B concentration of 0.2U/ml (figure 5.10, lane 2) an exon 3-containing catabolite of around 22 kDa is generated. At lower concentrations of cathepsin B, HumN appears to be undigested and appears to be similar to the undigested control in lane 1.

The results from digestion of bovine recombinant C-terminal PRG4 domain (BovC) with decreasing concentrations of cathepsin B is shown in figure 5.11. Digestion of BovC at higher concentrations of cathepsin B appeared to digest the two protein species as seen in figure 5.11, lane 2. Silver stain and Western blot intensity are both reduced in comparison to the undigested control in lane 1.

5.3.3.3 Digestion of recombinant PRG4 domains with plasmin

Figure 5.12 shows the digestion of human recombinant N-terminal PRG4 domain (HumN, figure 5.12A) and bovine recombinant C-terminal PRG4 domain (BovC, figure 5.12B) with decreasing concentrations of plasmin. At plasmin concentrations of 0.2 and 0.1 U/ml (figure 5.12, lanes 2 and 3), a HumN catabolite was produced of around 17 kDa that contains the epitope recognised by the anti-exon 3 antibody. This is accompanied by the loss of the 27 kDa species as viewed by both silver staining and Western blot with an anti-exon 3 antibody. Higher plasmin concentrations also resulted in increasing digestion of the 20 kDa BovC1 species as seen in 5.12B, lanes 7 and 8, where three species are visible by silver stain. Higher concentrations of plasmin also appear to reduce the anti-exon 12 antibody staining intensity of BovC compared to the undigested control (figure 5.12B).

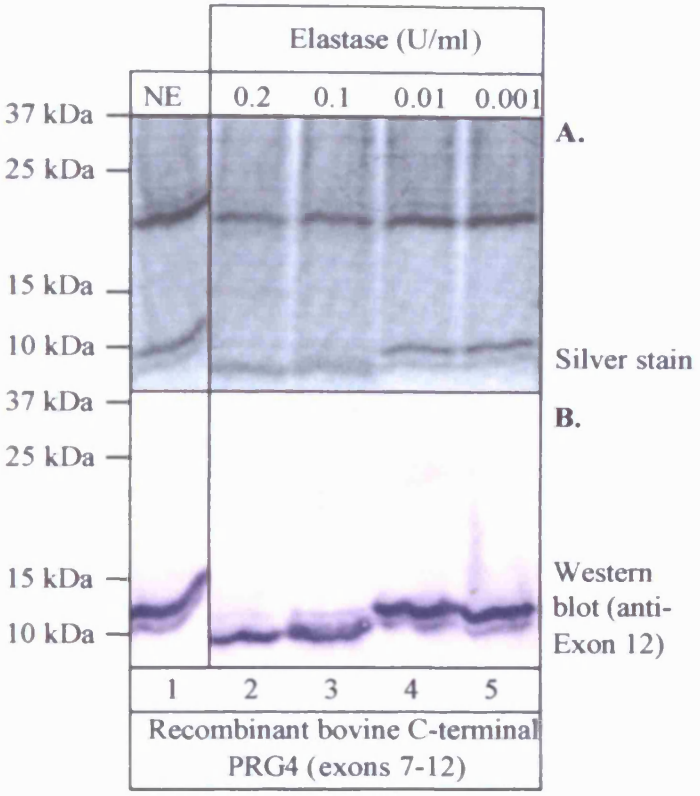


Figure 5.9 – Analysis of recombinant bovine C-terminal PRG4 domain after digestion with decreasing amounts of elastase. Reactions containing approximately 2.5µg PRG4 domain/50µl were incubated at 37°C for 2 hours. Reaction mixtures were separated by SDS-PAGE under reducing conditions (1.25µg/lane) and were visualised by silver stain (A) and Western blot with a polyclonal antibody to exon 12 of human PRG4 (B). A control reaction showing undigested bovine C-terminal domain is shown in lane 1(NE = no enzyme). Migration positions of molecular mass standards are shown to the left.

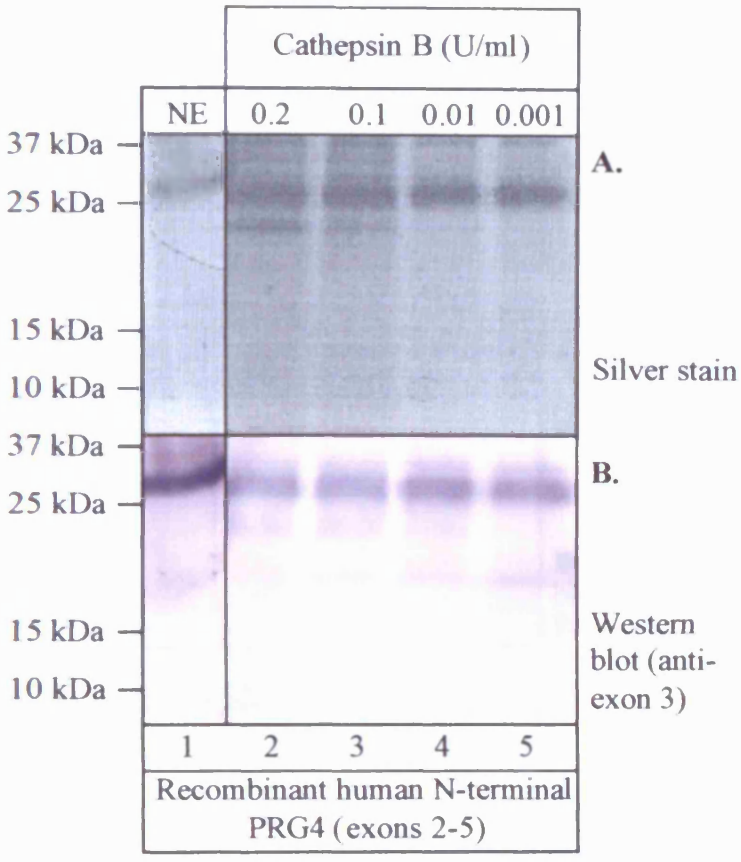


Figure 5.10 – Analysis of recombinant human N-terminal PRG4 domain after digestion with decreasing amounts of cathepsin B. Reactions containing approximately 2.5µg PRG4 domain/50µl were incubated at 37°C for 2 hours. Reaction mixtures were separated by SDS-PAGE under reducing conditions (1.25µg/lane) and were visualised by silver stain (A) and Western blot with a polyclonal antibody to exon 3 of human PRG4 (B). A control reaction showing undigested bovine C-terminal domain is shown in lane 1(NE = no enzyme). Migration positions of molecular mass standards are shown to the left.

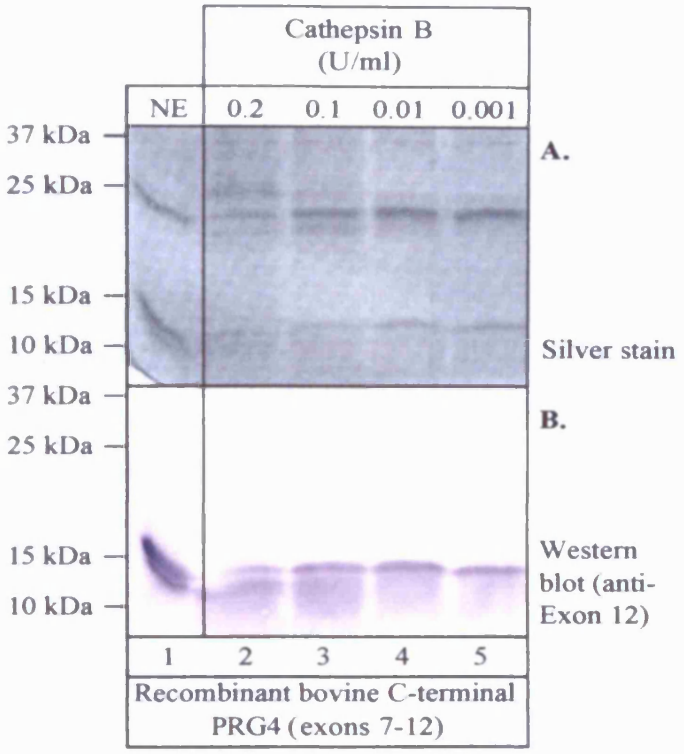


Figure 5.11 – Analysis of recombinant bovine C-terminal PRG4 domain after digestion with decreasing amounts of cathepsin B. Reactions containing approximately 2.5µg PRG4 domain/50µl were incubated at 37°C for 2 hours. Reaction mixtures were separated by SDS-PAGE under reducing conditions (1.25µg/lane) and were visualised by silver stain (A) and Western blot with a polyclonal antibody to exon 12 of human PRG4 (B). A control reaction showing undigested bovine C-terminal domain is shown in lane 1(NE = no enzyme). Migration positions of molecular mass standards are shown to the left.

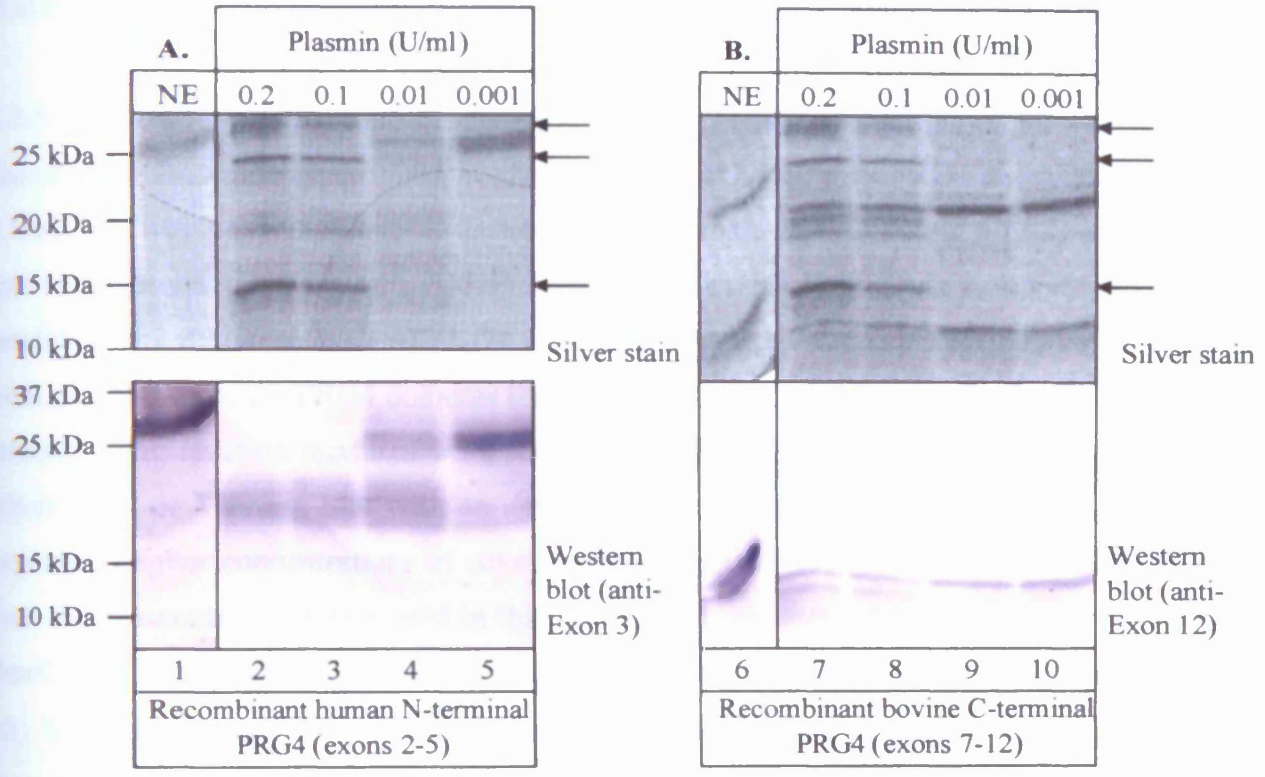


Figure 5.12 – Analysis of recombinant human N-terminal (A) and bovine C-terminal (B) PRG4 domains after digestion with decreasing amounts of plasmin. Reactions containing approximately 2.5µg PRG4 domain/50µl were incubated at 37°C for 2 hours. Reaction mixtures were separated by SDS-PAGE under reducing conditions (1.25µg/lane) and visualised by silver stain and Western blot with a polyclonal antibodies to either exon 3 (A) or exon 12 (B) of human PRG4. A control reaction showing undigested human PRG4 N-terminal domain is shown in lane 1, undigested bovine C-terminal domain is shown in lane 6. Migration positions of molecular mass standards are shown to the left.

It should be pointed out that the bands apparent by silver staining marked by arrows in figure 5.12A are present in both HumN and BovC digestions as viewed by silver stain, and their intensity increases with increased plasmin concentration. It is likely that these protein species are present in the plasmin preparation. The listed molecular weight of plasmin is 92 kDa.

5.3.3.4 Digestion of recombinant PRG4 domains with chymotrypsin

Figure 5.13 shows the digestion of human recombinant N-terminal PRG4 domain (HumN, figure 5.13A) and bovine recombinant C-terminal PRG4 domain (BovC, figure 5.13B) with decreasing concentrations of chymotrypsin. Although chymotrypsin is not particularly relevant as a potential candidate for the proteolysis of PRG4 *in vivo*, it was used to see if any interesting catabolites were derived from the PRG4 domains that could be used to further characterise the recombinant proteins. The reaction mixtures were separated by SDS-PAGE on 15% gels and visualised by silver stain or Western blot with an anti-exon 3 or an anti-exon 12 polyclonal antibody. As expected, higher concentrations of chymotrypsin resulted in fragmentation of HumN and BovC over the concentration range used in this experiment, as assessed by loss of antibody epitopes. BovC1 (20 kDa) was undigested at a chymotrypsin concentration of 0.2 μg/ml (figure 5.13, lane 10) but increasingly lower molecular weight forms were produced using chymotrypsin concentrations of 2.0 and 10 μg/ml. A 10 kDa BovC2 catabolite is generated, though this too is further degraded by higher chymotrypsin concentrations.

5.4 Discussion

One of the aims of this section of experimental work was to enzymatically digest native or recombinant PRG4 to generate different structural domains, such as the central mucin domain and the N- and C-terminal vitronectin-like domains. This would enable studies on the contribution of these domains on the functionality of PRG4: the lubricating function of the central mucin domain has been investigated (Jay *et al.*, 2001a), but as yet there have been no studies concerning the functions of the vitronectin-like domains either side of the mucin domain. Trypsin and chymotrypsin digestions of human recombinant and bovine native full-length PRG4 were carried out, using increasing concentrations of enzyme with a fixed concentration of substrate over a 2 hour incubation period.

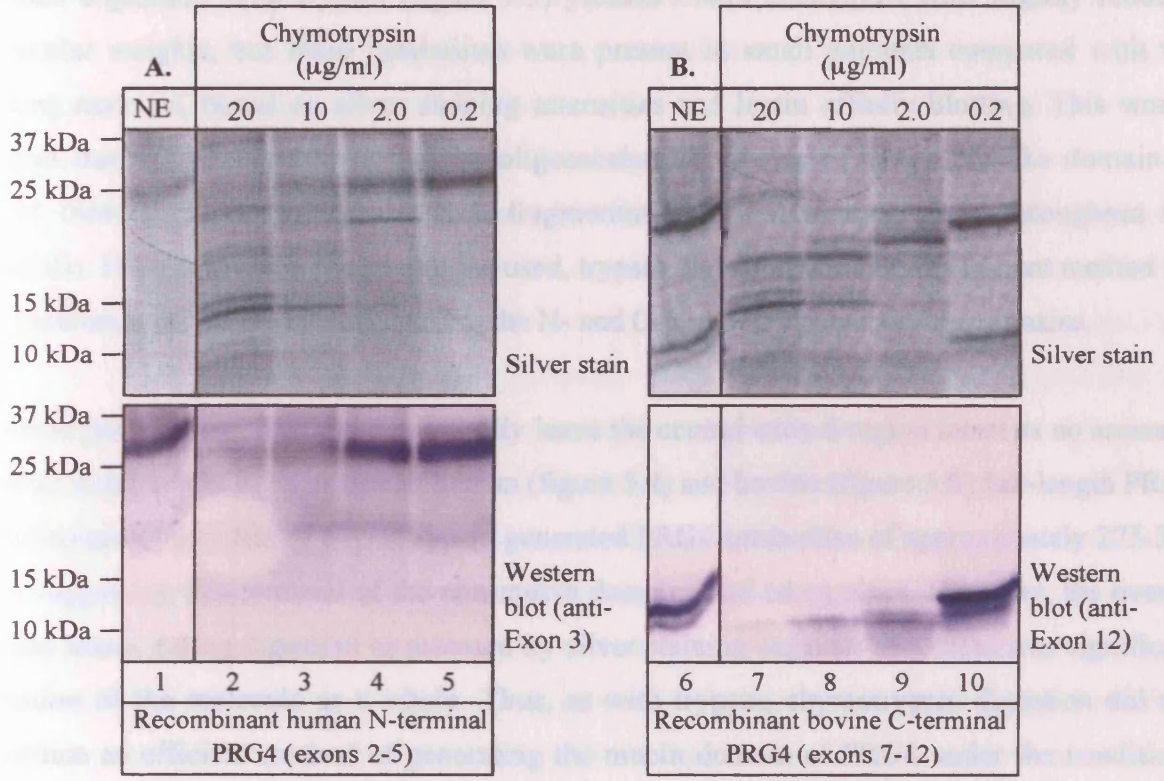


Figure 5.13 – Analysis of recombinant human N-terminal (A) and bovine C-terminal (B) PRG4 domains after digestion with decreasing amounts of chymotrypsin. Reactions containing approximately 2.5µg PRG4 domain/50µl were incubated at 37°C for 2 hours. Reaction mixtures were separated by SDS-PAGE under reducing conditions (1.25µg/lane) and visualised by silver stain and Western blot with a polyclonal antibodies to either exon 3 (A) or exon 12 (B) of human PRG4. A control reaction showing undigested human PRG4 N-terminal domain is shown in lane 1, undigested bovine C-terminal domain is shown in lane 6. Migration positions of molecular mass standards are shown to the left.

Limited digestions with trypsin (figure 5.3) yielded PRG4 catabolites with slightly reduced molecular weights, but these catabolites were present in small amounts compared with the starting material, based on silver staining intensities and lectin affinity blotting. This would suggest that whilst steric hindrance by oligosaccharides protected the mucin-like domain of PRG4 from digestion to some extent, fragmentation of PRG4 took place throughout the molecule. Therefore under the conditions used, trypsin digestion was not an efficient method for the generation of PRG4-domains lacking the N- and C-terminal vitronectin-like domains.

Chymotrypsin digestion would theoretically leave the central exon 6 region intact as no aromatic residues exist within it. Digestion of human (figure 5.4) and bovine (figure 5.6) full-length PRG4 with increasing amounts of chymotrypsin generated PRG4 catabolites of approximately 275-300 kDa, suggesting that removal of the non-mucin domains had taken place. However, the overall protein losses during digestion as assessed by silver staining suggests that there was significant digestion of the molecule as a whole. Thus, as with trypsin, chymotrypsin digestion did not constitute an efficient method of generating the mucin domain of PRG4 under the conditions used. Future experiments could be conducted in the presence of leupeptin and aprotinin, which would rule out the possibility that the commercial TLCK treatment of chymotrypsin incompletely inactivated contaminating trypsin in the enzyme preparation. Chymotrypsin digestions of human recombinant N-terminal PRG4 (HumN) and bovine recombinant C-terminal PRG4 (BovC) resulted in fragmentation of the recombinant proteins.

The other aim of this section was to assess the susceptibility of PRG4 to digestion by physiologically relevant enzymes found within the synovial joint. This was carried out by performing *in vitro* digests with PRG4 from a number of sources: full-length human recombinant and bovine native PRG4 (chapter 2) and recombinant human N- and bovine C-terminal domains (chapter 3). Articular cartilage chondrocytes are reported to secrete MMPs, serine and cysteine proteinases, with enzyme activities increased in OA (for a review, see Sandell & Aigner, 2001).

As seen in figure 5.6, elastase appeared to completely digest hrPRG4 under the conditions used. It is possible that digestion by elastase may be directed towards exon 6, acting on the bond between alanine and proline within the degenerate repeating sequence of KEPAPTT (Jay *et al.*,

2004). This hypothesis is supported by observations in this study, where full-length human recombinant PRG4 was completely digested by elastase. Neutrophil elastase is gaining credence as a candidate enzyme for the removal of the lubricating ability of synovial fluid via the removal of lubricin. The lubricating ability of lubricin has been shown to be labile in the presence of small amounts of elastase (Jay, 1992), and serine proteinases such as elastase are thought to play an important role during tissue inflammation (for a review see Hiemstra, 2002). A recent study has found an association between nucleated cell count and loss of lubricating ability in synovial fluid aspirated from patients with knee joint synovitis, which led the authors to suggest that the destruction of lubricin may be mediated by neutrophil elastase (Jay *et al.*, 2004). In experiments with stimulated human neutrophils, elastase was found to be the enzyme that was most relevant in cartilage degradation (Hilbert *et al.*, 2002). Levels of neutrophil elastase are elevated in the synovial fluid of RA cases as opposed to OA cases (Mohomara *et al.*, 1997), and high elastase activity has been found in the synovial fluid of patients with RA and non-infectious knee joint synovitis (Elsaid *et al.*, 2003). Another phenomenon that supports the hypothesis that the surface of articular cartilage is perturbed by neutrophil elastase is the reactivity of type II collagen antibodies with the articular surface following after *in vitro* treatment with elastase (Noyori *et al.*, 1994a). In other studies, the augmentation in type II collagen antibody reactivity with the articular surface is inhibited by the non-specific serine-esterase inhibitor PMSF, but not by the divalent metal chelator EDTA, whilst the elastase-specific inhibitor AAPVCMK also inhibited most of the PMN-induced increase in antibody binding (Jasin & Taurog, 1991). It is assumed that elastase, because of its comparatively low molecular weight of ~32 kDa, penetrates into cartilage to smooth the way for enzymes of higher molecular weights such as collagenase (Starkey *et al.*, 1977). The infiltration of joint tissues by elastase-producing polymorphonuclear neutrophil leukocytes (PMN) is a prominent feature of joint disease, particularly in RA (Edwards & Hallett, 1997). A recent study (Mitani *et al.*, 2001) has shown that PMN do not adhere to intact articular cartilage surfaces, whereas PMN adhesion occurs with cartilage explants digested with elastase and reconstituted with fibronectin. The study also demonstrated that PMN adhesion to plastic-bound fibronectin can be inhibited by a number of cartilage macromolecules, which would suggest that macromolecules normally present on the surface of articular cartilage act as a barrier against PMN adhesion. PRG4 may be part of this surface barrier and its degradation by elastase, shown *in vitro* in this study, could be partly responsible for an increase in PMN adhesion and the

associated cartilage damage in inflammatory arthritis. At present, very little is known regarding the potential ability of PRG4 to act as a barrier at the cartilage surface. It has been speculated (Flannery *et al.*, 1999) that the inhibition of cartilage PRG4 biosynthesis by IL-1 would remove the protective functions of the molecule, and may explain why, in rheumatoid arthritis, the proliferating synovial pannus can eventually invade the articular cartilage surface. Enzymatic degradation of PRG4 by elastase, which this study has shown is a possibility, may participate in this process.

PRG4 (both full-length and recombinant domains) was also found to be susceptible to plasmin, a serine proteinase that is upregulated in osteoarthritic cartilage (Martel-Pelletier *et al.*, 1991). Serine proteinases such as plasmin are also involved in the activation of proMMPs, although the interplay between them is unclear (Cawston & Rowan, 2002).

MMP-7 degrades hrPRG4 producing a catabolite of around 275 kDa. MMP-7, also known as matrilysin, is believed to degrade components of the extracellular matrix during tissue remodelling, and is reviewed in (Wilson & Matrisian, 1996). Among its substrates are fibronectin, type IV collagen, tenascin C, aggrecan and cartilage link protein. In osteoarthritic cartilage MMP-7 appears to be upregulated at the mRNA level, and immunohistochemistry has demonstrated its localisation to OA chondrocytes in the superficial and transitional zones (Ohta *et al.*, 1998). This would suggest that MMP-7 may play a role in the degradation of ECM macromolecules in OA cartilage, and PRG4 may be a substrate at the superficial zone of articular cartilage. Other data concerning MMP-7 in cartilage is less conclusive. A recent study detected MMP-7 mRNA in tibial articular chondrocytes in the STR/ort mouse model of OA but failed to detect protein expression (Flanelly *et al.*, 2002). A study of the MMP content of synovial fluid aspirated from OA and RA patients detected MMP-7 in 45% of RA patients and 20% of OA patients (Yoshihara *et al.*, 2000). The activation status of cartilage and synovial fluid MMP-7 is unclear, but this study has demonstrated that PRG4 is sensitive to digestion *in vitro* by MMP-7.

This study found no evidence to suggest that PRG4 is sensitive to degradation by calpain. Calpains (calcium-dependent cysteine proteinases) have been regarded as intracellular proteinases, but the presence of calpain in osteoarthritic synovial fluid has been documented,

together with its endogenous inhibitor calpastatin (Suzuki *et al.*, 1990). The calpain found in OA synovial fluid demonstrated proteoglycan-degrading activity, and so its potential for PRG4 proteolysis was investigated here. The extracellular appearance of calpain may be due to its secretion by synovial cells, and calpains may play a role in the cartilage damage associated with RA and OA (Yamamoto *et al.*, 1992).

Human recombinant and bovine native PRG4 appear to be sensitive to degradation by cathepsin B, and in the case of native bovine articular cartilage PRG4 a number of 6-A-1-positive catabolites are generated (figure 5.8). Sequencing of these catabolites may be useful in determining the epitope recognised by 6-A-1. The N- and C-terminal domains of PRG4 do not appear to be susceptible to digestion by cathepsin B. Cathepsin B, a cysteine proteinase (for a review see Mort & Buttle, 1997), may play a role during aggrecan breakdown (Fosang *et al.*, 1992) and has been proposed as an alternate enzyme for the generation of an aggrecan 'metalloproteinase' cleavage neoepitope (Mort *et al.*, 1998). Levels of cathepsin B are increased in human osteoarthritic cartilage as compared with normal tissue (Lang *et al.*, 2000). Cysteine proteases, along with elastase, have been found in increased levels in the synovial fluid of patients with inflammatory arthritis (Huet *et al.*, 1992).

These results demonstrate that PRG4 is susceptible to proteolytic digestion by a number of matrix proteinases, including matrix metalloproteinases (MMP-7), serine proteinases (elastase, plasmin) and cysteine proteinases (cathepsins B & D). However, the limitations of this work in the context of *in vitro* studies are that (i) not all of the enzymes shown to be capable of degrading PRG4 in preliminary experiments were used in fully titrated digestion experiments, and (ii) the full range of PRG4-specific antibodies need to be used individually in order to identify and characterise PRG4 catabolites. A full range of digestion experiments investigating the effects of altering the variables of enzyme concentration, substrate concentration and incubation time would provide a fuller picture of the effects of various matrix proteinases on cartilage PRG4. Also, it would be reductionist to assume that these digests represent the situation *in vivo* within a normal or inflamed synovial joint, as these are *in vitro* digests, preliminary in nature, with non-physiological concentrations of substrate and enzyme. Investigations into the breakdown of matrix molecules can lead towards a greater understanding of the events leading up to and

including cartilage degradation during inflammatory joint diseases, as seen with extensive studies with relatively clearly defined matrix molecules such as aggrecan (Caterson *et al.*, 2000). However, the role of PRG4 in healthy and diseased tissue is unclear. Matrix proteinase-mediated catabolism of PRG4 could affect the functionality of the molecule, thereby compromising the integrity and normal function of the surface of articular cartilage. The results presented in this chapter is preliminary data that demonstrates the sensitivity of PRG4 to digestion by certain enzymes, and catabolites produced in some instances may be useful in other applications. For example, sequenced catabolites could be used in the epitope mapping of existing PRG4 antibodies, in the production of novel antibodies, or in functional studies.

5.5 Chapter Summary

- Contrary to predictions, tryspsin and chymotrypsin digestion of full-length PRG4 substrate did not prove to be effective methods for the generation of the PRG4 central mucin domain
- Full-length human recombinant PRG4 appeared to be susceptible to proteolytic digestion by the proteinases cathepsin B, cathepsin D, elastase and MMP-7.
- In preliminary experiments, full-length bovine articular cartilage PRG4 was also susceptible to digestion by plasmin and cathepsin B.
- Recombinant PRG4 N-terminal and C-terminal domains are digested by plasmin and cathepsin B.

6. FUNCTIONAL ANALYSIS OF RECOMBINANT PRG4 DOMAINS

6.1 Introduction

Sequence analyses of cartilage PRG4 have indicated that it is a multidomain protein with the potential for a number of functions including lubrication, cytoprotection, matrix-binding, cell proliferation and self-aggregation (Flannery *et al.*, 1999). Chapter 3 describes the cloning, expression and purification of the non-mucin N-terminal and C-terminal domains of PRG4, which bear similarities to vitronectin and may possess a number of functions. For example, the N-terminal portion of MSF precursor protein (PRG4) has been shown to have cell proliferative effects (Merberg *et al.*, 1993). The somatomedin B-like domains in exons 2 and 3 of PRG4 are similar to the somatomedin B domain of vitronectin, which has been implicated in the binding of the serpin PAI-1 (Seiffert & Loskutoff, 1991). The C-terminal domain of PRG4 contains a haemopexin-type domain and an uneven number of cysteine residues that could mediate PRG4 self-aggregation and facilitate the trapping of the molecule at the interface between cartilage and the synovial cavity (Flannery *et al.*, 1999). Truncations in the C-terminal of PRG4 associated with CACP syndrome (Marcelino *et al.*, 1999) may result in PRG4 molecules with reduced functional capability. The aim of this work was to characterise purified recombinant PRG4 domains and utilising them in assays to investigate some of the potential functions of the molecule. In this chapter, experiments were performed to assay the binding of recombinant PRG4 domains to structural and functional extracellular matrix proteins and also to chondrocytes.

6.2 Materials & Methods

6.2.1 Materials

All chemicals were of an analytical grade and were purchased from Sigma-Aldrich unless indicated otherwise. A detailed list of the materials used in this research project and their suppliers is listed in Appendix A. The candidate binding proteins used are listed in table 6.1. Mutant PAI-1 incorporates four amino acid mutations to ensure structural stability for an extended period of time (Berkenpas *et al.*, 1995).

6.2.1.1 Preparation of recombinant proteins

Human and bovine recombinant N-terminal PRG4 domains (HumN & BovN) were produced and purified as described in Chapters 3 & 4 of this study. Where HumN1/BovN1 and HumN2/BovN2 species were used separately, heparin-agarose affinity chromatography was used to separate the two species following FLAG immunoaffinity chromatography. Recombinant bovine C-terminal PRG4 domain was purified by FLAG immunoaffinity chromatography as described in chapters 3 & 4, and included the 20 kDa and 12 kDa species.

Table 6.1 – Candidate binding proteins

Protein	Abbreviation	Source
Plasminogen Activator Inhibitor-1	PAI-1	Calbiochem, San Diego, CA
Human fibronectin, 70 kDa proteolytic fragment	Fn-70	Sigma-Aldrich, UK
Human fibronectin, 45 kDa proteolytic fragment	Fn-45	Sigma-Aldrich, UK
Human fibronectin, 30 kDa proteolytic fragment	Fn-30	Sigma-Aldrich, UK
Bovine fibronectin, full-length	Bov-Fn	Sigma-Aldrich, UK
Human vitronectin	Hum-Vn	Sigma-Aldrich, UK
Type II collagen, from bovine articular cartilage	Type II Coll	Prof. V. C. Duance, Cardiff University

6.2.2 Binding of recombinant PRG4 domains to candidate proteins (ELISA)

Microtitre plates (96-well, Elkay Products, Basingstoke, UK) were coated overnight (16 hours) at 4°C with 0.05 - 2μg candidate binding protein in PBS (100 μl of 0.5-20μg/ml). Candidate binding protein-coated wells and negative controls were then washed 3 times with PBS (300μl/well) and were incubated with 5% [w/v] BSA in PBS (200μl/well) for 1 hour at room temperature to block non-specific protein binding. After 3 washes with 300μl/well PBS, 1μg of purified recombinant PRG4 domain (N-terminal FLAG fusion protein) was added to each well (10μg/ml in PBS, 100μl/well) and incubated at room temperature for 1 hour. Unbound PRG4 protein was removed by washing with PBS (4 times) and bound protein was detected using anti-FLAG M2 alkaline phosphatase-conjugated monoclonal antibody. The mAb preparation was diluted by a factor of 1:10,000 in 1% [w/v] BSA in PBS, to give a protein concentration of ~10μg/ml, and the resultant solution was added to the microtitre plate (100μl/well) for 1 hour at room temperature. Unbound antibody was removed with 5 washes of PBS (300μl/well). The colourimetric alkaline phosphatase substrate ρ-nitrophenyl phosphate (PNPP, Sigma-Aldrich UK) was dissolved in substrate buffer (0.91M diethanolamine, 0.126mM $MgCl_2$, pH 9.8) according to the manufacturers instructions (1 tablet/5ml) and added to wells (100μl/well). Plates

were incubated with the substrate for 1 – 2 hours at 37°C and absorbance values were read at 405nm using a Labsystems Multiskan MS plate reader.

6.2.3 Co-immunoprecipitation of bovine recombinant PRG4 C-terminal domain with candidate binding proteins

Aliquots of purified recombinant bovine C-terminal PRG4 (30μg) were incubated with candidate binding proteins (30μg) in 0.5ml TBS (50mM Tris-HCl, pH 7.4, 0.15M NaCl) at 37°C for 2 hours, before addition to anti-FLAG M2 agarose (Sigma). Aliquots of FLAG M2 agarose (40μl) were prepared in 1.5ml Eppendorf tubes using 80μl of a 50% [v/v] stock solution. Prior to use with the sample, the resin was washed with TBS (3 x 0.5ml), pre-eluted with 3 portions of 0.5ml 0.1M glycine-HCl, pH 3.5 and washed again with TBS (3 x 0.5ml). For each wash and elution step, the resin was centrifuged at 10,000 x g for 5 seconds and allowed to settle for 1 – 2 minutes before the removal of supernatant with a syringe attached to a 19G 1/2 needle (Microlance), and for the acidic elution step the resin was incubated for 5 minutes at room temperature with gentle shaking before centrifugation. Following these pre-elution and washing procedures, the sample mixture was added to the resin and incubated at room temperature for one hour with gentle agitation. The sample was removed and the resin was washed 3 times with 0.5ml TBS to remove unbound protein. Bound proteins were eluted with 3 x 100μl aliquots of glycine-HCl, pH 3.5 and placed into a tube containing 5μl of 1.5M Tris-HCl, pH 8.8. Eluent fractions were separated on 15% Tris-glycine SDS-PAGE gels which were silver stained using the method described in section 2.2.9.

6.2.4 Analyses of interaction between recombinant PRG4 domains and HA by affinity blotting

Affinity blotting with biotinylated hyaluronan was performed as described in chapter 2, section 2.2.11. Briefly, preparations of purified or partially purified PRG4 domains were separated by SDS-PAGE (using 15% Tris-glycine gels) under reducing and non-reducing conditions and transferred to nitrocellulose. As a positive control for HA binding, 5μg of a preparation of link protein was used that runs at 45 kDa and binds HA under non-reducing conditions (bovine nasal cartilage A1D4 fraction, prepared by Dr. C. R. Flannery). Membranes were blocked and blotted as described in section 2.2.11, but with a six hour incubation in the presence of biotinylated HA,

and development was with NBT/BCIP substrate (chapter 2, section 2.2.1.7) as for Western blotting experiments.

6.2.5 Immunocytochemistry with bovine chondrocytes

6.2.5. Preparation of bovine superficial zone chondrocyte monolayer cultures for immunocytochemistry

Immunocytochemistry was used to study the binding of PRG4 domains to chondrocytes. Superficial zone chondrocytes were isolated from bovine metacarpophalangeal articular cartilage by pronase and collagenase digestion as described in chapter 2, section 2.2.2. Cells were then seeded at an initial density of 0.8 x 10^6 cells/ml in DMEM supplemented with 5% [v/v] FCS and 50μg/ml gentamicin. Cells were cultured on 100mm tissue culture dishes within the wells of FlexiPerm slides (In Vitro Systems & Services GmbH, Göttingen, Germany), which were attached in order to divide each dish into 8 culture chambers, each with a culture volume of 0.5ml. Chondrocytes were incubated at 37°C with 5% CO_2 for 48 hours prior to binding experiments, in order to allow the re-establishment of the cell surface glycocalyx that was likely to have been perturbed during the enzymatic isolation of the cells from the cartilage matrix.

6.2.5.2 Addition of recombinant PRG4 domains and FLAG immunocytochemistry

To remove serum proteins after pre-culture, each monolayer culture chamber was washed twice with serum free DMEM (0.5ml/chamber), incubated for 1 hour at 37°C with the same medium and washed a further two times. PRG4 domains and BSA for control experiments were added at concentrations of 10-50μg/ml in serum free DMEM with an incubation of 2 hours at 37°C. For competition experiments with heparin, PRG4 domains (10-50μg/ml) were incubated with 100μg/ml heparin (sodium salt from porcine intestinal mucosa, Sigma H3419) at 37°C for 1 hour before addition to chondrocytes. Cells were then washed twice with serum free DMEM and twice with PBS before fixation with ice-cold methanol/acetone [1:1 v/v]. Plates were allowed to air dry and were stored at -20°C. At this point part of the culture dish with fixed monolayers was excised from the rest of the culture dish with a hot scalpel to facilitate microscopy following immunocytochemistry. To detect bound PRG4 domains, cells were rehydrated with PBS/0.01% [v/v] Tween-20 (PBS-T) before the addition of alkaline phosphatase-conjugated anti-FLAG M2 monoclonal antibody (10μg/ml in PBS-T). Following an overnight incubation at 4°C unbound

antibody was removed by four 2 minute washes with PBS-T prior to addition of alkaline phosphatase substrate (Vector Red kit, Vector). Levamisole (Vector) was included in the reaction mixture to quench any endogenous alkaline phosphatase activity. Following development, cells were washed in water and increasing concentrations of methanol, cleared in xylene and mounted in DPX. Positive staining was viewed using a Nikon Labphot 2 microscope.

6.3 Results & Discussion

6.3.1 Analysis of recombinant N-terminal PRG4 domain interactions with other matrix molecules by ELISA

ELISA-based binding assays were used to examine the interactions between recombinant N-terminal PRG4 domain proteins and other matrix molecules. These included plasminogen activator inhibitor type 1 (PAI-1), fibronectin fragments and type II collagen. In all of these preliminary ELISA experiments, the candidate binding proteins were immobilised on a multiwell plate (solid phase) before the additions of recombinant PRG4 (solution phase).

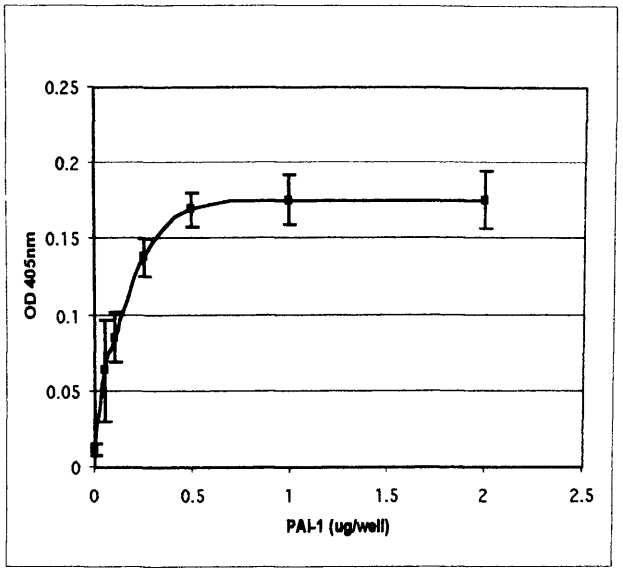
6.3.1.1Analysis of recombinant N-terminal PRG4 domain interactions with plasminogen activator inhibitor-1 (PAI-1)

Plasminogen activator inhibitor type 1 (PAI-1) is a serine proteinase inhibitor that is the primary inhibitor of both tissue- and urinary-type plasminogen activators (tPA and uPA). It has been implicated in the processes of haemostasis, angiogenesis, tissue remodelling and tumour metastasis. PAI-1 has a brief half-life as an inhibitor because of the spontaneous insertion of its reactive centre loop (RCL) into the main β-sheet of the molecule (Mottonen *et al.*, 1992). The biological activity of PAI-1 is stabilised by binding to the 70 kDa glycoprotein vitronectin, an interaction thought to be mediated by the somatomedin B (SMB) domain comprising of the first 44 residues of vitronectin (DePrada *et al.*, 2002; Seiffert & Loskutoff, 1991; Okumura *et al.*, 2002). The SMB domain has been isolated from human serum as a separate soluble protein (Standker *et al.*, 1996), and contains eight Cys residues arranged in four disulphide bonds, suggesting a compact structure. Treatment of the isolated SMB domain with reducing agents abolishes PAI-1 binding (Seiffert *et al.*, 1997), and conversion of any of the eight Cys residues to Ala also abolishes PAI-1 binding activity (Deng *et al.*, 1996a).

PRG4 contains two somatomedin B-like domains in exons 2 and 3 that display ~45% sequence homology with the SMB domain of vitronectin (Flannery *et al.*, 1999; Merberg *et al.*, 1993), and each contains eight cysteine residues in identical positions to the SMB domain of vitronectin (for a diagrammatic representation see chapter 1, figure 1.15). Flannery *et al.* (1999) suggested that the SMB-type domains of PRG4 could function as a PAI-1 binding region in a similar manner to the SMB domain of vitronectin. This was investigated in this study by means of an ELISA-based solid phase binding assay to examine the potential interaction between the recombinant N-terminal PRG4 domains (described in chapters 3 and 4) and recombinant PAI-1. Different amounts of human recombinant PAI-1 (Calbiochem) were immobilised on an ELISA plate before the addition of a constant amount of recombinant N-terminal PRG4. After removal of unbound recombinant PRG4, bound PRG4 domain protein was detected by an alkaline phosphatase conjugated anti-FLAG monoclonal antibody.

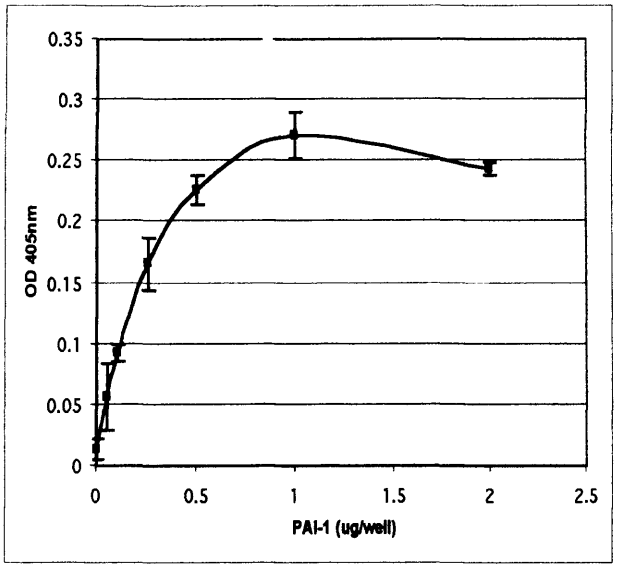
The results from the first of these experiments can be seen in figure 6.1. Recombinant human N-terminal (exons 2-5) PRG4 domain (HumN) was used in this case. The two HumN species described in chapter 3 were purified as two populations and used separately in identical binding assays. After initial purification of both species by FLAG immunoaffinity chromatography, the two species were separated by heparin-agarose affinity chromatography – HumN1 binds to heparin-agarose whereas HumN2 does not. Results from using the 27 kDa HumN1 species are shown in figure 6.1A, results using the 16 kDa HumN2 species are shown in figure 6.1B. The results of the PAI-1 binding assay with the individual species, as shown in figure 6.1, demonstrated that both HumN1 and HumN2 bind to immobilised PAI-1, and that binding of both human recombinant PRG4 N-terminal domain species increased in relation to increasing concentrations of PAI-1 immobilised on the plate. However, this interaction appeared to be saturable at a PAI-1 coating concentration of 1μg/well and above. Similar results were observed with the bovine recombinant N-terminal PRG4 domain protein species BovN1 and BovN2, as illustrated in figures 6.2A and 6.2B, respectively. The higher absorbency values observed with BovN2 were produced by a longer incubation time in the developing solution.

A.



HumN1

B.



HumN2

Figure 6.1 – Analysis of human recombinant PRG4 N-terminal domain (HumN) interactions with PAI-1 by ELISA. Different amounts of PAI-1 were immobilised before the addition of 1μg HumN1 (A) or HumN2 (B), followed by detection of recombinant PRG4 protein with AP-conjugated anti-FLAG M2 monoclonal antibody. Experiments were conducted in triplicate, and bars are included to illustrate standard error.

HumN1/BovN1 and HumN2/BovN2 contain an N-terminal FLAG epitope as assessed by Western blotting, and only HumN1/BovN1 contain a heparin binding site (as assessed by heparin-agarose affinity chromatography), so it is likely that both proteins contain exons 2 and 3 (see chapter 4, figure 4.9). Based on these predicted structures for the two recombinant N-terminal PRG4 domain species, an interaction between PAI-1 and the N-terminal domain of PRG4 may be mediated by the two SMB-type domains present within exons 2 and 3, and thus does not involve the heparin binding domain.

6.3.1.2Analysis of recombinant N-terminal PRG4 domain interactions with fibronectin fragments

Similar ELISA-based binding assays were used to assess the binding of recombinant N-terminal PRG4 domain proteins to commercially available proteolytic fragments of human fibronectin (Fn) as well as full-length bovine fibronectin. Fn is a classical example of a protein with a modular structure, possessing several functional domains that can be separated by limited proteolysis. Separated domains retain their functionality in isolation (Ruoslahti *et al.*, 1981), and may influence cellular activity independently (Woods *et al.*, 1986). Synthesis of fibronectin is upregulated in OA and RA, and proteolytic Fn fragments may potentially regulate cartilage metabolism (for a review see Homandberg, 1999). Homandberg and others showed that the addition of certain proteolytic fragments of Fn to bovine cartilage explant cultures induced the degradation of cartilage, as assessed by the release of MMPs and proteoglycan into the culture medium (Homandberg *et al.*, 1992). Intact fibronectin did not induce these effects. Levels of fibronectin in OA cartilage show a marked increase near the articular surface (Rees *et al.*, 1987) and in synovial fluid (Scott *et al.*, 1981). As PRG4 is present at the articular surface (Schumacher *et al.*, 1999) and in synovial fluid (Jay *et al.*, 2000), the potential interaction between fibronectin fragments and PRG4 was investigated in this study. Initially, a N-terminal 70 kDa fragment of human fibronectin was immobilised on an ELISA plate at increasing concentrations in the range of 0-2μg/well, and a constant amount (1μg) of recombinant N-terminal PRG4 domain was added. Bound PRG4 domains were detected with an AP-conjugated anti-FLAG M2 monoclonal antibody. Repeat experiments were conducted with the 45 kDa and 30 kDa fibronectin fragments.

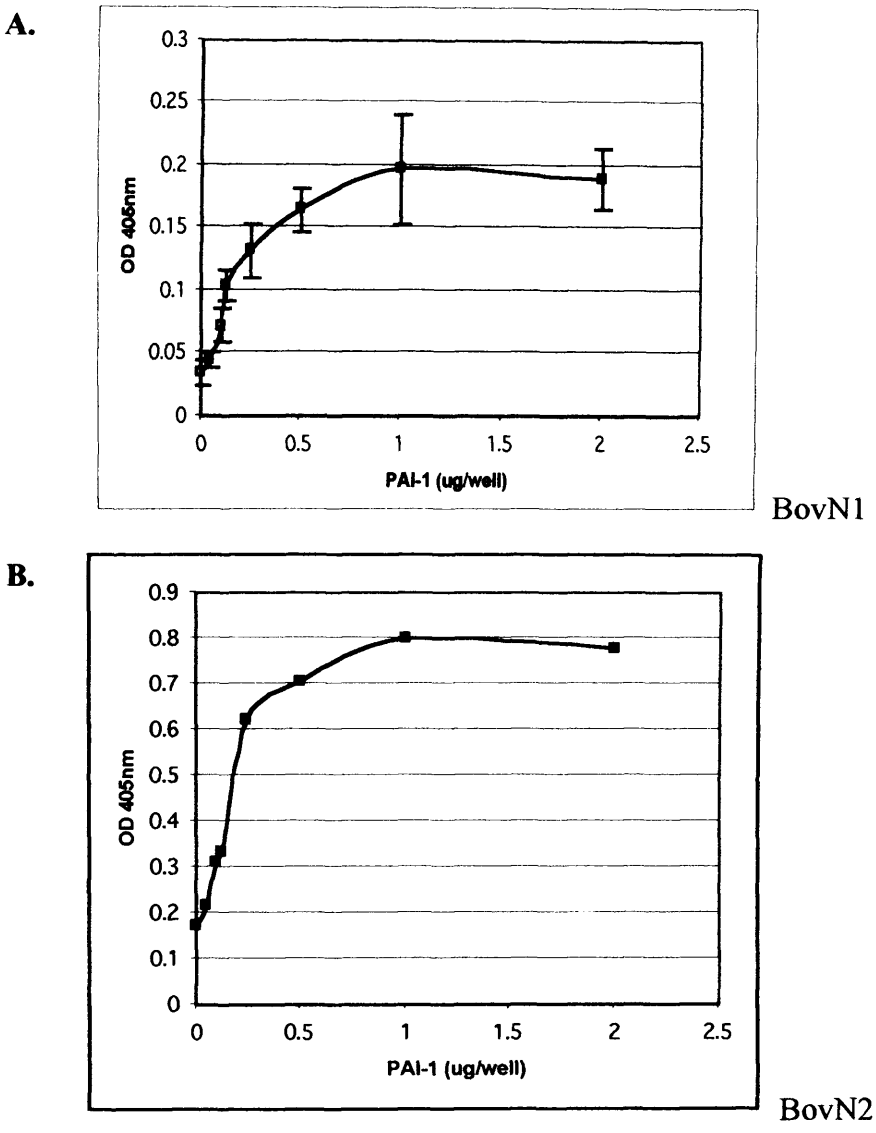


Figure 6.2 – Analysis of bovine recombinant PRG4 N-terminal domain (BovN) interactions with PAI-1 by ELISA. Different amounts of PAI-1 were immobilised before the addition of 1μg BovN1 (A) or BovN2 (B), followed by detection of recombinant PRG4 protein with AP-conjugated anti-FLAG M2 monoclonal antibody. Where experiments were conducted in triplicate, mean absorbency values were plotted and bars are included to illustrate standard error.

Figure 6.3 shows the results of these experiments with BovN1 (figure 6.3A) and HumN1 (figure 6.3B). Both BovN1 and HumN1 appeared to interact with the immobilised 70 kDa Fn fragment, with interaction increasing proportionally with the amount of Fn fragment immobilised. This interaction was not saturable with the protein concentrations used. It would be interesting to investigate the Fn fragment binding potential of the HumN2 and BovN2 recombinant N-terminal PRG4 domain species. Interestingly, no discernable interaction was observed between the recombinant PRG4 N-terminal domain species and full-length bovine fibronectin. Similar experiments with 45 kDa and 30 kDa proteolytic N-terminal fragments of human fibronectin have not revealed an interaction with recombinant N-terminal PRG4 proteins (see figure 6.3). These results are similar to work with cartilage oligomeric matrix protein, or COMP, which is seen to interact with the 70 kDa Fn fragment but not other isolated domains (DiCesare *et al.*, 2002).

6.3.1.3 Analysis of recombinant N-terminal PRG4 domain interactions with type II collagen

ELISA-based binding assays were also used to investigate the potential interaction between recombinant N-terminal PRG4 domains and immobilised bovine type II collagen, and the results are seen in figure 6.4. Type II collagen was immobilised in increasing amounts and a constant amount (1μg) of BovN1 (figure 6.4A) or HumN1 (figure 6.4B) was added, and bound recombinant protein was detected with an anti-FLAG monoclonal antibody. An interaction was observed, although more experiments need to be conducted to determine the strength of this interaction.

6.3.1.4 Analysis of recombinant N-terminal PRG4 domain interactions with vitronectin

Merberg and others, in their 1993 paper on MSF precursor protein, alluded to a potential interaction between PRG4 and vitronectin (Merberg *et al.*, 1993). In this study, vitronectin from human plasma (Sigma) was immobilised in increasing concentrations on a microtitre plate, and ELISA binding assays were conducted using similar conditions to the experiments described above. However, under these conditions no interaction was observed between recombinant N-terminal PRG4 domains and immobilised vitronectin (data not shown).

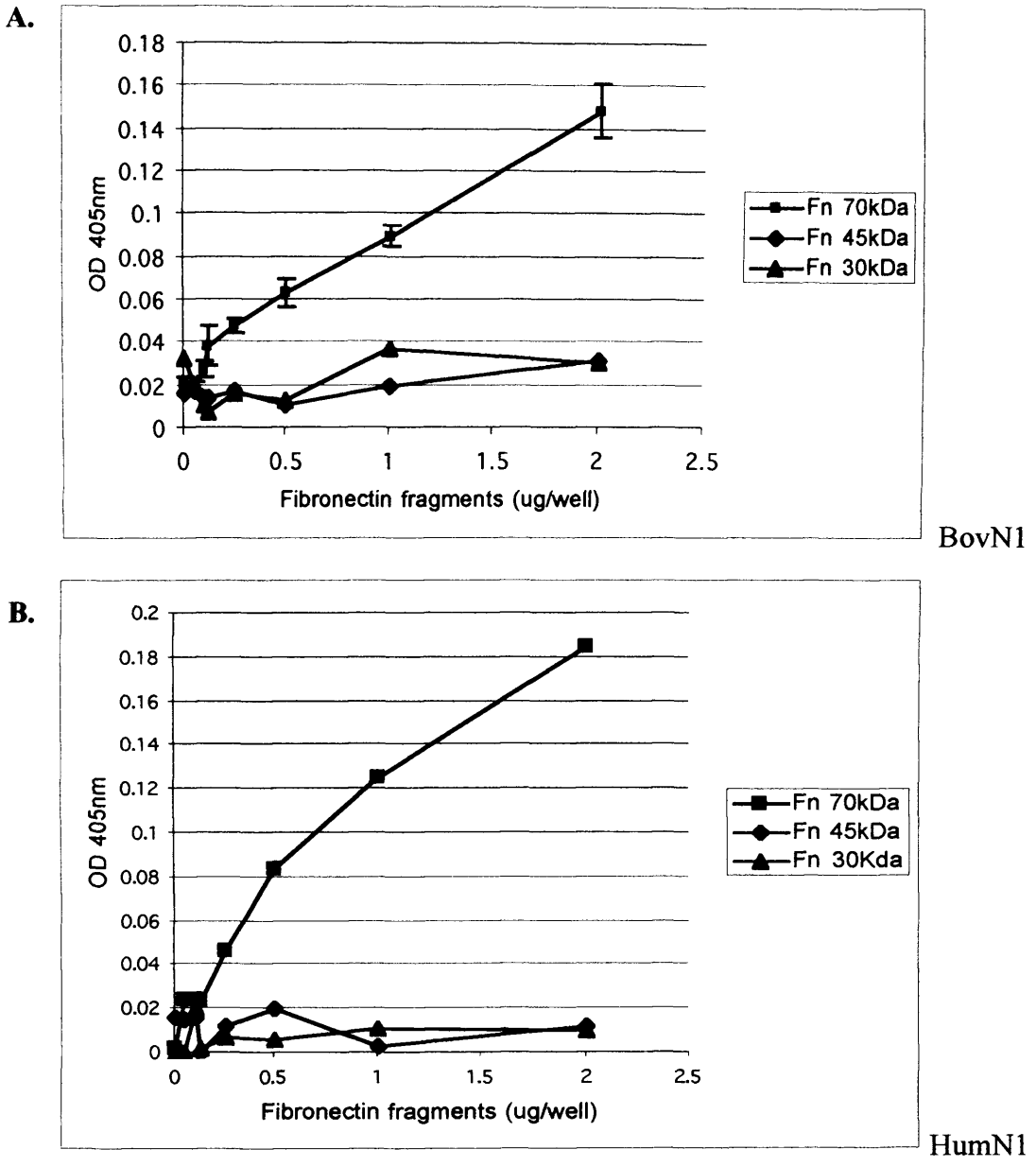


Figure 6.3 – Analysis of bovine and human recombinant PRG4 N-terminal domain interactions with proteolytic fragments of human fibronectin by ELISA. Fibronectin fragments were immobilised in increasing concentrations prior to the addition of 1μg BovN1 (A) or HumN1 (B), followed by detection of recombinant PRG4 protein with AP-conjugated anti-FLAG M2 monoclonal antibody. Where experiments were conducted in triplicate, mean absorbency values are plotted and bars are included to illustrate standard error.

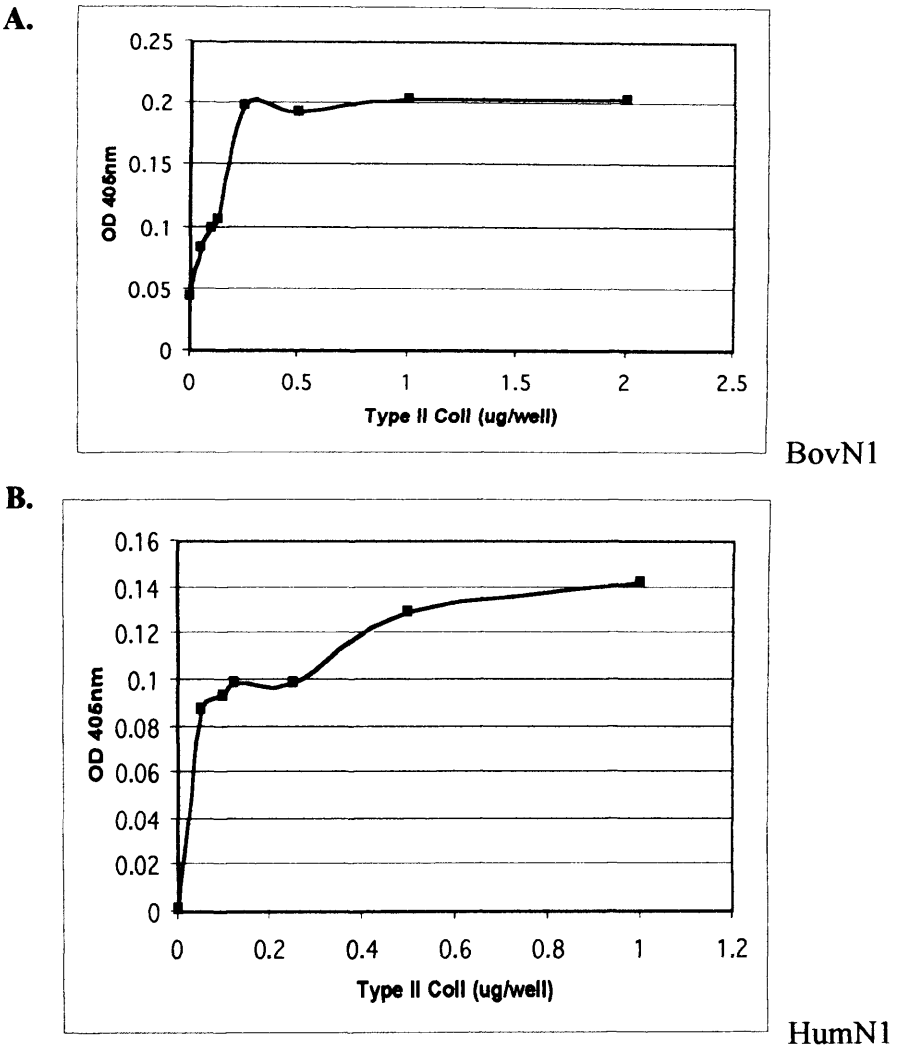


Figure 6.4 – Analysis of bovine and human recombinant PRG4 N-terminal domain interactions with bovine type II collagen by ELISA. Increasing concentrations (0-1μg/well) of bovine type II collagen were immobilised before the addition of 1μg BovN1 (A) or HumN1 (B), followed by detection of recombinant PRG4 protein with AP-conjugated anti-FLAG M2 monoclonal antibody.

6.3.2 Analysis of recombinant bovine C-terminal PRG4 domain interactions with other matrix molecules by ELISA

Similar assays to the experiments described above were used to study the interactions between the recombinant bovine C-terminal PRG4 domain and candidate binding proteins, but a high level of background binding activity was observed with the blocking reagents used, which included BSA, skimmed milk, ECL blocking reagent (Pierce) and deoxycholate (data not shown). This indicated that non-specific binding of the recombinant C-terminal PRG4 domain to the blocking reagents was occurring, ruling out the use of ELISA as a binding assay for this protein. Other methods were used to study the interactions of this protein, and these are described in the following section.

6.3.3 Co-immunoprecipitation of recombinant bovine C-terminal PRG4 domain with fibronectin fragments

As discussed in section 6.3.1.2, Fn fragments have the ability to affect cartilage metabolism, and are present in the synovial fluid and at the articular surface of osteoarthritis patients. The ability of Fn fragments to interact with the vitronectin-like, haemopexin domain-containing C-terminal region of PRG4 was investigated in this study by co-immunoprecipitation. Bovine recombinant C-terminal PRG4 (BovC, described in chapters 3 & 4) was incubated with the 30 kDa, 45 kDa or 70 kDa proteolytic fragments of human fibronectin before immunoprecipitation of BovC (and BovC-containing complexes) using anti-FLAG M2 agarose. Eluent fractions were separated by SDS-PAGE under reducing conditions and visualised by silver staining, and the results can be seen in figure 6.5. In all lanes there are two contaminating protein species running at 25 and 50 kDa, indicated with dashed arrows, and it is likely that these contaminants are the heavy and light chains of the immunoglobulin proteins that have leached from the FLAG immunoaffinity resin. Lane 4 shows a control precipitation with BovC alone, and the two BovC species at ~20 kDa and 12 kDa are visible. These two species can also be seen in lanes 5, 6 and 7, where they appear to have been co-precipitated with the 30 kDa, 45 kDa and 70 kDa fragments of fibronectin, respectively. The migration positions of Fn fragments are indicated with arrows. This demonstrates an interaction between the C-terminal domain of PRG4 and proteolytic fragments of fibronectin.

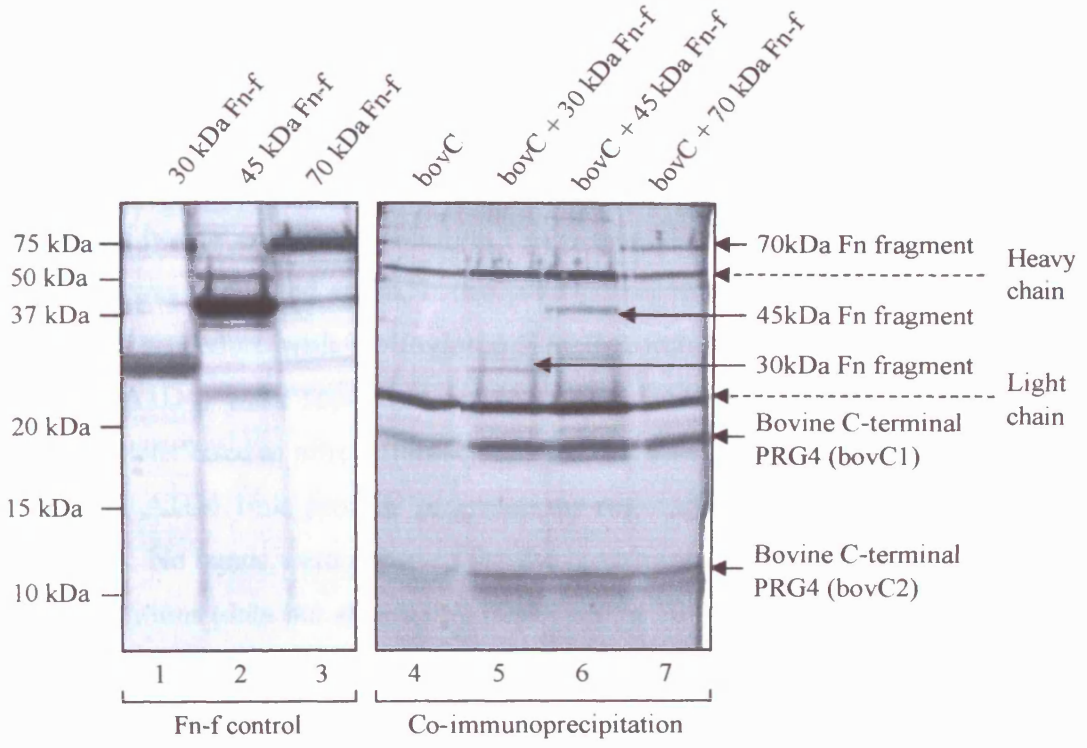


Figure 6.5 – Co-immunoprecipitation of bovine recombinant C-terminal PRG4 domain (BovC) and fibronectin (Fn) fragments. BovC was incubated with Fn fragments before immunoprecipitation using anti-FLAG M2 agarose. Eluents were separated by SDS-PAGE (15%) under reducing conditions and proteins were visualised by silver staining. Fibronectin fragments were run alone, these are shown in the left panel. Migration of molecular size standards is shown to the left.

6.3.4 Analysis of the interaction between recombinant PRG4 domains and HA by affinity blotting experiments

In chapter 2 of this study, the potential interaction between purified full-length PRG4 molecules and HA was examined by means of affinity blotting experiments using biotinylated HA (section 2.3.6), and in this procedure there was no visible interaction between HA and human recombinant and bovine cartilage full-length PRG4 molecules. The potential interaction between HA and PRG4 was examined again, using purified recombinant PRG4 domains in a similar affinity blotting procedure with biotinylated HA. Recombinant PRG4 domains and a positive control (BNC A1D4) were separated on 15% SDS-PAGE gels under native and reducing conditions and transferred to nitrocellulose before a HA affinity blotting procedure. Under native conditions, the A1D4 link protein preparation, running at around 45 kDa, reacted with biotinylated HA. No bands were apparent for the N-terminal PRG4 domains under either native or reduced conditions (data not shown). A faint band at 30 kDa was seen relating to the bovine recombinant C-terminal domain protein (BovC) under non-reducing conditions (data not shown). This is the region of PRG4 most likely to interact with HA: exons 8-9 of PRG4 contain haemopexin-like domains similar to vitronectin, and haemopexin is a serum haeme-scavenging protein that has been shown to interact with HA (Hrkal *et al.*, 1996). The procedure was repeated with different amounts of BovC (5μg and30μg), and the results are illustrated in figure 6.6, which shows BovC and A1D4 proteins under native and reduced conditions. Figure 6.6A shows an affinity blot with biotinylated HA, figure 6.6B shows a control blot performed in the absence of biotinylated HA. As seen in figure 6.6, lane 3, there was a strong interaction between link protein (~45 kDa) and biotinylated HA under native conditions. As shown in the control blot in 6.6B, there appeared to be background binding activity between the streptavidin-AP conjugate and BovC under reducing and non-reducing conditions. However, the intensity of staining is increased in the presence of biotinylated HA (figure 6.6A) under native and reduced conditions, which raises the possibility that there is an interaction between BovC and HA.

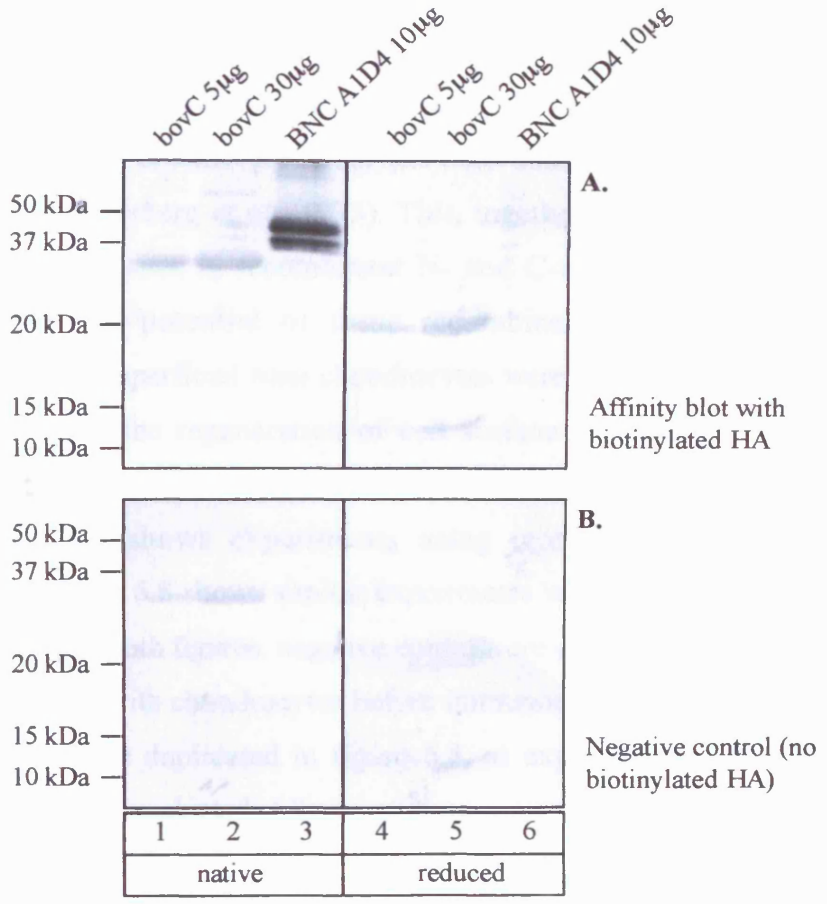


Figure 6.6 – Analysis of the interaction between recombinant bovine C-terminal PRG4 domain (BovC) and hyaluronan (HA) by affinity blotting. In (A), proteins were separated under native and reduced conditions and probed with biotinylated HA. A bovine nasal cartilage A1D4 preparation containing link protein is used as a positive control (lane 3, 5µg/lane). In (B), the procedure was repeated in the absence of biotinylated HA as a negative control. Migration of size standards is shown to the left.

6.3.5 An investigation into interactions between chondrocytes and recombinant PRG4 domains using immunocytochemistry

PRG4 is homologous to megakaryocyte stimulating factor (MSF) precursor protein, and the ~25 kDa N-terminal domain of MSF precursor has been shown to have cell proliferative effects on haemopoetic tissue (Merberg *et al.*, 1993). This, together with the observation in this study of active heparin-binding sites in recombinant N- and C-terminal PRG4 domains, prompted an investigation into the potential of these recombinant PRG4 domains to interact with chondrocytes. Bovine superficial zone chondrocytes were isolated and cultured in monolayer for 48 hours to allow for the regeneration of cell surface proteoglycans before the addition of recombinant PRG4 domains. Bound protein was detected using an anti-FLAG monoclonal antibody. Figure 6.7 shows experiments using recombinant PRG4 N-terminal domain preparations, and figure 6.8 shows similar experiments with the bovine recombinant C-terminal PRG4 domain. Within both figures, negative controls are shown where PBS alone or BSA diluted in PBS was incubated with chondrocytes before immunocytochemistry. The control experiments shown in figure 6.7 are duplicated in figure 6.8, as experiments with the N- and C-terminal domains of PRG4 were conducted at the same time.

6.3.5.1 Immunocytochemistry of bovine superficial zone chondrocyte monolayer cultures after incubation with human and bovine recombinant N-terminal domains

All four of the available recombinant N-terminal PRG4 domain species (HumN1, HumN2, BovN1 and BovN2) were used in this set of experiments. As seen in figure 6.7, no staining was observed when recombinant N-terminal domain species were incubated with superficial zone chondrocytes (panels A-D), as staining was at a level that is comparable seen with the BSA-supplemented and untreated controls shown in figure 6.7, panels E and F. This data suggests that there is no interaction between the N-terminal domain of PRG4 and superficial zone chondrocytes in monolayer culture.

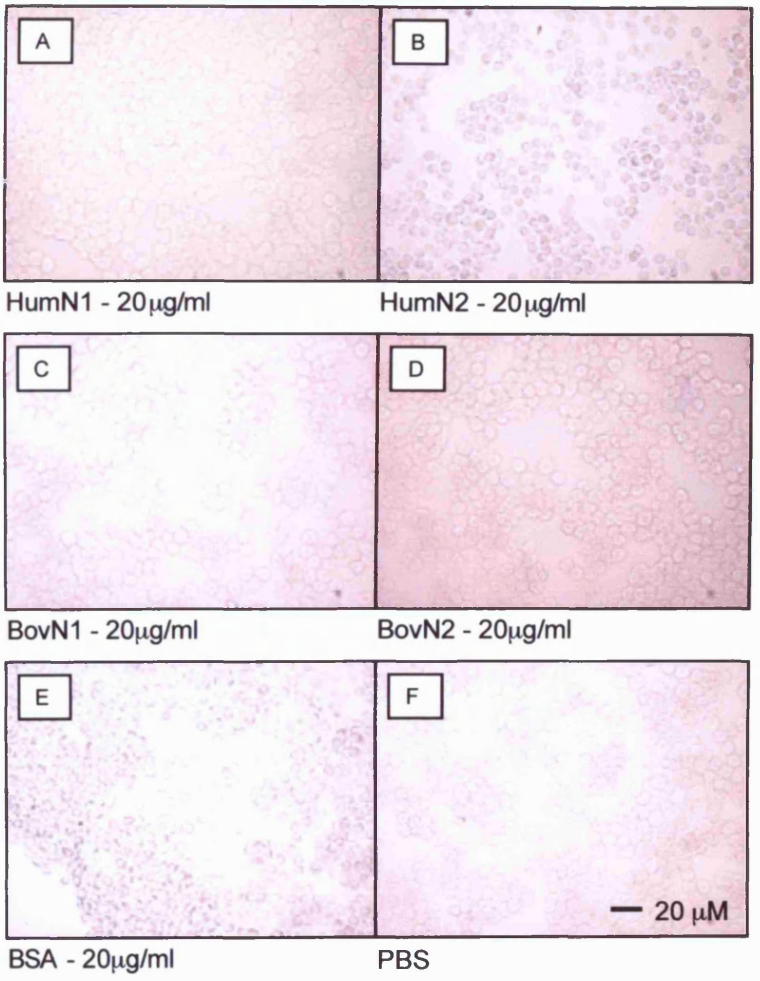


Figure 6.7 – Immunocytochemical analysis of bovine superficial zone chondrocyte monolayer cultures after incubation with recombinant PRG4 N-terminal domains. Monolayer cultures were incubated for 1 hour in the presence of recombinant PRG4 N-terminal domains, which were diluted in a final volume of 0.5ml PBS. Concentrations can be seen beneath each panel. Cells were fixed and immunocytochemistry was performed using an AP-conjugated anti-FLAG M2 monoclonal antibody to detect recombinant FLAG fusion proteins.

6.3.5.2 Immunocytochemistry of bovine superficial zone chondrocyte monolayer cultures after incubation with bovine recombinant C-terminal domains

The results of experiments with the recombinant bovine C-terminal domain (BovC) can be seen in figure 6.8. Staining was seen with BovC at 20μg/ml (figure 6.8, panel A), and this positive staining increased at a BovC concentration of 50μg/ml (panel B). This suggests that under the conditions used there is an interaction between BovC and superficial zone chondrocytes cultured as a monolayer. In a duplicate set of cell culture chambers, recombinant PRG4 domains were pre-incubated with 100μg/ml heparin at 37°C for 1 hour prior to their addition to the chondrocyte monolayers. This was performed in order to assess whether the heparin-binding ability of the PRG4 domains (documented in chapter 4) had any effect on the binding of the recombinant proteins to chondrocytes in monolayer. The pre-incubation with heparin was intended to saturate the heparin-binding sites of the recombinant proteins. In the experiments where BovC was pre-incubated with heparin, staining for the FLAG epitope appears to be greatly reduced in comparison with experiments with BovC in the absence of heparin (figure 6.8, panels C and D). This data suggests a potential interaction between the recombinant C-terminal domain of PRG4 and the chondrocyte, or matrix component(s) surrounding the chondrocyte, which may be mediated by cell surface heparan sulphate proteoglycans.

6.4 Discussion

PRG4, a protein that is known by many terms including lubricin, SZP, CACP protein and MSF precursor protein, is a protein/proteoglycan that is found in a number of tissues, including at the surface of articular cartilage and in synovial fluid. The determination of the amino acid sequence of PRG4 led to the prediction that it is, potentially, a multifunctional, multidomain protein. Chapters 3 and 4 of this study describe the production, purification and characterisation of the N- and C-terminal domains of PRG4. These are domains that have a number of similarities to functional domains in other proteins such as vitronectin, and some of these potential functions were preliminarily investigated in this chapter.

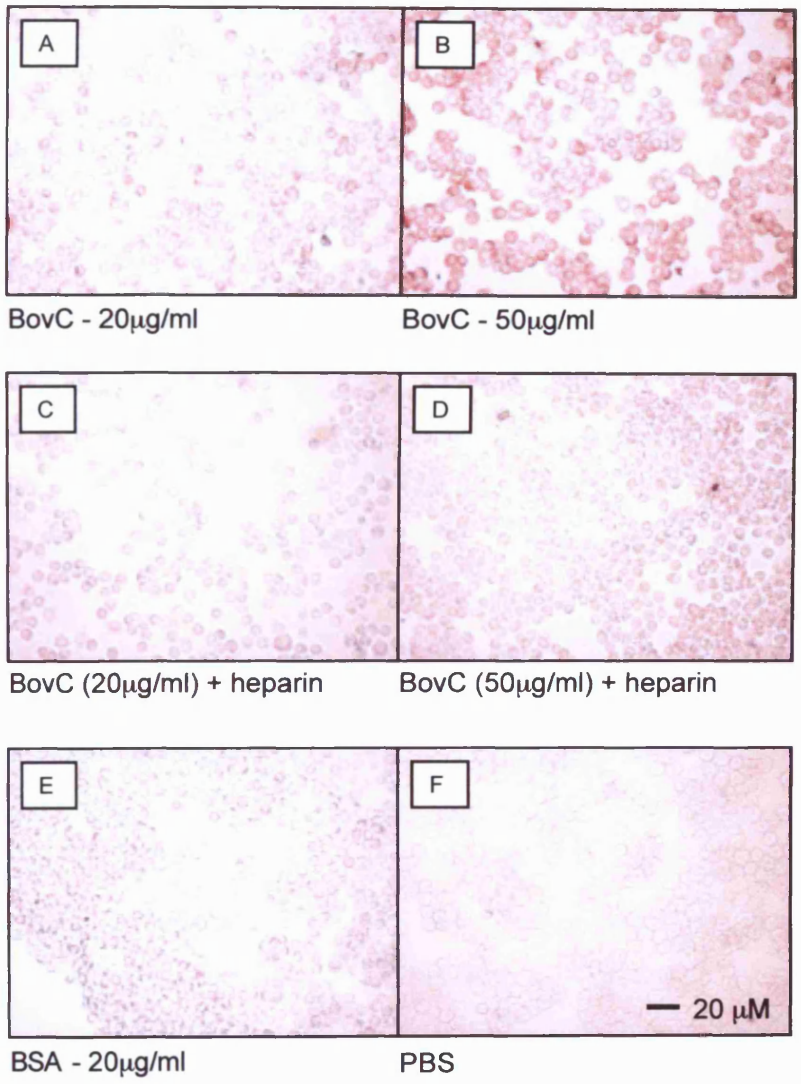


Figure 6.8 – Immunocytochemical analysis of bovine superficial zone chondrocyte monolayer cultures after incubation with recombinant bovine PRG4 C-terminal domain (BovC). Monolayer cultures were incubated for 1 hour in the presence of recombinant bovine PRG4 C-terminal domain diluted in a final volume of 0.5ml PBS, which in some instances were subjected to a pre-incubation with 100μg/ml heparin. PRG4 domain concentrations are displayed below each panel. Cells were fixed and immunocytochemistry was performed using an AP-conjugated anti-FLAG M2 monoclonal antibody to detect recombinant FLAG fusion proteins.

Plasminogen activator inhibitor type 1 (PAI-1) is a serine proteinase inhibitor that is the primary physiological inhibitor of tissue and urokinase-type plasminogen activators (tPA and uPA), and is an important regulator of proteolytic events in the circulation and in the extracellular matrix (for a review, see Wind *et al.*, 2002). PAI-1 is a conformationally labile molecule that is stabilised by an interaction with vitronectin. Other functions of PAI-1 include a modulation of cell motility based on its interaction with vitronectin, a result of the modulation of the interaction between vitronectin and the urokinase receptor uPAR caused by the binding of PAI-1 (Okumura *et al.*, 2002). PAI-1 is unstable in solution and decays into an inactive inhibitor ($T_{1/2} \sim 2$ hours), but is stabilised by binding to vitronectin such that its half-life is increased by up to four-fold, longer when vitronectin is incorporated into the ECM (Declerck *et al.*, 1988; Mimuro & Loskutoff, 1989). There is a bulk of evidence to suggest that this interaction is mediated by the somatomedin B domain of vitronectin (Seiffert & Loskutoff 1991; Deng *et al.*, 1996b; Seiffert, 1997; Deng *et al.*, 2001; Kamikubo *et al.*, 2002). The N-terminal portion of PRG4 contains 2 regions that display sequence homology with the SMB domain of vitronectin, and sequence analyses of PRG4 by Flannery (1999) and Tudor (2002) have proposed a vitronectin-like function for this region of PRG4. In this study, binding assays were conducted with immobilised human PAI-1 and solution-phase recombinant PRG4 N-terminal domain proteins, and a saturable interaction was demonstrated. These results describe an interaction between recombinant N-terminal domains of human and bovine PRG4 and the serpin PAI-1, which may be mediated by the 2 SMB-type domains in exons 2 and 3 of PRG4. More experimentation is necessary to determine whether or not this is the case, and work with the SMB domain of vitronectin provides a number of indicators in this respect. For example, mutational analyses of the SMB domain of vitronectin coupled with the use of monoclonal antibodies have pinpointed the region of the SMB domain that is critical for PAI-1 binding (Royle *et al.*, 2001), and crystal structure analysis of the PAI-1 – vitronectin complex has elucidated which residues on each molecule are involved in the interaction (Zhou *et al.*, 2003). The PRG4-PAI-1 interaction could also be studied by a competitive binding approach, as in the method used to examine the Vitronectin-PAI-1 interaction: solution phase PAI-1 was bound to immobilised vitronectin and displaced by the addition of solution phase vitronectin (Lawrence *et al.*, 1997). Sedimentation equilibrium ultracentrifugation has been used to assess the stoichiometry of the PAI-1 – vitronectin interaction, and a high molecular weight complex has been described involving four PAI-1

molecules and two vitronectin molecules (Podor *et al.*, 2000). Jensen and others have produced a recombinant PAI-1 mutant that lacks vitronectin binding activity (Jensen *et al.*, 2004), and it would be interesting to see if such a mutant also lacks PRG4 binding activity. A popular method of defining protein-protein interactions is BIAcore analysis (reviewed in Nedelkov & Nelson, 2003), and this could be applied to the potential PRG4–PAI-1 interaction as it has been for the vitronectin–PAI-1 interaction (Kamikubo *et al.*, 2002). Broadly speaking, a large number of methods to facilitate the study of serpin-ligand interactions exists (for a review see Patston *et al.*, 2004), and these techniques could be used in the context of PRG4 interactions with serpins.

Although slightly capricious at this stage, it is interesting to consider the role that a PRG4 – PAI-1 interaction could play in the synovial joint. PRG4 could stabilise PAI-1 in a manner similar to vitronectin, and may cause its localisation to specific regions in the synovial joint: synovial fluid and the articular surface. From these points, PAI-1 may have an effect on the fibrinolytic cascade, which may in turn influence the plasmin-mediated activation of MMPs. Osteoarthritic synoviocytes have been shown to produce PAI-1 and other fibrinolytic factors (Matucci-Cerinic *et al.*, 1998), and there is fibrinolytic activity in the synovial membrane of OA (Matucci-Cerinic *et al.*, 1990). PAI-1 production has been documented in chondrocytes (Treadwell *et al.*, 1991), and may be influenced by various cytokines and growth factors (Campbell *et al.*, 1991; Campbell *et al.*, 1994), although its exact role in healthy and diseased synovial tissue is yet to be elucidated. Urokinase has been shown to induce arthritis (Jin *et al.*, 2003), and the influence of PAI-1 on factors such as uPA, an active component of inflamed synovial fluid, may turn out to play a part in joint inflammation. The plasminogen activator/plasmin system is altered in RA (for a review, see Busso & Hamilton, 2002), as is apparent with the accumulation of fibrin in RA joints. Vitronectin synthesised by rheumatoid synovial cells inhibits plasmin generation (Tomasini-Johanssen *et al.*, 1998), which could be demonstrative of the potential effect of the Vitronectin-PAI-1 interaction. Increased levels of plasmin, uPA and PAI-1 have been demonstrated in OA chondrocyte cultures (Martel-Pelletier *et al.*, 1991). The expression and secretion of PAI-1 is attenuated in chondrocytes treated with IL-1, an effect that can be reversed with NSAID treatment (Sadowski & Steinmeyer, 2002). The expression of PRG4 has been shown to be downregulated by IL-1 (Flannery *et al.*, 1999; Tudor, 2002), and decreased levels of serpins in synovial fluid may induce cartilage breakdown. Plasminogen added to cartilage explants

increases degradation, which can be stopped by the addition of serpins (Olesyszyn & Augustine, 1996).

Recombinant bovine and human N-terminal PRG4 domains were found to interact with a 70 kDa proteolytic N-terminal fragment of fibronectin as assessed by an ELISA binding assay, with fibronectin in solid phase and PRG4 in solution phase. The N-terminal 70 kDa fibronectin fragment contains both heparin- and gelatin-binding domains, although no binding was observed with the isolated heparin (30 kDa) or gelatin (45 kDa) domains. This is similar to the COMP-fibronectin interaction (DiCesare *et al.*, 2002), and may suggest that N-terminal PRG4 binds to the junctions of the two fibronectin domains, or that the PRG4 binding site on the 70 kDa fragment is conformationally altered by proteolysis. No interaction was observe between PRG4 and intact bovine fibronectin. The bovine recombinant C-terminal domain of PRG4 (BovC) was co-purified with the 30 kDa, 45 kDa and 70 kDa proteolytic fragments of fibronectin in co-immunoprecipitation experiments using a FLAG agarose gel specific to the FLAG-tag of BovC. This suggests an interaction between three N-terminal proteolytic fragments of fibronectin and BovC. Fibronectin fragments are potential regulators of cartilage metabolism. Whilst intact fibronectin has little effect when added to chondrocytes *in vitro*, proteolytic fragments have been found to be active, and may contribute to the protease-mediated damage to cartilage (for a review see Homandberg, 1999). PRG4, present on boundary surfaces of the synovial joint and in synovial fluid, may have an anti-inflammatory effect in stopping fibronectin fragments from reaching cells. Co-localisation studies could be performed and other fragments of fibronectin could be used, such as intact fibronectin and the 170 kDa fibronectin fragment seen in arthritic synovial fluid (Peters *et al.*, 2003).

Preliminary ELISA-based binding assays demonstrated an interaction between bovine type II collagen and recombinant PRG4 N-terminal domain protein species. This interaction may be structurally important for the attachment of PRG4 to the articular surface, and such an interaction would provide the firm anchor that would be necessary for PRG4 to perform as a boundary lubricant. Binding assays should also be performed with other macromolecular components of the articular surface such as decorin, biglycan and fibromodulin (Noyori *et al.*, 1998). It may also be interesting to investigate a potential interaction between PRG4 and type I collagen, as another

macromolecule that has been shown to be present at the articular surface (Eyre, 1978; Duance, 1983).

Affinity blotting experiments with purified BovC and biotinylated HA indicate that there may well be an interaction between the C-terminal domain of PRG4 and HA. This interaction was proposed in 1992 following the retention of bovine synovial fluid lubricating factor (homologous to PRG4) with HA on a 0.22μM filter (Jay *et al.*, 1992). C-terminal truncating mutations of PRG4 are characteristic of the disease CACP (Marcelino *et al.*, 1999), and synovial fluid from patients with CACP displays a lower coefficient of friction than a lubricin-HA solution (Jay *et al.*, 2003). This has led Jay and co-workers to suggest that the loss of lubricating ability in CACP patients may be associated with a loss of PRG4-HA interaction caused by a C-terminal truncating mutation. PRG4 contains haemopexin-like domains in exons 8 and 9 of PRG4 that may be involved in a HA interaction (Jay *et al.*, 2000), and data presented here points towards this. A number of other methods could be utilised to further investigate the potential interaction between BovC and HA, and these are discussed in chapter 2, section 2.4. Intra-articular hyaluronate injections have recently been used for treatment of osteoarthritic knee joints, although a placebo effect makes the benefit of this procedure difficult to assess (for a review see Kirwan, 2001). Tentative discussions are taking place regarding the replacement of PRG4 in diseased joints, and if a HA-PRG4 interaction exists there is scope for intra-articular injections to develop in this manner.

Immunocytochemical analyses have demonstrated an interaction between BovC recombinant PRG4 domain and bovine superficial zone chondrocytes cultured in monolayer, an interaction that seems to be attenuated by the addition of heparin. However, further experimentation will help to elucidate the details of this potential interaction, such as the use of chondrocytes from the superficial, middle and deep zones of articular cartilage as well as other cell types. The precise reason for the observed interaction between the recombinant C-terminal domain of PRG4 and superficial zone chondrocyte monolayer cultures still needs further investigation, as there are a number of other possibilities that could explain this interaction. Other work suggests that superficial zone chondrocytes isolated by pronase and collagenase digestion retain some fibrous residual matrix in comparison to their counterparts from the deep zone (Pfister *et al.*, 2001). This

residual matrix contains a number of known matrix components including type VI collagen, link protein, CH65, annexin V, COMP and fibromodulin. So BovC added to monolayer cultures in this experiment may be binding to (a) the cell surface, or (b) components of a retained or newly synthesised matrix surrounding the chondrocytes. Similar culture systems and experimental procedures could be used to determine if PRG4 has any metabolic effects on cells, as predicted by a number of studies. Megakaryocyte stimulating factor (MSF) is in essence a ~25 kDa proteolytic N-terminal fragment of PRG4, and was first isolated from the urine of patients undergoing bone marrow transplantation during a period of acute thrombocytopaenia (Merberg *et al.*, 1993). This protein has been shown to stimulate growth in megakaryocyte colonies from bone marrow fibrin clot cultures, and it would be interesting to determine whether or not the N-terminal PRG4 domain produced in this study has a similar proliferative effect on megakaryocytes, or on other cell types, including chondrocytes.

Whilst the experiments described in this chapter raise a number of interesting questions regarding the interactions of PRG4 with other molecules, a more detailed determination of the structure and molecular weights of the recombinant PRG4 proteins used in this study would assist in the description of the interactions. For example, accurate molecular weights for the proteins would allow the calculation of K_D values for each interaction. It might also be interesting to look at full-length PRG4 or some of the different PRG4 splice variants known to be present within the synovial joint with respect to their binding capabilities, especially as different splice variants may possess different binding activities. Different expression constructs could be assembled that represent different splice variants of PRG4 that have been seen in various tissues and cell types, such as synovial fluid, synoviocytes, monocytes, chondrocytes, heart, liver, lung and brain. The functional implications of these different splice variants may well indicate that PRG4 is a modular protein, in a similar way to fibronectin. Mutational analysis of PRG4 and domains thereof, used in conjunction with binding assays, could localise protein-protein binding sites. The PRG4 domain catabolites described in chapter 5 of this study could also be used in binding assays following characterisation. In summary, the results presented in this chapter point towards the interaction of the N- and C-terminal domains of PRG4 with structural and functional molecules. These properties would be in addition to the well-documented boundary lubricating activity of the central mucinous region of PRG4 (Jay *et al.*, 2001a; Swann *et al.*, 1985).

6.5 Chapter Summary

- Preliminary ELISA data provided in this chapter suggests that there are potential interactions between:

  - the recombinant N-terminal domain of bovine and human PRG4 and human PAI-1, and that these interactions may be mediated by a somatomedin B domain

  - the recombinant N-terminal domain of PRG4 and the 70 kDa proteolytic fragment of human fibronectin, but not intact fibronectin or 45 kDa/30 kDa proteolytic fragments

  - the N-terminal domain of PRG4 and type II collagen, the major structural component of articular cartilage

- Co-immunoprecipitation experiments demonstrated potential interactions between the recombinant C-terminal domain of bovine PRG4 and the 30 kDa, 45 kDa and 70 kDa proteolytic fragments of human fibronectin.
- Affinity blotting experiments have revealed a potential interaction between recombinant C-terminal domain of bovine PRG4 and hyaluronan, which may be important in pathologies that directly involve PRG4
- The recombinant C-terminal domain of bovine PRG4 appears to interact with superficial zone chondrocytes from bovine articular cartilage

7. GENERAL DISCUSSION

PRG4 was fist described as a lubricating fraction from bovine synovial fluid (Radin *et al.*, 1970; Swann & Radin, 1972), and was later given the term 'lubricin' (Swann *et al.*, 1985). Although the initial descriptions of the lubricin occurred in the early 1970s, lubricin research has only seemed to gather pace in the last 10-12 years. Charles W. McCutchen, a crucial theorist in the field of joint lubrication, ascribes this lull in lubricin research to the existence of a National Institutes of Health (NIH) study section "cabal in joint lubrication" (McCutchen, 1995). In a lecture given in 1990, McCutchen stated that "in 1984, NIH decided to end its support of David Swann's research on lubricin..... For NIH to support no work on lubricin is grotesque" (McCutchen, 1990). Publications concerning lubricin started to re-appear in the early 1990s. Since then, lubricin has been found to be homologous to megakaryocyte stimulating factor precursor protein (Jay *et al.*, 2000) and cartilage superficial zone proteoglycan (Flannery *et al.*, 1999; Jay *et al.*, 2001), which are products of the HGNC-named PRG4 (proteoglycan 4) gene. Sequence analyses of this gene (Merberg *et al.*, 1993; Flannery *et al.*, 1999) have indicated that the PRG4 gene product may have functions other than boundary lubrication, as vitronectin-like domains lie either side of the central mucin domain. The aims of this study were to (i) investigate the potential multifunctionality of the PRG4 molecule using recombinant non-mucin domains of PRG4, (ii) to further characterise full length PRG4, and (iii) to determine the susceptibility of PRG4 to a variety of matrix proteinases that may be present in an arthritic joint.

Broadly speaking, recent PRG4/SZP/lubricin/MSF precursor protein/CACP protein publications have been centred on one or more of the following aspects:

- The boundary lubricating function of PRG4/lubricin at the surface of articular cartilage, as documented within initial papers by Swann and others, and more recently in numerous publications from the laboratories of Gregory D. Jay;

Immunolocalisation of PRG4 in tissues and body fluids using monoclonal antibodies;

- Analysis of PRG4 mRNA expression in various tissues including the identification of splice variants;
- Sequence analyses of PRG4;
- The effect of cytokines and growth factors on regulation of PRG4 expression;
- Description of CACP syndrome, the disease resulting from truncated mutations of PRG4.

Functional analyses of PRG4 have, to date, primarily been concerned with the boundary lubricating properties of the molecule at the surface of articular cartilage (Radin *et al.*, 1970; Swann *et al.*, 1972; Jay *et al.*, 1998). The two schools of thought with respect to lubrication by PRG4 are (i) that the molecule is the primary boundary lubricant in the synovial joint (Jay *et al*, 2001b) and (ii) that PRG4 is a carrier for surface-active phospholipid (SAPL), which is the true boundary lubricant (Hills *et al.*, 2003). Sequence analyses of the PRG4 gene by Merberg *et al.* (1993) and Flannery *et al.* (1999) have ascertained that PRG4 displays some sequence homology to the serum adhesive glycoprotein vitronectin, indicating that PRG4 may have other functional properties besides lubrication. One of the aims of this study was to investigate these vitronectin-like domains using different types of PRG4 species. Summaries of the major papers concerning PRG4/SZP/MSF/lubricin/CACP protein are shown in chronological order in table 7.1, and the findings of this thesis are summarised at the bottom of the table. Figure 7 shows a schematic representation of the full-length PRG4 molecule, and annotations detail the potential functions of the molecule as determined by this and other studies.

Data shown in this study demonstrate the heparin-binding capability of PRG4. Full length human recombinant, bovine articular cartilage and bovine tendon PRG4 were able to bind to a heparin-agarose affinity chromatography matrix. Heparin affinity purification of native bovine tendon PRG4 from the conditioned medium of deep digital flexor tendon explant cultures represents a novel method for obtaining native PRG4. Both the N- and C-terminal structural domains of PRG4 contain heparin-binding consensus sequences (Cardin & Weintraub, 1989), and experiments with recombinant PRG4 domains have shown that both these sites are functionally active. Heparin-binding sites within PRG4 may serve to anchor PRG4 to molecules within the *lamina splendens* of articular cartilage as proposed by Jay and others (Jay *et al.*, 2001b). It may also contribute to an interaction between PRG4 and chondrocytes: data in chapter 6 of this study shows that the recombinant C-terminal region of bovine PRG4 was able to interact with bovine superficial zone chondrocytes, and that this interaction was perturbed by the addition of heparin. It is interesting to consider the existence of PRG4 molecules that lack one or both of the heparin-binding sites in exons 4 & 10. A PRG4 gene product has been found in synovial fluid that contains only exons 6-9 (Jay *et al.*, 2000), and PRG4 mRNA lacking exons 4 & 5 has been found in OA chondrocytes (Flannery *et al.*, 1999) and tendon fibroblasts (Rees *et al.*, 2002). Further

Table 7.1 – Summary of published articles/abstracts concerning PRG4

Author(s)	Year	Summary
Radin *et al.*	1970	Separation of a lubricating fraction from synovial fluid
Swann & Radin	1972	Purification and characterisation of lubricating fraction from bovine synovial fluid
Swann *et al.*	1981	Lubricating glycoprotein-1: purification, lubricating ability, binding to cartilage
Swann *et al.*	1985	Lubricin from different synovial fluids: lubricating ability and molecular structure
Jay	1992	Lubricating ability of purified synovial lubricating factor (PSLF)
Jay & Hong	1992	Purified synovial lubricating factor lubricates, salivary mucin does not
Jay *et al.*	1992	Retention of purified synovial lubricating factor with HA on a 0.22μm filter
Merberg *et al.*	1993	Comparison of structural and functional aspects of MSF and vitronectin
Schumacher *et al.*	1994	SZP isolated from culture medium of bovine SZ cartilage explant cultures
Bahabri *et al.*	1998	Description of CACP syndrome; genetic mapping to chromosome 1q25-31
Jay *et al.*	1998	Healon (rooster comb HA) displays less boundary lubricating ability than lubricin
Schwarz & Hills	1998	Surface-active phospholipid (SAPL) as a lubricating component of lubricin
Flannery *et al.*	1999	Homology of cartilage SZP & MSF; analyses of amino acid sequence & potential functions; inhibition of biosyntheis by IL-1, upregulation by TGFβ and IGF-1
Marcelino *et al.*	1999	Description of truncating mutations in CACP protein (PRG4) in CACP syndrome
Schumacher *et al.*	1999	Monoclonal antibodies to bovine SZP (6-A-1 & 3-A-4); immunodetection of SZP at articular surface, synovium, synovial fluid and predominantly SZ chondrocytes
Ikegawa *et al.*	2000	PRG4 expression in cartilage, liver, lung, heart, bone; tissue-specific splice variants
Jay *et al.*	2000	Homology of lubricin from synovial fibroblasts with exons 6-9 of MSF
Jay *et al.*	2001a	Lubrication by lubricin mediated by O-linked β(1-3)GalNAc oligosaccharides
Jay *et al.*	2001b	Homology of SZP, lubricin and MSF; localisation to chromosome 1q25
Schmid *et al.*	2001	SZP concentration in human serum ~126μg/ml, normal synovial fluid ~159μg/ml
Su *et al.*	2001	Cross-species SZP mAbs; SZP immunodetection in synovial fluid, plasma, serum
Rees *et al.*	2002	Detection of SZP on the surface of mature fibrocartilaginous bovine tendon
Schmid *et al.*	2002	Purified SZP binds to fibronectin, BSA, HA (ELISA binding assays)
Tudor	2002	Immunohistochemical analyses, purification & characterisation of PRG4
El-Garf *et al.*	2003	Description of CACP syndrome among Egyptians
Jay *et al.*	2003a	Altered frictional characteristics of CACP knockout mice joints
Jay *et al.*	2003b	Synovial fluid from CACP syndrome patients lacks boundary lubricating ability
Klein *et al.*	2003	Engineering of articular cartilage from chondrocyte subpopulations; SZP detection
Morawietz *et al.*	2003	Lubricin overexpression: a factor in prosthesis loosening?
Schmid *et al.*	2003	Distribution of SZP in OA cartilage: SZP in regions of matrix damage
Schumacher *et al.*	2003	Immunolocalisation of SZP in meniscus
Benya *et al.*	2003	Upregulation of SZP synthesis by TGFβ and TAK-1a (TGFβ activated kinase-1)
Brass *et al.*	2003	SZP promoter region analyses: IL-1β may suppress SZP expression
Berthelot	2004	Hypothesis: could RA result from abnormal T cell response to SZP/lubricin?
Kutsumi *et al.*	2004	Lubricin in canine intrasynovial tendon; improves gliding of extrasynovial tendon
Schmidt *et al.*	2004	Detection of SZP in cartilage explants: inhibition by IL-1α, upregulation by TGFβ
Schumacher *et al.*	2004	Localisation of SZP in osteoarthritic cartilage: expression appears to be upregulated
Jones	2004	Full length PRG4: heparin binding, glycosylation profiles, purification of tendon PRG4 from explant cultures, degradation by candidate proteinases Recombinant domains of PRG4: heparin binding, glycosylation profiling, interaction with matrix proteins (fibronectin fragments, PAI-1), possible interaction with HA, interaction with SZ chondrocytes, degradation by proteinases

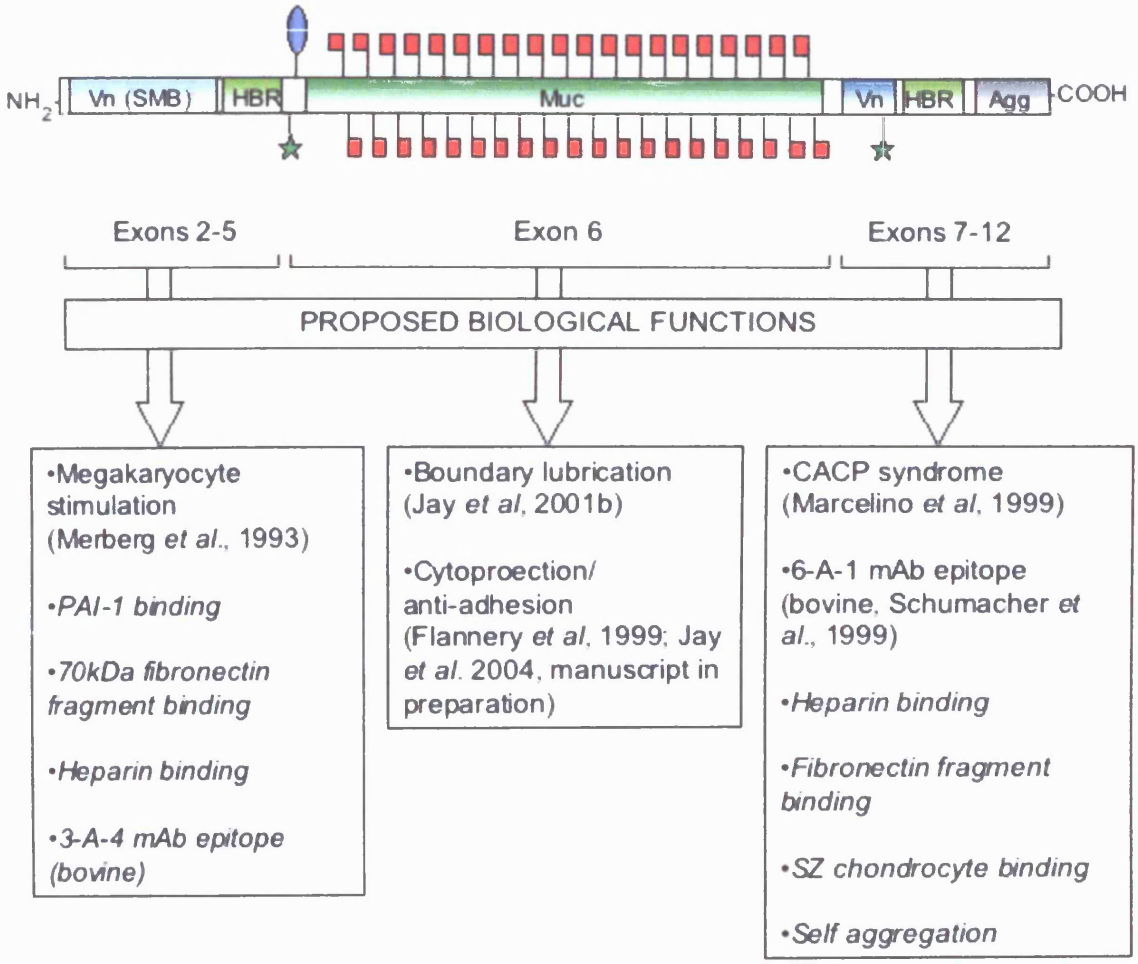


Figure 7 – Proposed functions of PRG4/SZP/lubricin/MSF precursor protein/CACP protein, illustrated using a schematic representation of the molecule. Functions proposed by this study are shown in italics. Abbreviations: Vn – vitronectin-like domain; SMB – somatomedin B homology; HBR – heparin binding region; Muc – mucin-like repeat region; Agg – potential aggregation domain (uneven number of cysteines); mAb – monoclonal antibody.

studies could examine the PRG4-heparin interaction more closely using techniques such as BIAcore analysis.

Data from lectin affinity blot experiments with full length PRG4 from three different sources (bovine cartilage, bovine tendon & human recombinant) have established that different full length PRG4 species share similar patterns of glycosylation. In particular, both human recombinant and bovine cartilage PRG4 displayed affinity with the lectin PNA, which recognises galactose linked β(1-3) to N-Acetylgalactosamine, and this interaction was used in the purification of PRG4 using a PNA lectin-agarose affinity chromatography matrix. Sequence analyses of the recombinant PRG4 domains produced by this study indicated likelihood of O- and N-linked oligosacchride substitutions, and these were investigated by lectin affinity blotting and deglycosylation experiments. Deglycosylation experiments documented the presence of an N-linked oligosaccharide in the C-terminal domain of PRG4 whilst lectin affinity blotting data showed that O-linked oligosaccharides are present in the N-terminal domain. These data show that PRG4 is glycosylated outside of the central mucin domain.

In this study, a number of interactions between PRG4 and structural/functional molecules have been described by using N-terminal (exons 2-5) and C-terminal (exons 7-12) recombinant domains of PRG4 in binding assays. Recombinant proteins were secreted as N-terminal FLAG-fusion proteins using an insect cell-based expression system. The PCR cloning stage of this recombinant protein expression strategy enabled the deposition of novel PRG4 sequence to GenBank. Solid-phase binding assays (ELISA) have demonstrated an interaction between the N-terminal structural domain of PRG4 and PAI-1, fibronectin fragments, and type II collagen. Immunoprecipitation experiments have demonstrated an interaction between the C-terminal structural domain of PRG4 and fibronectin fragments, and an interaction with HA in this region is also a possibility. The C-terminal region of PRG4 also displays self-aggregation properties. Although preliminary in nature these results support the sequence analyses of Flannery (1999), who surmised that the molecule has potential matrix-binding and self-aggregating properties. The work would benefit from knowledge of the 3-D structure of PRG4, and the use of other methods for determining binding kinetics. For example, the binding of PRG4 N-terminal domain to PAI-1 could localise PAI-1 to the articular surface, and could hold the molecule in an active

conformation in a similar manner to vitronectin (Mimuro & Loskutoff, 1989). The vitronectin – PAI-1 interaction is mediated by the somatomedin B domain of vitronectin (Seiffert & Loskutoff, 1991), and mutational studies could be used to determine whether or not the two somatomedin B domains of PRG4 stabilise a similar interaction. Sulphated GAGs have an effect on the PAI-1 – vitronectin interaction (Seiffert, 1997), and may affect a PRG4 – PAI-1 interaction. A number of methods exist for the examination of serpin-ligand interactions, reviewed in Patston *et al.* (2004).

The potential for PRG4 in cytoprotection and cell proliferation (Flannery *et al.*, 1999) have not been investigated in this study, although a good place from which to start might be the use of recombinant PRG4 domains in culture systems to look for these effects – immunocytochemical data in this study has demonstrated an interaction between superficial zone chondrocytes and the C-terminal structural domain of PRG4. The addition of fibronectin fragments to cartilage cultures has been shown to promote proteoglycan degradation (reviewed by Homandberg, 1999), and the addition of fibronectin fragment-binding PRG4 domains to this system may have modulative effects.

It is interesting to consider the hypothesis that different types of PRG4 gene product perform different functions based on their location. As can be seen in table 7.1, the tissue distribution of PRG4 is widespread, and there are a number of different PRG4 molecular weight species and splice variants. The synovial joint is probably the best example to use in the expansion of this hypothesis, although PRG4 expression has been documented in bone, liver, heart, lung, brain (Ikegawa *et al.*, 2000) and body fluids such as serum (Schmid *et al.*, 2001). Immunolocalisation studies have detailed the presence of PRG4 at the surface of articular cartilage, in synovial fibroblasts (Schumacher *et al.*, 1999), in chondrocytes of the superficial zone (Schumacher *et al.*, 1994), in synovial fluid (Schumacher *et al.*, 1999), in meniscus (Schumacher *et al.*, 2003) and on the surface of mature compressed tendon (Rees *et al*, 2002). A series of PRG4 mRNA splice variants have been found to be expressed by articular chondrocytes and synovial fibroblasts (Jay *et al.*, 2001b), and a PRG4 mRNA variant lacking exons 4 & 5 is expressed by osteoarthritic chondrocytes (Flannery *et al.*, 1999). Several PRG4 species of different molecular weights have been purified from synovial joint tissue. A 345 kDa isoform can be purified from superficial zone chondrocyte (Schumacher *et al.*, 1994) and tendon explant cultures (chapter 2 of this study), and

isoforms of 315 kDa (Schumacher *et al.*, 1999) and 280 kDa (Jay *et al.*, 2001b) have been purified from synovial fluid. Lower molecular weight isoforms (100-150 kDa) have been detected in the culture medium of superficial zone chondrocytes (Tudor, 2002). The significance of these different forms of PRG4 is yet to be understood. This study has contributed to the understanding of this situation, as functions have been preliminarily ascribed to the N-terminal (exons 2-5) and C-terminal (exons 7-12) structural domains of PRG4 by the use of recombinant proteins. This approach could be augmented as and when novel forms of PRG4 are described in the synovial joint: expression constructs could be made in order to produce recombinant PRG4 molecules that mimic PRG4 species seen *in vivo*. It would also be interesting to investigate the functional properties of recombinant PRG4 proteins that are truncated in a similar way to proteins seen in CACP syndrome (Marcelino *et al.*, 1999). For example, a symptom of CACP syndrome is synovial hyperplasia, so the effect of differently truncated recombinant PRG4 variants on synovial fibroblasts could be investigated. The effect of different physiological factors on the expression of PRG4 gene products, such as the influence of cytokines and growth factors, is also an area that has not been extensively investigated. Future studies will no doubt expand on the data already available, that IL-1 downregulates and TGFβ upregulates expression of cartilage PRG4 (Flannery *et al.*, 1999). Preliminary studies into the PRG4 promoter region have already been conducted using the popular luciferase reporter system (Brass *et al.*, 2003).

The cellular source of PRG4 at various locations in the synovial joint is unclear. For example, the layer of PRG4 on the surface of articular cartilage may equally be from chondrocytes or synovial fibroblasts. It may be that there is a population of PRG4 that is adsorbed onto the surface of articular cartilage through a cartilage-binding domain, and that there is another population of PRG4 in synovial fluid that lacks this domain. However, it may be that there is a 'floating' population of PRG4 within synovial fluid, and that a certain amount of this material is adsorbed on the surface of tissues such as synovium, meniscus and cartilage. Jay has proposed an interaction between PRG4 and hyaluronic acid (HA) (Jay *et al.*, 2003b). HA is abundant in synovial fluid and is used as a treatment for arthritis in the form of intra-articular injections. If PRG4 is depleted in diseased synovial fluid and cartilage, it may be useful within intra-articular injections in terms of working towards an injection that would help to revert synovial fluid back to a 'normal' physiological composition. However, there is much debate as to the efficicacy of

intra-articular HA injections, and the proteolytic conditions within an inflamed joint may cause injected PRG4 to be quickly degraded.

Although the role of PRG4 in arthritic diseases is unclear, its presence on the surface of cartilage, meniscus and synovium, at the boundary between joint tissue and synovial fluid, infers significance for the molecule, particularly in osteoarthritis where cartilage wears out from the surface downwards. In this thesis, preliminary data is present that documents the degradation of full length PRG4 by a number of enzymes that could be present in the synovial joint, namely elastase, MMP-7, plasmin and cathepsin B. Matrix proteinase-mediated digestion of PRG4 could affect the functional properties of the molecule, and could therefore compromise the integrity of the surface of articular cartilage. However, it would be capricious to speculate too much as to the significance of these *in vitro* digests in relation to the situation *in vivo*. Attempts in this study to selectively digest the non-mucin domains of full length PRG4 with chymotrypsin were unsuccessful, as degradation of the molecule as a whole occurred. This may explain the results seen by Jay and others (Jay *et al.*, 2001b), where PRG4 digested with chymotrypsin displayed an increased coefficient of friction in comparison to undigested PRG4. Jay *et al.* proposed that chymotrypsin digestion would remove non-mucin domains of PRG4, and that these domains also contribute to the boundary lubricating properties of PRG4. Whilst this may still be true, data in this study shows that chymotrypsin digests PRG4 as a whole.

Collectively, the data in this thesis provides novel information concerning the biochemical functions of PRG4 gene products. With the exception of lubrication, the specific functional properties of PRG4 have not been fully investigated within the synovial joint, and these findings advocate a potential multifunctional role for PRG4. This work points towards the interaction of PRG4 with both structural and functional molecules including heparin, fibronectin fragments and PAI-1. The future challenge is to relate these properties, along with the lubricating properties of the central mucinous region of PRG4, to healthy and diseased tissues in which the molecule is present such as articular cartilage.

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10. APPENDICES

APPENDIX A -LIST OF SUPPLIERS

Table A1 – List of materials used in this project and their suppliers

Supplier	Materials/Equipment
Anachem Ltd. Luton, UK	0.5ml & 1.5ml Eppendorf tubes
Applied Biosystems Perkin-Elmer UK Warrington, UK	RT-PCR reagents, sequencing reagents and machinery
Amersham Pharmacia Biotech St.Albans, Herts., UK	UltroSpec2000 spectrophotometer
Beckman Instruments Ltd. High Wycombe, UK	Chromatography columns, Western blot transfer tanks & power packs, agarose gel electrophoresis power packs
Bio-Rad Laboratories Ltd. Hemel Hemstead, UK	J-25 centrifuge, L-60 ultracentrifuge
Boehringer-Mannheim (Roche) Lewes, UK	Pronase from *Streptomyces griseus*
Calbiochem (CN Biosciences Ltd.)	Human mutant plasminogen activator inhibitor-1, calpain II

Beeston, UK	
Cambio Cambridge, UK	6X running buffer for agarose gel electrophoresis, DNA molecular markers
Cambrex Ltd. Wokingham, UK	SeaKem agarose
Corning Costar Ltd. High Wycombe, UK	Tissue culture plastic
Fisher Scientific Loughborough, UK	Acetic acid, hydrochloric acid, sodium hydroxide, methanol, ethanol, industrial methylated spirit, xylene
Invitrogen Ltd. Paisley, UK	Oligonucleotide primers, *Drosophila* S2 cell line, Drosophila Expression system (DES), pMT/BiP/V5-His A, B, C plasmid vectors, pCoHYGRO plasmid vector, calcium phosphate transfection kit, TOP10 *E. Coli*, FLAG M2 agarose, FLAG M2 AP conjugate mAb, Precast polyacrylamide gels & running equipment. All tissue culture media, serum and supplements
In vitro Systems and Services GmBH Gottingen, Germany	Flexiperm slides
Millipore Ltd. Watford, UK	All 0.22μM filters, Milli-Q Plus 185 unit, Milli-RO unit, Immobilon P^{SQ} transfer membrane, Centriplus YM10 centrifugal filters
Nalgene Milton Keynes, UK	Cryovials
National Diagnostics Hessle, UK	Acrylagel and bis-acrylagel
Novagen (CN Biosciences Ltd.) Beeston, UK	Enterokinase
Oxoid Basingstoke, UK	PBS tablets
Promega Corporation Southampton, UK	Restriction endonucleases, Wizard plasmid miniprep kits, *Taq* polymerase, T4 DNA ligase, anti-mouse secondary antibody (AP conjugate), BCIP/NBT substrate for AP
Qiagen Ltd. Crawley, UK	EndoFree plasmid prep kits, QIAquick gel extraction kit, RNeasy RNA isolation kit
R & D Systems Ltd. Abingdon, UK	pTAg plasmid vector
Roche Diagnostics Ltd. Lewes, UK	DIG Glycan differentiation kit
Schleicher and Schuell Ltd. London, UK	Protran BA nitrocellulose transfer membrane
Sigma-Aldrich Ltd. Poole, UK	General use laboratory chemicals, SOC medium, ampicillin, tri-reagent, fibronectin, EDGLY protein deglycosylation kit, anti-rabbit secondary antibody (AP conjugate), trypsin, chymotrypsin, plasmin, elastase, cathepsins B and D, heparin (sodium salt),
Spectrum Laboratories Rancho Dominguez, CA, USA	Spectra/Por dialysis membrane
Techne Burlington, NJ, USA	Technegene thermal cycler
Vector Laboratories Ltd.	Levamisole, Vectastain ABC-AP kit
Wothington Biochemical Corp. NJ, USA	Type II collagenase from *Clostridium histolyticum*
Wyeth Cambridge, MA, USA	Human recombinant ADAM TS-4 and –5, CHO-MegK and CHO-MegN141 cell lines, polyclonal anti PRG4 antibodies 06A10,

APPENDIX B - VECTOR DATA

Table B1 – Index of vectors described in this section, together with their supplier, uses, and figure number reference

Vector name	Supplier	Features	Figure
pTAg	R & D Systems	TA cloning vector, incorporating Amp^R and *LacZ* genes to confer ampicillin resistance and to facilitate blue-white screening of transformants	B1
pMT/BiP/V5-His A, B, C	Invitrogen	Expression vector for use in the *Drosophila* Expression System (Invitrogen). Transformants are ampicillin-resistant. Metallothionein promoter[a] and BiP signal sequence allow for inducible, secreted expression of recombinant protein	B2
pCoHYGRO	Invitrogen	Transformants are ampicillin-resistant. When co-transfected with pMT vector, can generate stable *Drosophila* S2 cell lines expressing recombinant protein[b]	B3

[a]The *Drosophila* metallothionein promoter is described in (Bunch *et al.*, 1988).

[b]Vector confers resistance to Hygromycin B, an aminocyclitol antibiotic that inhibits protein synthesis through a dual effect on mRNA translation (Cabanas *et al.*, 1978). Resistance is conferred by the hygromycin B phosphotransferase gene, which catalyses the phosphorylation of the 4-hydroxyl group on the cyclitol ring (hyosamine), producing 7'-O-phosphoryl-hygromycin B which lacks biological activity (Pardo *et al.*, 1985).

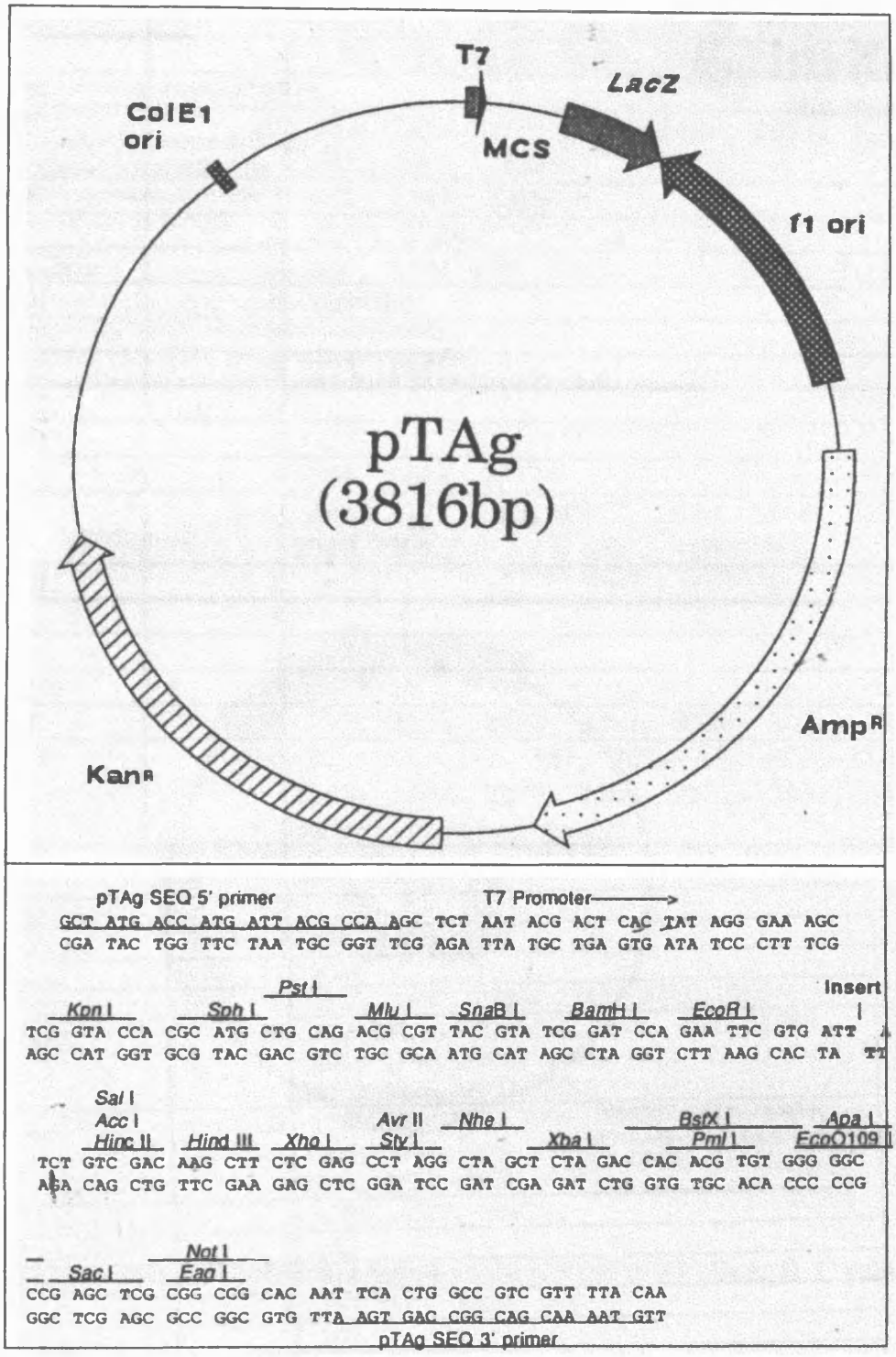


Figure B1 – Vector map and polylinker details of the pTAg vector

Comments for pMT/BiP/V5-His A
3642 nucleotides

Metallothionein promoter: bases 412-778
Start of transcription: base 778
MT Forward priming site: bases 814-831
BiP signal sequence: bases 851-904
Multiple cloning site: bases 906-999
V5 epitope tag: bases 1003-1044
Polyhistidine region: bases 1054-1074
BGH Reverse priming site: bases 1094-1111
SV40 late polyadenylation signal: bases 1267-1272
pUC origin: bases 1765-2438 (complementary strand)
bla promoter: bases 3444-3542 (complementary strand)
Ampicillin (*bla*) resistance gene ORF: bases 2583-3443 (complementary strand)

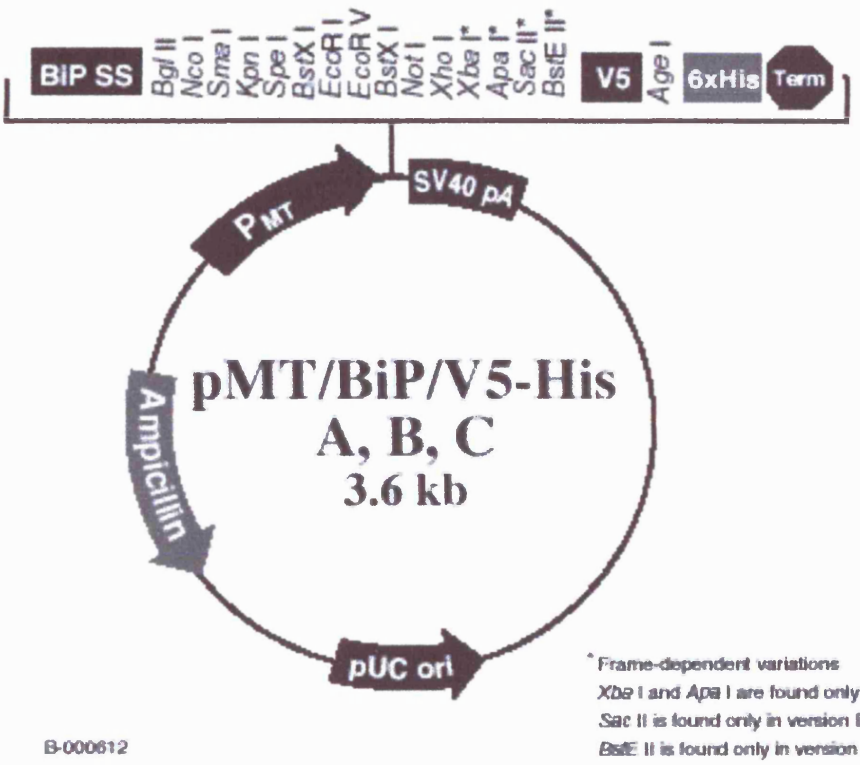
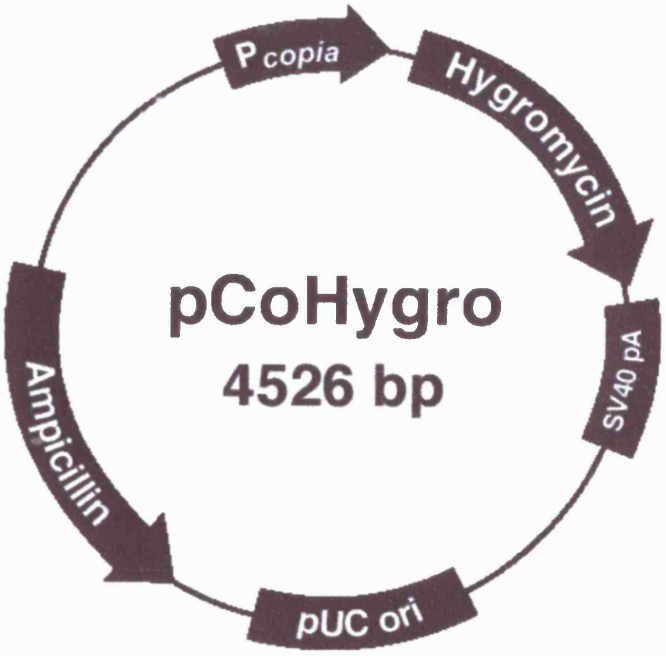


Figure B2 – Vector map and polylinker details of the pMT/BiP/V5-His A, B, C vector



Comments for pCoHygro
4526 nucleotides

copia promoter: bases 500-746
Hygromycin resistance gene: bases 781-1830
SV40 early polyadenylation sequence: bases 2146-2280
pUC origin: bases 2648-3321 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 3466-4326 (complementary strand)
bla promoter: bases 4327-4425 (complementary strand)

Figure B3 – Vector map of the pCoHYGRO selection vector

APPENDIX C – ABSTRACTS & PRESENTATIONS

ABSTRACTS

Jones A.R.C., Hughes C.E., Wainwright S.D., Flannery C.R., Little C.B. & Caterson B. (2003) Degradation of PRG4/SZP by matrix proteases. *Transactions of the Orthopaedic Research Society* **28**: 133

Jones A.R.C., Hughes C.E., Wainwright S.D., Flannery C.R. & Caterson B. (2003) PRG4 N- and C-terminal domains: cloning, expression and characterisation. *Transactions of the Orthopaedic Research Society* **29**: 576

ORAL PRESENTATIONS

677th Meeting of the Biochemical Society, Cardiff University, Wales, June 2002

XVIIIth Meeting of the Federation of European Connective Tissue Societies, Brighton, UK, July 2002

678th Meeting of the Biochemical Society, Imperial College, London, UK, December 2002

49th Meeting of the Orthopaedic Research Society, New Orleans, USA, February 2003