Recombinant rat complement regulatory proteins as therapeutic agents in inflammatory disease

By

Natalie Jayne Hepburn

A thesis submitted to Cardiff University in
Candidature for the Degree of Doctor of Philosophy.

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My acknowledgements would be incomplete if I did not mention the One who sustains me.
"My soul finds rest in God alone; my salvation comes from Him. He alone is my rock and my salvation; He is my fortress, I shall never be shaken" Psalm 62:1-2.

Finally, James,

I dedicate this thesis to you.

Thank you for your love, support and patience

through all of this,

you alone have known my true 'ups and downs'.

Remember, I want to grow old with you!

Abbreviations

AChR	Acetylcholine receptor
AP	Alternative pathway
ARDS	Adult respiratory distress syndrome
BSA	Bovine serum albumin
BBS	Borate buffered saline
C	Complement
C1-inh	C1 inhibitor
$C3_{MA}$	Methylamine inactivated C3, a C3b-like molecule
C4bp	C4b binding protein
CAB-2	Complement activation blocker-2
cDNA	Complementary DNA
CFD	Complement fixation diluent
CH	Constant region of the heavy chain
CH50	Concentration of serum to cause 50% lysis of target cells
CHO	Chinese hamster ovary
CNS	Central nervous system
CO_2	Carbon dioxide
CP	Classical pathway
cpm	Counts per minute
CR1	Complement receptor 1
CR2	Complement receptor 2
CRD	Carbohydrate recognition domains
CReg	Complement regulator
Crry	Complement receptor 1 related protein/gene Y
C-terminus/ terminal	Carboxy-terminus/ terminal
CV	Column volumes
CVF	Cobra venom factor
DAF	Decay accelerating factor
DepC	Diethylpyrocarbonate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid

dNTPs	Deoxy-nucleotide-tri-phosphates
DTT	Dithiothreitol
E	Erythrocytes
EA	Antibody-sensitised erythrocytes
EAMG	Experimental autoimmune myasthenia gravis
EC	Extracellular
ECACC	European collection of animal cell cultures
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescent assisted cell sorting
fB	Factor B
FBS	Foetal bovine serum
fD	Factor D
fH	Factor H
fI	Factor I
GPE	Guinea pig erythrocytes
GPI	Glycosyl-phosphatidylinositol
HAE	Hereditary angioedema
H&E	Haematoxylin and Eosin
HRPO	Horseradish peroxidase
IC	Intercellular
Ig	Immunoglobulin
IH50	Concentration of inhibitor required to inhibit lysis of target cells by 50%
i.p	Intraperitoneal
I/R	Ischaemia reperfusion injury
i.v	Intravenously
KD	Dissociation equilibrium constant
LB	Lauria-Bertani
LHR	Long homologous repeat
LP	Lectin pathway
MAC	Membrane attack complex

MASPs	MBL-associated serine protease
MBL	Mannan-binding lectin
MCP	Membrane cofactor protein
MG	Myasthenia gravis
MMPs	Matrix metalloproteinases
N_2	Nitrogen
N-terminus/ terminal	Amino-terminus/ terminal
O_2	Oxygen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PET	Polyethylene teraphthalate
RCA	Regulators of complement activation
RT-PCR	Reverse transcriptase polymerase chain reaction
RU	Resonance unit
SCR	Short consensus repeat
sCR1	Soluble complement receptor 1
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STP	Serine/ threonine/ proline
TAE	Tris acetate EDTA
TBS	Tris buffered saline
TCA	Trichloro-acetic acid
UV	Ultraviolet
v/v	Volume to volume
w/v	Weight to volume

Suppliers

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B Braun Biotch	via Sartorious AG, Gottingen, Germany
BDH	Lutterworth, UK
Becton Dickinson	Oxford, UK
Behring Diagnostics	Marburg, Germany
Biacore	Uppsala, Sweden
Binding site	Birmingham, UK
Bioline	London, UK
Bio-Rad	Hemel Hempstead, UK
Biotecx	Houston, Texas, USA
Charles River Laboratories	Wilmington, Massachusetts, USA
Dako	Ely, UK
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Nunc	via Fisher Scientific, Loughborough, UK
Nuncon	via Fisher Scientific, Loughborough, UK
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Summary

The complement system has become a therapeutic target due to its involvement in a number of inflammatory conditions. Soluble recombinant forms of the membrane-associated regulators (CReg) have been created as therapeutics. However, these reagents are limited by their short half-lives in vivo and their tendency to systemically inhibit complement. To extend their circulating half-lives Fc fusion proteins have been generated while targeting reagents to sites of inflammation or inhibiting specific parts of complement, such as the terminal pathway, has been used to overcome systemic complement inhibition. Many of the reagents generated to date are based upon human proteins and are therefore immunogenic in rats, preventing their testing in rat models of chronic disease. To enable the testing of anti-complement therapeutics in rat models of chronic disease, various CReg-Fc containing a rat Fc and different portions of rat Crry were generated in this study. Functional analysis of the generated reagents was carried out to identify two different Crry-Fc. One would retain full activity while the other would address the issues of systemic complement inhibition by having negligible activity in the circulation, due to steric hindrance imparted on the Crry by the Fc, but could be cleaved at inflammatory sites to unleash an active regulator. A Crry-Fc that retained full activity was identified and tested in vivo. This reagent had a long circulating half-life, low immunogenicity and markedly reduced disease severity in a rat model of myasthenia gravis. Attempts to generate a Crry-Fc that had negligible activity in the circulation but could be cleaved at inflammatory sites to unleash anti-complement activity were unsuccessful. The generation of the active Crry-Fc reagent paves the way to testing anti-complement therapeutics in rat models of chronic disease.

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Chapter 1: Introduction.

1.1 The Complement System

The complement (C) system, discovered as a heat-labile component of blood plasma, that "complemented" the action of antibodies, consists of more than 30 proteins in plasma or on cell surfaces. The proteins are part of the innate immune system, which acts as an early defence against infectious organisms. The C system is a cascade mechanism that brings about cell death or damage, opsonisation of pathogens, induction of inflammatory responses and clearance of immune complexes. It involves three activation pathways, known as the classical, alternative and lectin pathways, these all lead into a common terminal pathway. The classical activation pathway (CP) is initiated by the binding of the first C component, C1q, to aggregated IgG or IgM on a pathogen's surface. The alternative pathway (AP) proceeds in the absence of a specific antibody and is initiated by the target surface itself; this pathway can also act as an amplification loop for both the classical and lectin pathway. The lectin pathway (LP) is triggered by mannan-binding lectin or ficolins binding to sugar residues that are abundant in bacterial cell walls. The terminal pathway results in the formation of the membrane attack complex (MAC), a lytic pore that brings about osmotic lysis of the cell. The C system is summarised in figure 1.1 and the pathways are described in more detail below.

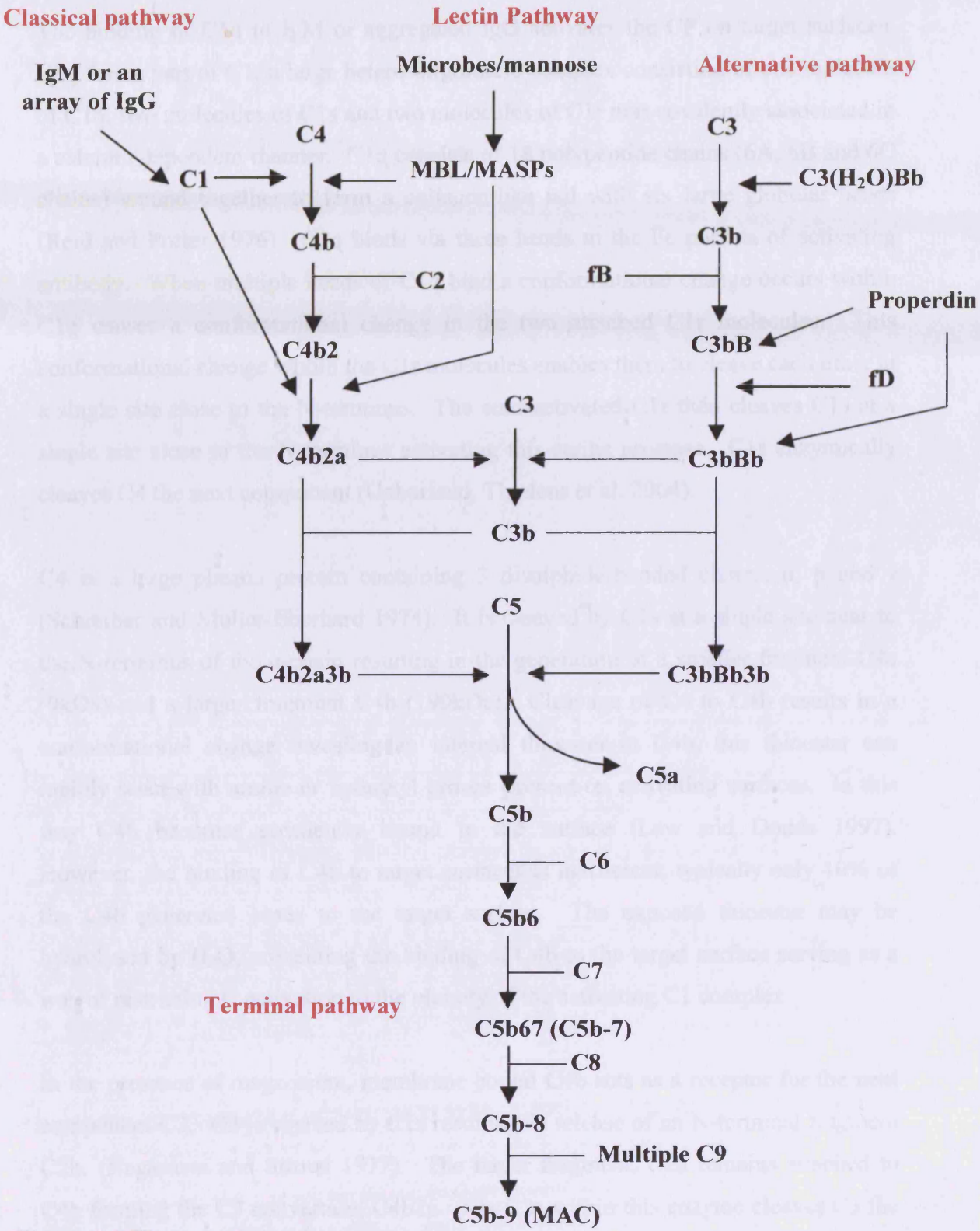


Figure 1.1 The complement system.

A schematic diagram of the activation and terminal pathways of the C system. Activation of the C system can occur via three pathways; the classical, alternative or lectin pathway. All three activation pathways lead into a common terminal pathway that results in the formation of a lytic pore, the membrane attack complex (MAC).

1.1.1 The classical activation pathway

The binding of C1q to IgM or aggregated IgG activates the CP on target surfaces. C1q forms part of C1, a large hetero-oligomeric complex consisting of one molecule of C1q, two molecules of C1s and two molecules of C1r non-covalently associated in a calcium dependent manner. C1q consists of 18 polypeptide chains (6A, 6B and 6C chains) wound together to form a collagen-like tail with six large globular heads (Reid and Porter 1976). C1q binds via these heads to the Fc portion of activating antibody. When multiple heads of C1q bind a conformational change occurs within C1q causes a conformational change in the two attached C1r molecules. This conformational change within the C1r molecules enables them to cleave each other at a single site close to the N-terminus. The auto-activated C1r then cleaves C1s at a single site close to the N-terminus activating this serine protease. C1s enzymically cleaves C4 the next component (Gaboriaud, Thielens et al. 2004).

C4 is a large plasma protein containing 3 disulphide-bonded chains; α, β and γ (Schreiber and Muller-Eberhard 1974). It is cleaved by C1s at a single site near to the N-terminus of the α-chain resulting in the generation of a smaller fragment C4a (9kDa) and a larger fragment C4b (190kDa). Cleavage of C4 to C4b results in a conformational change revealing an internal thioester in C4b, this thioester can rapidly react with amine or hydroxyl groups present on activating surfaces. In this way C4b becomes covalently bound to the surface (Law and Dodds 1997). However, the binding of C4b to target surfaces is inefficient; typically only 10% of the C4b generated binds to the target surface. The exposed thioester may be hydrolysed by H_2O, preventing the binding of C4b to the target surface serving as a way of restricting C activation to the vicinity of the activating C1 complex.

In the presence of magnesium, membrane bound C4b acts as a receptor for the next component C2. C2 is cleaved by C1s resulting in release of an N-terminal fragment C2b. (Nagasawa and Stroud 1977). The larger fragment, C2a remains attached to C4b forming the C3 convertase, C4b2a. The C2a within this enzyme cleaves C3 the next C component. C3 consists of two disulphide-bonded chains, α and β, and is cleaved in the α-chain releasing C3a (9kDa), a pro-inflammatory peptide (Lambris 1988). An internal thioester is exposed in C3b, the remaining fragment, which binds through the thioester to the C3 convertase to form a C5 cleaving enzyme, C4b2a3b.

The C3b may also bind to the adjacent membrane (Law and Dodds 1997). The coating of membranes with C3b is called opsonisation and mediates interactions with phagocytic cells (see section 1.2.1)

C5 is the first component of the terminal pathway and is a two-chain plasma protein that binds non-covalently to the C5 convertase. C5 is cleaved by C2a at a single site in the α-chain, releasing a small pro-inflammatory fragment C5a (11kDa). The larger fragment C5b (181kDa) contains a labile hydrophobic surface binding site and a binding site for C6 (Loos 1985) (figure 1.1).

1.1.2 The alternative pathway

The AP is antibody independent and is initiated by the binding of C3b to an activating surface. This C3b may arise from the cleavage of C3 through classical or lectin pathway activation and in this way the AP serves as an amplification loop for these pathways. Alternatively, the C3b may arise due to a process called 'tickover'. In 'tickover' the internal thioester of C3 undergoes spontaneous hydrolysis at a slow rate, about 1% of the total C3 in plasma per hour. A conformationally altered C3 arises called C3(H_2O), a C3b-like molecule. This C3b-like molecule can bind factor B (fB; 93kDa) in solution enabling a constitutively active serum protease, factor D (fD; 26kDa), to cleave fB (Volanakis and Narayana 1996). Factor B is cleaved at a single site resulting in release of a smaller fragment Ba (30kDa) and the generation of a larger fragment Bb (63kDa). The cleavage into Bb activates a serine protease domain within the molecule which forms a fluid phase C3 convertase, C3(H_2O)Bb. This enzyme can bind and cleave C3 resulting in deposition of C3b on adjacent surfaces and AP activation. The generated C3b binds fB in a magnesium dependent manner rendering it susceptible to cleavage by fD as before and results in the generation of C3bBb, the AP C3 convertase. This convertase is stabilised and its lifetime extended by the binding of properdin, the only known positive regulator of C (Hourcade 2006). The resultant C3b from this convertase may become surface bound acting to recruit more fB, amplifying the C3 convertase formation, or it may bind to the C3 convertase to form a C5 convertase, C3bBbC3b. C3bBbC3b can bind C5 allowing the cleavage of C5 by Bb and thereby marking the beginning of the terminal pathway (Xu, Narayana et al. 2001) (figure 1.1).

1.1.3 The lectin pathway

The LP is activated when mannan-binding lectin (MBL) or ficolins bind to sugar residues in bacterial cell walls. MBL is a serum C-type lectin structurally similar to C1q, consisting of multiple copies of a single subunit composed of three identical polypeptide chains. The polypeptide chains form a helical collagen-like domain and C-terminal carbohydrate recognition domains (CRD). Through the CRD, MBL binds carbohydrates in the presence of calcium while it is associated with MBL-associated serine proteases (MASPs) through the collagen like domain. Ficolins consist of a collagen like domain, through which they are also associated with MASPs, and a fibrinogen like domain through which they bind to carbohydrates (Endo, Takahashi et al. 2006). There are three MASPs which are associated with MBL and ficolins: MASP-1, MASP-2 and MASP-3. MASP-3 is an alternatively spliced form of MASP-1 while MASP-2 has an alternatively spliced form, sMAP which lacks the serine protease domain and is believed to compete with MASPs for binding to MBL and ficolins. The binding of MASPs to MBL or ficolin activates MASPs by promoting their auto-activation through cleavage at a single site, analogous to the activation of C1r and C1s when bound to C1q. While three MASPs exist, MASP-2 is sufficient for LP activation. The function of MASP-1 and 3 remain unclear. MASP-2 cleaves C4 and C2, as C1s does in the CP, generating the C3 convertase (Hajela, Kojima et al. 2002; Sorensen, Thiel et al. 2005). The pathway then follows the same cascade as the CP (figure 1.1).

1.1.4 The terminal pathway

All three activation pathways lead into the membrane attack or terminal pathway. Whilst still attached to the C5 convertase, C5b binds C6, a single chain protein, resulting in a conformational change in C6 that exposes a C7 binding site. Once C7 attaches to the complex a further conformational change occurs resulting in the detachment of C5b67 from the convertase. The fluid phase C5b-7 complex contains a transient, high-affinity lipid binding site that is susceptible to inactivation by hydrolysis or interaction with other plasma proteins, C5b-7 that does encounter the membrane physically associates with it. The next component C8 consists of 3 chains (α, β and γ). C8 binds to the C5b-7 complex through its β-chain. The C5b-8

complex can cause slow lysis of erythrocytes as the α-chain of C8 inserts through the membrane causing them to become leaky (Schreck, Parker et al. 2000). The final component, C9, binds to the α-chain of C8 and unfolds, inserts into the membrane and increases leakiness. The unfolding of C9 enables polymerisation of further C9 molecules which also insert into the membrane forming a pore-like structure called the membrane attack complex (MAC) (Podack and Tschopp 1984). The MAC increases permeability leading to osmotic lysis of simple cells such as erythrocytes or in bacteria it disrupts the outer membrane increasing permeability and inducing lethal changes in the inner membrane.

1.2 The physiological roles of C

C not only mediates the killing of invading pathogens either directly through the formation of the MAC or indirectly through enhancement of phagocytosis, but is also involved in the solubilisation of immune complexes, the induction of humoral and cellular immune systems, the clearance of apoptotic cells and the activation of certain cell types.

1.2.1 Bacterial killing

C can kill pathogens through the formation of the MAC on the cell surface (discussed 1.14) or through the induction of phagocytosis. Pathogens or cellular debris opsonised with C3b or its breakdown products, iC3b and C3dg, are recognised by phagocytic cells via complement receptors expressed on these cells (Taylor 1983). C3a and C5a, released during C activation, enhance phagocytosis by stimulating the migration of neutrophils, eosinophils, basophils and monocytes which express receptors for these fragments (Hugli 1981). These anaphylatoxins can also activate cells increasing vascular permeability, causing smooth muscle contraction and histamine release from mast cells and basophilic leukocytes.

1.2.2 Immune complex clearance

Immune complexes, antigen-antibody complexes, become more insoluble as they grow resulting in precipitation and inflammation. C plays several roles in clearing

immune complexes. Firstly, the complex itself can activate C resulting in opsonisation of the complex preventing further growth by masking antigenic sites. Opsonisation of large immune complexes with C3b can break apart the complex resulting in it disaggregating. Secondly, C3b and C4b on the immune complex can bind CR1 (CD35), a complement receptor expressed on erythrocytes. Binding to erythrocytes via CR1 results in the removal of the complex from the circulation as the complexes are carried to the liver and spleen where they are internalised and degraded (Schifferli 1996).

1.2.3 Humoral immune response

The discovery that a transient reduction in C3 led to impairment in antibody response suggested that C was involved in adaptive immunity (Pepys, Mirjah et al. 1976). Opsonisation of antigen containing complexes with C3b enhances B cell immunity. The complement receptor CR2 (CD21) binds C3b opsonised antigens and forms a receptor complex with CD19 and CD81, the B cell receptor. Co-engagement of CD21-CD19-CD81 lowers the threshold for B cell activation (Dempsey, Allison et al. 1996). The expression of CR1 and CR2 on follicular dendritic cells (FDCs) serves to retain C3-coated antigen within the lymphoid compartment where antigen is presented to B and T lymphocytes (Carroll 2004).

1.2.4 Apoptosis

C influences apoptosis in diverse ways. Firstly, C can protect cells from undergoing apoptosis. C5a inhibits spontaneous apoptosis in neutrophils while the MAC can inhibit apoptosis of cells in the central nervous system by up-regulating anti-apoptotic genes and down-regulating pro-apoptotic genes (Lee, Whyte et al. 1993; Rus, Niculescu et al. 2001). Secondly, C plays a role in the clearance of apoptotic cells. C1q binds apoptotic cells and via specific receptors on phagocytes including CD91 and calreticulin it initiates phagocytosis (Ogden, deCathelineau et al. 2001). The opsonisation of apoptotic bodies with fragments of C4 and C3 also aids the recognition and clearance via interaction of the C fragments with C receptors on phagocytes.

1.2.5 Cell activation

C activation at a cell surface does not always result in cell death. Cells are protected by the expression of regulators (discussed section 1.3), inactivation of C components by hydrolysis, proteolysis or phosphorylation at the cell surface, or due to the active removal of MAC by a cell. MAC can be removed from cell membranes via shedding on membrane vesicles or internalisation and degradation (Hansch, Betz et al. 1984). The deposition of MAC on a cell membrane that does not result in cell death is referred to as sub-lytic attack. Sub-lytic attack activates phagocytes resulting in release of inflammatory mediators such as prostaglandins, thromboxanes, leukotrienes and reactive oxygen species. On aortic smooth muscle cells and Schwann cells sub-lytic attack stimulates cell proliferation (Niculescu, Badea et al. 1999; Dashiell, Rus et al. 2000).

1.3 Complement Regulation

To prevent inappropriate C attack on self-cells an array of C regulators (CReg) exist. A group of over 10 proteins regulate either in the fluid-phase or on the membrane of host cells. These regulators inhibit the activation pathways and the terminal pathway of C. The CRegs found in humans are summarised in table 1.1 and a schematic of their sites of action is shown in figure 1.2.

Regulator	Location	Ligand	Function
C1-inh	Plasma	C1	Dissociates the activated C1 complex and acts as a chaperone for C1 in plasma preventing spontaneous fluid phase activation.
C4bp	Plasma	C4b	Accelerates the decay of the CP convertases and acts as a cofactor for the factor I mediated cleavage of C4b.
Factor H	Plasma	C3b	Accelerates decay of AP convertases and acts as cofactor for factor I mediated cleavages of C3b.
Factor I	Plasma	C4b, C3b	In conjunction with a cofactor cleaves C3b and C4b inactivating them.
CR1 (CD35)	Membrane	C4b, C3b, iC3b	Possesses decay accelerating activity and cofactor activity.
DAF (CD55)	Membrane	C4b, C3b	Accelerates decay of the convertases.
MCP (CD46)	Membrane	C4b, C3b	Acts as a cofactor for factor I mediated cleavages of C4b and C3b.
S-Protein	Plasma	C5b-7	Binds C5b-7 in the fluid phase preventing binding to a membrane.
Clusterin	Plasma	C5b-7	Binds C5b-7 in the fluid phase masking the membrane-binding site.
CD59	Membrane	C5b-8	Binds to C5b-8 complex preventing multiple C9 from binding and forming the MAC
Rat regulators			
Rat DAF	Membrane	C4b, C3b	Accelerates decay of the convertases.
Rat MCP	Membrane (testis specific)	C3b, C4b	Can act as a cofactor for factor I but expression pattern suggests it has more of a role in reproduction.
Rat Crry	Membrane	C3b, C4b	Acts as a decay accelerator of the convertases and as a cofactor for factor I.
Rat CD59	Membrane	C5b-8	Binds to C5b-8 complex preventing multiple C9 from binding and forming the MAC

Table 1.1 Complement regulatory proteins.

A summary of the CReg found in humans as well as the membrane-associated regulators found in rats.

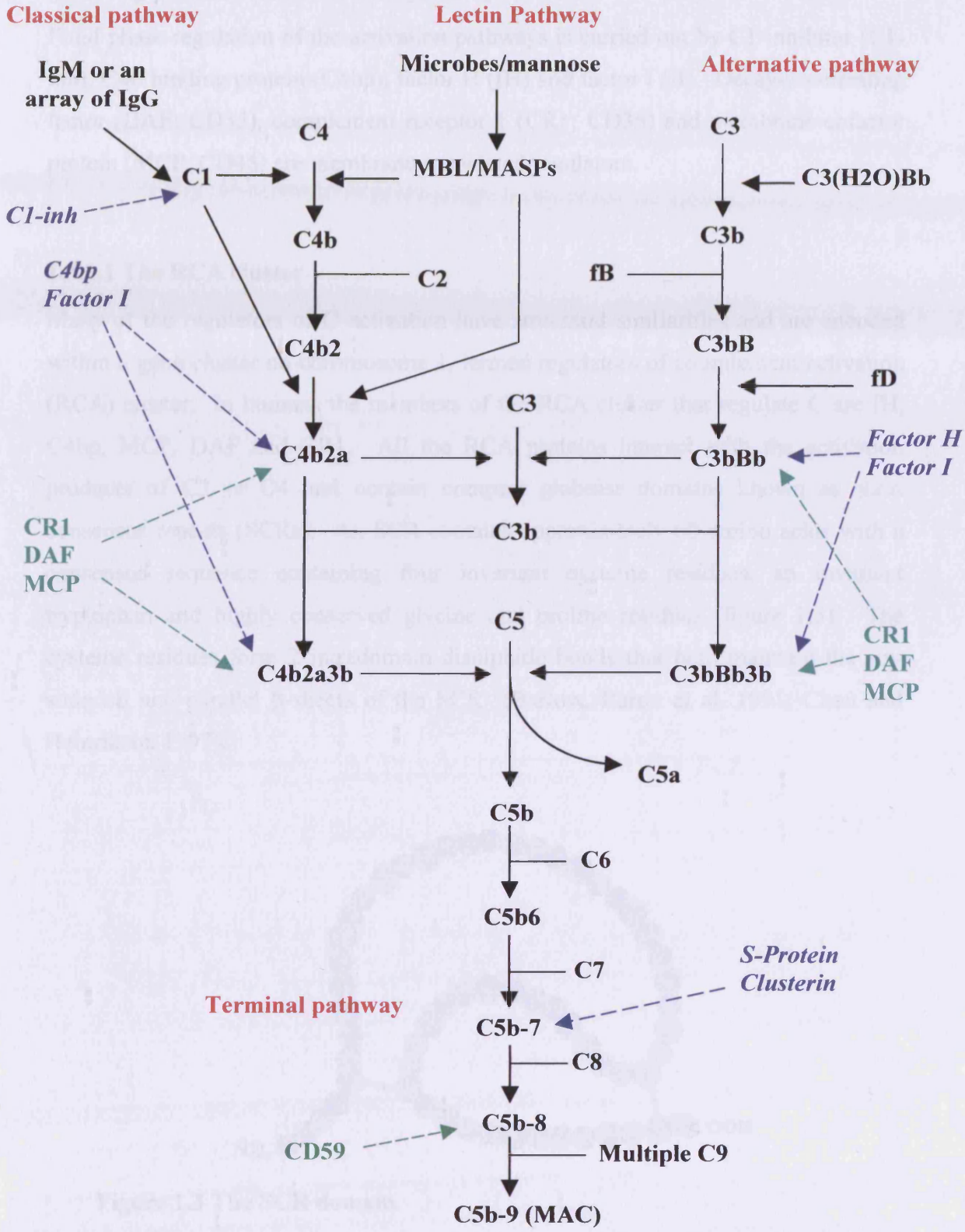


Figure 1.2 Regulation of C.

A schematic showing the site of action of the CReg on the activation and terminal pathways (dashed arrows). Fluid phase regulators are shown in blue in italics, whilst the membrane bound are in green.

1.3.1 Regulation of the activation pathways

Fluid phase regulation of the activation pathways is carried out by C1-inhibitor (C1-inh), C4b binding protein (C4bp), factor H (fH) and factor I (fI). Decay-accelerating factor (DAF; CD55), complement receptor 1 (CR1; CD35) and membrane cofactor protein (MCP; CD46) are membrane-associated regulators.

1.3.1.1 The RCA cluster

Many of the regulators of C activation have structural similarities and are encoded within a gene cluster on chromosome 1, termed regulators of complement activation (RCA) cluster. In humans the members of the RCA cluster that regulate C are fH, C4bp, MCP, DAF and CR1. All the RCA proteins interact with the activation products of C3 or C4 and contain compact globular domains known as short consensus repeats (SCRs). An SCR contains approximately 60 amino acids with a consensus sequence containing four invariant cysteine residues, an invariant tryptophan and highly conserved glycine and proline residues (figure 1.3). The cysteine residues form 2 intradomain disulphide bonds that help maintain the six-stranded anti-parallel β-sheets of the SCR (Barlow, Baron et al. 1991; Chou and Heinrikson 1997).

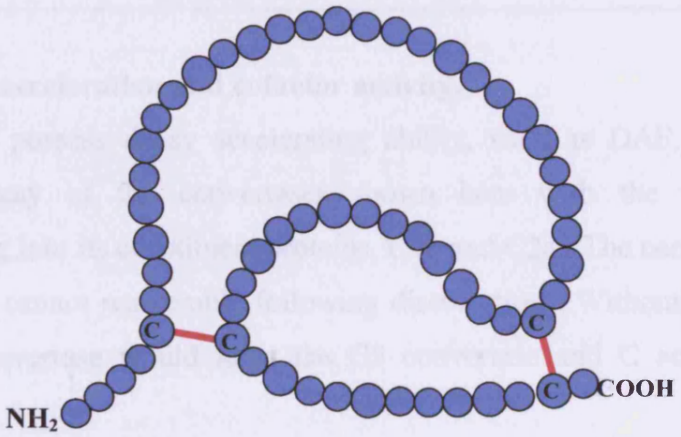


Figure 1.3 The SCR domain.

A diagram showing the compact globular nature of an SCR, the disulphide bonds (red) maintain the structure of the SCR. Amino acids are shown in blue with the cysteine residues labelled (C).

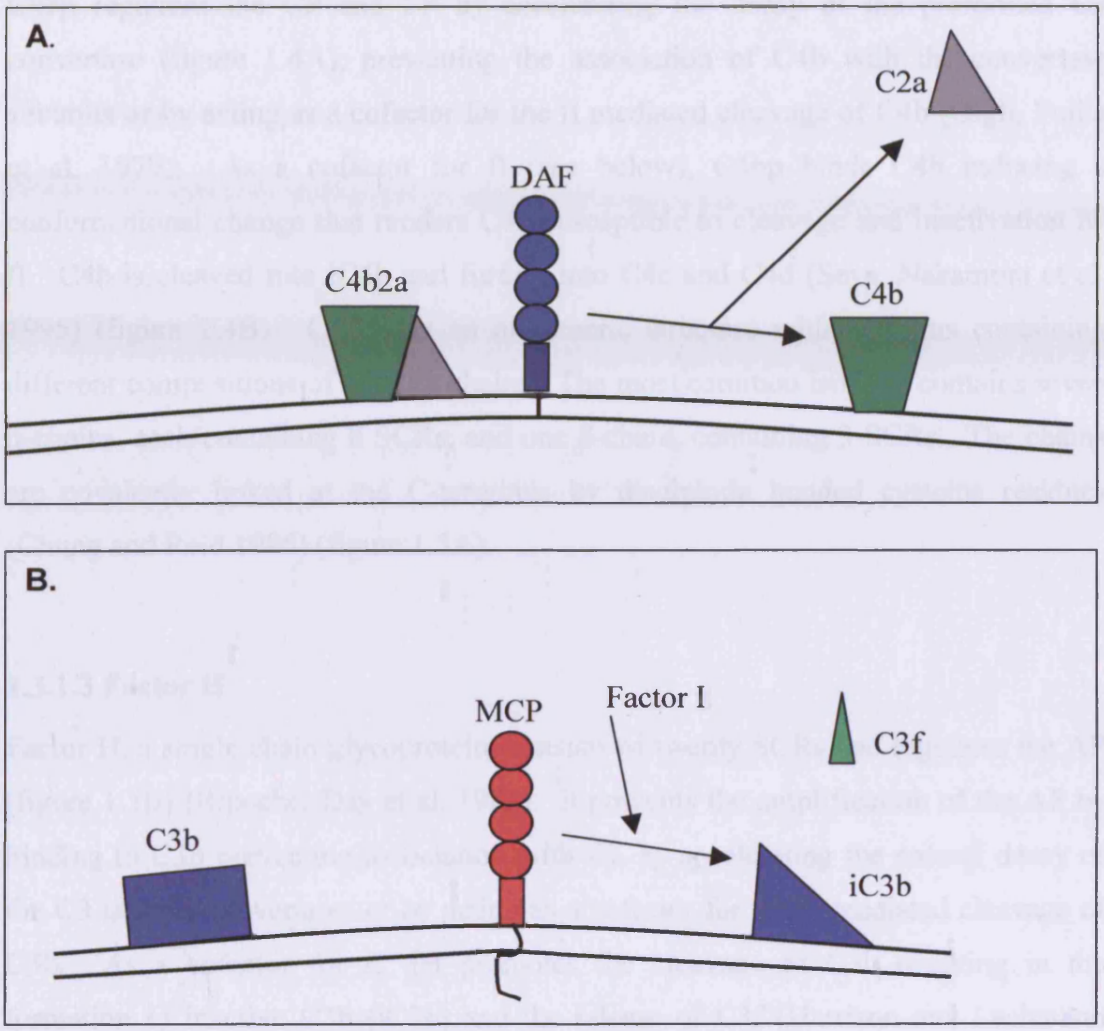


Figure 1.4 Decay acceleration and cofactor activity.

A. CReg that possess decay accelerating ability, such as DAF, accelerate the natural decay of the convertases shown here with the C3 convertase dissociating into its constituent proteins, C4b and C2a. The components of the convertase cannot reassemble following dissociation. Without this regulation the C3 convertase would form the C5 convertase and C activation would proceed.

B. CReg that act as cofactors, such as MCP, promote the inactivation of C3b and C4b by enabling their cleavage by factor I. Here, C3b has been cleaved into iC3b and C3f by the action of factor I with MCP.

1.3.1.2 C4b binding protein

C4bp regulates the CP and LP by accelerating the decay of the preformed C3 convertase (figure 1.4A), preventing the association of C4b with the convertase subunits or by acting as a cofactor for the fI mediated cleavage of C4b (Gigli, Fujita et al. 1979). As a cofactor for fI (see below), C4bp binds C4b inducing a conformational change that renders C4b susceptible to cleavage and inactivation by fI. C4b is cleaved into iC4b and further into C4c and C4d (Seya, Nakamura et al. 1995) (figure 1.4B). C4bp has an oligomeric structure with isoforms containing different compositions of α and β chains. The most common isoform contains seven α-chains, each containing 8 SCRs, and one β-chain, containing 3 SCRs. The chains are covalently linked at the C-terminus by disulphide bonded cysteine residues (Chung and Reid 1985) (figure 1.5A).

1.3.1.3 Factor H

Factor H, a single chain glycoprotein, consists of twenty SCRs and regulates the AP (figure 1.5B) (Ripoche, Day et al. 1988). It prevents the amplification of the AP by binding to C3b preventing association with fB, by accelerating the natural decay of the C3 and C5 convertases or by acting as a cofactor for the fI mediated cleavage of C3b. As a cofactor for fI, fH promotes the cleavage of C3b resulting in the formation of inactive C3b (iC3b) and the release of C3f (Harrison and Lachmann 1980).

1.3.1.4 C1 inhibitor.

C1-inh, a member of the serine protease inhibitor family (serpin), inactivates several different proteases. It inactivates C1r, C1s and MASPs in the C system in addition to Factor XII and kallikrein in the contact system, Factor XI and thrombin in the coagulation system and tissue plasminogen activator and plasmin in the fibrinolytic system. An exposed reactive loop within C1-inh acts as a pseudo-substrate for the protease. Cleavage of this loop by a protease results in a conformational change trapping the protease in a complex with C1inh (Bos, Hack et al. 2002). In addition to acting as a substrate for C1r, C1s and MASPs resulting in their inactivation, C1-inh

acts as a chaperone for C1 in plasma preventing spontaneous activation (Tenner and Frank 1986).

1.3.1.5 Factor I

Factor I is a two-chain serine protease that, in the presence of a cofactor (C4bp, fH, MCP or CR1), cleaves C3b or C4b within their α'-chains thereby inactivating them (figure 1.4B) (Tsiftsoglou, Willis et al. 2005). C3b is cleaved into iC3b and C3f (Harrison and Lachmann 1980). In the presence of CR1, C3 can be degraded further into C3c and C3dg (Ross, Lambris et al. 1982). The cleavage of C4b proceeds via iC4b, which is rapidly cleaved to give C4c and C4d (Shiraishi and Stroud 1975; Nagasawa, Ichihara et al. 1980)

1.3.1.6 Complement receptor 1

CR1, a transmembrane protein, regulates both the CP and AP. Four allelic variants of CR1 exist; the most common consists of 30 SCRs and has a molecular weight of 190kDa. Based on a degree of internal homology, all except the two C-terminal SCRs are arranged into larger units of 7 SCRs called Long Homologous Repeats (LHRs). CR1 serves to clear immune complexes from the circulation (discussed 1.2.2) and also regulates C by accelerating the decay of the C3 and C5 convertases in addition to acting as a cofactor for the fI-mediated cleavages of both C3b and C4b. CR1 is the only cofactor in humans to act as a cofactor for the third fI-mediated cleavage of C3b into C3dg (Krych-Goldberg and Atkinson 2001).

1.3.1.7 Decay Accelerating Factor (DAF)

DAF, a 70-80kDa glycoprotein, accelerates the natural decay of the C3 and C5 convertases of both the CP and AP. DAF consists of four SCRS, a region rich in serine, proline and threonine (STP) residues and a GPI-anchor (figure 1.6). SCRs 2 and 3 regulate the CP while SCRs 2-4 are necessary to regulate the AP (Coyne, Hall et al. 1992). A hydrophobic patch between SCRs 2 and 3 appears to be necessary for function (Lukacik, Roversi et al. 2004). DAF interacts with the individual components of the convertase (i.e. C3b and Bb or C2a) with a much lower affinity

than for the whole convertase enabling DAF to selectively bind and decay the active convertase (Harris, Abbott et al. 2005).

1.3.1.8 Membrane Cofactor Protein (MCP)

MCP is an abundantly expressed cofactor for the fI-mediated cleavage of C3b into iC3b and C4b into C4c and C4d (Seya, Turner et al. 1986; Seya and Atkinson 1989). MCP is capable of regulating all the activation pathways of C but evidence suggests it has a major role in regulating the AP of C and can only act on C3b that is not part of a convertase (Barilla-LaBarca, Liszewski et al. 2002). MCP consists of 4 N-terminal SCR domains, a heavily glycosylated STP region, a small segment of 12 amino acids of unknown function followed by a transmembrane region and a cytoplasmic domain (figure 1.6) (Lublin, Liszewski et al. 1988). Alternative splicing gives rise to 3 different STP regions, A, B and C, as well as different cytoplasmic tails including cyt-1 (16 amino acids) and cyt-2 (23 amino acids) (Post, Liszewski et al. 1991). C4b binding and cofactor activity has been located to SCRs 2-4 although SCR 1 is required for optimal activity while C3b cofactor activity resides in SCRs 2-4 with SCRS 3 and 4 binding C3b (Adams, Brown et al. 1991).

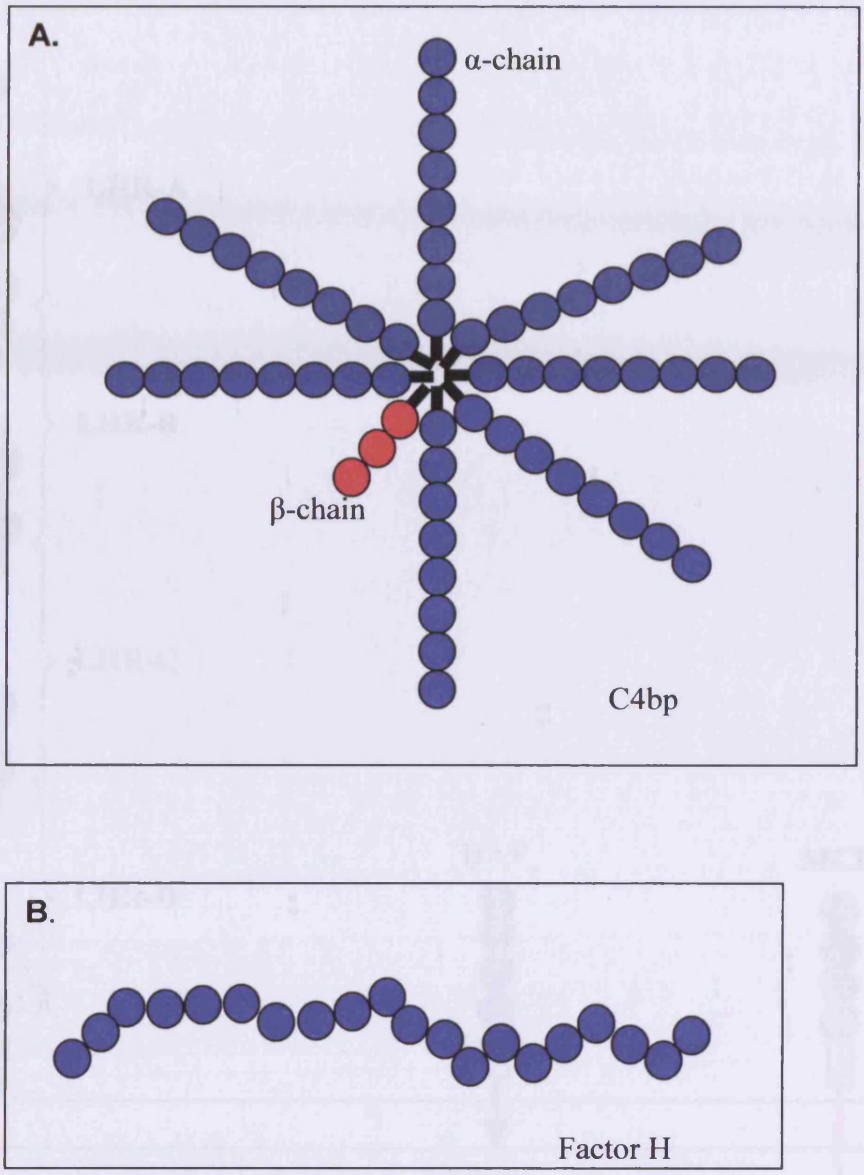


Figure 1.5 The structure of fluid phase regulators of complement activation that are encoded within the RCA cluster.

A. The structure of the most common isoform of C4bp. It consists of 7 α-chains (blue) each containing 8 SCRs and 1 β-chain (red) containing 3 SCRs covalently linked at the C-terminus by disulphide bonded cysteine residues.

B. The structure of fH consists solely of 20 SCRs (blue circles).

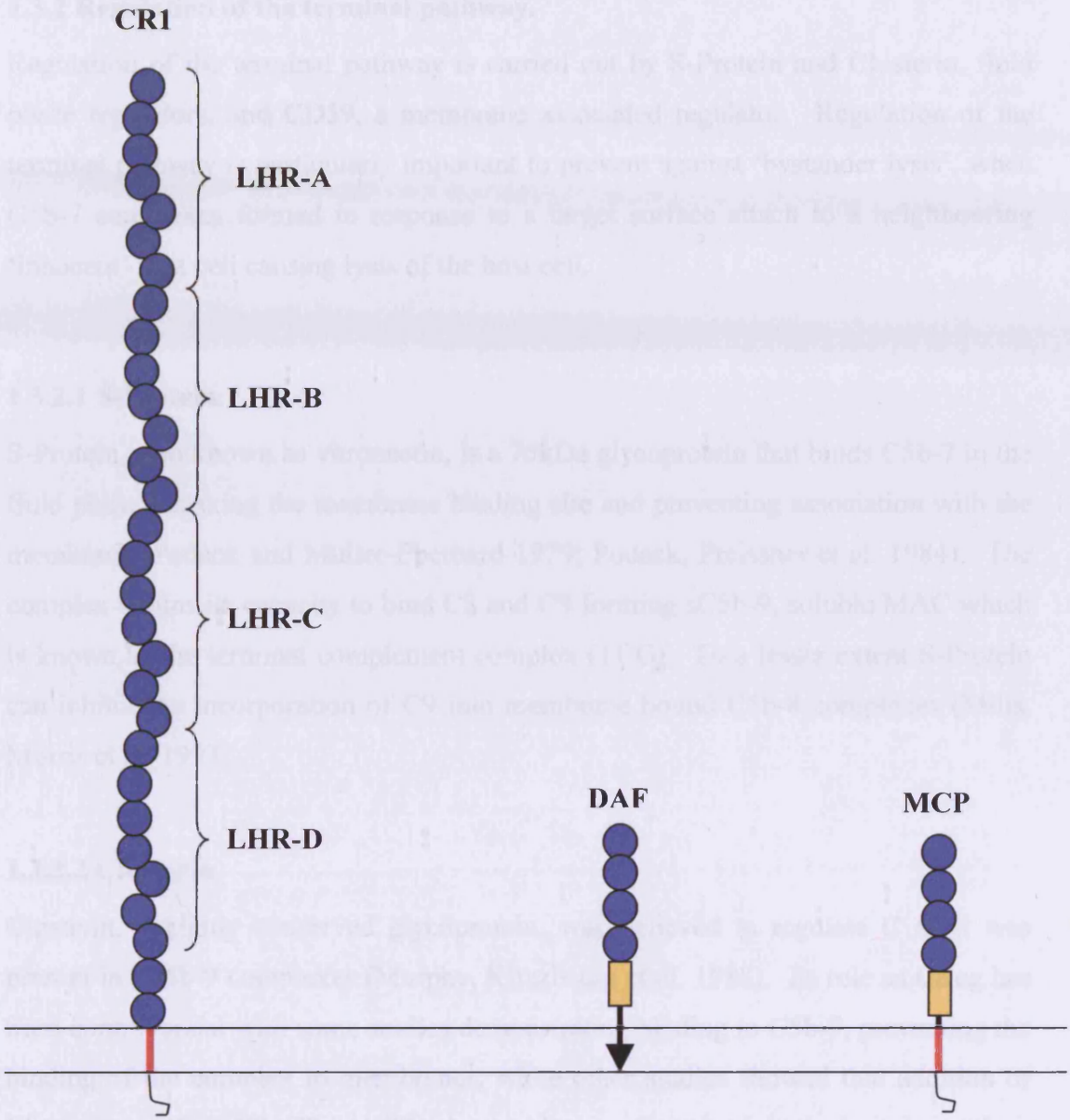


Figure 1.6 The structure of the membrane bound regulators of C activation encoded within the RCA cluster.

The structure of complement receptor 1 (CR1), decay accelerating factor (DAF) and membrane cofactor protein (MCP). The constituent SCRs (blue circles) are shown along with serine/threonine/proline rich regions (STP, yellow box) and the mechanism for membrane attachment. Transmembrane domains are shown in red while GPI-anchor attachment sequences are shown as arrows. The constituent long homologous repeats (LHRs) of CR1 are shown.

1.3.2 Regulation of the terminal pathway.

Regulation of the terminal pathway is carried out by S-Protein and Clusterin, fluid phase regulators, and CD59, a membrane associated regulator. Regulation of the terminal pathway is particularly important to prevent against 'bystander lysis', when C5b-7 complexes formed in response to a target surface attach to a neighbouring 'innocent' host cell causing lysis of the host cell.

1.3.2.1 S-Protein.

S-Protein, also known as vitronectin, is a 75kDa glycoprotein that binds C5b-7 in the fluid phase, masking the membrane binding site and preventing association with the membrane (Podack and Muller-Eberhard 1979; Podack, Preissner et al. 1984). The complex retains its capacity to bind C8 and C9 forming sC5b-9, soluble MAC which is known as the terminal complement complex (TCC). To a lesser extent S-Protein can inhibit the incorporation of C9 into membrane bound C5b-8 complexes (Milis, Morris et al. 1993)

1.3.2.2 Clusterin

Clusterin, a highly conserved glycoprotein, was believed to regulate C as it was present in sC5b-9 complexes (Murphy, Kirszbaum et al. 1988). Its role as CReg has been controversial with some studies demonstrating binding to C5b-7, preventing the binding of the complex to membranes, while other studies showed that addition of Clusterin to C5b6, C7, C8 and C9 enhanced lysis or that physiological concentrations of Clusterin did not regulate C (Choi, Mazda et al. 1989; Jenne and Tschopp 1989; Hochgrebe, Humphreys et al. 1999). The emerging picture is that Clusterin acts as an extracellular chaperone, serving as a hydrophobic 'sink' to remove toxic or damaged molecules away from cells (Wilson and Easterbrook-Smith 2000). In this way Clusterin binds terminal C complexes through their hydrophobic regions clearing them from the circulation rather than directly regulating C.

1.3.2.3 CD59

CD59, a widely expressed GPI anchored membrane glycoprotein, has a variety of names including membrane inhibitor of reactive lysis, HRF-20, protectin and MAC inhibitory factor. It inhibits MAC formation by binding to the α-chain of C8 within the C5b8 complex allowing only a single copy of C9 to bind, preventing the unfolding, polymerisation and insertion of C9 into the membrane (figure 1.7) (Meri, Morgan et al. 1990; Rollins and Sims 1990). CD59 contains 5 intrachain disulphide bonds that maintain the disc shaped structure of the molecule (Sugita, Nakano et al. 1993; Fletcher, Harrison et al. 1994). Molecular engineering was used to locate the active site, which was found to be on the upper face of the disc around a hydrophobic groove formed between two anti-parallel β-sheets (Bodian, Davis et al. 1997).

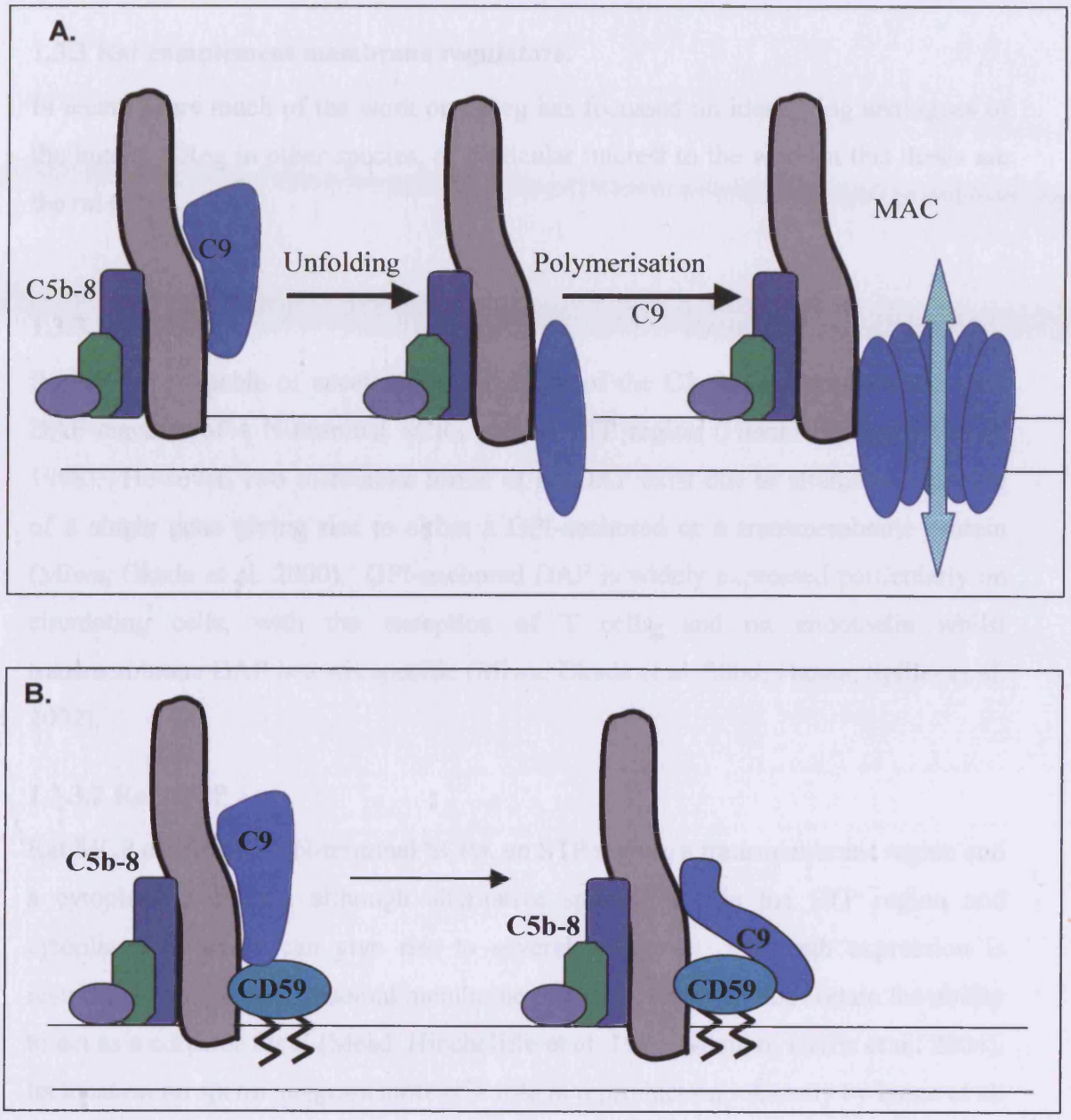


Figure 1.7 Mechanism of action of CD59.

A. In the absence of CD59, C9 binds to the C5b-8 complex, unfolds and polymerises with further C9 molecules which unfold to form the MAC.

B. CD59 binds to the C5b-8 complex allowing only one C9 molecule to bind preventing the unfolding and polymerisation of multiple C9 that form the MAC.

Modified from a figure kindly provided by Dr Claire Harris, Department of Medical Biochemistry and Immunology, Cardiff University.

1.3.3 Rat complement membrane regulators.

In recent years much of the work on CReg has focussed on identifying analogues of the human CReg in other species, of particular interest to the work in this thesis are the rat CReg.

1.3.3.1 Rat DAF

Rat DAF is capable of accelerating the decay of the C3 and C5 convertases. Rat DAF consists of 4 N-terminal SCRs and an STP region (Hinchliffe, Spiller et al. 1998). However, two membrane forms of rat DAF exist due to alternative splicing of a single gene giving rise to either a GPI-anchored or a transmembrane protein (Miwa, Okada et al. 2000). GPI-anchored DAF is widely expressed particularly on circulating cells, with the exception of T cells, and on endothelia whilst transmembrane DAF is testis specific (Miwa, Okada et al. 2000; Hanna, Spiller et al. 2002).

1.3.3.2 Rat MCP

Rat MCP consists of 4 N-terminal SCRs, an STP region, a transmembrane region and a cytoplasmic domain although alternative splicing within the STP region and cytoplasmic domain can give rise to several isoforms. Although expression is restricted to the inner acrosomal membrane of sperm, rat MCP does retain the ability to act as a cofactor for fI (Mead, Hinchcliffe et al. 1999; Mizuno, Harris et al. 2004). Its location on sperm suggests more of a role in reproduction. A study by Inoue et al. suggested MCP had a suppressive role in reproduction as MCP deficient mice had accelerated acrosome reactions and enhanced fertility (Inoue, Ikawa et al. 2003).

1.3.3.3 Rat Crry

Rat Crry is a widely expressed CReg unique to rodents, Crry was first discovered in mice and then two groups identified rat Crry (Wong and Fearon 1985; Quigg, Galishoff et al. 1993; Takizawa, Okada et al. 1994). Crry inhibits C activation of both the CP and AP, although in vivo data suggests it has a more dominant role in regulating the AP (Molina, Miwa et al. 2002). Crry possesses both fI cofactor

activity and decay accelerating activity, suggesting it was a functional homologue of both DAF and MCP in rodents (Kim, Kinoshita et al. 1995). However, the sequence more closely resembles human CR1 and so Crry is considered a CR1 homologue (Molina, Wong et al. 1992).

Crry consists of SCRs, a transmembrane domain and a cytoplasmic tail. Two forms have been identified in the rat, one containing 6 SCRS and the other 7 (Quigg, Lo et al. 1995). Although the exact functional site of Crry has not been identified, it is believed that function lies within SCRs 1-4 as they are homologous to SCRs 1-4 within mouse and human CR1 where CReg activity resides (Kalli and Fearon 1994; Molina, Kinoshita et al. 1994). SCR 5 of Crry is homologous to SCR 30 of human CR1 while the remaining SCRs, 6 and 7, are homologous to SCR 6 in human CR1. Crry appears to be the dominant CReg in rodents as Crry deficiency is embyronically lethal due to uncontrolled C activation and it has been demonstrated that Crry is necessary for the protection of both T cells and erythrocytes in vivo (Xu, Mao et al. 2000; Hanna, Spiller et al. 2002; Miwa, Zhou et al. 2002). The blocking of Crry by monoclonal antibodies in vivo caused increased vascular permeability, leucocytopenia and thrombocytopenia (Matsuo, Ichida et al. 1994).

1.3.3.4 Rat CD59

Rat CD59 resembles human CD59 in terms of molecular weight, membrane anchorage and stage of C inhibition (Hughes, Meri et al. 1993). It is broadly expressed, although it is not expressed on T lymphocytes and platelets, and shows 44% homology with human CD59 at the amino acid level (Funabashi, Okada et al. 1994; Hanna, Spiller et al. 2002). All 10 of the cysteine residues are conserved as well as several other stretches.

1.4 The role of complement in pathology

Under normal conditions CReg provide sufficient protection of self-cells from C attack. However, C can be activated to an excessive degree or in an inappropriate site and as a result has been implicated in the pathology of a number of inflammatory diseases or states. These diseases include immune complex and autoimmune diseases such as systemic lupus erythematosus and autoimmune arthritis, ischaemia-reperfusion (I/R) injuries, sepsis, multiple trauma and neurodegenerative disorders such as Alzheimer's disease, multiple sclerosis and Guillian-Barré syndrome. Diseases involving C and evidence for the role C activation in these diseases are given in table 1.2. In autoimmune diseases C can be activated by antibodies that recognise self-antigens leading to C deposition and destruction of host cells and tissue (Semple and Freedman 2005). In I/R injuries it is believed that C is activated by membrane phospholipids and mitochondrial proteins that become exposed due to the ischaemia, following reperfusion these serve as neoantigens to activate C (Austen, Kobzik et al. 2003; Chan, Ibrahim et al. 2003). In therapeutic interventions, exposure of blood to a foreign surface whether that be in dialysis or transplantation, can activate C via the AP.

C mediates injury either directly by the MAC or indirectly by the generation of the anaphylatoxins C3a and C5a. The MAC causes tissue damage by lysing unprotected cells as well as having pro-inflammatory activity, inducing cell activation resulting in production of inflammatory chemokines and expression of P- and E-selectins and intercellular adhesion molecules (Kilgore, Shen et al. 1995; Kilgore, Schmid et al. 1997; Tedesco, Pausa et al. 1997). The peptides, C3a and C5a, induce damage through their effects upon neutrophils, eosinophils and mast cells (Wetsel 1995). C5a acts as a potent chemoattractant in addition it induces the production of a wide range of inflammatory mediators. C3a acts as a chemoattractant for eosinophils and mast cells and activates these cells to produce inflammatory mediators.

Disease	Evidence	References
Rheumatoid arthritis	C activation products in synovial fluid and on synovial membrane. Systemic C inhibition or C deficiency suppresses disease in various animal models. Treatment of patients with an anti-C5 antibody lessened disease severity.	Brodeur, Ruddy et al. 1991; Kemp, Spragg et al. 1992; Wang, Rollins et al. 1995; Goodfellow, Williams et al. 2000; Kaplan 2002
Glomerulonephritis	Abundant deposits of C activation products in glomeruli. C activation products found in urine of patients with glomerular disease C inhibition or deficiency suppresses disease in models.	Davis and Cavallo 1976; Adler, Baker et al. 1984; Couser, Johnson et al. 1995; Nangaku, Pippin et al. 1999; Morita, Ikeguchi et al. 2000
Multiple sclerosis	C activation products in cerebrospinal fluid and around areas of demyelination in the CNS. C inhibition suppresses disease in animal models.	Morgan, Campbell et al. 1984; Compston, Morgan et al. 1989; Piddlesden, Storch et al. 1994; Davoust, Nataf et al. 1999
Alzheimer's disease	C deposition in and around plaques in the CNS. Up-regulation of C components in diseased brain. Deposited plaques capable of activating C.	Rogers, Cooper et al. 1992; Jiang, Burdick et al. 1994; Shen, Li et al. 1997; Terai, Walker et al. 1997; Yasojima, Schwab et al. 1999
Myocardial infarction	Abundant C deposits in and around infarcts. Reperfusion associated with C activation in ischaemic area. C inhibition at time of reperfusion in models reduces infarct size. Reduced serum C levels after myocardial infarction.	Pinckard, Olson et al. 1975; Pinckard, O'Rourke et al. 1980; Shandelya, Kuppusamy et al. 1993; Mathey, Schofer et al. 1994; Vakeva, Agah et al. 1998
Stroke	Abundant C deposits in and around affected area. C deficient animals have reduced infarct size. C inhibition at time of reperfusion shown to reduce damage.	Davis and Brey 1992; Huang, Kim et al. 1999; Imm, Feldhoff et al. 2002; Figueroa, Gordon et al. 2005
Guillian-Barré syndrome	C deposition found at nerve and activation products found in spinal fluid. C depletion and anti-C therapy reduces disease severity.	Sanders, Koski et al. 1986; Hartung, Schwenke et al. 1987; Jung, Toyka et al. 1995; Vriesendorp, Flynn et al. 1995; Putzu, Figarella-Branger et al. 2000

Table 2.1 continued overleaf

Disease	Evidence	References
Adult respiratory distress syndrome (ARDS)	C activation products found in lungs and plasma. Anti-C therapy therapeutically effective in condition.	Robbins, Russ et al. 1987; Zilow, Joka et al. 1992; Zimmerman, Dellinger et al. 2000
Systemic lupus erythematosus	C involved in tissue damage with C activation products found in lesions and in plasma. C inhibition in animal models decreases proteinuria in associated renal disease. C also protective as C1q and C4 deficiency predisposes to disease development, early stages of C necessary for clearance of apoptotic bodies and immune complexes.	Belmont, Hopkins et al. 1986; Helm and Peters 1993; Wang, Hu et al. 1996; Stone, Williams et al. 2000; Rupert, Moulds et al. 2002
Myasthenia gravis	C activation products on post-synaptic membrane. C consumption in vivo C deficiency or inhibition reduces disease severity in animal models.	Sahashi, Engel et al. 1978; Sahashi, Engel et al. 1980; Engel and Arahata 1987; Christadoss 1988; Piddlesden, Jiang et al. 1996; Romi, Kristoffersen et al. 2005
Cardiopulmonary bypass and haemodialysis	C activation products in plasma. Coating circuits found to reduce C activation. Inhibition of C in vivo inhibits reaction to extracorpeal circuits.	Amadori, Candi et al. 1983; Cheung, Parker et al. 1994; Rinder, Rinder et al. 1995; Tamim, Demircin et al. 1999
Hyperacute rejection	C activation products found in transplanted tissue. C deficiency and anti-C therapy prolongs graft survival.	Forbes, Pinto-Blonde et al. 1978; Forbes and Guttmann 1982; Pruitt and Bollinger 1991; Brauer, Baldwin et al. 1995; Abe, Sawada et al. 2003

Table 1.2 Complement in pathology.

A summary of some of the diseases associated with C activation along with evidence for the role of C in these disease states.

1.5 Anti-complement therapeutics

Although C is not always the primary cause of many of the diseases listed in table 1.2 it has been associated with, it does contribute to the resultant pro-inflammatory cycle and tissue damage and has therefore become a therapeutic target. Numerous agents have been tested as potential anti-complement therapeutics; these include polyanionic reagents, peptides, antibodies and recombinant proteins.

1.5.1 Cobra venom factor

Cobra venom factor (CVF) was the first anti-C therapeutic to be tested in models. CVF binds fB forming a stabilised AP C3 convertase (CVFBb). Compared to C3bBb the complex has an extended half-life, 7 hours compared to 1.5 minutes, and is resistant to fluid phase regulation (Vogel, Bredehorst et al. 1996). CVFBb continuously activates the AP thereby consuming C3 and obliterating C activity in experimental animals for between 24 and 72 hours. The use of this reagent has however been limited as it is strongly antigenic resulting in the generation of neutralising antibodies. CVF has been a useful proof of principle demonstrating that anti-C therapy is relevant to many pathologies. Recently, human C3 was used to generate chimeric derivatives of CVF that stabilised the C3 convertase whilst reducing the immunogenicity of CVF paving the way to use a CVF-like molecule therapeutically (Kolln, Spillner et al. 2004).

1.5.2 Polyanionic agents

A number of polyanionic molecules including heparin, dextran sulphate, polyvinyl sulphate, polylysine and suramin have been shown to inhibit C in vitro (Asghar 1984). Heparin has been shown to inhibit C at various points including inactivating C1, blocking assembly of the C3 convertase and interfering with MAC assembly (Baker, Lint et al. 1975; Hughes-Jones and Gardner 1978; Weiler 1983). In vivo, heparin is widely used as an anti-coagulant in patients with shock syndromes and to coat extracorpeal circuits in dialysis and cardiopulmonary bypass where it serves to prevent coagulation and C activation (Ovrum, Mollnes et al. 1995; Heyer, Lee et al. 2002). Its role as an anti-C therapeutic in vivo has not been extensively addressed.

1.5.3 Small molecule inhibitors

A number of natural and synthetic molecules have been shown to inhibit C in vitro as well as in vivo. For a summary of these molecules see table 1.3. Whilst the small molecule inhibitors are cost-effective, have good tissue penetration and can be developed for oral use, they often have non-specific effects affecting more than just the C system. They are also limited by their short in vivo half-lives resulting in their rapid clearance from the body making them unsuitable for long term anti-C therapy in chronic inflammatory conditions.

Molecule	Synthetic/ natural	Action and uses	References
K76COOH	Natural, a fungal metabolite	Inhibits C at the C5 stage. Effective in vivo in animal models.	Konno and Tsurufuji 1983; Iida, Izumino et al. 1987; Yamada, Kudoh et al. 1997
FUT-175	Synthetic organic molecule	Broad-spectrum serine protease, acts as an inhibitor for C1s, fD and the C3/C5 convertases. Effective in various animal models where it proved to be anti-inflammatory. Effective in glomerulonephritis patients.	Issekutz, Roland et al. 1990; Inagi, Miyata et al. 1991; Fujita, Inoue et al. 1993
BCX-1470	Synthetic organic molecule	Inhibits fD, C1s as well as thrombin, factor Xa and trypsin. Inhibited development of reverse passive Arthus reaction induced oedema in rats.	Szalai, Digerness et al. 2000
Compstatin	Synthetic cyclic peptide	Binds C3 and prevents cleavage. Effective in an ex vivo model of kidney xenotransplantation, inhibited C activation on extracorporeal circuits and abrogated C activation induced by heparin-protamine complexes in Baboons.	Sahu, Kay et al. 1996; Nilsson, Larsson et al. 1998; Fiane, Mollnes et al. 1999; Soulika, Khan et al. 2000
F-OpdChaWR	Synthetic cyclic peptide	C5aR antagonist. Effective in vivo including inhibiting C5a-meditated neutropenia in rats.	Paczkowski, Finch et al. 1999; Short, Wong et al. 1999

Table 1.3 Small molecule inhibitors of C.

A summary of the small molecules used to inhibit C with their site of action, uses and some key references.

1.5.4 Regulation of C by therapeutic antibodies

Several antibodies have been developed against C5 that inhibit C by preventing the cleavage of C5 (Wurzner, Schulze et al. 1991). Anti-C5 monoclonal antibodies have been tested in mouse models of immune complex nephritis and collagen-induced arthritis, and a rat model of myocardial I/R injury (Wang, Rollins et al. 1995; Wang, Hu et al. 1996; Vakeva, Agah et al. 1998). In all these models anti-C5 antibodies proved therapeutically effective. To enable testing of these reagents in humans, a humanised antibody was created and a single-chain Fv fragment (scFv) derived from the antibody (Thomas, Rollins et al. 1996). The scFv antibody significantly reduced C activation and therefore myocardial damage in patients on cardiopulmonary bypass (Fitch, Rollins et al. 1999). This reagent, termed Pexelizumab^{TM}, is now in advanced stages of development for acute conditions such as cardiopulmonary bypass, myocardial infarction and stroke, and chronic situations including rheumatoid arthritis, nephritis, dermatomyositis and pemphigus (Kaplan 2002; Whiss 2002). Phase II trials of Pexelizumab in acute myocardial infarction have been disappointing as it did not reduce infarct size or adverse clinical outcomes despite blocking C activity in vivo (Mahaffey, Granger et al. 2003). However, the observation that it reduced mortality in myocardial infarction patients and patients undergoing cardiopulmonary bypass means that it warrants further investigation as a viable anti-C therapeutic (Granger, Mahaffey et al. 2003; Shernan, Fitch et al. 2004).

1.5.5 Soluble complement regulators

Many of the fluid phase regulators are at such a high concentration in plasma that large quantities would have to be used to see any therapeutic affect. However, C1-inh purified from plasma has been used therapeutically in hereditary angioedema (HAE), which results from a C1-inh deficiency (Donaldson and Evans 1963). By inhibiting the activity of C1 and other proteolytic cascades, C1-inh resolved the oedema. Experimental models have also demonstrated the therapeutic benefit of C1-inh in the treatment of septic shock, acute pancreatitis, myocardial infarction and liver I/R injury (Buerke, Murohara et al. 1995; Niederau, Brinsa et al. 1995; Horstick, Heimann et al. 1997; Heijnen, Straatsburg et al. 2006). In murine cerebral I/R injury, C1-inh reduced ischaemic volume and neurological deficits by down-regulating the expression of adhesion molecules and pro-inflammatory cytokines

whilst up-regulating protective cytokines (De Simoni, Storini et al. 2003; Storini, Rossi et al. 2005). Recently, recombinant C1-inh has been produced and reached phase I clinical trials in HAE (van Doorn, Burggraaf et al. 2005).

1.5.6 Recombinant membrane regulators

1.5.6.1 Recombinant soluble CR1

The first and best-characterised soluble recombinant form of a membrane-associated regulator is soluble CR1 (sCR1). sCR1 is comprised of the extracellular domains of CR1 (30 SCRs) and inhibits the formation of the C3 and C5 convertases by acting as a decay accelerator and a cofactor for the factor I mediated cleavages of C3b and C4b (figure 1.8A). sCR1 has been shown to be protective against tissue injury in several animal models of acute and chronic inflammatory conditions including myasthenia gravis, multiple sclerosis, rheumatoid arthritis, glomerulonephritis and I/R injuries (Weisman, Bartow et al. 1990; Piddlesden, Storch et al. 1994; Couser, Johnson et al. 1995; Goodfellow, Williams et al. 1997; Eror, Stojadinovic et al. 1999; Goodfellow, Williams et al. 2000). sCR1 has been tested in human with ARDS and in patients on cardiopulmonary bypass where the agent was well tolerated and effectively inhibited C activation (Zimmerman, Dellinger et al. 2000; Rioux 2001). This reagent is still undergoing development, particularly for males on cardiopulmonary bypass as it provided more clinically effective in males than females.

Various modifications have been carried out in order to improve the therapeutic efficacy of sCR1 and its in vivo half-life. Removal of LHR-A (the N-terminal 7 SCRs) created a regulator specific for the AP of C (figure 1.8B). This reagent has been used to dissect the relative contributions of the CP and AP in I/R injuries and has the therapeutic advantage of still allowing C activation via the CP and immune complex handling (Murohara, Guo et al. 1995; Scesney, Makrides et al. 1996). sCR1 has also been expressed containing an albumin-binding domain from streptococcal protein G at the C-terminus. By enabling the attachment of the protein to albumin in the circulation this approach resulted in an increased in vivo half-life of sCR1 whilst not inhibiting function (Makrides, Nygren et al. 1996). Addition of Siayl Lewis X

(sLeX) tetrasaccharide groups to sCR1 created a bifunctional molecule that localised to sites of inflammation by binding P-, E- and L-selectins where it not only acted as a CReg but also inhibited selectin-dependent leukocyte adhesion (Mulligan, Warner et al. 1999; Rittershaus, Thomas et al. 1999). Perhaps the most promising sCR1 modification is APT070, a membrane targeted form of sCR1 containing only the 3 N-terminal SCRs (figure 1.8C). This molecule has been targeted to membranes by the addition of a lipid targeting peptide at the C-terminus that contains positively charged amino acids that bind the phospholipid membrane and a terminal myristoyl group that inserts into the membrane bilayer (Smith and Smith 2001). APT070 showed an enhanced C inhibitory activity compared to a non-membrane targeted form of the protein and is effective in inhibiting disease in models of arthritis and renal transplantation (Linton, Williams et al. 2000). Current studies are being carried out to assess the role of APT070 in rheumatoid arthritis and renal transplantation (Smith 2002). This lipid targeting strategy has been applied to other CReg including rat Crry where it increased C inhibitory activity of the molecule and rat CD59 where it created a reagent that suppressed disease in a model of rheumatoid arthritis (Fraser, Harris et al. 2002; Fraser, Harris et al. 2003).

1.5.6.2 Recombinant forms of other membrane regulators

CR1 is not the only membrane regulator to be expressed as a recombinant form and tested for therapeutic efficacy. Soluble forms of both DAF and MCP have been expressed and shown to inhibit C activation in vitro as well as in in vivo models (Moran, Beasley et al. 1992; Christiansen, Milland et al. 1996). However, the effectiveness of sDAF and sMCP is less than sCR1 presumably because sCR1 contains both decay accelerating activity and fI cofactor activity. Studies using sDAF and sMCP show that they are much more effective together than alone and therefore a hybrid molecule containing 4 SCRs of MCP and 4 SCRs of DAF called C-activation blocker-2 (CAB-2) was generated (Christiansen, Milland et al. 1996; Higgins, Ko et al. 1997). This molecule was therapeutically effective in a model of the Arthus reaction and Forssman shock in vivo and reduced myocardial injury in an ex vivo model of I/R injury (Kroshus, Salerno et al. 2000). More recently CAB-2 was shown to prolong graft survival in pig to primate cardiac xenografts (Salerno, Kulick et al. 2002).

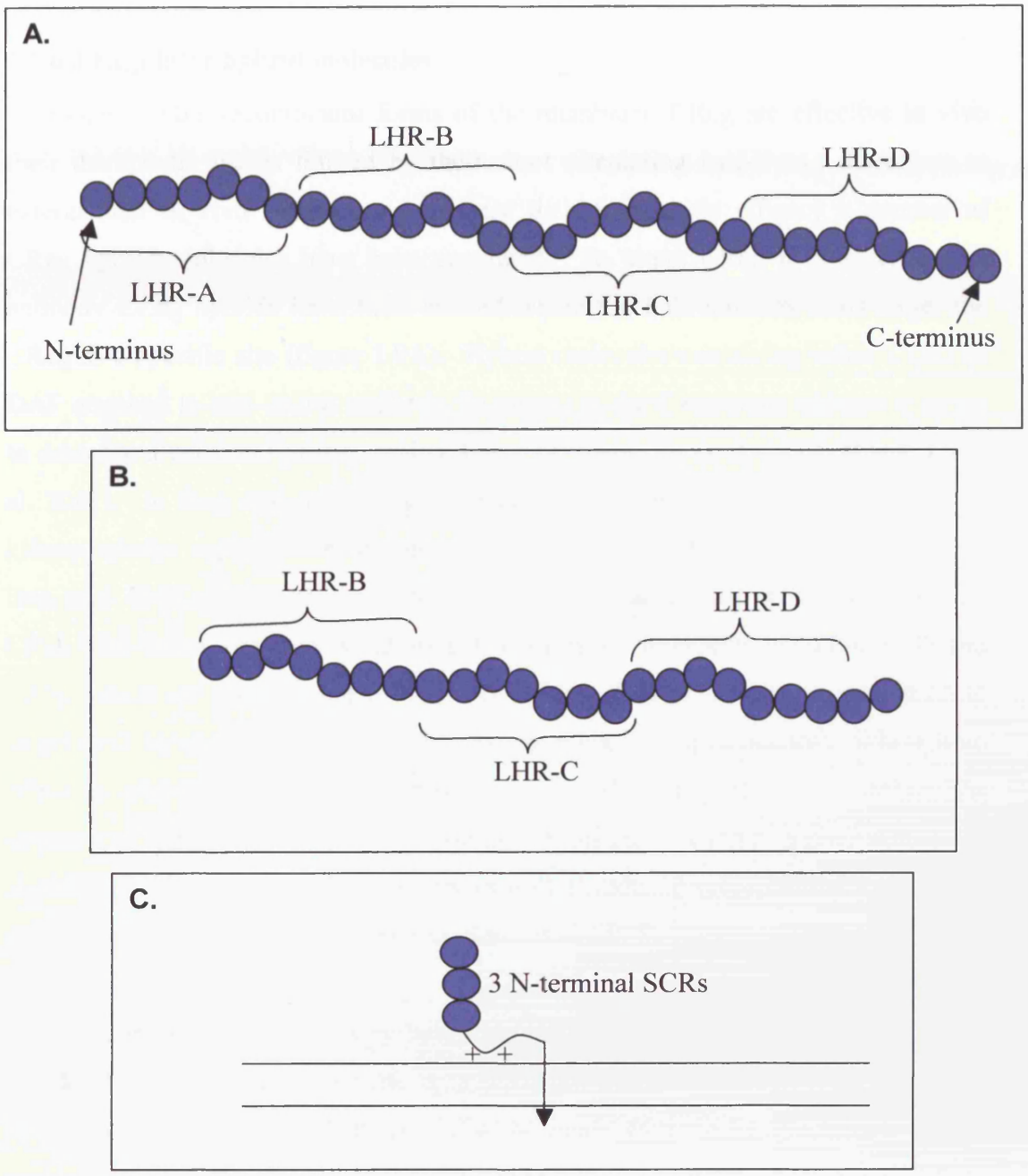


Figure 1.8 Soluble recombinant forms of CR1.

A diagram showing the recombinant forms of CR1 generated.

A. sCR1 consisting of the 30 amino-terminal SCRs of CR1.

B. Removal of the 7 N-terminal SCRs (LHR-A) of sCR1 generated an AP specific regulator.

C. Expression of the 3 N-terminal SCRs from LHR-A of sCR1 with a membrane targeting peptide tail consisting of positively charged amino acids (+) and a myristoyl group (arrow) generated APT070.

1.5.6.3 Regulator hybrid molecules

Although soluble recombinant forms of the membrane CReg are effective in vivo their therapeutic use is limited by their short circulating half-lives. Therefore to extend their in vivo half-life and increase their therapeutic efficacy a number of CReg hybrid molecules have been generated. To target CReg to a specific site, antibody CReg hybrids have been created where the Fab antibody arms target the CReg to a specific site (figure 1.9A). Hybrid molecules containing either CD59 or DAF attached to anti-dansyl antibody fragments at the C-terminus efficiently target to dansylated cells and protect against C lysis (Zhang, Yu et al. 1999; Zhang, Lu et al. 2001). In vivo this strategy has been used to target rat Crry and rat CD59 to kidney tubular epithelia where these reagents reduced tubulointestinal injury (He, Imai et al. 2005). CReg have also been targeted to sites of C activation by generating CReg-CR2 fusion proteins. CR2 binds C3 activation fragments including iC3b and C3dg, which are present at sites of C activation. This approach has been used to target DAF to sites of C activation in a mouse model of lupus nephritis although no effect on clinical severity was assessed in this study (Song, He et al. 2003). The targeting of Crry to sites of C activation in an intestinal I/R injury model using CR2 significantly reduced C-mediated injury as well as reduced susceptibility to infection due to systemic C inhibition (Atkinson, Song et al. 2005).

A second type of CReg-antibody hybrid molecule utilises the Fc of an antibody to confer a long circulating half-life upon attached molecules (Pugsley 2001). The CReg domains replace the Fab arms of an antibody with the Fc attaching to the C-terminus of the CReg (figure 1.9B). This approach was used to create a mouse Crry fusion protein containing the Fc of mouse IgG1 (Quigg, Kozono et al. 1998). This reagent had an increased in vivo half-life and was therapeutically effective in glomerulonephritis, intestinal I/R injury and traumatic brain injury (Quigg, Kozono et al. 1998; Rehrig, Fleming et al. 2001; Leinhase, Schmidt et al. 2006). Both DAF and CD59-Fc fusion proteins have been generated containing a human Fc (Harris, Williams et al. 2002). These reagents were effective in vitro at inhibiting C and the DAF-Fc was shown to inhibit disease in a rat model of antigen-induced arthritis. Such reagents are still undergoing development.

1.5.6.4 'Prodrug' regulators

DAF-Fc and CD59-Fc reagents were shown to have a reduction in function compared to sDAF and sCD59 respectively (Harris, Williams et al. 2002). The function was improved by insertion of 'spacing' domains between the CReg and the Fc and full function was totally restored when the Fc domains were removed with papain. Hence, the CReg function was reduced by steric restraint imparted by the Fc. This observation resulted in the design of a CReg 'prodrug'. In the proof-of-concept molecule the minimal functional domains of human DAF (3 N-terminal SCRs) were fused to the rigid Fc of human IgG2a in order to maximise the steric hindrance of the CReg (Harris, Hughes et al. 2003). A short enzyme site was inserted between the CReg and the Fc; the enzyme site was specific for either aggrecanase or matrix metalloproteinases (MMPs). These enzymes are at high levels in arthritic joints and cleave aggrecan, a major component of cartilage that is destroyed during arthritis (Little, Hughes et al. 2002). The resultant DAF-Fc 'prodrug' had negligible C regulatory activity but was cleaved by purified enzymes or enzymes present in synovial fluid to release an active CReg. Thus a reagent with little or no systemic activity was designed that could unleash powerful anti-C activity at target sites (figure 1.9C). This reagent has not been tested therapeutically in vivo.

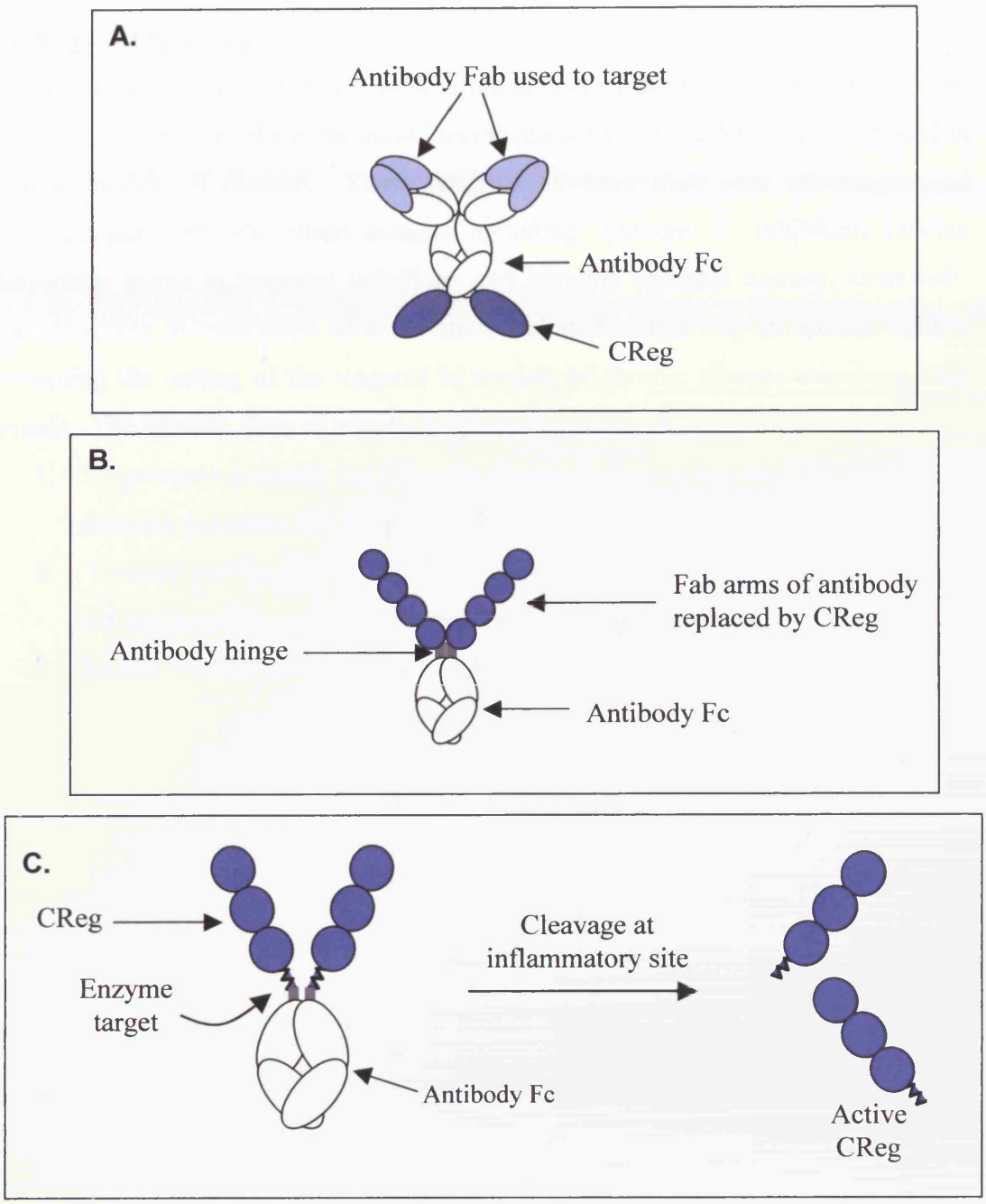


Figure 1.9 CReg hybrid molecules.

A. CReg-Fab hybrid molecules. The Fab arms of an antibody are used to target the CReg to specific sites.

B. CReg-Fc hybrid molecules. The Fc confers an extended half-life upon the attached CReg.

C. CReg 'prodrug', a sterically hindered CReg-Fc molecule that is cleaved at sites of inflammation by specific enzymes resulting in the release of an active regulator.

1.6 Aims of this thesis

The overall aim of this work was to design and develop better inhibitors of C for use in rats. To date many different anti-C therapeutics have been designed and tested in various models of disease. These reagents all have their own advantages and disadvantages with the disadvantages including systemic C inhibition leaving individuals prone to bacterial infections and immune complex disease, short half-lives resulting in continued administration and immunogenicity in animal models preventing the testing of the reagents in models of chronic disease over long time periods. The specific aims of this study where therefore:

1. To generate a totally rat CReg-Fc fusion protein using rat Crry and rat Fc domains to reduce the immunogenicity of such reagents in rats.
2. To carry out functional analysis of rat Crry to locate the functional domains and enable the generation of a rat Crry 'prodrug'.
3. To test both the active Crry-Fc and 'prodrug' in rat models of disease.

Chapter 2: Materials and methods.

Unless otherwise stated all chemicals were from Sigma Chemical Company or Fisher Scientific UK Ltd.

2.1 Molecular Biology

The cloning of recombinant rat Crry and the Fc of rat IgG2a into expression vectors.

All RNA manipulations were carried out using Diethylpyrocarbonate (DepC) treated autoclaved tips and vials. All DNA manipulations were carried out using autoclaved tips, vials and solutions. All polymerase chain reactions (PCRs) were carried out using a Dyad DNA engine (MJ Research Ltd).

2.1.1 Buffers and Broths

Buffer	Contents
1x TAE:	40mM Tris, 40mM acetic acid, 1mM EDTA pH 7.2
Agarose gel loading buffer:	20% glycerol (v/v) in 10x TAE with bromophenol blue.
Lauria-Bertani (LB) broth:	1% tryptone (w/v), 1% NaCl (w/v), 0.5% (w/v) yeast extract in water
LB Amp broth:	100ug/ml ampillicin in LB broth cooled to 50°C
LB Amp plates:	As LB amp broth except the broth contained 1.5% agar
SOC:	2% tryptone (w/v), 0.5% yeast extract (w/v), 0.05% NaCl (w/v) and 2.5mM KCl at pH 7. After autoclaving 10mM $MgCl_2$ and 20mM glucose was added and the solution sterile filtered using a 0.22μM filter

2.1.2 RNA isolation

Rat oligodendroglioma cells (33B; ECACC) were harvested from a T80 tissue culture flask, resuspended in 1ml UltraSpec™ RNA (Biotecx) and transferred to a

DepC treated vial. Following incubation on ice for 5 minutes, 0.3ml chloroform was added. Following vortexing to mix, the vial was incubated on ice for 5 minutes. Following centrifugation at 12,000g for 15 minutes to separate the DNA and protein phase from the RNA phase, the aqueous phase (RNA) was transferred to a fresh tube and an equal volume of isopropanol was added to precipitate the RNA. The tube was incubated on ice for 10 minutes and then centrifuged at 12,000g for 10 minutes. The supernatant was removed and the pellet was washed twice in 70% (v/v) ethanol diluted using DepC treated water. The washes involved vortexing the pellet to resuspend and centrifugation at 7500g for 5 minutes. The pellet was briefly dried on a heated block and dissolved in 100µl DepC treated water. The concentration of the RNA was established using its absorbance at 260nm.

2.1.3 Reverse transcriptase-PCR (RT-PCR)

cDNA was prepared from the RNA isolated from the 33B cells.
The reaction consisted of:
1.5µg RNA,
2µl pdN6 primers (Invitrogen Life Technologies),
1.5µl 25mM dNTPs (Bioline),
6µl 5x 1st strand buffer (Invitrogen Life Technologies),
1.5µl RNAsin (Promega)
3µl DTT (Invitrogen Life Technologies).
DepC treated dH_2O to take the reaction volume to 30µl.

After incubation at 65°C for 10 minutes followed by 10 minutes on ice, 1.5µl Superscript II RNase H Reverse Transcriptase (Invitrogen Life Technologies) was added. The reaction mix was incubated at 25°C for 10 minutes to allow the primers to anneal, 42°C for 2.5 hours to extend the DNA and 99°C for 2 minutes to denature the enzyme. The cDNA was stored at -20°C.

2.1.4 PCR

A number of different PCRs were carried out to clone the different proteins for this project. PCRs were carried out using either Taq (Bioline) or Vent proofreading

(New England Biolabs) polymerase. For the cloning of DNA encoding 5SCRs of rat Crry a two step PCR reaction was used, this DNA was then used as a template to clone the DNA for the other Crry proteins. Details of which method was used to clone each construct as well as the source of DNA used is given in table 2.1. The primers used for amplification, screening and sequencing of DNA were obtained from Invitrogen Life Technologies. The primers were reconstituted using dH_2O to achieve a concentration of 100pmol/µl and then diluted to 10pmol/µl. Table 2.2 describes the primers used in each PCR.

Product/ Purpose	Primers	PCR reaction	cDNA source
Fc of rat IgG2a	IgG.1 and IgG.2	Vent PCR	Fc within a pDR2ΔEF1α plasmid that had been previously made in the lab.
5SCR (for Fc fusion protein)	Crry1, Crry2, CrryA and CrryB	Two stage PCR to eliminate a BamH1 site	Rat oligodendroglioma cells.
4SCR (for Fc fusion protein)	Crry1 and 4SCRFc	Vent PCR	Plasmid containing cDNA encoding 5SCRs of Crry
3SCR (for Fc fusion protein)	Crry1 and 3SCRFc	Vent PCR	Plasmid containing cDNA encoding 5SCRs of Crry
4SCR (non-Fc fusion protein)	Crry1 and SCR4	Vent PCR	Plasmid containing cDNA encoding 5SCRs of Crry
3SCR (non-Fc fusion protein)	Crry1 and SCR3	Vent PCR	Plasmid containing cDNA encoding 5SCRs of Crry
Bacterial colony screening	EF1α5', EF1α3' and IgG3'	Taq PCR	Bacterial lysate used as templates for PCR.

Table 2.1 The primers, PCR reaction and sources of cDNA used to clone the recombinant rat Crry proteins and the Fc of rat IgG2a.

Primer name	Primer sequence	Modifications/ restriction site
Crry1	5'CGCTCTAGAGAGGACCGCTGTGAGGG3'	Xba-1
CrryA	3'GTAGGCCAAACCCAGGAGATAG5'	T→C mutation to remove an internal BamH1 site
CrryB	5'CATCCGGTTTGGGTCCTCTATC3'	A→G mutation to remove BamH1 site
Crry2	3'GGACACACATTGTTCACTATCCTAGGCGC5'	BamH1
4SCRFc	3'TCGACGAAGTTCCACTTTAGGCCTAGGGCG5'	BamH1
3SCRFc	3'GAGGAGTCAGTAACTTGAGCCTAGGGCG5'	BamH1
SCR4	3'GGTTCGACGAAGTTCCACTTTATCCCTAGGGAG5'	BamH1 and stop codon
SCR3	3'GAGGAGTCACGTAACTTGAGATCCCTAGGGCG5'	BamH1 and stop codon
IgG.1	5'GCCGGATCCGCCAGCAGCACCAAGTGG3'	BamH1
IgG.2	3'GGATTTCCTACGGTTGTGGATATCATG5'	EcoRV
EF1α5'	5'CAAGCCTCAGACAGTGGTTC3'	.
EF1α3'	3'ATGTCTGGATCGGTGCGGGGC5'	
IgG 3'	3'GGGAAGATGAAGACAGATG5'	

Table 2.2 Primer sequences used in the cloning of the recombinant forms of rat Crry and the Fc of IgG2a.

The primer name is shown along side the sequence with any modifications to the cDNA or introduced restriction sites shown in the right hand column. Restriction sites are shown in the primer sequence in blue, the base mutation to eliminate an internal BamH1 site in grey and any introduced stop codons in red.

2.1.4.1 Taq PCR

Where Taq polymerase (Bioline) was used as the enzyme each PCR contained the following reagents:

100ng template DNA,

10pmol of forward primer,

10pmol of reverse primer,

2.5µl 10x NH_4 buffer (Bioline; 160mM $(NH_4)_2SO_4$, 670mM Tris-HCl, 0.1% (v/v) Tween-20), 1µl 50mM $MgCl_2$ (Bioline),

5µl 1mM dNTPs (Bioline),

dH_2O to take the reaction volume to 24.5µl.

The reaction was heated at 94°C for 3 minutes to denature the DNA and then 0.5µl Taq polymerase (Bioline, 5U/µl) was added.

Each PCR consisted of the 30 cycles of:

Denaturation:	95°C for 30 seconds.
Annealing:	58°C for 30 seconds (annealing temperature is dependant upon the specific primer used).
Extension:	72°C for 2 minutes

Followed by a final extension at 72°C for 15 minutes

2.1.4.2 Vent PCR

In the reactions where Vent polymerase (New England Biolabs) was used the reaction consisted of:

100ng template DNA,

15pmol of forward primer,

15pmol of reverse primer,

2.5µl 10x ThermoPol reaction buffer (New England Biolabs; 100mM KCl, 100mM $(NH_4)_2SO_4$, 200mM Tris-HCl pH 8.8, 20mM $MgSO_4$, 0.1% (v/v) Triton X-100),

7.5µl 1mM dNTPs (Bioline),

dH_2O to take the reaction volume to 24.5µl.

The reaction was heated to 94°C for 3 minutes prior to the addition of 0.5µl Vent DNA polymerase (New England Biolabs, 2U/µl) to denature the DNA.

Each PCR consisted of the same steps as the Taq polymerase reaction detailed above (section 2.1.4.1).

2.1.4.3 Two step PCR

To clone the DNA encoding the 5SCRs of rat Crry a two step PCR reaction was carried out to remove an internal BamH1 restriction site that would interfere later with the cloning procedure. The cloning strategy is demonstrated in figure 2.1. In step 1 the DNA was amplified in two sections following method 2.1.4.1. For section 1 primers Crry1 and CrryA (table 2.2) were used to amplify a 723 base pair fragment of DNA. Primer CrryA contained an A →G base mutation at residue 248 conserving the amino acid coded as a glycine but removing the BamH1 site. Primer Crry1 incorporated an Xba1 restriction site at the 5' end. For section 2 primers Crry2 and CrryB (table 2.2) were used to amplify a 363 base pair fragment of Crry. The CrryB primer was complementary to CrryA and also included the mutation at residue 248. Primer Crry2 incorporated a BamH1 restriction site.

In step 2, the two DNA sections from step 1 were allowed to anneal through their complementary sequence and were used as a template to amplify DNA encoding the entire 5SCRs of Crry. In this reaction 200ng of each of the products from step 1 were mixed together along with 5µl 10x ThermoPol reaction buffer (New England Biolabs), 15µl 1mM dNTPs (Bioline) and dH_2O to bring the reaction volume to 43.5µl. The reaction was heated to 94°C for 4 minutes and 0.5µl Vent DNA polymerase (New England Biolabs) was added.

Five cycles of the following steps were then carried out to enable the fragments to come together and the full length DNA be created:

Denaturation:	94°C for 30 seconds
Annealing:	58°C for 60 seconds
Extension:	72°C for 3 minutes

3µl of the Crry1 primer and Crry2 primer (both at 10pmol/µl) were then added whilst the reaction was held at 94°C. This enabled the cDNA encoding the 5SCRs of Crry to be amplified using 30 cycles of the following scheme:

Denaturation:	94°C for 20 seconds
Annealing:	58°C for 45 seconds
Extension:	72°C for 2 minutes

2.1.5 Agarose gel electrophoresis

DNA fragments and PCR products were visualised by agarose gel electrophoresis. 1% (w/v) agarose (Invitrogen Life Technologies) in 1x TAE buffer was melted in a microwave and allowed to cool before the addition of 100ng/ml ethidium bromide (final concentration). The agarose was poured into a gel casting tray (Thermo Electron Corporation), an appropriate size comb was inserted and the agarose was allowed to set. The comb was removed and the gel immersed in 1x TAE in an electrophoresis tank (Thermo electron corporation). DNA samples containing 10% (v/v) agarose gel loading dye were loaded along with DNA standard size markers. Gels were run at 60V for 1-2 hours depending on the resolution required. The DNA within the gel was visualised using a UV transilluminator (Bio-Rad, Chemi-Doc), the images were captured on a digital camera using the Bio-Rad Gel Documentation system. To quantify DNA using an agarose gel, the gels were prepared as above except that Smart Ladder DNA markers (Eurogentec) were loaded.

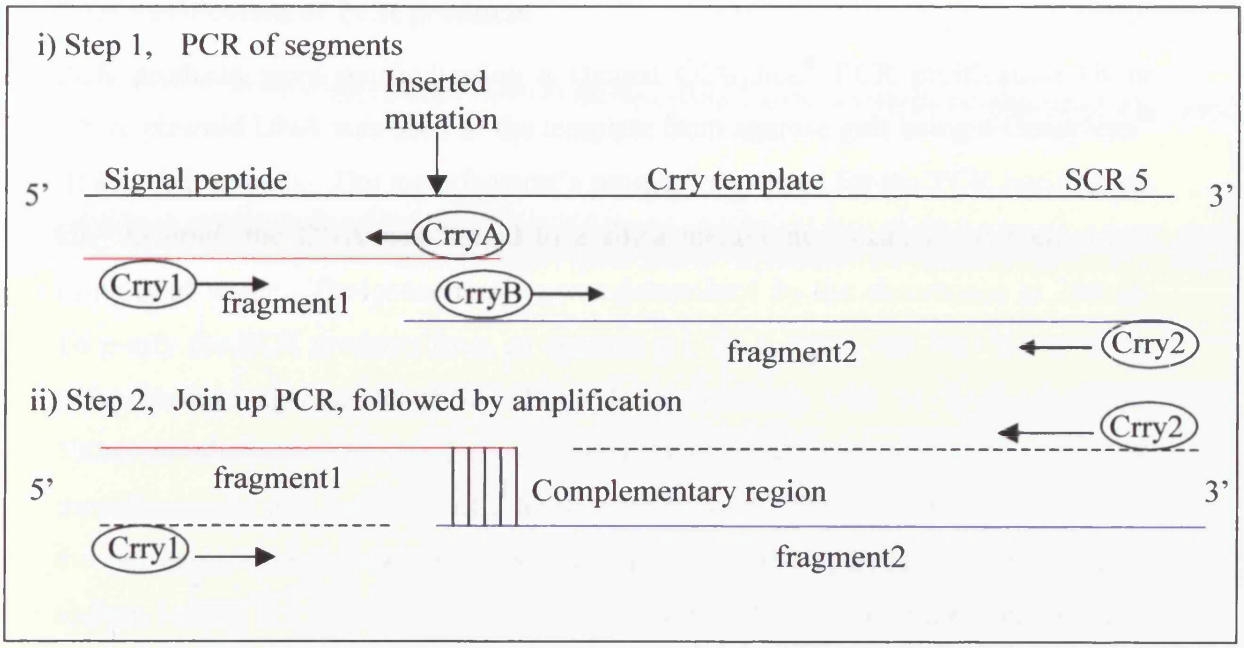


Figure 2.1 Two step PCR to generated DNA encoding 5SCRs of Crry.

In step 1 of the PCR, two fragments of DNA were generated. Primers Crry1 and CrryA were used to amplify fragment 1 whilst primers Crry B and Crry2 were used to amplify fragment 2. In step 2 of the PCR, DNA fragments 1 and 2 were allowed to anneal to each other through the complementary region. Full length cDNA encoding the 5SCRs of Crry was generated in a PCR reaction using primers Crry1 and Crry2

2.1.6 Purification of PCR products

PCR products were purified using a Qiagen QIAquick® PCR purification kit or where plasmid DNA was used as the template from agarose gels using a Geneclean® III kit (Q-Biogene). The manufacturer's protocol was used for the PCR purification kit. In brief, the DNA was bound to a silica membrane, washed and then eluted using 50µl water. The concentration was determined by the absorbance at 260nm. To purify the PCR products from an agarose gel, the product was loaded into a 3% (w/v) Nusieve agarose gel (FMC Bioproducts) made up with 1x TAE containing 100ng/ml ethidium bromide. Following electrophoresis the gel was placed on a UV transilluminator and the bands of interest were excised. The DNA was purified from the gel using the manufacturer's protocol in which the agarose was dissolved in sodium iodide, the DNA bound to a silica matrix, washed and eluted into 10µl of distilled water. The concentration was determined as above.

2.1.7 Restriction digests

The PCR products and plasmid were digested using the relevant restriction enzymes. All enzymes were from G E Healthcare (previously Amersham Biosciences). In each digest 5µl of the relevant enzyme was used with 7µl of the corresponding buffer, 5µg DNA (PCR product or plasmid) and the reaction volume was taken to 70µl with distilled water. For BamH1 Buffer K was used, for EcoRV Buffer H and for Xba1 Buffer M along with 7µl 0.1% (w/v) BSA. BamH1 digests were incubated at 30°C while EcoRV and Xba1 digests were incubated at 37°C. All digests were incubated for 2 hours. Following each digest the DNA was purified using a QIAquick® PCR purification kit and eluted using 60µl distilled water. To quantify the DNA yield, the products were loaded onto an agarose gel and run against Smart Ladder markers as described previously.

2.1.8 Ligation

The digested PCR products were ligated into the pDR2ΔEF1α plasmid or the pDR2-Fc plasmid using T4 DNA ligase (Promega). The ligation reaction was incubated at 16°C overnight and consisted of

100ng plasmid and a 3-fold molar excess of insert calculated by:

3 x (100 x size (kb) of insert)/ size (kb) of vector

along with 1μl 10x ligase buffer (Promega, 300mM Tris-HCl pH 7.8, 100mM $MgCl_2$, 100mM DTT and 10mM ATP), 1μl T4 DNA ligase and dH_2O to take the reaction volume to 10μl.

Ligations were stored at –20°C until required. The ligated DNA was transformed into bacteria directly from the ligation mix.

2.1.9 Transformation

Chemically competent DH5α (Invitrogen Life Technologies) were transformed with the ligated DNA by heat shock. Ligated DNA (2μl) was added to 50μl of chemically competent DH5α on ice and mixed. The bacteria were left on ice for 20 minutes and then incubated at 42°C for 45 seconds. Following this they were incubated on ice for a further 2 minutes and 950μl SOC medium was added. The bacteria were allowed to recover by incubating at 37°C for 1.5 hours. The bacteria (either 100 or 200μl) were then aliquoted onto LB amp plates and incubated overnight at 37°C.

2.1.10 Bacterial colony screening

Bacterial colonies were screened by PCR to detect successful ligation of DNA into the plasmid. The primers used in the screening are described in table 2.2. Primers EF1α5' and EF1α3' bound to the plasmid either side of the inserted DNA and were used to screen the pDR2ΔEF1α plasmid for ligation of DNA encoding the Fc or the 3 or 4 SCRs of rat Crry for the non-fusion proteins. Primers EF1α5' and IgG3' were used to screen the pDR2-Fc plasmid for the insertion of DNA encoding the 5, 4 or 3SCRs of rat Crry. Single colonies were picked with a sterile pipette tip and placed in 20μl of dH_2O in a 0.5ml tube. After gentle pipetting to mix, 1μl of the mix was spotted onto an LB amp plate. The remaining 19μl was boiled for 10 minutes on a

heating block to lyse the bacteria. The tubes were then incubated on ice for 5 minutes and the lysed bacteria pelleted by centrifugation at 12,000 g for 5 minutes. 2µl of the bacterial lysate was used as the template in a Taq PCR reaction as described in section 2.1.4.1. Following PCR, 10µl of each reaction was analysed by agarose gel electrophoresis and clones that contained inserts of the correct size were identified.

2.1.11 Plasmid isolation

Following the PCR screen, a positive colony was picked from the LB amp plate using a sterile loop. The colony was transferred to 10ml LB amp broth and incubated overnight at 37°C with shaking. The bacteria were pelleted by centrifugation at 1300 g for 10 minutes and the supernatant discarded. The DNA was isolated from the bacteria using the QIAprep® Spin Miniprep kit (Qiagen) according to the manufacturer's protocol using a microcentrifuge. In brief, the bacteria were lysed and the DNA bound to a silica matrix, following a wash, the DNA was eluted with 70µl dH_2O and stored at –20°C.

2.1.12 DNA sequencing

The cDNA constructs generated were sequenced using the Big Dye™ Terminator Cycle Sequencing Kit (Perkin Elmer). The sequencing reaction contained:
300ng of template DNA,
2.5pmol of either the forward or reverse primer,
1µl Big Dye Premix containing Ampli Taq DNA polymerase,
5µl Better Buffer (Web scientific) and
dH_2O to make the reaction volume up to 15µl.

The primers that were used for screening the bacterial colonies were also used in the sequencing reaction. For instance EF1α5' and EF1α3' were used to screen for insertion of DNA encoding the Fc so in the 5' sequencing reaction, EF1α5' was used while EF1α3' was used in the 3' sequencing reaction.

Reactions consisted of 25 cycles of the following steps:

Denaturation:	96°C for 30 seconds,
Annealing:	50°C for 15 seconds
Extension:	60°C for 4 minutes.

Following the reaction, 5µl dH_2O was added to the reaction tube and the DNA was precipitated by addition of 2µl 3M Sodium acetate pH 5.2 to the reaction tube along with 50µl ethanol. After mixing and incubation on ice for 20 minutes, the DNA was pelleted by centrifugation at 12,000 g for 25 minutes. The pellet was washed with 200µl 70% (v/v) ethanol. Sequencing was carried out in house (Central Biotechnology Services) using an ABI model 377 DNA sequencer (Perkin Elmer).

2.1.13 Expression vectors

The expression vector used within this project, pDR2ΔEF1α, was a gift from Dr I. Anegon (INSERM, U437, Nantes, France) (Charreau, Cassard et al. 1994). This vector is a eukaryotic expression vector that contains a multiple cloning site next to the Elongation Factor 1 alpha (EF1α) promoter, which drives very high levels of expression in eukaryotic cells (Mizushima and Nagata 1990). This vector also includes an ampillicin and a hygromycin resistance gene allowing for selection in both bacteria and eukaryotic cells. When the DNA encoding the Fc of rat IgG2a was ligated into this vector it was referred to as pDR2-Fc.

2.2 Eukaryotic expression and cell maintenance

The transfection of CHO cells with the expression plasmids and the maintenance of CHO and hybridoma lines.

All tissue culture was carried out under sterile conditions in a flow cabinet using sterile flasks, tubes and other equipment. All tissue culture reagents and equipment were obtained from Invitrogen Life Technologies unless otherwise stated. All flasks were incubated in a humid atmosphere at 37°C, 5% CO_2 unless otherwise stated.

2.2.1 Tissue culture cells and media

Cells	Suppliers/ source
Chinese Hamster Ovary (CHO) cells:	Obtained from ECACC.
Hybridomas:	TLD1C11 hybridoma cell line provided by Dr W. F. Hickey (Dartmouth College. Hanover, NH, USA).

RPMI-1640 was used as cell culture medium and was supplemented with 50U/ml penicillin/streptomycin, 1μg/ml amphotericin B, 2mM L-glutamine and 1mM sodium pyruvate. Depending upon the cell type being cultured different amounts of foetal bovine serum (FBS) were added. Transfected CHO cells were cultured in the presence of Hygromycin B. Details of the FBS content and Hygromycin B content of the cell culture media is given below:

Media	Use	FBS and Hygromycin B content
Untransfected CHO media	Culture of untransfected CHO	5% (v/v) FBS
Selection media	Selection of transfected CHO cells	5% (v/v) FBS, 400μg/ml Hygromycin B.
Transfected cell media	Culture of transfected CHO	5% (v/v) FBS, 100μg/ml Hygromycin B.
Bulk CHO media	Culture of CL AD1000 flasks, section 2.2.4.2	0% (v/v) FBS, 100μg/ml Hygromycin B.
Hybridoma media	Culture of hybridoma lines	10% (v/v) FBS.
Bulk hybridoma media	Culture of CL1000 flasks, section 2.2.5.2	No supplements
High density hybridoma media	Culture of CL1000 flasks, section 2.2.5.2	15% (v/v) Ultra low bovine immunoglobulin FBS
Freezing media	Freeze cells	10% (v/v) DMSO in foetal bovine serum

2.2.2 Transfection of CHO

CHO cells were transfected using LipofectAMINE™ PLUS reagent in T25 tissue culture flasks. Transfection was carried out according to manufacturer's instructions; 2μg plasmid DNA was pre-complexed with PLUS reagent in serum free media (opti-MEM® with GlutaMAX™) at room temperature for 15 minutes. The pre-complex was combined with the LipofectAMINE™ reagent diluted in opti-MEM® and incubated for a further 15 minutes at room temperature. The cells were washed twice in opti-MEM® and the transfection mix was applied to the cells. The cells were incubated with the transfection mix in to 2ml of opti-MEM® for 5 hours at 37°C, the mix was then replaced with untransfected CHO medium. The cells were placed in selection medium 24 hours later to select for transfected cells. A flask of mock-transfected cells (had undergone transfection conditions without plasmid DNA) were

also placed under selection. When these non-transfected cells had all died, as determined by microscopy, the CHO cells were maintained in transfected cell medium. The supernatant from the transfected cells was retained for Western blot analysis to detect for expression of the protein.

2.2.3 Cloning of CHO cells

In order to isolate high expressing transfected CHO cells, the cells were cloned out by limiting dilution in 96-well plates. Cells were harvested from a tissue culture flask and resuspended to 1000 cells/ml medium. 100µl of medium was placed in the wells of a 96 well plate. To the first column 100µl of cells was added, the cells were diluted in an 8-step 1:2 serial dilution across the plate to achieve dilution of approximately 1 cell per well. After two weeks in culture, the supernatant from the wells containing approximately 1 cell per well was screened by ELISA (section 2.4.9). Cells from the well where the supernatant had the highest level of protein were taken on and expanded.

2.2.4 Maintenance of CHO cells

Prior to transfection, an aliquot of CHO cells was taken from frozen stocks, rapidly thawed and resuspended in pre-warmed (37°C) untransfected CHO medium. The cells were centrifuged at 300g for 5 minutes, resuspended in 10mls of untransfected CHO media and transferred to a tissue culture flask. To sub-clone adherent cells, the cells were washed twice with sterile saline and lifted from the flask using 1ml Trypsin-EDTA. Once the cells had detached from the flask they were diluted in fresh medium and transferred to the desired number of flasks. To freeze down CHO cells, a T80 flask was trypsinised as before, centrifuged at 300g for 5 minutes and resuspended into 1ml freezing medium. The cells were placed in a Cyrovial (Greiner) and frozen in a Cyro 1° Freezing Container (Nalgene) to -80°C. The container ensured that the temperature would decrease by 1° per minute and in conjunction with the DMSO within the freezing medium prevented the formation of ice crystals within the cells. After 24 hours at -80°C, the cells were transferred to liquid N_2 for long term storage. After transfection a number of different cell culture methods were used to maintain the CHO cells.

2.2.4.1 T80 flasks

Transfected CHO cells were maintained in T80 flasks (Nunc) using transfected cell medium. The cells were sub-cloned and replenished regularly in order to maintain cell viability. The supernatant harvested from the cells was kept in sterile bottles for purification of the Crry proteins.

2.2.4.2 CELLine flasks

Transfected cells were also maintained in CL AD1000 flasks (Integra Biosciences). These are cell culture flasks which consist of two compartments separated by a 10kDa semi-permeable membrane (figure 2.2A). In one compartment, containing a polyethylene teraphthalate (PET) matrix, the cells were maintained in transfected cell medium whilst the other compartment contained bulk CHO medium (described section 2.2.1). The cells, cellular products and proteins within the bovine serum necessary for cellular growth were retained in the cell compartment by the membrane while nutrients and metabolic waste products diffused through the membrane. To inoculate the flasks, the membrane was wetted by placing 25ml bulk media in the medium compartment while the cell compartment was inoculated with $2.5x10^7$ cells in 15ml transfected cell medium. A further 975ml of bulk CHO medium was added to the medium compartment. Following inoculation the media in both compartments was changed on day 10 and then every seven days. The medium harvested from the cell compartment was centrifuged at 900g for 5 minutes and the supernatant retained for purification of the Crry proteins. The media from the medium compartment was discarded.

2.2.4.3 Fermenter

The CHO cells were also maintained in a BioStat B Plus fermenter (B Braun Biotech). This consisted of a stirred glass vessel in which the cells were incubated and a control unit which adjusted temperature, pH, pO2 levels and stirring speed within the glass vessel (figure 2.2B). Cytodex 1 beads (GE healthcare) were swollen in PBS as per manufacturer's instruction; 2g of beads were used per litre of culture.

The beads were washed in PBS and autoclaved. The beads were washed in transfected cell medium and the relevant number of cells (1.5 - 2 x 10^8 cells/litre of culture) were added. The cells were incubated with the beads to allow their attachment to the beads. There were two ways in which the cells were attached to the beads. In method one, the beads were autoclaved within the culture vessel, the transfected cell medium was added and the vessel stabilised at the culture conditions. The cells were inoculated into the culture vessel and the culture stirred for 5 minutes every hour for the first 3 hours after which full stirring was commenced. In method two, the beads were prepared and autoclaved in a separate siliconised 500ml glass bottle. The beads were washed into 200ml transfected cell medium within this bottle and the cells added. The cells were incubated with the beads in the bottle overnight at 37°C in 5% CO_2. The beads with the cells attached were then added to the culture vessel containing 800ml transfected cell medium and the fermentation commenced. With both methods the pH and pO_2 meters had previously been calibrated and the vessel autoclaved. The fermenter was stirred between 50-60RPM at 37°C, pH 7.4. The pH was maintained using CO_2 or 0.1M sodium carbonate (autoclaved), sterile filtered air was pumped into the system.

To assess cell number and viability a 10ml sample was removed from the culture vessel, centrifuged at 300g for 5 minutes, the supernatant was discarded and the bead/cell pellet was washed twice with sterile saline. The cells were trypsinised from the beads using Trypsin-EDTA and separated from the beads using a 100μm cell sieve (Nuncon). The cells were pelleted by centrifugation at 300g for 5 minutes and resuspended in 1ml transfected cell medium. The total number of cells were counted using a haemocytometer. To establish viability, cells were mixed 1:1 with Trypan blue and the live cells (not stained) were counted. When cell viability or number dropped either the fermentation was stopped or a medium change was carried out. To change the medium the stirrer was switched off to allow the cells to settle, the system pumps were then used to remove expired medium and add fresh medium.

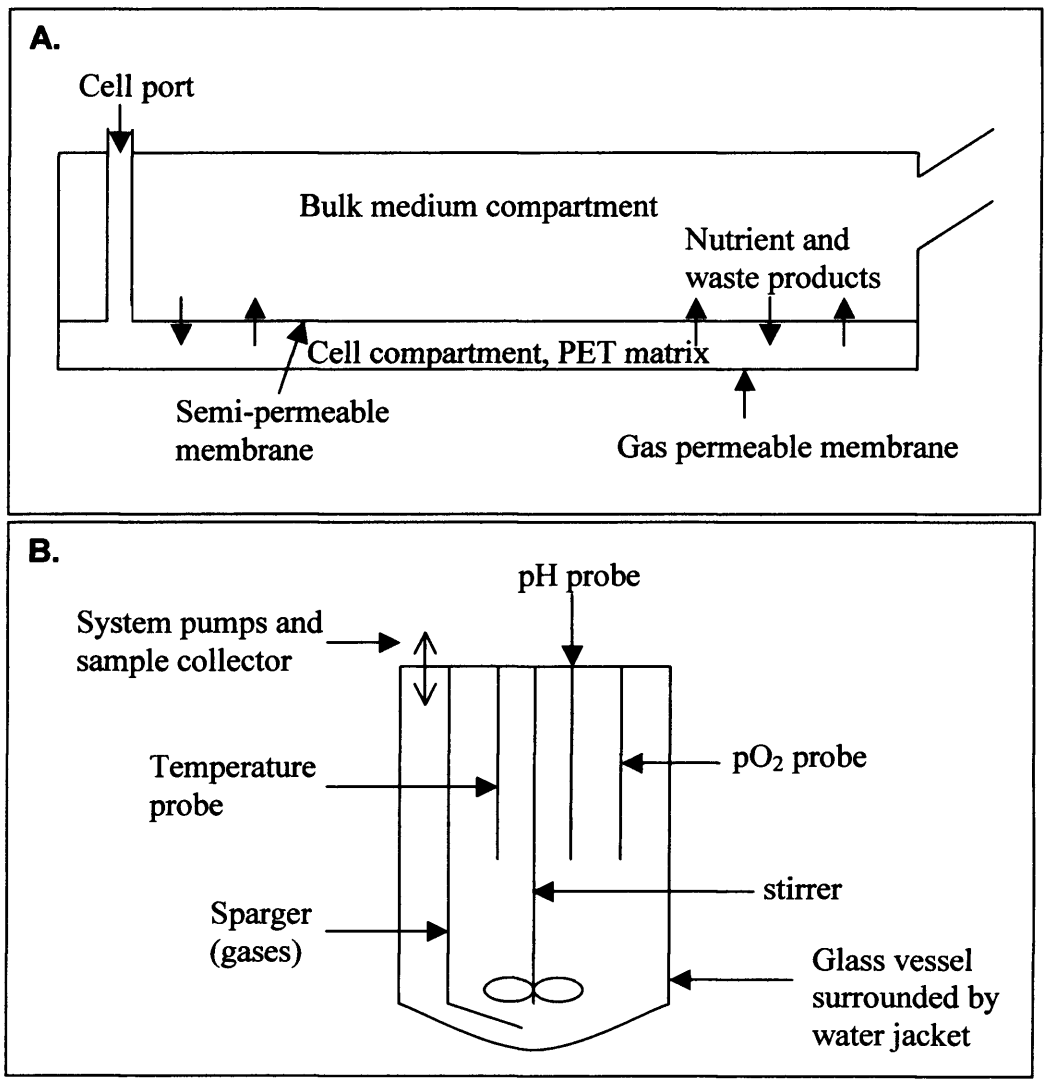


Figure 2.2 Diagrams of the CL AD1000 flask and fermenter system.

A. The CL AD1000 flask. Cells are placed into the cell compartment containing the PET matrix through the cell port. Nutrients pass into the cell compartment whilst waste products pass out into the bulk medium compartment through the semi-permeable membrane, the cut off of the membrane retains cellular products within the cell compartment. Gas enters the cell compartment through the gas permeable membrane while the medium was gassed via the lid.

B. The fermenter culture vessel. The vessel is surrounded by a water jacket and contains a stirrer, a temperature probe, a pH probe and a pO_2 probe. Gas enters the culture in fine bubbles through the sparger. The lid of the vessel contains inlets and outlets which can be attached to the system pumps to change media and add acids or bases to maintain pH.

2.2.5 Maintenance of hybridoma lines

Cyrovials were removed from liquid nitrogen storage and thawed as CHO cells (section 2.2.4) except the cells were washed into hybridoma medium and when bought up from storage they were incubated in T25 flasks which had been pre-seeded with mouse peritoneal macrophages. To obtain high expressing cells, the hybridoma cells were cloned out into 96 well plates pre-seeded with macrophages. The cloning was identical to section 2.2.3 other than hybridoma medium was used. Expression level was assessed using an ELISA (section 2.4.9).

2.2.5.1 T80 flasks

Hybridoma cells were maintained in T80 tissue culture flasks (Nunc) using hybridoma medium. As hybridoma cells are only weakly adherent they did not require trypsinisation for sub-cloning but were lifted by gentle knocking of the flask. The cells were then split between the required number of flasks. The cells were sub-cloned and the medium replenished regularly to keep cells healthy and to ensure secretion of antibody. The media harvested from the cells were kept in sterile bottles for purification of the antibody.

2.2.4.2 CELLine flasks

Hybridoma cells were maintained in CL1000 flasks (Integra Biosciences). These are similar to CL AD1000 flasks used to culture CHO cells except that they do not contain a PET matrix in the cell compartment. The membrane was wetted by placing 25ml bulk hybridoma medium in the medium compartment. The cell compartment of the flask was inoculated with $2.5x10^7$ cells in 15ml high density hybridoma medium and a further 975ml of bulk hybridoma medium was added to the medium compartment. The media of both compartments was changed on day 10 following inoculation and then every seven days. When harvesting from the cell compartment, cells were reseeded by mixing 3ml of the harvest with 12ml fresh high density hybridoma medium before re-inoculation. The remaining harvest from the cell compartment was centrifuged at 900g for 5 minutes and the supernatant was retained

for antibody purification. The bulk media from the medium compartment was discarded. The cell number was established by diluting the cell harvest 1 in 100 in fresh medium and then counting the cells using a haemocytometer. The viability was established by diluting the cell harvest in the same manner and then mixing the cells 1:1 with Trypan blue. The live cells (not stained) were counted.

2.2.5.3 Tecnomouse

A Tecnomouse (Integra Biosciences) hollow fibre culture system was used to culture hybridoma cells. The tecnomouse comprises a culture cassette consisting of a cultivation chamber for the cells and hollow fibres through which medium is pumped. The membrane of the hollow fibres allows nutrients to diffuse into the cultivation chamber and metabolic waste products to diffuse out of the chamber into the hollow fibres (figure 2.3). Cellular protein products are retained within the cultivation chamber. A cassette was prepared by attaching it to the control unit and washing the extracellular (EC) space with 1500ml non-supplemented RPMI-1640 overnight using the system pump. The intracellular space (IC) was flushed with 50ml high density hybridoma medium. Hybridoma cells ($6x10^8$) were harvested from tissue culture flasks, washed and resuspended in 10ml high density hybridoma medium. The IC space was inoculated with these cells and 2000ml bulk hybridoma medium was circulated in the EC space using the system pump. The cells were incubated at 37°C using a thermohood for the Tecnomouse and in 5% CO_2 to maintain pH. The media was replaced in both compartments every seven days. In the harvest, 3ml from the IC compartment was added to 12ml fresh high density hybridoma medium and replaced in the IC space to maintain cell numbers. The remaining IC harvest was centrifuged at 900g for 5 minutes and the supernatant retained for antibody purification. The medium from the EC space was discarded. The cell number and viability was established using the same method as for the CELLine flask (section 2.2.5.2).

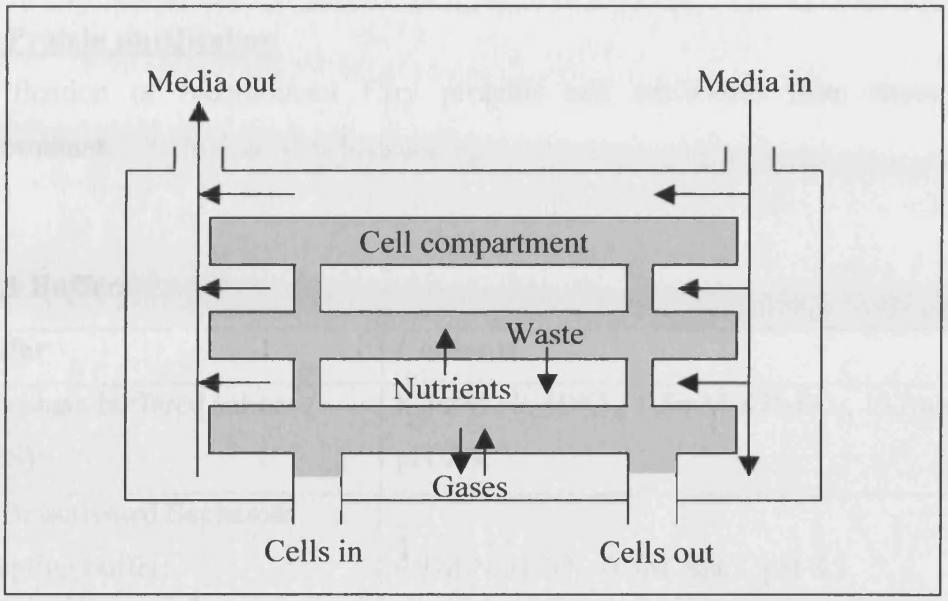


Figure 2.3 Diagram of the Tecnomouse cassette.

The cell compartment (intracellular space) is shown in grey, nutrients move into this compartment whilst metabolic waste products move out into the hollow fibres (extracellular space). Gases diffuse in and out of the intracellular space whilst medium is pumped around the extracellular space (Lukacik, Roversi et al. 2004).

2.3 Protein purification

Purification of recombinant Crry proteins and antibodies from tissue culture supernatant.

2.3.1 Buffers

Buffer	Contents
Phosphate buffered saline (PBS):	8.2mM Na_2HPO_4, 1.5mM KH_2PO_4, 137mM NaCl, pH 7.4.
CNBr-activated Sepharose Coupling buffer:	0.1M $NaHCO_3$, 0.5M NaCl, pH 8.3
Borate buffered saline (BBS):	100mM Boric acid, 25mM Sodium Borate, 75mM NaCl, pH 8.3
Tris buffered saline (TBS):	10mM Tris, 150mM NaCl, 0.01% (w/v) azide, pH 7.4
Malonate buffer:	50mM Malonic acid, 37.5mM NaOH, 4mM Betaine monohydrate pH 5.4

2.3.2 Clarification of supernatant prior to purification

Prior to any purification technique cell culture supernatant was clarified by centrifugation at 6000 g for 15 minutes at 4°C

2.3.3 Protein A and G chromatography

Both protein A and G are bacterial proteins which have been utilised in the purification of many antibody isotypes from a variety of species. Here, both protein A and G chromatography were compared as methods to purify the Crry-Fc proteins and these methods were also used to purify TLD1C11. For both columns a pre-column was used to remove any proteins which would non-specifically bind to the Sepharose matrix. The pre-column consisting of Sepharose CL4B (GE healthcare) was packed under gravity and washed with PBS to remove the ethanol preservative. The pre-column was then connected in-line before the protein A or G column. Prior to elution of bound protein from the column, the pre-column was taken out of line.

Table 2.3 reports the binding buffer, wash buffer and elution buffer used in the protein A and G chromatography of Crry-Fc and TLD1C11.

Method	Binding buffer	Wash buffer	Elution buffer
Crry-Fc purification by protein A chromatography	1.5M glycine/NaOH, 3M NaCl pH 9.	Same as binding buffer	0.1M glycine/HCl pH 2.5
Crry-Fc purification by protein G chromatography	None	PBS	0.1M glycine/HCl pH 2.5
TLD1C11 monoclonal antibody purification by protein A chromatography	1M glycine/NaOH, 0.15M NaCl pH 8.6	Binding buffer followed by 0.1M citrate pH 6.	0.1M citrate pH 3
TLD1C11 monoclonal antibody purification by protein G chromatography	None	PBS	0.1M glycine/HCl pH 2.5

Table 2.3 Protein A and G chromatography methods along with the binding buffer, wash buffer and elution buffer used.

2.3.3.1 Crry-Fc purification

For protein A purification of 5SCR-Fc, cell culture supernatant was mixed 1: 1 with the binding buffer (table 2.3). The supernatant was passed over a 10ml protein A column (ProSep A, Millipore). The columns were then washed, the pre-column disconnected and bound protein was eluted. The protein was eluted in 3ml fractions neutralised with 300µl 1M Tris/HCl pH 9. Fractions were assessed for protein content by their absorbance at 280nm in a spectrophotometer.

For protein G purification, 5SCR-Fc containing supernatant was not mixed with a binding buffer but was passed over a 10ml protein G column (ProSep G, Millipore).

The column was washed and bound protein was eluted, neutralised and screened as above.

2.3.3.2 TLD1C11 purification

The method used to purify the TLD1C11 monoclonal antibody from hybridoma supernatant varied depending upon which culture method was used. Supernatant generated using the Tecnomouse or CELLine flask contained ultra low bovine immunoglobulin FBS. In this case no bovine antibodies which would co-purify on protein G were present in the supernatant so protein G chromatography was used. The supernatant was run over the pre-column and protein G column connected in series. The columns were washed and bound protein was eluted as above. When supernatant generated from conventional T80 flasks was used, the TLD1C11 was purified using protein A chromatography to limit bovine antibody contamination. The supernatant was mixed 1:1 with binding buffer (table 2.3). The supernatant was passed over the pre-column and protein A connected in series. The columns were washed with the binding buffer, the pre-column disconnected and the protein A column washed with 0.1M citrate pH 6 to reduce bovine immunoglobulin contamination. Bound antibody was eluted as described previously.

2.3.4 Generation of affinity columns

Antibody affinity columns of TLD1C11, 5I2 and anti-rat Ig were generated using CNBr-activated Sepharose 4B (GE Healthcare). A TLD1C11 affinity column was also generated using the Affi-Gel® Hz matrix (Bio-Rad).

2.3.4.1 CNBr-activated Sepharose

The antibody to be bound to the matrix was dialysed into coupling buffer overnight at 4°C. The CNBr-activated Sepharose was swollen in 1mM HCl, 1g (dry weight) of Sepharose was used per 3mg of protein to be coupled. The Sepharose was washed with 1mM HCl, 200ml/g of Sepharose. The Sepharose was equilibrated in coupling buffer then added to the antibody in a covered vessel. The Sepharose and antibody were placed on a roller for two hours at room temperature. The supernatant was then

removed from the Sepharose and the remaining active sites were blocked by incubating the Sepharose in 0.1M Tris/HCl pH 8 for two hours at room temperature. The Sepharose was packed into a column (2.5cm diameter) under gravity and washed with 3 cycles of 5 gel volumes of 0.1M sodium acetate, 0.5M NaCl pH 4 followed by 0.1M Tris/HCl, 0.5m NaCl pH 8. The column was then equilibrated in PBS 0.01% (w/v) azide and stored at 4°C.

2.3.4.2 Affi-gel Hz matrix

Antibody affinity columns were also made using an Affi-Gel ® Hz immunoaffinity kit (Bio-Rad). The antibody to be coupled to the matrix was dialysed into the supplied coupling buffer. To oxidise the carbohydrate groups on the antibody, 1ml of sodium periodate (20mg) was added per 10ml of antibody (1mg/ml) and incubated on a roller at room temperature for 1 hour, the incubation was carried out in the dark. Glycerol (200mM) was added and the reaction mixed for 10 minutes. The antibody was dialysed into the provided coupling buffer to remove the sodium periodate. Affi-gel Hz matrix (5ml) was equilibrated in coupling buffer to remove the alcohol preservative and the matrix was incubated with the antibody for 24 hours at room temperature. The matrix was then packed into a column under gravity and washed with PBS. The column was stored at 4°C in PBS 0.01% (w/v) azide

2.3.5 Use of affinity columns

To purify all Crry proteins from cell culture supernatant, clarified cell culture supernatant was passed over a pre-column connected in line with the relevant affinity column as described previously. The columns were then washed with PBS and bound protein was eluted from the affinity column using 0.1M glycine/HCl pH 2.5. Fractions (3ml) were neutralised with 300µl 1M Tris/HCl pH 9 and assessed for protein content as described previously.

To automate the affinity purification of the Crry proteins the TLD1C11 (anti-Crry) affinity column was packed into a XK-16 column (G E Healthcare) under gravity. The column was run using an AKTA Prime system (G E Healthcare). Each run consisted of a 2 column volume (40ml) equilibration with TBS, a supernatant load, a

3 column volume wash with TBS and a 3 column volume elution with 0.1M glycine/ HCl pH 2.5. The elution was collected in 2 ml fractions neutralised with 200µl 1M Tris/ HCl pH 9. All buffers were filtered through a 0.22µm filter prior to use and the supernatant was filtered through a 0.45µm filter.

2.3.6 Ammonium sulphate cuts

Ammonium sulphate was used to precipitate 5SCR-Fc from cell culture supernatant. Solid ammonium sulphate was added to cell culture supernatant which was stirred at 4°C. To achieve a cut of 40% saturation, 24g per 100ml supernatant was added. To increase the cut to 45% saturation a further 3g was added, another 3g was added to increase the cut to 50% saturation. Following each cut, the supernatant was stirred at 4°C for 2 hours to allow complete protein precipitation. The precipitate was then harvested by centrifugation at 12,000g for 15 minutes. Following the protein harvest, the supernatant was subjected to the increasing cuts while the pellet was dissolved in PBS.

2.3.7 Anion and cation exchange chromatography

Ion exchange methods separate proteins based upon their differences in charge, anion exchange columns bind negatively charged proteins while cation exchange columns bind positively charged proteins. Proteins were separated using an increasing salt gradient. For the purification of 5SCR-Fc pre-packed Mono Q and Mono S columns (G E Healthcare) were used as the anion and cation exchange columns respectively. For the purification of rat C3 a Source Q column (GE Healthcare) was used as an anion exchange column. The columns were connected to an AKTA Purifier Liquid Chromatography System (GE Healthcare) with an automated Frac 901 fraction collector and UV absorbance meter.

2.3.7.1 Anion exchange of 5SCR-Fc

Following a cut of 50% saturation on 5SCR-Fc cell culture supernatant, the pellet was resuspended in 5mM sodium phosphate pH 8 and loaded onto a Mono Q column. The proteins were separated 0-150mM NaCl gradient over 25 column

volumes followed by 150-500mM over a further 20 column volumes.

2.3.7.2 Cation exchange of 5SCR-Fc

An ammonium sulphate cut of 50% saturation was carried out on 5SCR-Fc cell culture supernatant and the pellet resuspended in malonate buffer. The resuspended pellet was loaded onto a Mono S column and the proteins were separated using an increasing salt gradient. The gradient was 0-250mM NaCl over 30 column followed by 250mM- 500mM NaCl over a further 10 column volumes.

2.3.7.3 Rat C3

Fresh rat serum (100ml) was mixed with 900ml 4% (w/v) PEG 6000, 5mM EDTA in PBS and incubated at 4°C for 30 minutes. The pellet was harvested by centrifugation at 10,000g for 30 minutes. The supernatant was retained and mixed with 385ml 30% (w/v) PEG 6000, 5mM EDTA in PBS. After incubation at 4°C for 30 minutes, the pellet was harvested at 10,000g for 30 minutes. The supernatant was discarded and the pellet resuspended in 5mM KPO_4, 5mM EDTA, 5mM benzamidine, pH 7.8. The pellet was loaded onto a Source Q column and the proteins were separated by a 0-0.5M NaCl gradient over 20 column volumes. The C3 containing peak was identified by dot blot and dialysed into 50mM $NaPO_4$ pH 6. The C3 peak was loaded onto a Mono S column. The proteins were separated by a 0-1M NaCl gradient over 20 column volumes. C3 was identified by dot blot and dialysed into 10mM HEPES, 150mM NaCl pH 7.4 for storage at -80°C and further studies. In order to generate C3b-like C3b ($C3_{MA}$), the rat C3 was methylamine inactivated. For this the C3 was dialysed against Borate Buffered Saline (BBS) while methylamine was diluted to 0.2M in BBS. The C3 was incubated 1:1 with the 0.2M methylamine (0.1M final) for 2 hours at 37°C. The $C3_{MA}$ was dialysed against PBS to remove the methylamine and stored at -80°C.

2.3.8 Cibacron Blue

Affi-gel® blue affinity gel (Bio-Rad) was used as a source of Cibacron Blue F3GA dye which binds albumin. A 3ml column was poured under gravity and equilibrated

in 20mM sodium phosphate pH 7.1. The protein sample was applied to the column and the column was washed with 20mM sodium phosphate, pH 7.1. The column was then washed in the 20mM sodium phosphate, 1.4M NaCl, pH 7.1 to elute bound albumin. For all the washes 2ml fractions were collected.

2.3.9 Gel filtration

Gel filtration separates proteins based upon size. Gel filtration columns consist of beads which contain pores; small proteins are able to enter these pores and are retained by the column. While large proteins are unable to enter the pores so pass through the column more quickly. For gel filtration of the proteins described here, a Superdex 200 (GE Healthcare) was used on the AKTA Purifier. Generally, protein was loaded with a maximum volume of 2.5% of the column volume (CV) i.e. 0.5ml loaded onto a 20ml column, and proteins were separated with a 1.5 column volume isocratic elution.

2.3.10 Dialysis and concentration of the proteins

Following purification of the protein of interest, fractions containing the protein were combined and dialysed into PBS unless otherwise stated. For the dialysis of all proteins, the dialysis tubing (Medicell International Ltd) used had a molecular weight cut off of 12-14 kDa. Following dialysis the proteins were concentrated to between 0.5-1mg/ml using an appropriate size Amicon ultrafiltration cell (Millipore). Table 2.4 shows the membrane cut off used to concentrate each protein.

Protein	Molecular weight cut off (kDa)
5SCR-Fc	100
4SCR-Fc	100
3SCR-Fc	100
4SCR	10
3SCR	10
TLD1C11	100
C3	100

Table 2.4 Proteins purified and the molecular weight cut off of the membrane used for concentration.

2.4 Protein analysis

Determination of protein concentration and purity using SDS PAGE and ELISA analysis.

2.4.1 Buffers

Buffer	Contents
Sodium Dodecyl Sulphate (SDS) stacking gel buffer:	0.5M Tris, 0.4% (w/v) SDS, pH 6.8.
SDS resolving gel buffer:	1.5M Tris, 0.4% (w/v) SDS, pH 8.8.
Non-reducing loading buffer:	0.1M Tris, 10% (v/v) glycerol, 2% (w/v) SDS, 0.01% bromophenol blue, pH 6.8.
Reducing loading buffer:	5% (v/v) β-mercaptoethanol in non-reducing buffer.
Running buffer:	25mM Tris, 191mM glycine, 1% (w/v) SDS
Coomassie stain:	0.2% (w/v) Coomassie blue R250 in 45% (v/v) methanol, 10% (v/v) acetic acid in dH_2O.
Destain:	20% (v/v) methanol, 8% (v/v) acetic acid in dH_2O.
Gel drying buffer:	4% (v/v) glycerol, 20% (v/v) methanol in dH_2O.
Transfer buffer:	25mM Tris, 191mM glycine, 20% (v/v) methanol.
ELISA coating buffer:	0.2M Na_2CO_3, pH 9.6.
ELISA developing solution:	4 OPD tablets (Dako) dissolved in 12ml dH_2O plus 5µl of 30% (v/v) H_2O_2.

2.4.2 Protein concentration

The concentration of purified proteins was obtained by measuring their absorbance a 280nm in a spectrophotometer (Jenway). The concentration was calculated using the following equation:

$C = A / \varepsilon$ where C is concentration (mg/ml), A is absorbance and ε is the extinction coefficient when the pathlength is 1cm.

Table 2.5 shows the extinction coefficients used for each protein.

Protein	Extinction coefficient
TLD1C11	1.4
5SCR-Fc	1.3
4SCR-Fc	1.2
3SCR-Fc	1.3
4SCR	1.2
3SCR	1.3
C3	1.0

Table 2.5 The extinction coefficients of the proteins purified in this thesis.

2.4.3 Antibodies

The antibodies used within this project for Western blot, dot blot and ELISA analysis are detailed below in table 2.6.

Name	Antigen recognised	Isotype	Species	Source
TLD1C11	Rat Crry	IgG1	Mouse	Prof. W. Hickie, (Dartmouth, USA)
5I2	Rat Crry	IgG1	Mouse	Prof. H. Okada, (Nagoya, Japan)
Anti-rat Ig	Rat Ig	Polyclonal	Sheep	Binding Site
Anti-rat Crry	Rat Crry	Polyclonal	Rabbit	In house
Anti-rat Ig HRPO	Rat Ig	Polyclonal	Donkey	Jackson Laboratories
Anti-mouse Ig HRPO	Mouse Ig	Polyclonal	Goat	Jackson Laboratories
Anti-rabbit Ig HRPO	Rabbit Ig	Polyclonal	Donkey	Jackson Laboratories
Anti-human C3c	Human C3c, also cross reacts with rat C3c	Polyclonal	Sheep	Binding Site
Anti-sheep Ig HRPO	Sheep Ig	Polyclonal	Donkey	Binding Site
Anti-bovine Ig HRPO	Bovine Ig	Polyclonal	Donkey	Jackson Laboratories
Anti-rat IgG1 HRPO	Rat IgG1	IgG1	Mouse	Southern Biotech
Anti-rat IgG2a HRPO	Rat IgG2a	IgG1	Mouse	Southern Biotech
Anti-rat IgG2b HRPO	Rat IgG2b	IgG2b	Mouse	Southern Biotech
Anti-rat IgG2c HRPO	Rat IgG2c	IgG2a	Mouse	Southern Biotech
Anti-rat IgM HRPO	Rat IgM	IgG1	Mouse	Southern Biotech

Table 2.6 A summary of the antibodies used within this project.

2.4.4 SDS PAGE analysis

Electrophoretic separation of proteins was carried out using the Hoefer mighty small gel apparatus. Gels were poured with an acrylamide concentration according to the size of the proteins to be resolved, low molecular weight proteins were resolved on higher percentage gels while high molecular weight proteins were resolved on lower

percentage gels. Typically gels were poured with a 5% acrylamide stacking gel and a 7.5-12.5% acrylamide resolving gel. Table 2.7 gives the gel recipes.

Percentage gel →	**5% (stacking)**	**7.5% (resolving)**	**10%**	**11%**	**12.5%**
Stacking gel Buffer (ml)	2.4	-	-	-	-
Resolving gel Buffer (ml)	-	7.5	7.5	7.5	7.5
40% Acrylamide (ml)	1.3	5.6	7.5	8.4	9
10% Ammonium Persulphate (µl)	100	300	300	300	300
dH_2O (ml)	6.1	16.4	14.5	13.6	13.0
TEMED (µl)	10	30	30	30	30

Table 2.7 Gel recipes for stacking and resolving SDS PAGE mini-gels, recipe makes 4 mini-gels.

In general, the protein or protein mixture of interest was mixed with either 15µl of non-reducing or reducing loading buffer and boiled for 5 minutes. The samples were loaded into the wells of the SDS PAGE gel within a running tank (Hoefer) containing running buffer. The gels were subjected to electrophoresis at 200V for between 45-60 minutes. Time depended upon the percentage gel being used.

2.4.5 Silver staining

In order to detect between 100ng-2µg of protein, SDS PAGE gels were silver stained (Morrissey 1981). Gels were:

- rinsed in dH_2O
- soaked for 30 minutes in 50% (v/v) methanol, 10% (v/v) acetic acid
- soaked in 5% (v/v) methanol, 7% (v/v) acetic acid for 30 minutes
- rinsed with dH_2O
- soaked in 5% (v/v) glutaraldehyde for 30 minutes
- washed in dH_2O overnight
- soaked in DTT (5µg/ml in dH_2O) for 30 minutes
- rinsed with dH_2O
- stained with 0.1% (w/v) $AgNO_3$ in dH_2O for 30 minutes

- rinsed in dH_2O
- developed in 0.28M Na_2CO_3 containing 0.0185% (v/v) formaldehyde.
- development was stopped by adding solid citric acid (~1g/50ml developing solution).

All steps were carried out with agitation. Following the silver staining procedure, gels were soaked in gel drying buffer and dried as described below.

2.4.6 Coomassie staining

To detect 2-5μg of protein SDS PAGE gels were Coomassie stained. Following electrophoresis, the gels were soaked in Coomassie stain with agitation for 30 minutes. Excess stain was removed and the gels soaked in destain solution with agitation until the background staining was minimised and the bands were clearly visible. Gels were soaked in gel drying buffer for at least 30 minutes. The gels were placed between pre-soaked acetate gel drying film (Promega) and stretched within a gel drying frame overnight.

2.4.7 Western blotting

To identify specific proteins of interest within a SDS PAGE gel, Western blotting was used. Table 2.8 gives details of the Western blots carried out in this thesis. Following electrophoresis, the gel was soaked in transfer buffer along with nitrocellulose (VWR), blotting paper and sponges. The nitrocellulose was placed on top of the gel and these were sandwiched between blotting paper between sponges within a cassette. The cassette was placed within a blotting tank (Hoefer system) with the nitrocellulose closest to the anode and the gel closest to the cathode. The transfer was run at 100V for 1 hour. The nitrocellulose was removed from the tank and cassette, and blocked with PBS, 5% (w/v) non-fat dried milk (PBS milk) for 30 minutes at room temperature. Following blocking, the blot was probed with a relevant antibody at 1μg/ml in PBS milk overnight at 4°C. The blot was washed and probed with a horseradish peroxidase (HRPO) conjugated secondary antibody (1/1000 – 1/10000) in PBS milk. In some instances, for example when detecting a rat Fc domain, the primary antibody was directly conjugated to the enzyme and this incubation was carried out for an hour at room temperature. In between the

incubations with primary and secondary antibodies as well as after the secondary antibody incubation the blots were washed 3 times for 10 minutes in PBS, 0.1% (v/v) Tween followed by 3 times 10 minute washes in PBS. Blots were developed using Super Signal Reagent (Pierce) by mixing the reagents 1:1 and incubating the blot in them for 3 minutes. The Super Signal Reagent was removed from the blot and the blot was exposed to X-OMAT UV-film (Kodak) and developed in a Compact X2 developer (X-Ograph Ltd).

Western	**Primary antibody**	**Secondary antibody**
Detection of Crry fusion proteins	-	Anti-rat Ig HRPO
Detection of all Crry proteins	TLD1C11	Anti-mouse Ig HRPO
C3 detection	Anti-human C3c	Anti- sheep Ig HRPO
Detection of TLD1C11	-	Anti-mouse Ig HRPO
Bovine antibody detection	-	Anti-bovine Ig HRPO
Cofactor assay	Anti-human C3c	Anti-sheep Ig HRPO

Table 2.8 Western blots carried out along with the detection antibodies used.

2.4.8 Dot blots

Proteins (1-2µl depending upon concentration) were spotted directly onto nitrocellulose. The nitrocellulose was left to air dry and then was blocked at room temperature for 1 hour in PBS milk. Dot blots were probed and developed as with Western blots.

2.4.9 ELISA

ELISAs were used to identify and quantify the Crry proteins and TLD1C11, table 2.9. ELISA plates (MP Biomedicals) were used for the ELISAs. All steps were carried out for 1 hour at 37°C and were separated by washing the wells of the plate 3 times with 150µl PBS, 0.1% (v/v) Tween. In brief, the plate was coated with 100µl per well of a capture protein or antibody at 1µg/ml in ELISA coating buffer, blocked with 150µl per well of PBS, 5% (w/v) milk and incubated with the sample of interest

or standard in an appropriate dilution (100µl per well). The plate was incubated with 100µl per well of detection antibody at 1µg/ml followed by 100µl per well of a HRPO conjugated secondary antibody at 1/1000 or with the conjugated antibody alone. The ELISAs were developed with 100µl per well of ELISA developing solution and stopped with 50µl of 10% H_2SO_4 when background began to increase. Absorbance was read at 490nm in a microplate reader.

ELISA	Capture	Sample	Detection antibody	Secondary antibody
Quantification and detection of Crry-Fc proteins	TLD1C11	Cell culture supernatant or pure protein	-	Anti-rat Ig HRPO
Quantification and detection of non-Fc Crry proteins	TLD1C11	Cell culture supernatant or pure protein	anti-Crry (polyclonal antiserum)	Anti-rabbit Ig HRPO
Quantification and detection of TLD1C11	5SCR-Fc	Cell culture supernatant or pure protein	-	Anti-mouse Ig HRPO
DAF-Fc immune response	Rat DAF-human Fc	Serum	-	HRPO conjugated anti-rat IgM, IgG1, IgG2a, IgG2b or IgG2c
Crry-cys immune response	Crry-cys	Serum	-	HRPO conjugated anti-rat IgM, IgG1, IgG2a, IgG2b or IgG2c
Crry-Fc immune response	Crry-Fc	Serum	-	HRPO conjugated anti-rat IgM, IgG1, IgG2a, IgG2b or IgG2c
CD59-Fc response	CD59-Fc	Serum	-	HRPO conjugated anti-rat IgM, IgG1, IgG2a, IgG2b or IgG2c

Table 2.9 ELISAs used along with capture, sample and detection antibodies.

2.5 Functional analysis

The functional analysis of the Crry proteins.

2.5.1 Buffers and reagents

Buffer	Contents
Complement Fixation Diluent (CFD; Oxoid):	2.8mM Barbituric acid, 145.5mM NaCl, 0.8mM $MgCl_2$, 0.8mM $CaCl_2$, 0.9mM Sodium Barbital, pH 7.2
Alternative pathway (AP) buffer:	5mM sodium barbitone, 150mM NaCl, 7mM $MgCl_2$, 10mM EGTA
HBS-P (Biacore):	10mM HEPES, 150mM NaCl, 0.005% (v/v) Surfactant P20, pH 7.4
HBS-EP (Biacore):	10mM HEPES, 150mM NaCl, 0.005% (v/v) Surfactant P20, 3mM EDTA, pH 7.4
PBS;	8.2mM Na_2HPO_4, 1.5mM KH_2PO_4, 137mM NaCl, pH 7.4.

2.5.2 Classical pathway haemolysis assay

2.5.2.1 Preparation of sheep erythrocytes

Sheep erythrocytes (TCS) were washed into PBS and diluted to 4% (v/v). Amboceptor (rabbit anti-sheep erythrocytes, Behring Diagnostics) was diluted 1 in 250 in PBS and incubated 1:1 with the sheep erythrocytes for 30 minutes at 37°C. The antibody coated sheep erythrocytes (E) were washed into CFD and diluted to 2% (v/v).

2.5.2.2 Titre of rat serum

To establish the amount of rat serum required to cause 60% lysis an assay was carried out using a serum titration. Two 10-step 1:2 serial dilutions of rat serum were set up, one beginning at 1/10 and the other at 1/15. Fifty micro-litres of each concentration was incubated with 50µl antibody coated sheep E and 50µl CFD in a round-bottomed 96-well plate. Control wells were included for 100% lysis in which

the rat serum was substituted for 50µl 0.1% (v/v) NP40 and 0% lysis in which the rat serum was substituted for 50µl CFD. Each concentration was carried out in duplicate. Following incubation at 37°C for 30 minutes, the remaining sheep E were pelleted by centrifugation at 900g for 5 minutes. The supernatant (50µl) was removed from each well and added to 100µl dH_2O in a flat-bottomed 96-well plate. The absorbance at 415nm was measured and was used to calculate the degree of lysis (proportion of haemoglobin released)

Percentage lysis was calculated using the following equation:

$\%\ \text{lysis} = 100 \times (A_{415}^{\text{Test}} - A_{415}^{0\%}) / (A_{415}^{100\%} - A_{415}^{0\%})$

The concentration of serum giving 60% lysis was used in subsequent assays.

2.5.2.3 Function of Crry proteins

To compare the function of the Crry proteins they were diluted in a 10-step 1:2 serial dilution beginning at 140nM of Crry. As a control, a 10-step 1:2 serial dilution of rat immunoglobulins (Ig) was used. In the assay 50µl antibody coated sheep E were incubated with 50µl of Crry from the dilution series and 50µl rat serum diluted to give 60% lysis in the absence of inhibition. Controls for 100% and 0% lysis were included. Each incubation was carried out in triplicate. The assay was incubated at 37°C for 30 minutes. Following the incubation the remaining sheep E were pelleted, the absorbance at 415nm measured and the percentage lysis calculated. The following equation was used to calculate percentage inhibition of lysis.

Inhibition of lysis (%) = 100 x (%lysis with rat Ig- % lysis with Crry) / % lysis rat Ig

Percentage inhibition of lysis (y-axis) was plotted against the log of concentration (x-axis) and was used to calculate the concentration of each Crry protein that caused 50% inhibition of lysis (IH50).

2.5.3 Alternative pathway haemolysis

2.5.3.1 Titre of rat serum

Guinea pig erythrocytes (GPE) were washed into AP buffer and diluted to 1.5% (v/v). To establish the amount of serum needed to cause 60% lysis, rat serum diluted in two 10-fold 1:2 serial dilutions beginning at 1 in 2 and 1 in 3. Fifty micro-litres of serum from the dilution series was incubated with 50µl GPE and 50µl AP buffer.

Control wells contained 50µl 0.1% NP40 instead of serum for 100% lysis or 50µl AP buffer instead of the serum for 0% lysis. The assay was carried out in duplicate. The assay was incubated at 37°C for 30 minutes, the absorbance at 415nm was measured and percentage lysis calculated as described for the classical pathway haemolysis assay.

2.5.3.2 Function of Crry proteins

To compare the Crry proteins they were diluted in a 10-fold 1:2 serial dilution beginning at 560nM Crry. GPE (50µl) were incubated with 50µl Crry proteins from the concentration titre and 50µl rat serum (at a concentration that caused 60% lysis in the absence of inhibition). Controls included a titre of rat Ig beginning at 560nM, 100% lysis and 0% lysis as described previously. Following the incubation at 37°C for 30 minutes, the absorbance at 415nm was measured. The percentage lysis, percentage inhibition of lysis and the concentration of the Crry proteins required to cause 50% inhibition of lysis (IH50) was calculated as described above.

2.5.4 Cofactor assay

Rat $C3_{MA}$ (2.5µg) was incubated with 0.5µg rat factor I and 2µg of individual Crry proteins, the reaction volume was taken to 50µl with PBS. The following control incubations were included:

Control	Purpose
$C3_{MA}$ (2.5µg) only	Rule out contamination with both factor I and cofactors
$C3_{MA}$ (2.5µg) and factor I (0.5µg) only	Rule out contamination of either with cofactors
$C3_{MA}$ (2.5µg), factor I (0.5µg) and human soluble complement receptor 1 (sCR1, 2µg)	Positive control
$C3_{MA}$ (2.5µg) and human sCR1 (2µg)	Rule out contamination of CR1 and $C3_{MA}$ with factor I
5SCR-Fc, 4SCR-Fc, 3SCR-Fc, 4SCR or 3SCR with $C3_{MA}$ (2.5µg)	Rule out contamination of Crry proteins and $C3_{MA}$ with factor I

All incubations were carried out at 37°C for 4 hours. Following incubation 6µl of each reaction was loaded onto a 10% SDS PAGE gel. The gel was either silver stained or Western blotted and probed for C3.

2.5.5 Decay accelerating function

The ability of the Crry proteins to decay the alternative pathway C3 convertase was assessed by surface plasmon resonance using the Biacore® 3000. The Biacore® utilises a gold covered chip coated with dextran, molecular interactions occur on this dextran. Any molecular interactions causing mass changes alter the read out of the machine (Malmqvist 1999).

2.5.5.1 Surface preparation

One hundred RU of human C3b dimer (100ng/ml in 10mM citrate pH4) was amine coupled to the chip surface (CM5 chip, Biacore®). The remaining active sites on the chip surface were blocked with 0.1M ethanolamine pH 8.5 (figure 2.4). The covalently immobilised human C3b was used to form the AP C3 convertase with factors B and D. This convertase was used to cleave rat C3 resulting in the deposition of rat C3b on the surface. HBS-P supplemented with 1mM $MgCl_2$ was used as the running buffer for the system, the flow rate was 5µl/min. Following immobilisation of human C3b, human factors B and D (Quidel, 200µg/ml and 12µg/ml respectively) were mixed 1:1 and 20µl was injected across the chip surface immediately followed by 20µl rat C3 (0.9mg/ml). The factors B and D formed C3bBb which cleaved rat C3 resulting in the deposition of rat C3b on the surface through nucleophilic attack upon the thioester group within C3b by amine groups on the chip surface. The cycle of C3bBb formation followed by rat C3b deposition was repeated until 5000RU of rat C3b had been deposited. The surface was stabilised by a 10µl injection of 50mM diethylamine pH 11 to remove any loosely bound rat C3b.

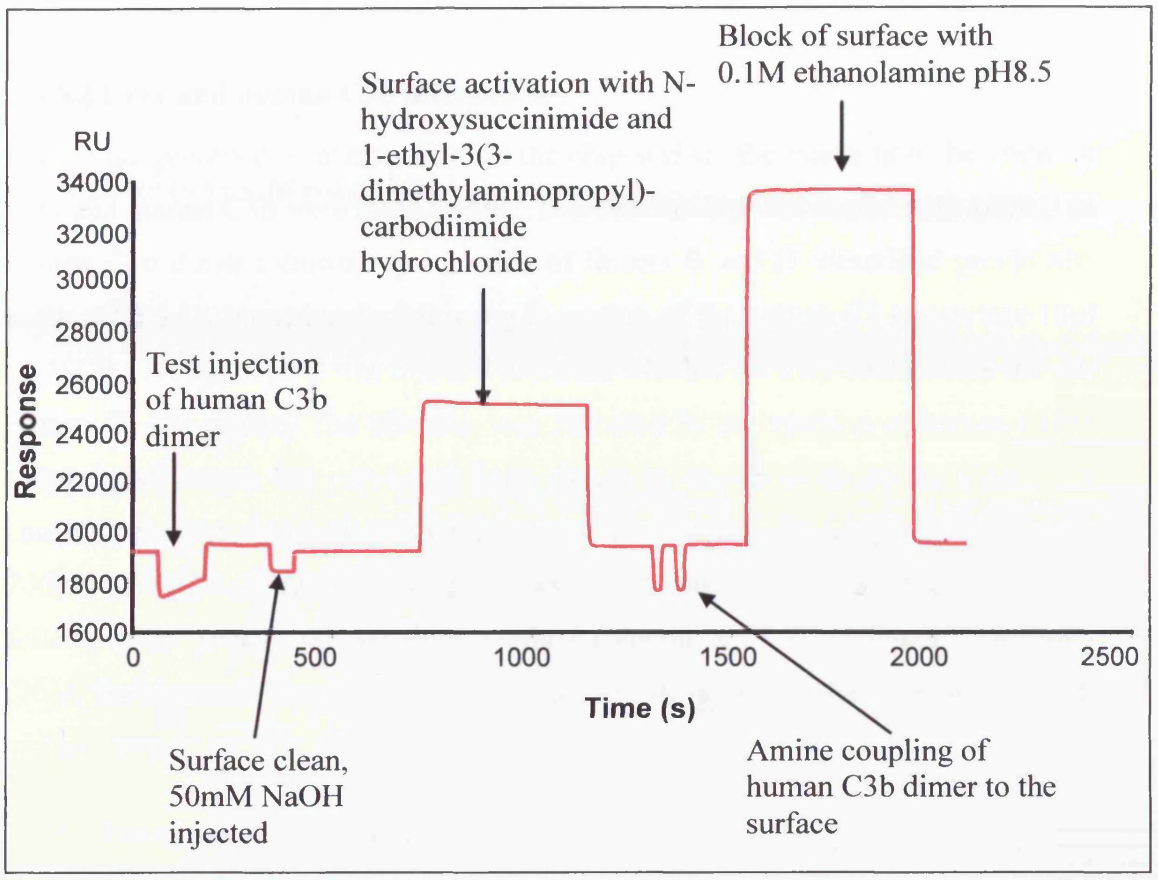


Figure 2.4 Sensogram of the preparation of the chip surface.

The association of human C3b dimer with the chip was first tested by injection of human C3b dimer (10µl). The non-specifically bound human C3b dimer was removed from the surface by injection of 5µl 50mM NaOH. The surface was activated with 35µl of N-hydroxysuccinimide and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (mixed 1:1). The human C3b dimer was injected in 2µl injections until 100RU was reached. The surface was blocked with 35µl 0.1M ethanolamine pH 8.5.

2.5.5.2 Crry and human C3b interaction

Due to the presence of human C3b on the chip surface the interactions between rat Crry and human C3b were investigated. Therefore a chip was coated with 100RU of human C3b dimer followed by injection of factors B and D (described previously, section 2.5.5.1). Immediately following formation of the human C3 convertase 10µl of 4SCR (700µg, 25µM) was injected to assess whether rat Crry could decay the all-human C3 convertase. The injection was followed by an injection of human CD55 (10µl, 1µM) which has previously been shown to shown to decay the human C3 convertase on the chip surface (Harris, Abbott et al. 2005). On a separate CM5 chip, 200RU of 4SCR (25µg/ml in 10mM citrate pH 4) was amine coupled to the surface following the previously described method (section 2.5.5.1). Human C3b dimer (20µl, 5mM) was injected across the surface to assess binding between human C3b and rat Crry.

2.5.5.3 Decay acceleration assay

The deposited rat C3b was used to form the C3bBb convertase using a 30µl injection of human factors B and D in the concentrations described previously. The convertase was allowed to decay naturally (in the presence of no complement regulators) or 10µl of a Crry protein from a 5-fold 1:2 serial dilution beginning at 1.8µM was injected. A separate convertase was built up on the surface for each concentration of each protein. Prior to each convertase formation 10µl 50mM diethylamine pH 11 was injected to ensure that no Crry from the previous injection remained on the surface. By measuring the amount of convertase remaining on the chip surface following decay and comparing with the amount of convertase in the absence of any regulation, percentage decay was calculated as:

Percentage decay = 100 x (y axis natural - y axis after Crry) / y axis natural.

The percentage of decay (y-axis) was plotted against concentration (x –axis) and the concentration of inhibitor to decay 50% of the convertase was calculated.

2.5.6 Affinity analysis

2.5.6.1 Affinity analysis 500RU coat

The affinity of the 4SCR and 3SCR Crry proteins for rat C3b was assessed using surface plasmon resonance and the Biacore® 3000. HBS-P (Biacore®) was used as the buffer. Fifty RU of human C3b dimer was immobilised on a CM5 chip as described previously (section 2.5.5.1). Human factors B and D (100μg/ml and 6μg/ml respectively) were used in 15μl injections to form the C3 convertase and with injections of rat C3 deposited 500RU of rat C3b on the surface. The surface was stabilised by a 10μl injection of 50mM diethylamine pH 11 to remove loosely bound rat C3b. Both 3SCR and 4SCR were diluted in a 6-fold 1:2 serial dilution and each concentration was injected across the surface with a 90 second contact time followed by a 60 second dissociation in buffer, flow rate 10μl/min. The 4SCR concentration began at 350μg/ml (12.5μM) while 3SCR began at 700μg/ml (33.3μM). Each concentration was injected in duplicate. The dissociation equilibrium constant (KD) was calculated by plotting the response (y-axis) against concentration (x-axis). The point at which response no longer increases with concentration is the Rmax. The concentration corresponding to 50% Rmax is the KD (figure 2.5). In Biacore the chi^2 shows how well the data fits to the computer-modelled interaction, the smaller the chi^2 the more reliable the data. Chi^2 is calculated using the following equation:

$X^2 = \Sigma (O - E)^2 / E$

where O is the observed value and E is the expected value

2.5.6.2 Affinity analysis 1000RU coat

The affinity analysis was repeated on the same chip with the coat of rat C3b increased to 1000RU by forming the C3bBb convertase by injecting human factors B and D followed by rat C3 as described above. The surface was stabilised as before and the injections of 4SCR and 3SCR repeated. With this experiment the 4SCR serial dilution began at 700μg/ml (25μM) and the 3SCR serial dilution began at 700μg/ml (33.3μM). The dissociation equilibrium constant (KD) was calculated as described above

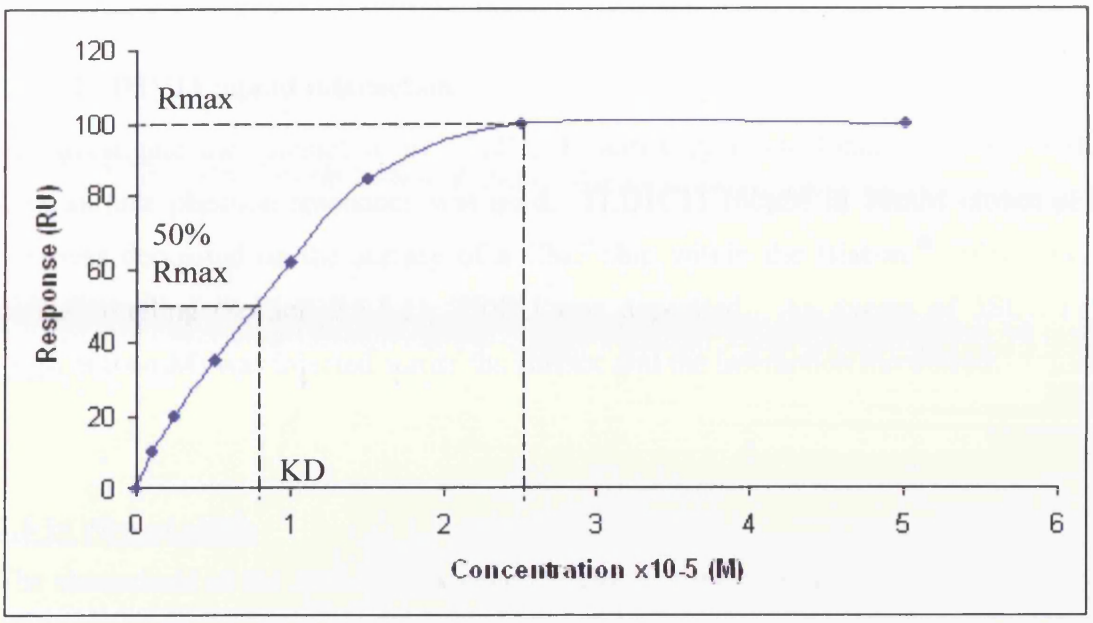


Figure 2.5 Calculation of Rmax and KD

The point at which response no longer increases with concentration is the Rmax. Half of Rmax (50% Rmax) corresponds to the dissociation equilibrium constant (KD).

2.5.7 TLD1C11 ligand interaction

To investigate the interaction of TLD1C11 (anti-Crry monoclonal antibody) with Crry surface plasmon resonance was used. TLD1C11 (60μM in 10mM citrate pH 4.5) was deposited on the surface of a CM5 chip within the Biacore® 3000 using amine coupling (section 2.5.5.1), 750RU was deposited. An excess of 5SCR-Fc (20μl at 0.6mM) was injected across the surface and the interaction monitored.

2.6 In vivo analysis

The assessment of the half-life, immunogenicity, in vivo complement inhibition and therapeutic efficacy of 4SCR-Fc and 4SCR.

2.6.1 Buffers and reagents

Buffer	Contents
PBS (phosphate buffered saline)	8.2mM Na_2HPO_4, 1.5mM KH_2PO_4, 137mM NaCl, pH 7.4.
FACS buffer	PBS, 1% (w/v)BSA, 10mM EDTA, 0.01% (w/v) sodium azide, pH 7.4
CFD	2.8mM Barbituric acid, 145.5mM NaCl, 0.8mM $MgCl_2$, 0.8mM $CaCl_2$, 0.9mM Sodium Barbital, pH 7.2

All animals were obtained from Charles River Laboratories and maintained according to Home Office guidelines within the Biomedical Services Unit (BSU) at the School of Medicine, Cardiff University.

2.6.2 In vivo half-life

2.6.2.1 Iodination

4SCR-Fc and 4SCR (1mg of each) were labelled with ^{125}I using Iodobeads (Pierce) according to the method of Fraker and Speck (Fraker and Speck 1978). Following iodination, the proteins were isolated from the reaction mix by gel filtration into PBS using PD10 columns (G E Healthcare), 600µl fractions were collected. The fractions corresponding to the highest protein concentration were combined and used in the subsequent experiment. The iodination was carried out by Prof. B. P. Morgan, Department of Medical Biochemistry and Immunology.

2.6.2.2 In vivo experiment

Two groups of 5 female Lewis rats were used. Group A were injected i.v. with 150µl 4SCR-Fc whilst group B were injected i.v. with 150µl 4SCR, approximately 12 x 10^6 cpm of each protein. The animals were tail bled at the following time points 1, 3, 5, 15 and 30 minutes, 1, 2, 4, 6, 8, 24, 36, 48, 72 and 96 hours post injection. For the bleeds until 2 hours post injection, 20µl of blood was collected into 0.5ml 200mM EDTA to prevent clotting. To enable accurate detection of decreasing amounts of radioactivity the blood volume collected was increased to 30µl for the 4-10 hours post injection bleeds, 50µl for the 24 and 48 hours post injection bleeds and 100µl for the 72 hour post injection bleed, all bleeds were taken into 0.5ml 200mM EDTA. At 96 hours post injection, 1ml of blood was collected into 100µl 200mM EDTA. The in vivo experiment was carried out in collaboration with Dr Anwen Williams, Department of Rheumatology, who carried out the i.v injections and collected the tail bleeds.

2.6.2.3 Determination of half-life

The amount of radioactivity in the injection was counted by adding 10µl of the injection to 1ml foetal bovine serum (FBS) and 9ml PBS. One millilitre was counted to a Wallac Multigamma Gamma counter to give the total counts/ml. To calculate protein bound counts, 1ml 20% (v/v) trichloroacetic acid (TCA) was added to precipitate the protein and the supernatant isolated by centrifugation at 900g for 10

minutes. One millilitre of the supernatant was counted to give the free iodine measurement. Protein bound counts was calculated as follows:
Protein bound counts = total counts – (2 x free iodine counts)

To ascertain the total counts from the bleeds, 0.5ml FBS was added to each bleed and the total counts were obtained above. The free iodine was measured by adding 1ml 20% TCA, centrifuging to pellet the precipitated protein and measuring the counts of 1ml of the supernatant. The protein bound counts were calculated as described previously.

For each time point the protein bound counts per ml of blood was calculated and expressed relative to the protein bound counts at 5 minutes post administration to account for the initial distribution through the body as follows:
% of 5 minute level = 100 x protein bound counts at time, T / protein bound counts at 5 minutes
The percentage counts (y-axis) were plotted against the log of time in hours (x-axis). The half-life was calculated from the slope of the linear region of the line.
β-half-life = 0.693 / -slope

2.6.3 Immunogenicity study

2.6.3.1 In vivo experiment and measurement of immune response

Three groups of 5 female Wistar rats were injected i.v. with 1mg/kg of sCrry-cys (group A), rat DAF-human Fc (group B) or Crry-Fc (group C) on day 0, 3, 7, 14, 21 and 28. The animals were tail bled (200μl) on day 0 (pre-bleed), 7, 14, 21, 28 and 35. The blood was allowed to clot at room temperature for 30 minutes, stored on ice for 30 minutes and the serum was harvested by centrifugation at 12000g for 10 minutes. The immune response was measured by detecting antibody generation using ELISAs. Section 2.4.9 describes the ELISA set up. This experiment was carried out in collaboration with Dr Anwen Williams, Department of Rheumatology, who carried out the injections and collected the blood samples.

2.6.3.2 Characterisation of the immune response

To determine if the Crry-Fc immune response recognised the whole molecule or just part of the molecule an ELISA was carried out as described in section 2.4.9 coating with Crry-Fc, sCrry-cys or CD59-Fc (same Fc domain; rat IgG2a). An ELISA was also used to calculate the concentration of Crry-Fc remaining in the serum taken at each time point. The concentration of Crry-Fc at each time point was calculated from the standard curve, a 10-fold 1:2 dilution beginning at 1μg/ml.

The affect of the immune response on the function of Crry was judged using CHO cells expressing rat Crry on their surface. These cells had been generated in house previously by transfecting CHO cells with the pDR2ΔEF1α vector containing DNA encoding the full length of rat Crry including the transmembrane region. Deposition of antibodies from the immune sera was detected by incubating 50μl of cells at 2 x 10^6/ml in FACs buffer with either 50μl of immune serum at 10% (v/v) in FACs buffer, TLD1C11 (anti-Crry monoclonal antibody at 1μg/ml) or normal rat serum at 10% (v/v) in FACs buffer for 30 minutes at 4°C. The cells were washed three times by centrifugation at 300g for 3 minutes and resuspension in FACs buffer. Deposition of anti-Crry antibodies was detected by incubating the cells with 5% donkey anti-mouse Ig RPE (Dako; cross reacts with rat Ig) for 1 hour at 4°C. The cells were washed as previously described and fixed in 1.5% (v/v) formaldehyde prior to processing on a FACSCalibur (Becton Dickinson).

Changes in C3 deposition on the Crry expressing CHO cells were used to assess whether the deposited antibodies inhibited the function of Crry. The CHO cells (50μl at 2 x 10^6/ml in FACs buffer) were incubated with 50μl rabbit polyclonal anti-CHO antiserum (heat-inactivated) at 10% (v/v) in FACs buffer at 4°C for 30 minutes. Cells that were not antibody coated were incubated in FACS buffer for the same period. The cells were washed as described previously and were incubated with either 100μl immune response serum from the day 35 bleeds, normal rat serum, normal rabbit serum or rabbit polyclonal anti-Crry antiserum. All were heat inactivated and at 20% (v/v) in FACs buffer, the incubation was carried out for 30 minutes at 4°C. The cells were washed in CFD three times by centrifuging the cells at 300g for 3 minutes and resuspending in CFD. The cells were incubated in 100μl of 2% (v/v) rat serum for 30 minutes at 37°C. The deposition of C3 was detected

with 100µl of mouse ascites anti-human C3b monoclonal antibody (C3/30; cross reacts with rat C3) at 0.2% (v/v) in CFD, the incubation was carried out at 4°C for 30 minutes. The anti-C3b antibody was detected with 100µl of donkey anti-mouse Ig RPE at 1% (v/v) by incubation at 4°c for 1 hour. The cells were fixed in 1.5% formaldehyde and processed as above.

2.6.4 In vivo complement inhibitory activity

The in vivo complement inhibitory activity (CH50) of 4SCR-Fc and 4SCR was investigated in rats with EAMG (experimental autoimmune myasthenia gravis; see section 2.6.5). The rats were tail bled (100µl) pre-disease induction and then at the following time points: 2, 4, 6, 24, 48, 72 and 96 hours post induction. The blood was allowed to clot at room temperature for 30 minutes, stood on ice for 30 minutes and then centrifuged at 12,000g for 10 minutes. The serum was isolated from the clot.

2.6.4.1 CH50 assay

For each animal the serum was first diluted to 2% (v/v) in CFD and then this diluted serum was further diluted: 95%, 90%, 87.5%, 83%, 75%, 50%, 25%, 17.5%, 12.5%, 10%, 5%, 2.5% and 1.3% (v/v). Serum (50µl) from each concentration was incubated with 50µl antibody coated sheep erythrocytes (described section 2.5.2) in a round-bottomed 96-well plate for 30 minutes at 37°C. To establish 0% lysis, 50µl sheep erythrocytes were incubated with 50µl CFD and to establish 100% lysis, 50µl cells were incubated with 50µl dH_2O. The assay was carried out in triplicate. Following the incubation 150µl CFD was added and the cells pelleted by centrifugation at 900g for 5 minutes. Supernatant (150µl) from each well was transferred to a flat-bottomed 96-well plate and the absorbance at 415nm was measured.

For each time point percentage lysis was calculated as described in section 2.5.2 and was plotted against log of serum volume used (µl). The volume of serum to bring about 50% lysis was calculated. The volume of serum to bring about 50% lysis at each time point was expressed relative to the volume of serum from the pre-bleed calculated to cause 50% lysis as follows:

% haemolytic activity = 100 x (volume of serum to cause 50% lysis for the pre-bleed/ volume of serum to cause 50% lysis at time (T))

Percentage haemolytic activity (y-axis) was plotted against time in hours (x –axis).

2.6.5 Therapeutic activity

2.6.5.1 EAMG disease induction

On day 0, 3 groups of 6 female Lewis rats (160-200g) were injected i.p with 1mg/kg of mAb35, a rat monoclonal anti-acetylcholine receptor antibody (Piddlesden, Jiang et al. 1996). The animals were also administered an appropriate therapeutic agent i.v. Animals in group A were given PBS (1ml/animal), those in group B 4SCR at 10mg/kg and those in group C 4SCR-Fc at 20mg/kg. Prior to injection a pre-bleed was taken by tail tipping, further bleeds were taken at 2, 4, 6, 24, 48, 72 and 96 hours post induction and processed at described in section 2.6.4.

Animals were assessed daily for evidence of EAMG. The weights of the animals were measured as well as the clinical score. The clinical score was measured by the ability of the animals to grasp and lift the lid of a mouse cage.

0. no disease
1. reduced grip strength in front paws, could grip but not lift
2. loss of grip in front paws
3. loss of grip and hind limb weakness and wasting
4. loss of grip and hind limb paralysis

After the onset of clinical symptoms rats were given pre-wetted food. The animals were sacrificed by a schedule 1 method when weight loss was equal to or greater than 20% of the original body weight, or when the clinical score reached 4. This experiment was carried out in collaboration with Dr Anwen Williams, Department of Rheumatology, who administered the i.v injections and Dr Jayne Chamberlain-Banoub who administered the i.p injections, collected the blood samples, clinically scored the animals and carried out the histology (method 2.6.5.2).

2.6.5.2 Histological analysis

The soleus muscle was isolated from the sacrificed rats and subjected to flash freezing in isopentane cooled on dry ice, the muscles were stored at -80°C. The muscles were embedded in OCT medium (Agar Scientific) and 10μm sections were cut on a cryostat and transferred to slides (Surgipath). Slides were acetone fixed for 5 minutes and stained with Bungarotoxin (BuTx)-rhodamine conjugate (Molecular probes) at 0.5% (v/v) in PBS 1% (w/v) BSA for 40 minutes at room temperature in a humid chamber. The sections were also stained for C3 deposition, C9 deposition and macrophage infiltration with an appropriate antibody, see below.

Purpose	**Antibody**	**Supplier**	**Concentration**	**Working dilution**
C3 deposition	Goat anti-rat C3c	Nordic laboratories	8mg/ml	1:400
C9 deposition	Sheep anti-human C9	In house	n/a, polyclonal antiserum	1:400
Macrophage infiltration	Mouse anti-rat CD68	Serotec	0.5mg/ml	1:400

In between antibody incubations, the slides were washed 3 times in PBS for 5 minutes each. Secondary antibodies at 0.5% (v/v) (donkey anti-mouse Ig FITC or donkey anti-goat/sheep Ig FITC both Jackson) were incubated with the slides in PBS 1% (w/v) BSA for 40 minutes at room temperature in a humid chamber. Following washings in PBS the sections were mounted in Vectashield (Vector Laboratories) to prevent fading of the fluorescent signal and analysed on a fluorescent microscope.

To assess inflammatory infiltration by Haematoxylin and Eosin (H and E) staining, the isolated soleus muscle was fixed in 10% (v/v) formaldehyde overnight at 4°C and paraffin-wax embedded by the Department of Histology. The embedded muscle was cut into 8μm sections and transferred to glass slides. Sections were dried at room temperature for at least an hour followed by 60°C overnight. The sections were placed in 100% xylene for 10 minutes followed by fresh xylene for 5 minutes to remove the paraffin. Sequential 5 minute incubations in 100% ethanol, 95% (v/v)

ethanol, 70% (v/v) ethanol and tap water were carried out. Sections were stained with Harris' Haematoxylin for 5 minutes, 'blued' in Scott's tap water and then washed in ordinary tap water. The staining was differentiated in 1% acid alcohol (70% (v/v) ethanol, 0.04% (v/v) hydrochloric acid) for 1-2 seconds and washed in tap water. The sections were stained in Eosin for 2 minutes, washed in tap water, dehydrated by sequential 5 minute incubations in 70% (v/v) ethanol and 95 % (v/v) ethanol, cleared in 100% xylene for 10 minutes and mounted with XAM neutral mounting medium (BDH).

Chapter 3: Generation of recombinant forms of rat Crry.

3.1 Introduction

Since its emergence in the 1970's recombinant DNA technology has enabled enormous advances to occur in the understanding of gene structure and protein function, and it has revolutionised protein production. To produce recombinant proteins the relevant piece of DNA can be amplified using the polymerase chain reaction (PCR). This DNA can be cut (restricted) and ligated into an expression vector using enzymes. The vector can then be used to transfect a host system, which is used to produce large quantities of the protein of interest. The protein of interest can then be purified and used in protein interaction studies or in the case of this project as a therapeutic reagent.

Recombinant DNA technology has revolutionised the possibilities for drugs as large amounts of pure protein can be created and purified. Such technology has been used in many situations such as the generation of tissue plasminogen activator for administration after a myocardial infarction, the production of insulin for administration in type I diabetes and the generation of factor VIII to treat haemophiliacs (Lubiniecki and Lupker 1994). A number of different host systems are available including bacteria, yeast and eukaryotic cells. Each system has its own advantages and disadvantages and the choice of system depends upon the protein to be produced and final use of the protein. For this project, eukaryotic cells have been employed to generate all the proteins. In particular Chinese Hamster Ovary (CHO) cells were used. This host was chosen despite the lower production rates compared to bacteria, as eukaryotic cells carry out appropriate post-translational modifications, glycosylate and correctly fold proteins, where as bacteria do not. These post-translational modifications may be important when generating a therapeutic as different modifications may cause proteins to have neoepitopes which are immunogenic and result in the reagent's enhanced clearance and/or a reduction in function. Glycosylation is also crucial to producing Fc fusion proteins as without correct glycosylation Fc fail to fold correctly and function (Leatherbarrow, Rademacher et al. 1985; Tao and Morrison 1989; Walker, Lund et al. 1989). The

ability of eukaryotic cells to carry out post-translational modifications including folding also makes the downstream processes of purification easier.

For this project the aim was to generate two kinds of Crry-Fc: one that functioned effectively in its original form to inhibit complement, and a second that could be further engineered into a 'prodrug' (see section 1.5.6.4). This latter form would need the active site to be close to the Fc so that the function of the Crry would be sterically hindered. The functional site of rat Crry has not yet been identified however, it is known that the first 4 N-terminal SCRs are active when expressed as a soluble protein (Fraser, Harris et al. 2002). To identify the SCRs of rat Crry in which function resides and to identify suitable constructs for the above reagents, SCR deletion mutants were generated consisting of the N-terminal SCRs. The reagents generated were 5SCR-Fc, 4SCR-Fc, 3SCR-Fc, 4SCR and 3SCR. The Fc fusion proteins and non-Fc proteins were generated so that any reduction in function could either be attributed to the deletion of an active site or steric restriction from the Fc.

The Fc used to generate these proteins was the Fc of rat IgG2a. Use of a rat Fc should result in the generation of a less or non-immunogenic molecule compared to those containing a human Fc used for preliminary studies (Harris, Williams et al. 2002). IgG2a has a very short upper hinge, the polypeptide stretch between the end of CH1 and the first cysteine of the inter-heavy chain disulphide bond (Schneider, Wensel et al. 1988; Tan, Shopes et al. 1990; Brekke, Michaelsen et al. 1995). The length of the upper hinge in part dictates antibody flexibility, a shorter hinge being less flexible. A lack of flexibility in a Crry-Fc protein will maximise steric hindrance and be advantageous in the generation of a 'prodrug'.

The reagents generated in this chapter contain the N-terminal SCRs of Crry with the numbering beginning with the SCR at the N-terminus. Therefore 5SCR-Fc contains the 5 N-terminal SCRs of rat Crry fused to the Fc of rat IgG2a, 4SCR-Fc contains only the 4 N-terminal SCRs fused to the Fc while 3SCR-Fc contains the 3 N-terminal SCRs and the Fc. Similarly 4SCRs and 3SCRs contain the 4 and 3 N-terminal SCRs of Crry without the Fc (figures 3.1 and 3.2).

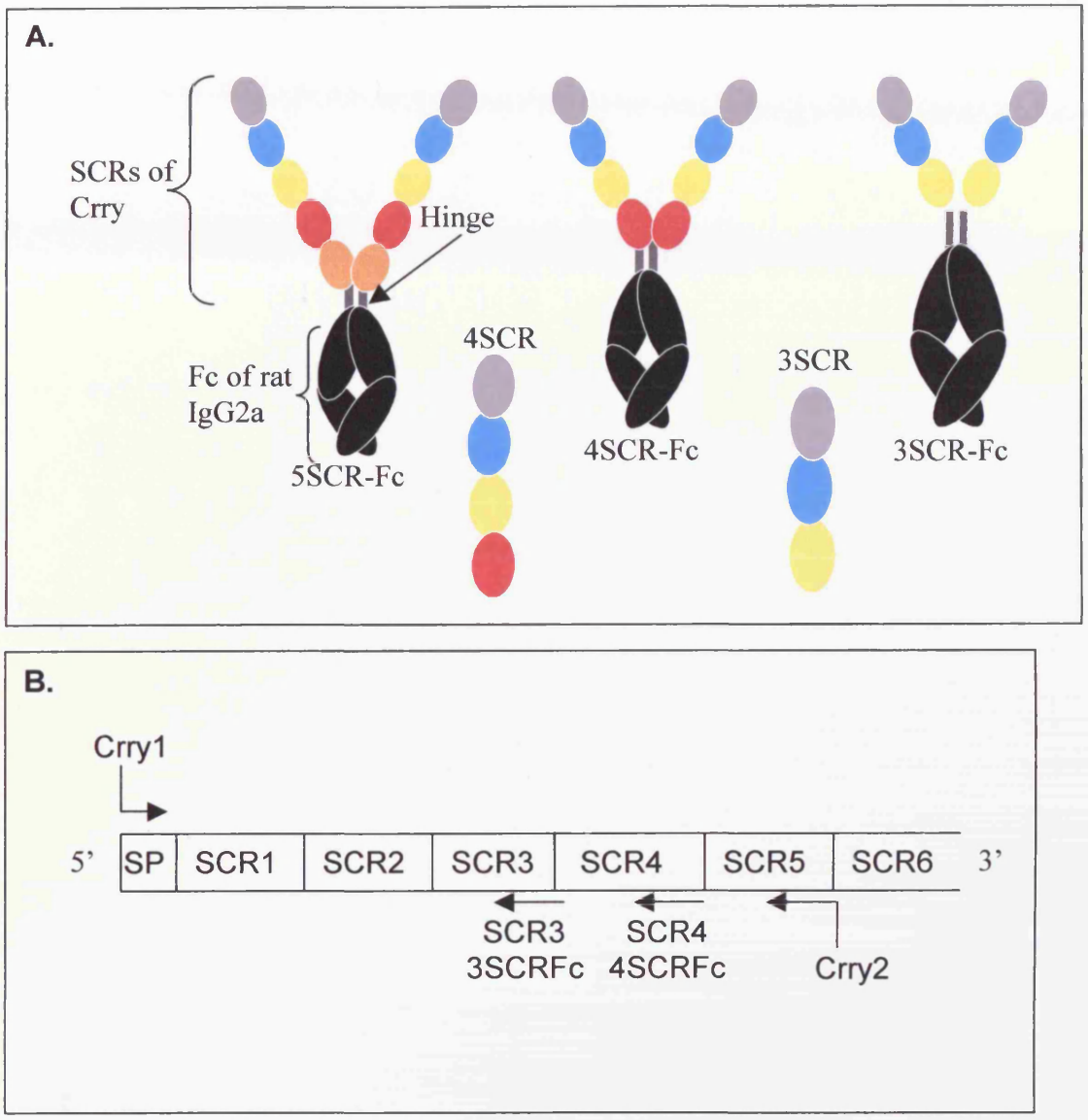


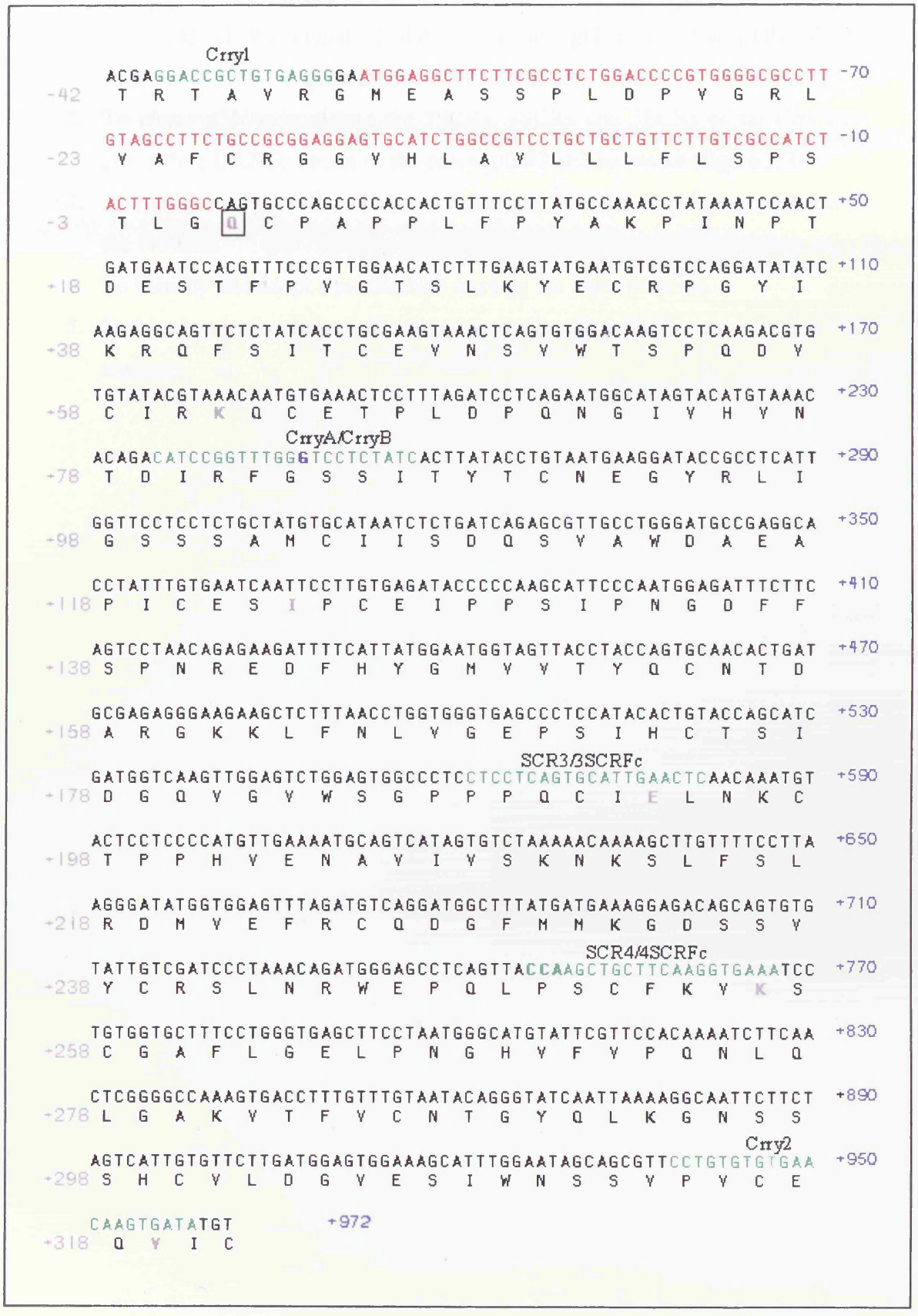
Figure 3.1 Crry reagents generated.

A. Schematic of the Crry reagents generated. Fc fusion proteins containing 5, 4 and 3 SCRs of rat Crry were generated and non-Fc proteins containing the 4 and 3 SCRs of rat Crry. The Fc fusion proteins consisted of the rat Fc, linked to SCRs of Crry through the antibody hinge.

B. Representation of DNA encoding Crry showing the position of the primers (arrows) used to amplify the Crry proteins. For all reagents primer Crry1 was used. This primer was used along with primers Crry2, 4SCRFc, 3SCRFc, SCR4 or SCR3 to generate 5SCR-Fc, 4SCR-Fc, 3SCR-Fc, 4SCR or 3SCR respectively. Primers used to generate 4SCR and 3SCR included a stop codon, more details are given in table 2.1. SP signal peptide, SCR short consensus repeat.

Figure 3.2 Nucleotide and amino acid sequence of the first five N-terminal SCRs of rat Crry.

The DNA sequence of the first 5 N-terminal SCRs of rat Crry and the amino acid sequence of the polypeptide is shown opposite. The nucleotide number is given in blue on the right hand side with the first nucleotide of the mature protein assigned to position 1, the amino acid number is given in grey on the left hand side, the first amino acid of the mature protein is assigned to position 1 (boxed), sequence encoding the signal peptide is highlighted in red, the introduced mutation is shown in bold in blue and the SCR divisions are shown by the first amino acid of each SCR in bold in grey. The positions of the primers are shown in green in the nucleotide sequence, and are labelled above. Primers SCR3 and 3SCRFc contained the same sequence except for the presence of a stop codon within the SCR3 primer. Primers SCR4 and 4SCRFc also contained the same sequence except for additional nucleotides in the 4SCRFc primer, shown in bold, and primer SCR4 contained a stop codon. Primers CrryA and CrryB used to clone the 5SCRs of Crry in the two step PCR were complementary, CrryA was the sense primer whilst CrryB was the antisense primer.


Crry1
-70
-42 T R T A V R G M E A S S P L D P V G R L
-10
-23 V A F C R G G V H L A V L L L F L S P S
+50
-3 T L G Q C P A P P L F P Y A K P I N P T
+110
+18 D E S T F P V G T S L K Y E C R P G Y I
+170
+38 K R Q F S I T C E V N S V W T S P Q D V
+230
+58 C I R K Q C E T P L D P Q N G I V H V N
CrryA/CrryB
+290
+78 T D I R F G S S I T Y T C N E G Y R L I
+350
+98 G S S S A M C I I S D Q S V A W D A E A
+410
+118 P I C E S I P C E I P P S I P N G D F F
+470
+138 S P N R E D F H Y G M V V T Y Q C N T D
+530
+158 A R G K K L F N L V G E P S I H C T S I
SCR3/3SCRFc
+590
+178 D G Q V G V W S G P P P Q C I E L N K C
+650
+198 T P P H V E N A V I V S K N K S L F S L
+710
+218 R D M V E F R C Q D G F M M K G D S S Y
SCR4/4SCRFc
+770
+238 Y C R S L N R W E P Q L P S C F K V K S
+830
+258 C G A F L G E L P N G H V F Y P Q N L Q
+890
+278 L G A K V T F V C N T G Y Q L K G N S S
Crry2
+950
+298 S H C V L D G V E S I W N S S V P V C E
+972
+318 Q Y I C

The aims of the work described in this chapter are:

1. To clone cDNA encoding the Fc of rat IgG2a into the pDR2ΔEF1α expression vector to generate the pDR2-Fc vector.
2. To clone cDNAs encoding the 3SCRs, 4SCRs and 5SCRs of rat Crry into either the pDR2-Fc vector or the parent pDR2ΔEF1α vector (figure 3.3).
3. To transfect CHO cells with these constructs in order to obtain expression of the proteins.
4. To identify and adopt a purification strategy for these proteins.
5. To biochemically characterise the proteins by SDS-PAGE and Western blot analysis.

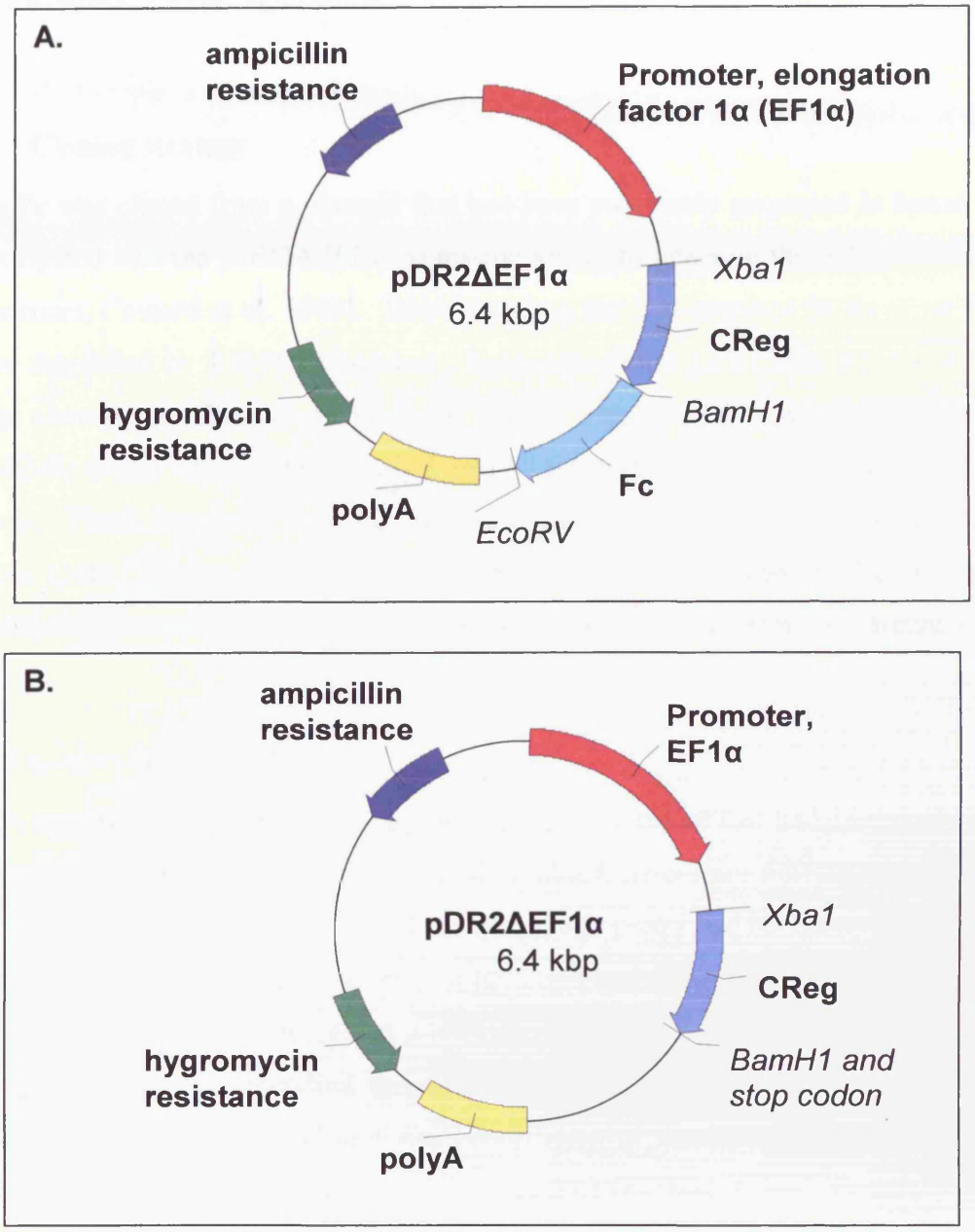


Figure 3.3 Schematic of the plasmids used to generate the Crry constructs.

A. To generate the Crry-Fc reagents, the Crry cDNA was ligated into a plasmid in frame with the cDNA sequence encoding the Fc portion of an antibody. The Crry cDNA and Fc were joined together by the common BamH1 digestion site.

B. To generate the non-Fc reagents the cDNA was ligated into a vector without cDNA encoding the Fc, the cDNA terminated by a stop codon.

3.2 Specific methods and results

3.2.1 Cloning strategy

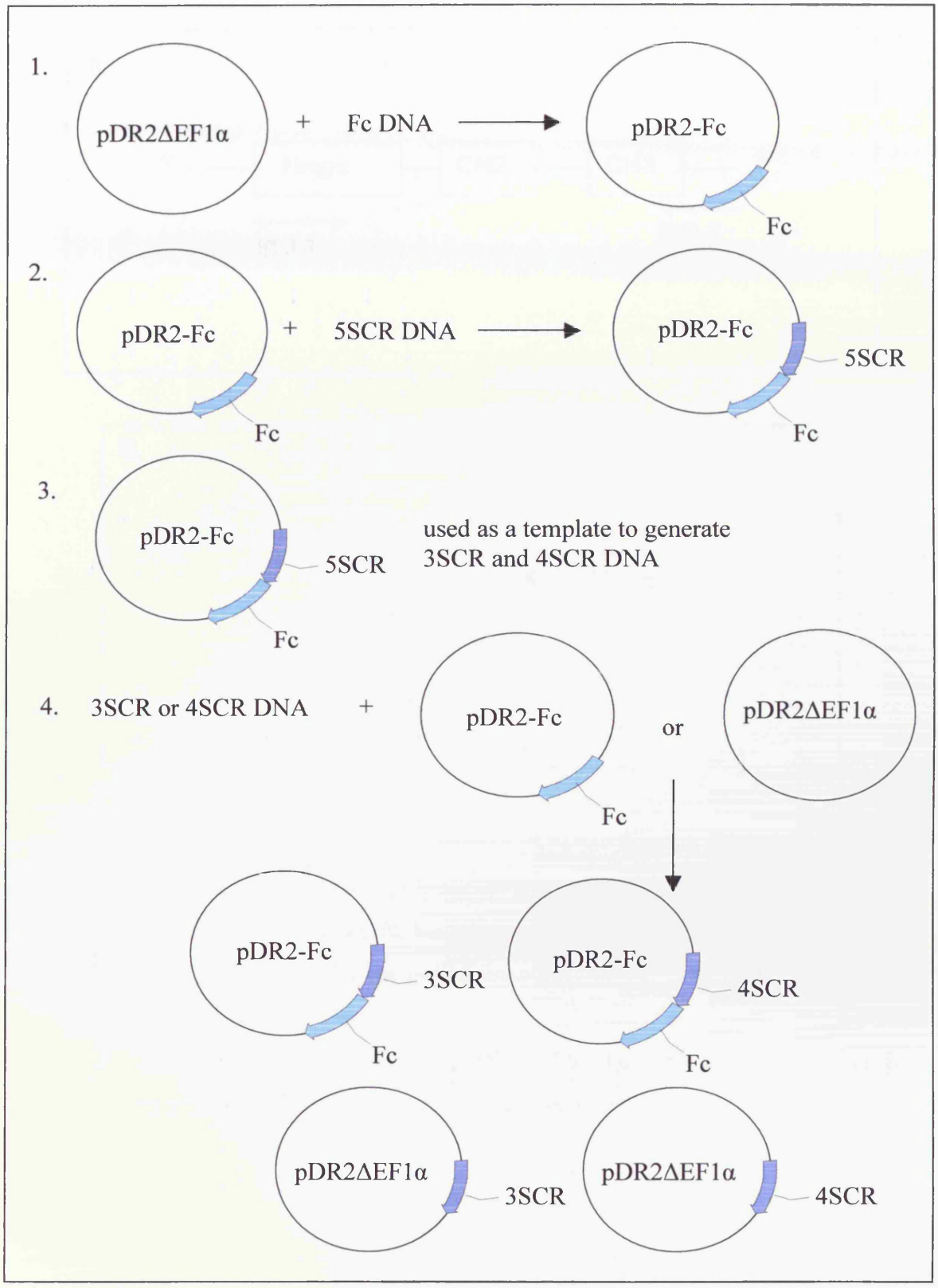
The Fc was cloned from a plasmid that had been previously generated in house and was ligated into the pDR2ΔEF1α expression vector to generate the pDR2-Fc vector (Charreau, Cassard et al. 1994). DNA encoding the 5 N-terminal SCRs of rat Crry were amplified by RT-PCR from rat oligodendroglioma cell (33B) RNA. A two stage cloning step eliminated an internal BamH1 site. This DNA was ligated into the pDR2-Fc vector which subsequently formed a template for PCR of the 3 and 4 SCR forms. These fragments were ligated into either the pDR2-Fc vector or the parent vector (pDR2ΔEF1α) to generate an expression system for either the Fc or non-Fc proteins respectively. The cloning strategy is pictorially demonstrated in figure 3.4.

3.2.2 Generation of the Fc.

DNA encoding the Fc of IgG2a was amplified from a vector that had previously been engineered in house to contain the Fc; this enabled the correct restriction sites to be introduced into the construct. The DNA within this vector had been amplified from a hybridoma line secreting monoclonal rat IgG2a. The Fc was amplified using primers IgG.1 and IgG.2, IgG.1 contained a BamH1 restriction digest site at the 5' end and overlapped the DNA encoding the hinge region while IgG.2 contained an EcoRV restriction site at the 3' end and was downstream of the DNA encoding the CH3 region after the stop codon (figure 3.5A and 3.6) (method 2.1.4). Once the PCR product had been viewed by agarose gel electrophoresis (figure 3.5B), it was purified, digested with the relevant restriction enzymes and ligated into pDR2ΔEF1α (method 2.1.5- 2.1.8). Chemically competent DH5α were then transformed using this vector by heat shock and bacterial colonies were screened by PCR for expression of the insert (method 2.1.10). A positive bacterial colony was selected, grown up and the plasmid purified by a QIAquick® spin miniprep purification kit (Qiagen) (method 2.1.11). The fidelity of the PCR reaction was checked by sequencing the product (method 2.1.12).

Figure 3.4 The cloning strategy used to generate recombinant Crry.

1. The DNA encoding the Fc was ligated into the pDR2ΔEF1α vector to create the pDR2-Fc vector.
2. DNA encoding the 5SCRs of rat Crry was ligated into the pDR2-Fc vector.
3. The 5SCR-Fc vector was used as a template to generate the DNA encoding the 3 and 4 SCRs of rat Crry.
4. The DNA encoding the 3 and 4 SCRs was ligated into either the pDR2ΔEF1α vector or the pDR2-Fc vector resulting in the generation of 4 different vectors.


1.
pDR2ΔEF1α
+
Fc DNA
pDR2-Fc
Fc
2.
pDR2-Fc
+
5SCR DNA
pDR2-Fc
5SCR
Fc
Fc
3.
pDR2-Fc
5SCR
Fc
used as a template to generate
3SCR and 4SCR DNA
4.
3SCR or 4SCR DNA
+
pDR2-Fc
Fc
or
pDR2ΔEF1α
pDR2-Fc
3SCR
Fc
pDR2-Fc
4SCR
Fc
pDR2ΔEF1α
3SCR
pDR2ΔEF1α
4SCR

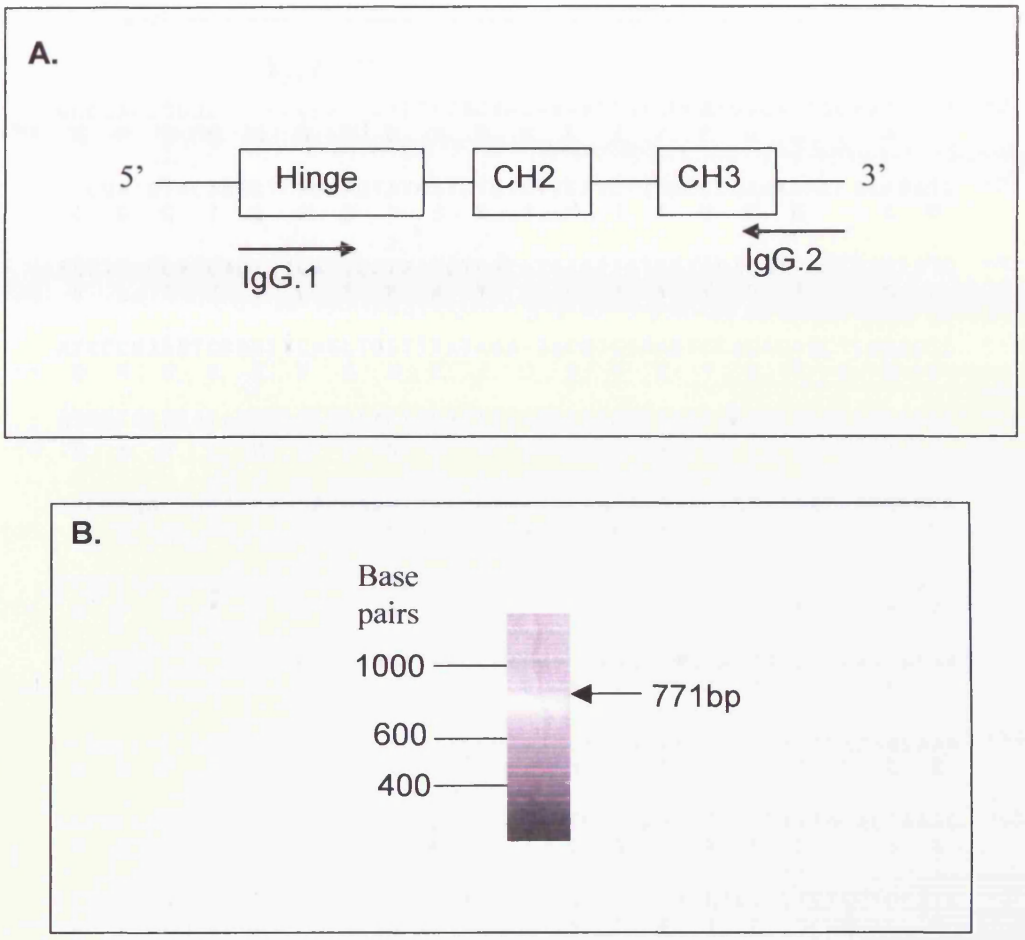


Figure 3.5 Amplification of the Fc of rat IgG2a for ligation into pDR2ΔEF1α.

A. Representation of the DNA encoding the Fc showing the positions of the primers used to clone the Fc. Primers IgG.1 and IgG.2, shown as arrows, were used to amplify the cDNA encoding the Fc. The product included a stop codon at the 3'end.

B. A band of 771bp encoding the cDNA of the Fc of rat IgG2a was amplified by PCR and visualised by agarose gel electrophoresis.

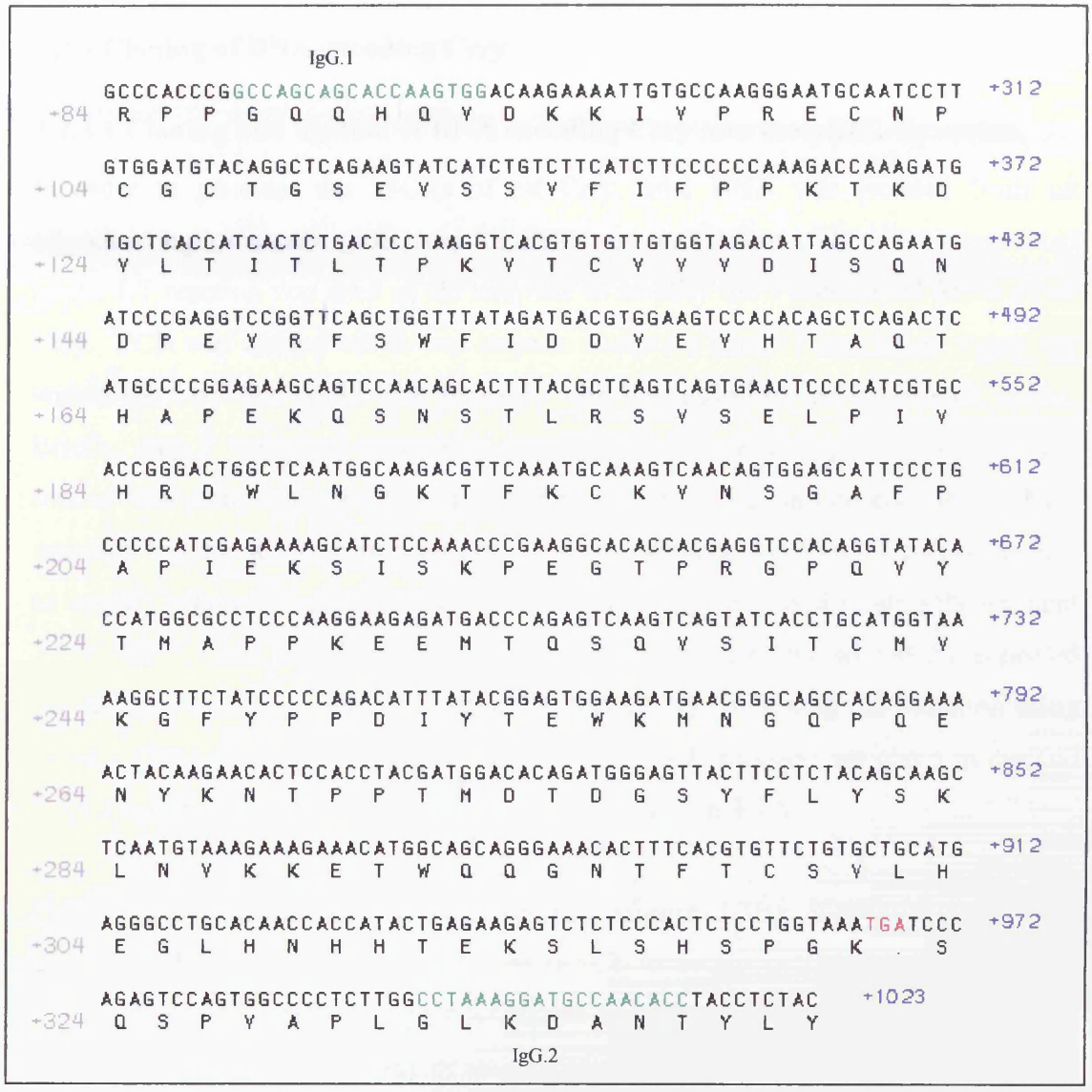


Figure 3.6 Nucleotide and amino acid sequence of the Fc of rat IgG2a.

The sequence of the Fc DNA construct cloned into pDR2ΔEF1α and the amino acid sequence of the polypeptide is shown above. The nucleotide number is given in blue with the first nucleotide of the mature protein assigned position 1, the amino acid number is given in grey, the first amino acid of the CH1 domain is assigned position 1. The positions of the primers are shown in green in the nucleotide sequence; IgG.1 is labelled above the nucleotide sequence while IgG.2 is labelled below the amino acid sequence. The stop codon in highlighted in red.

3.2.3 Cloning of DNA encoding Crry

3.2.3.1 Cloning and ligation of DNA encoding Crry into the pDR2-Fc vector.

In order to generate the 5SCRs of rat Crry, total RNA was isolated from rat oligodendroglioma cells (33B) and RT-PCR was undertaken. The cDNA generated by the RT reaction was used as the template to amplify the 5 N-terminal SCRs of rat Crry. PCR was carried out in two steps to remove a BamH1 restriction digest site within the Crry that could later interfere with generation of the expression vector. Briefly, step 1 was used to amplify the DNA in two sections; the 3' end of one section was complementary to the 5' end of the other section and contained a base mutation to remove the restriction site. In step 1 primers Crry1 and CrryA were used to amplify segment 1 while primers CrryB and Crry2 were used to amplify segment 2. In step 2, these complementary regions were allowed to anneal and the annealed product was used to amplify the entire 5SCRs of Crry containing the mutation using primers Crry1 and Crry2. Further details of this PCR reaction are given in method 2.1.4.3 and the PCR is pictorially demonstrated in figure 3.7A.

Following purification of the PCR product (figure 3.7B), it was digested with BamH1 and Xba1 and ligated into the pDR2-Fc vector in frame with the Fc of IgG2a. The common BamH1 restriction site in both the Fc and Crry cDNA formed the link between the DNA fragments. Following digestion and ligation all cloning reactions described here and subsequently were treated in the same way: DH5α chemically competent bacteria were transformed with the vector by heat shock and bacterial colonies were screened by PCR. A positive bacterial colony was selected, grown up and the DNA was purified using a QIAprep® miniprep spin purification kit (Qiagen) (method 2.1.6- 2.1.12). The sequences were checked for fidelity.

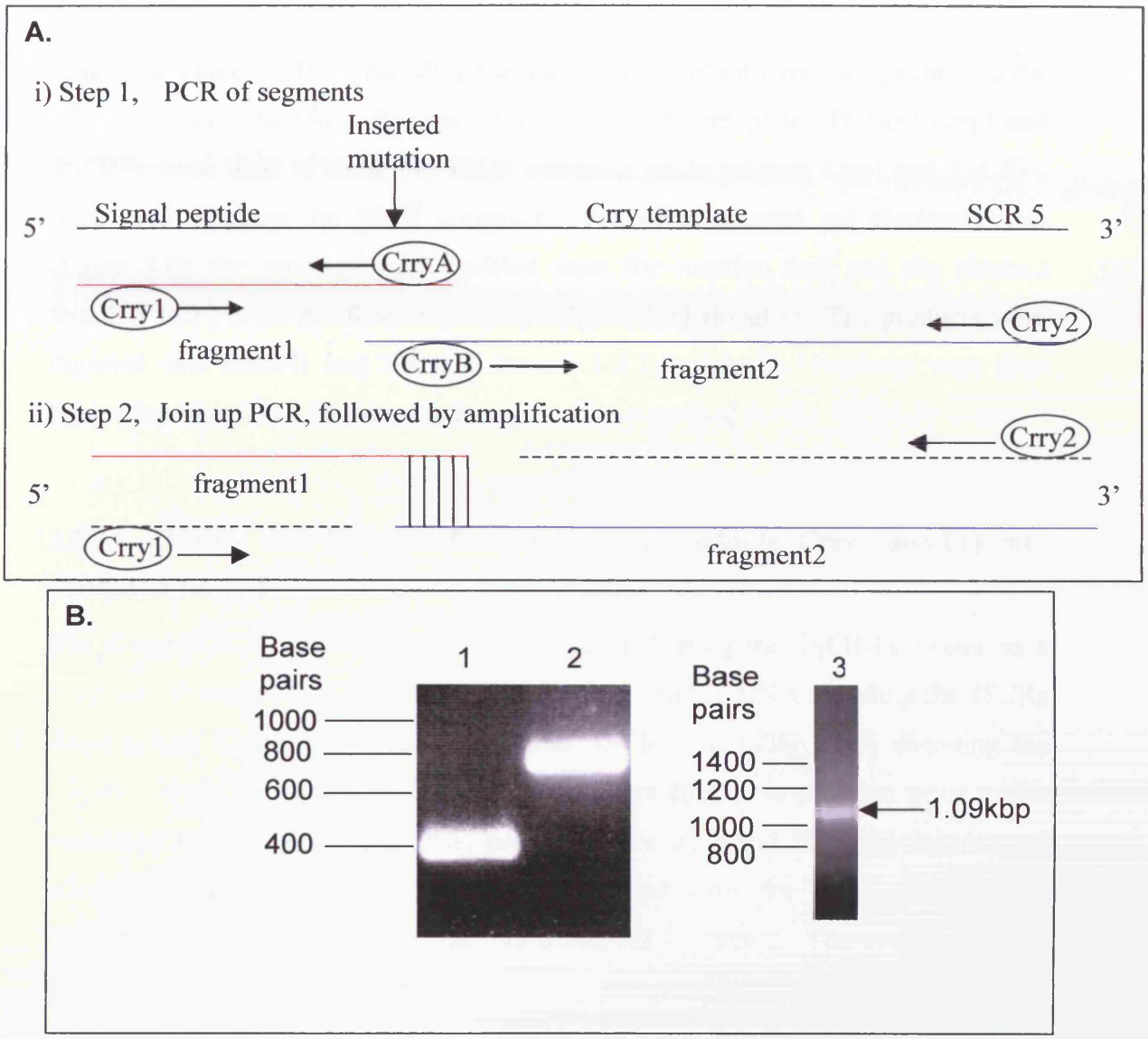


Figure 3.7 Amplification of 5SCRs Crry.

A. In step 1 of the PCR to generate 5SCRs Crry cDNA, two primers were used to amplify fragment 1 (Crry1 and CrryA) whilst two other primers were used to amplify fragment 2 (CrryB and Crry2). In step 2 of the PCR, DNA fragments 1 and 2 were allowed to anneal to each other through the complementary region. Full length cDNA encoding the 5SCRs of Crry was generated in a PCR reaction using primers Crry1 and Crry2

B. In step 1 bands of 383bp and 708bp encoding fragments 1 and 2, respectively, were amplified by PCR and visualised by gel electrophoresis (lanes 1 and 2). In step 2 a band of 1.09kbp encoding the full length 5SCRs cDNA was amplified by PCR and also visualised by gel electrophoresis (lane 3).

In order to clone the DNA encoding the 4 and 3 SCRs of rat Crry for ligation into the pDR2-Fc vector the 5SCR-Fc plasmid was used as the template. Primers Crry1 and 4SCRFc were used to clone the 4SCR construct while primers Crryl and 3SCRFc were used to clone the 3SCR construct. Following agarose gel electrophoresis (figure 3.8), the products were purified from the reaction mix and the plasmid template using a gel purification kit (Geneclean III, QBiogene). The products were digested with BamH1 and Xba1 as for the 5SCR construct. Products were then ligated into the pDR2-Fc vector and processed as described for the 5SCR-Fc vector.

3.2.3.2 Cloning and ligation of DNA encoding soluble Crry (non-Fc) into pDR2ΔEF1α.

The 4 and 3 SCR Crry constructs were generated using the 5SCR-Fc vector as a template. Primers Crry1 and SCR4 were used to amplify DNA encoding the 4SCRs while primer Crry1 was used with primer 3SCR to amplify DNA encoding the 3SCRs. Both 3' primers, 3SCR and 4SCR, contained a stop codon prior to the restriction enzyme site. The PCR products were extracted from the agarose gel (Geneclean III, QBiogene) (figure 3.8), digested with the Xba1 and BamH1 restriction enzymes and ligated into the pDR2ΔEF1α vector. The constructs were processed as described previously.

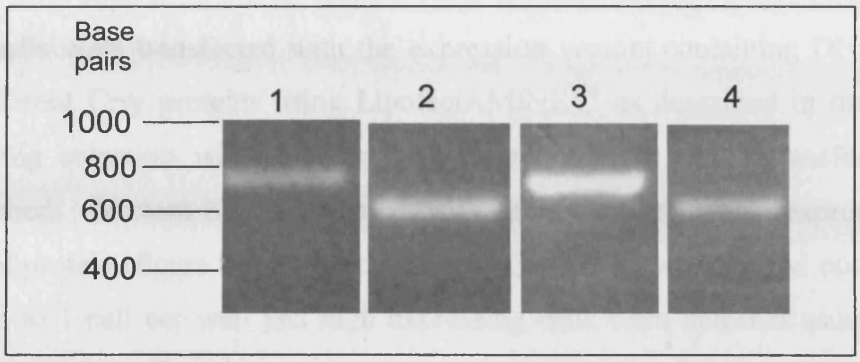


Figure 3.8 Amplification of DNA encoding the 4 and 3SCR forms of Crry for ligation into the pDR2-Fc vector or the pDR2ΔEF1α vector.

cDNA encoding the 4SCRs and 3SCRs of rat Crry to create the Fc and non-Fc constructs were amplified by PCR and visualised by agarose gel electrophoresis. For ligation into the pDR2-Fc vector, amplification of the 4SCR gave a product of 768bp, lane 1, while amplification of 3SCR gave a product of 582bp (lane 2). In order to generate the non-Fc proteins the antisense primers for both 3SCR and 4SCR contained an additional stop codon, this gave a product of 771bp for 4SCR, lane 3, and for 3SCR a product of 585bp, lane 4.

3.2.4 Transfection

CHO cells were transfected with the expression vectors containing DNA encoding the different Crry proteins using LipofectAMINE^{TM} as described in method 2.2.2. Following selection with 400μg/ml of Hygromycin B stable transfectants were established. Western blot analysis of cell supernatant confirmed expression of the relevant protein (figure 3.9). The transfected CHO cells were cloned out by limiting dilution to 1 cell per well and high expressing cells were detected using an ELISA (method 2.4.9). The clones with the greatest secretion levels were selected and expanded. Cell culture supernatant was collected from tissue culture flasks or the adopted tissue culture growing system (see chapter 5) and a method of purifying the proteins was established.

3.2.5 Optimisation of Purification

There were a number of different possible methods for purifying the proteins so these were compared to find the most efficient method, which gave a high purity product in high yields with the least number of steps. The methods were all investigated using 5SCR-Fc cell culture supernatant. The first approach was to purify the Fc proteins via the Fc group, using either protein A or protein G chromatography. Methods using classical column chromatography were also investigated as a method of purifying both the Fc and non-Fc proteins. Antibody affinity methods were also tested and finally optimised.

3.2.5.1 Protein A

Many antibody Fc domains have a high affinity for protein A. However, the literature documents that rat IgG2a Fc has a very low affinity for protein A (Akerstrom, Brodin et al. 1985). Nevertheless, affinity of certain Fc domains for protein A can be increased by using high salt and/or high pH binding buffers (Palmer 2002). To investigate whether such an approach could be used to purify 5SCR-Fc, cell culture supernatant was mixed 1: 1 with the appropriate binding buffer (1.5M glycine/NaOH, 3M NaCl pH 9) and passed over a protein A column (ProSep A,

Millipore). The column was washed and bound protein was eluted using the elution buffer (table 2.3). Fractions containing protein were identified by their absorbance at 280nm, combined, dialysed against PBS and concentrated using an Amicon ultrafiltration cell. Although protein was eluted from the protein A column, SDS-PAGE analysis revealed that the protein consisted of multiple bands, Western blotting revealed that none of these bands were 5SCR-Fc (figure 3.10).

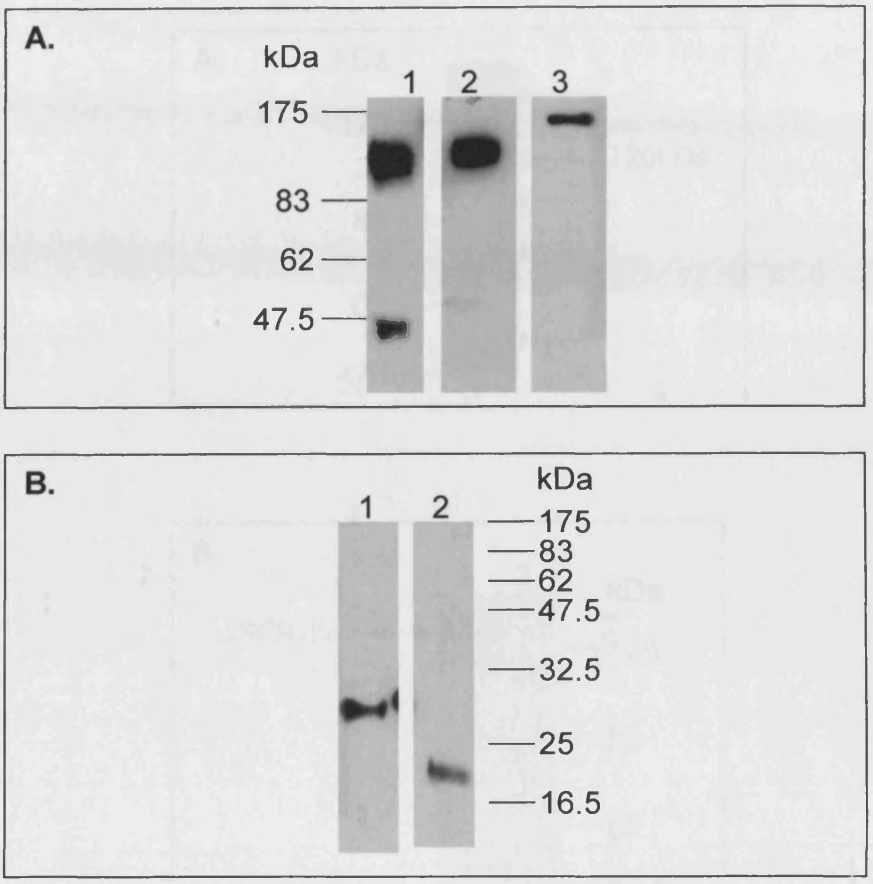


Figure 3.9 Western blot analysis of cells expressing Crry proteins.

A. Supernatants harvested from CHO cells expressing 5SCR-Fc, 4SCR-Fc and 3SCR-Fc, proteins were separated on a 7.5 % gel and Western blotted onto nitrocellulose. The Western was probed with anti-rat IgG HRPO. 3SCR-Fc (1) gave a band of 120kDa with a smaller form visible at 40kDa. 4SCR-Fc (2) gave a band of 140kDa and 5SCR-Fc (1) gave a band of 160kDa.

B. The expression of the non-Fc proteins was analysed by loading an 11% gel with supernatant taken from transfected CHO cells expressing 4SCR and 3SCR Crry. Following electrophoresis the gels were Western blotted and probed with TLD1C11, a monoclonal anti-Crry antibody, followed by anti-mouse IgG HRPO. 4SCR (1) gave a band at 28kDa whilst 3SCR (2) gave a band at 21kDa.

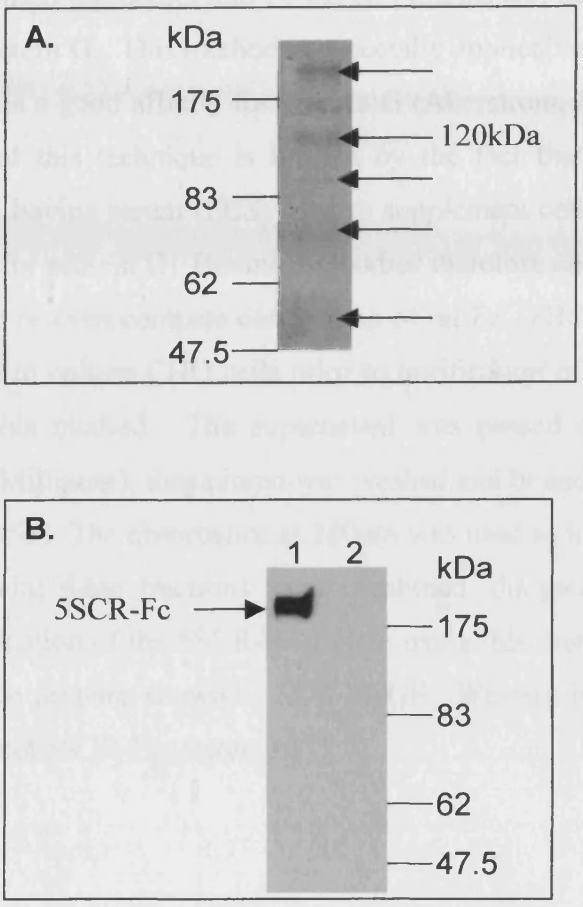


Figure 3.10 SDS PAGE analysis of the protein A product.

A. The product from the elution of the protein A column was resolved on a 7.5 % gel and Coomassie stained. The product was a mix of proteins (arrows) consisting of a protein that only just entered the gel, a protein with a mass of 120kDa and a number of smaller proteins.

B. To test whether the product contained 5SCR-Fc, protein was resolved on a 7.5 % gel (lane 2) along side 5SCR-Fc supernatant (lane 1) as a positive control. Following Western blotting, onto nitrocellulose it was probed with anti-rat IgG HRPO. 5SCR-Fc was detected in the supernatant showing that the probing and development of the Western was successful but no 5SCR-Fc was detected in the product revealing that 5SCR-Fc was not purified using protein A chromatography.

3.2.5.2 Protein G

As with protein A, many antibodies and Fc fusion proteins can be purified due to the affinity of Fc for protein G. This method is especially applicable to rat IgG2a as the Fc of this isotype has a good affinity for protein G (Akerstrom, Brodin et al. 1985). However, the use of this technique is limited by the fact that bovine antibodies present in the foetal bovine serum (FBS) used to supplement cell culture media also have a high affinity for protein G. Bovine antibodies therefore co-purify and become a major contaminant or even compete out binding of rat Fc. FBS depleted of bovine antibodies was used to culture CHO cells prior to purification of 5SCR-Fc from the supernatant using this method. The supernatant was passed over the protein G column (ProSep G, Millipore), the column was washed and bound protein was eluted using the elution buffer. The absorbance at 280nm was used to identify the fractions that contained protein; these fractions were combined, dialysed against PBS and concentrated. Purification of the 5SCR-Fc protein using this method gave a product consisting of multiple proteins shown by SDS-PAGE. Western blotting showed that these proteins were not 5SCR-Fc (figure 3.11).

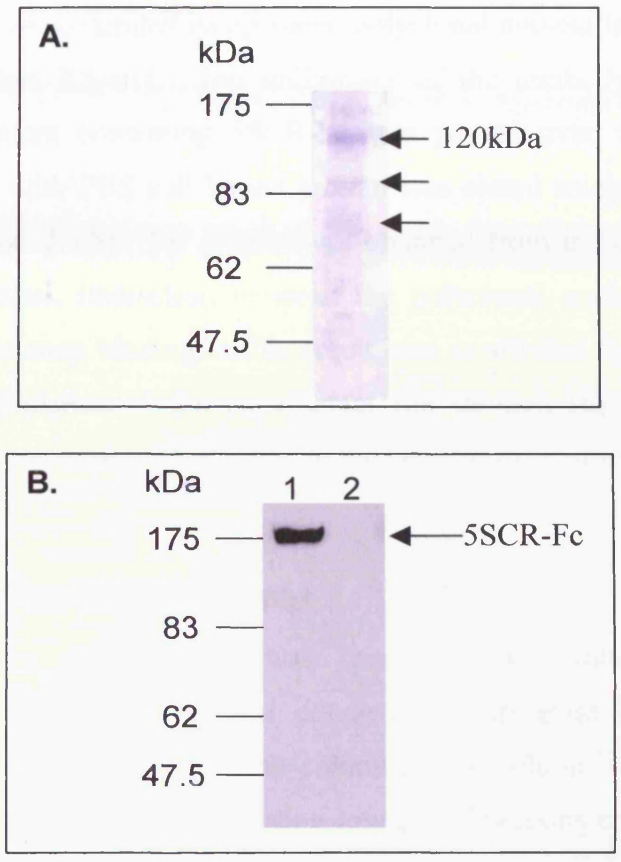


Figure 3.11 SDS PAGE of the protein G product.

A. The product eluted from the protein G column was resolved on a 7.5% gel and stained with Coomassie. The product consisted of a number of proteins (arrows), the most prevalent of which was at 120kDa.

B. To ascertain if the elution contained 5SCR-Fc, the product (lane 2) and 5SCR-Fc cell culture supernatant (lane 1) to act as a positive control were resolved on a 7.5 % gel. Following Western blotting, it was probed with anti-rat IgG HRPO. The positive control developed but the product from the protein G column did not indicating that 5SCR-Fc was not purified by protein G.

3.2.5.3 Anti-rat immunoglobulin affinity chromatography

An affinity column was generated using sheep polyclonal anti-rat immunoglobulins (Binding site) (method 2.3.4.1). Ten milligrams of the antibody was bound to Sepharose. Supernatant containing 5SCR-Fc was passed over the column, the column was washed with PBS and bound protein was eluted using the appropriate elution buffer (method 2.3.5). No protein was obtained from the elution; this was probably due to a weak interaction between the polyclonal antibody and ligand resulting in low efficiency binding. This result was confirmed by ELISA, which showed very little depletion of the supernatant run through (figure 3.11). Any protein that did bind was most probably lost in the wash of the column.

3.2.5.4 Anti-Crry affinity chromatography

Monoclonal anti-Crry (TLD1C11) was purified by protein G affinity chromatography. Ten milligrams was coupled to Sepharose and supernatant containing 5SCR-Fc was passed over the column. The column was washed with PBS and the bound protein was eluted using low pH. Fractions containing protein were identified and processed as with the previous methods. This method resulted in the purification of 5SCR-Fc (figure 3.12A). However, the yield was low with each run producing 0.5mg of protein. An ELISA (method 2.4.9) using serial dilutions of supernatant from before the column and run through revealed that the low yield was not due to low expression levels of the protein but a low capacity of the column as the purification did not deplete the supernatant of 5SCR-Fc (figure 3.12C). Another antibody affinity column was generated using a different monoclonal anti-Crry antibody, 5I2, which had previously been purified in house. An 8mg column was generated and used to purify 5SCR-Fc from cell culture supernatant following the above protocol. This column also resulted in the purification of 5SCR-Fc, SDS-PAGE analysis revealed that the eluted protein was pure (figure 3.12B). However, the yield (600μg) was also low using this column. As the yield was low, antibody affinity methods appeared unlikely to generate the large amount of Crry proteins required for this project. This project requires at least 40mg of protein to enable therapeutic testing and functional characterisation. Whilst these methods gave pure protein, to achieve the amount needed multiple purification runs would be necessary.

Therefore classical methods were investigated as a way of producing pure protein in high yields.

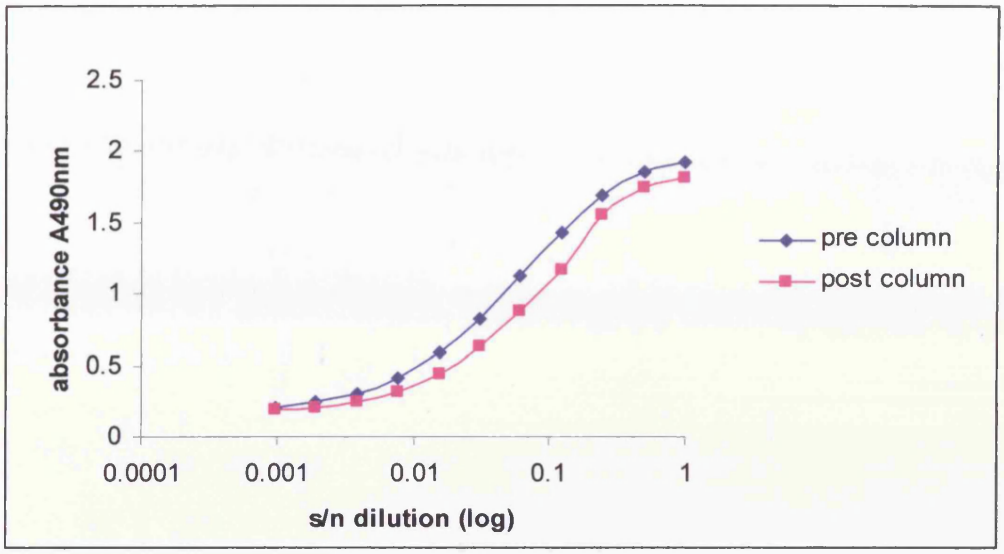


Figure 3.11 ELISA analysis of the purification of 5SCR-Fc by the anti-rat immunoglobulin (Ig) column.

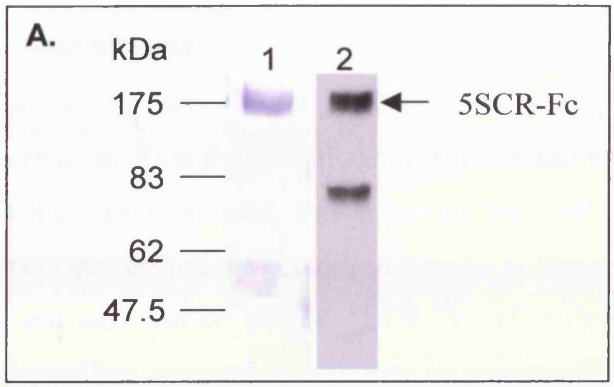
To assess the degree of supernatant depletion bought about by the anti-rat Ig column and hence the expected yield, an ELISA was carried out as follows. The plate was coated with TLD1C11, blocked and incubated with a sample of supernatant from before and after the column (1:2 serial dilutions). The plate was probed with anti-rat IgG HRPO. 5SCR-Fc was detected in both the pre column supernatant and the run through. Very little difference was seen between the two supernatants revealing that the lack of yield from this method was due to a lack of binding to the column and not low expression levels of the protein.

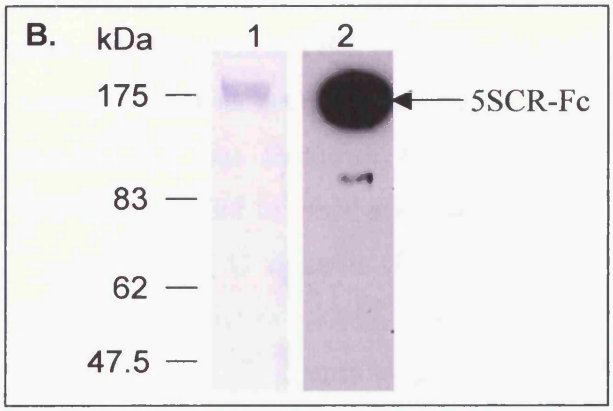
Figure 3.12 SDS PAGE and ELISA analysis of purification by anti-Crry chromatography.

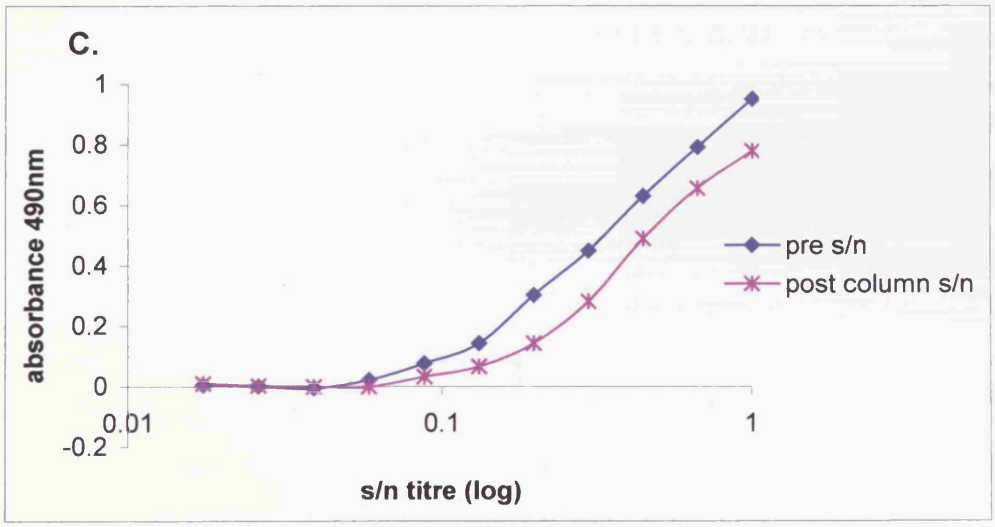
A. The product from the TLD1C11 anti-Crry affinity column was resolved on a 7.5% gel and Coomassie stained (lane 1). The product had a mass of 160kDa, the correct mass for 5SCR-Fc. To confirm that the product was 5SCR-Fc a Western blot was carried out probing with anti-rat IgG HRPO (lane 2). The band at 160kDa developed confirming that the product was 5SCR-Fc.

B. The product of the 5I2 column was separated on a 7.5% gel. Lane 1 was Coomassie stained and revealed a product of 160kDa, the correct mass for 5SCR-Fc. Lane 2 was Western blotted and probed with anti-rat IgG HRPO. A band developed confirming that the product was 5SCR-Fc.

C. To assess the degree of supernatant depletion by the TLD1C11 anti-Crry column an ELISA plate was coated with TLD1C11. Following blocking, the plate was incubated with a sample of supernatant from before the column and a sample of run through, both in a serial dilution. The plate was probed with anti-rat IgG HRPO and developed. 5SCR-Fc was detected in both the pre and post column supernatant revealing that the low yield was due to the low capacity of the column as opposed to low expression levels of 5SCR-Fc.


A.
kDa
1
2
175
83
62
47.5
5SCR-Fc

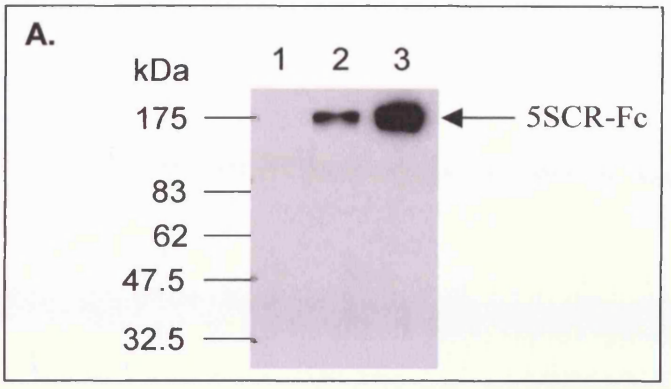

B.
kDa
1
2
175
83
62
47.5
5SCR-Fc


C.
absorbance 490nm
1
0.8
0.6
0.4
0.2
0
-0.2
0.01
0.1
1
s/n titre (log)
pre s/n
post column s/n

3.2.5.5 Classical chromatography

Both anion and cation exchange were investigated as methods for purifying 5SCR-Fc from cell culture supernatant. First the percentage of ammonium sulphate required to precipitate the 5SCR-Fc was optimised, this removed the bulk of contaminating protein and allowed resuspension of the protein in a small volume. A 40% saturated ammonium sulphate cut was carried out on 5SCR-Fc cell culture supernatant by gradual addition of crystalline ammonium sulphate to supernatant at 4°C. The cut was then increased to 45% and then to 50%. On each occasion the pellet was isolated by centrifugation and dissolved in PBS. Analysis of the cuts by SDS PAGE revealed that a 50% saturated cut was necessary to precipitate the 5SCR-Fc (figure 3.13A). A 50% saturated cut was therefore carried out on 500ml 5SCR-Fc supernatant and the pellet resuspended in 5mM sodium phosphate pH 8. The pellet (1.5mg) was loaded onto a Mono Q column (GE Healthcare) and the proteins separated by a segmented gradient (figure 3.13B). The proteins were separated using a gradient of 0-150mM NaCl over 25 column volumes followed by a gradient of 150-500mM NaCl over a further 20 column volumes. Analysis of the fractions by SDS PAGE revealed that whilst 5SCR-Fc was present in peaks from the column, the yield was low and it was not pure (figure 3.14A and B), the major contaminant appeared to be albumin. Adjusting the gradient to resolve the different proteins did not decrease the level of contamination.

Cibacron Blue, a dye which has an affinity for albumin, was employed as a method for removing the albumin contamination (Travis, Bowen et al. 1976; Leatherbarrow and Dean 1980). The fractions containing 5SCR-Fc from the Mono Q column were pooled and dialysed against 20mM sodium phosphate pH 7.1. This was then passed over the Cibacron Blue column (Bio Rad) and the protein run through collected in fractions. The column was then washed with 1.4M NaCl in 20mM sodium phosphate at pH 7.1 to remove the albumin. SDS PAGE analysis of the eluted fractions revealed that the 5SCR-Fc protein also bound to the column and was eluted with the albumin (figure 3.14C). This method was therefore not suitable for removing albumin contamination.



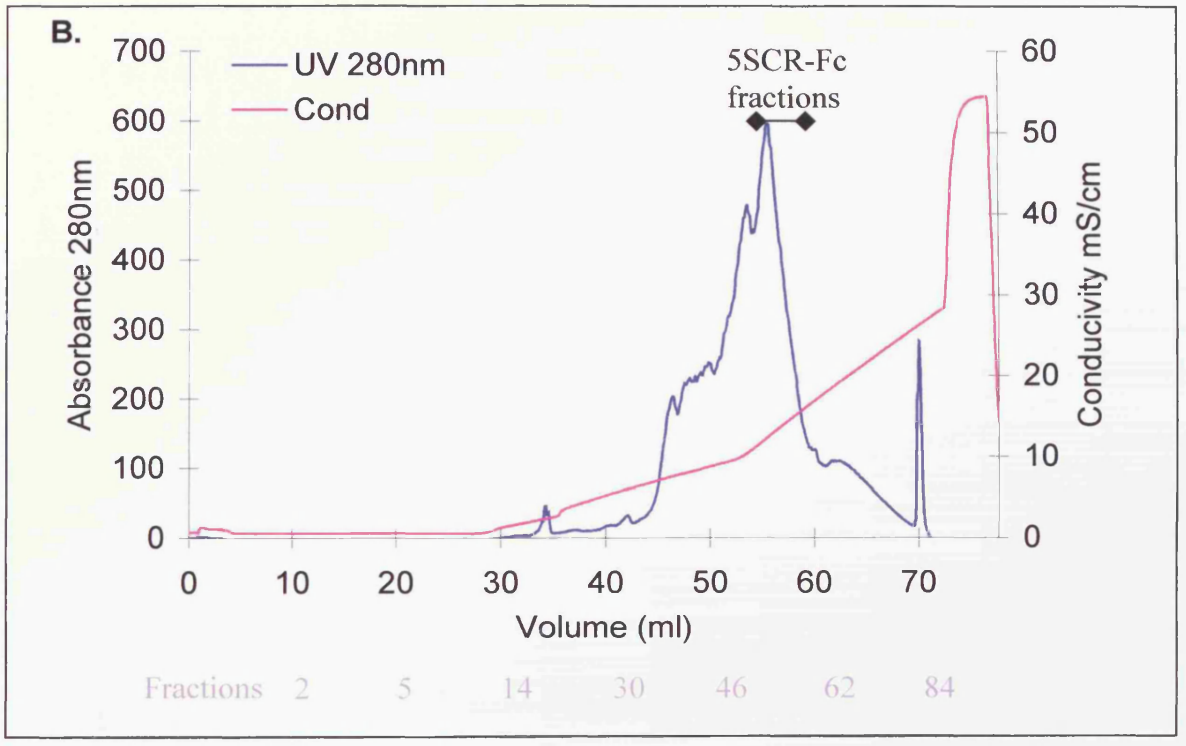


Figure 3.13 Purification of 5SCR-Fc by anion exchange chromatography.

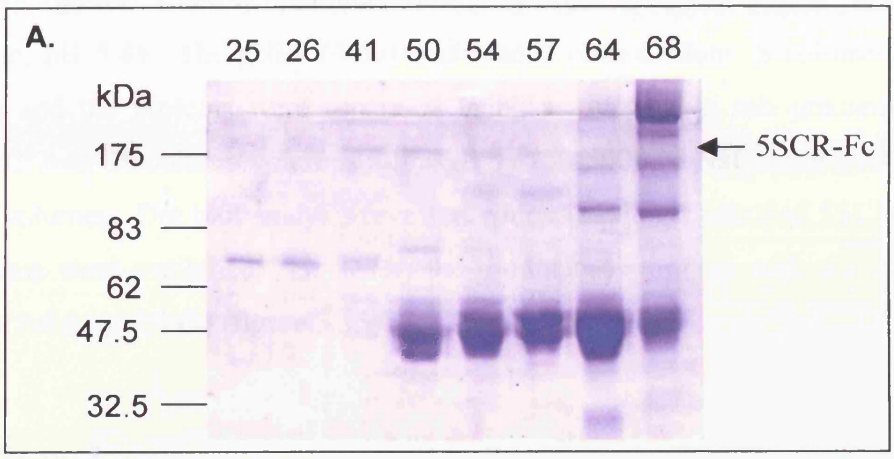
A. Equal amounts of protein from the 40, 45 and 50% saturated ammonium sulphate cuts of the cell culture supernatant were separated on a 7.5% SDS PAGE gel in lanes 1, 2 and 3 respectively. Following electrophoresis and transfer to nitrocellulose, the blot was probed with anti-rat IgG HRPO. 5SCR-Fc was detected in both the 45 and 50% cuts.

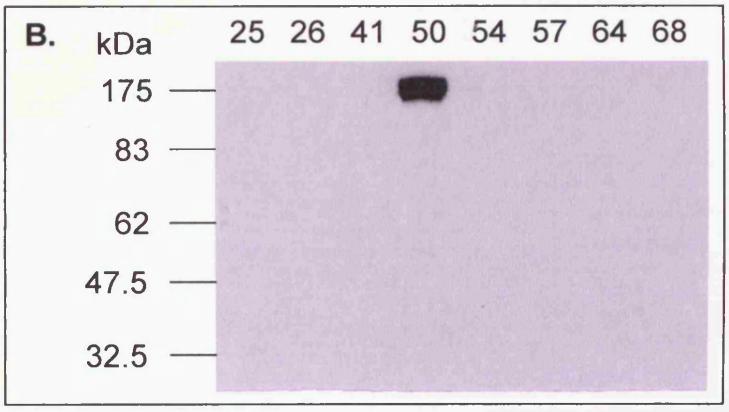
B. Chromatogram of the anion exchange of the resuspended pellet containing 5SCR-Fc. The Mono Q column was loaded with protein from the 50% ammonium sulphate cut and separated using an increasing salt gradient. The UV trace is shown in blue with the gradient illustrated by conductivity in pink, the fractions are indicated in grey.

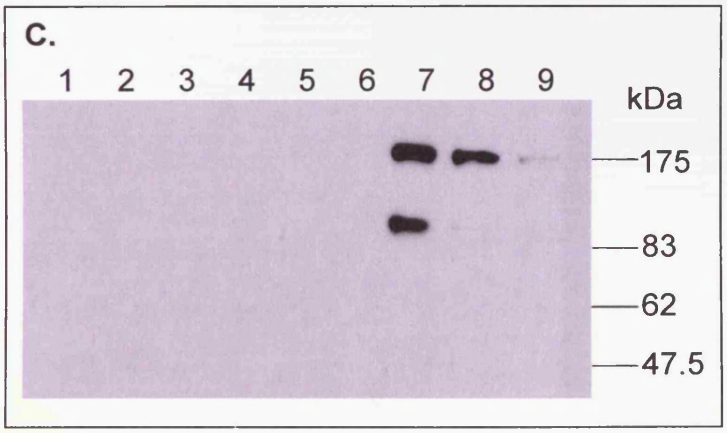
Figure 3.14 SDS PAGE analysis of anion exchange purification of 5SCR-Fc

A. and B. Fractions 25, 26, 41, 50, 54, 57, 64 and 68 from the anion exchange of the 5SCR-Fc pellet were resolved on 7.5% gels. The gel was either Coomassie stained (A) or Western blotted onto nitrocellulose and probed with anti-rat IgG HRPO (B). The Western blot showed that 5SCR-Fc was present in fraction 50, however, the Coomassie stained gel showed that the product was not pure, the major contaminant being at 47.5kDa.

C. Fractions collected of the run through (lanes 1-6) and the elution (lanes 7-9) of the Cibacron Blue column were run on a 7.5% gel. The gel was Western blotted onto nitrocellulose and probed with anti-rat IgG HRPO. 5SCR-Fc was detected in the elution fractions along with albumin.


A.
25 26 41 50 54 57 64 68
kDa
175
83
62
47.5
32.5
5SCR-Fc


B.
kDa
25 26 41 50 54 57 64 68
175
83
62
47.5
32.5


C.
1 2 3 4 5 6 7 8 9
kDa
175
83
62
47.5

Cation exchange was also investigated as a method of purifying 5SCR-Fc from cell culture supernatant. A 50% saturated ammonium sulphate cut was carried out on the cell culture supernatant, the pellet was harvested by centrifugation and resuspended in malonate buffer (50mM malonic acid, 37.5mM NaOH, 4mM betaine monohydrate, pH 5.4). The pellet (4mg) was loaded onto a Mono S column (GE Healthcare) and the proteins were separated using an increasing salt gradient: 0-250mM NaCl over 30 column volumes followed by 250-500mM NaCl over a further 10 column volumes. Dot blot analysis revealed which fractions contained 5SCR-Fc, these fractions were combined. However, the product was impure with the major contaminant being at 55kDa (figure 3.15).

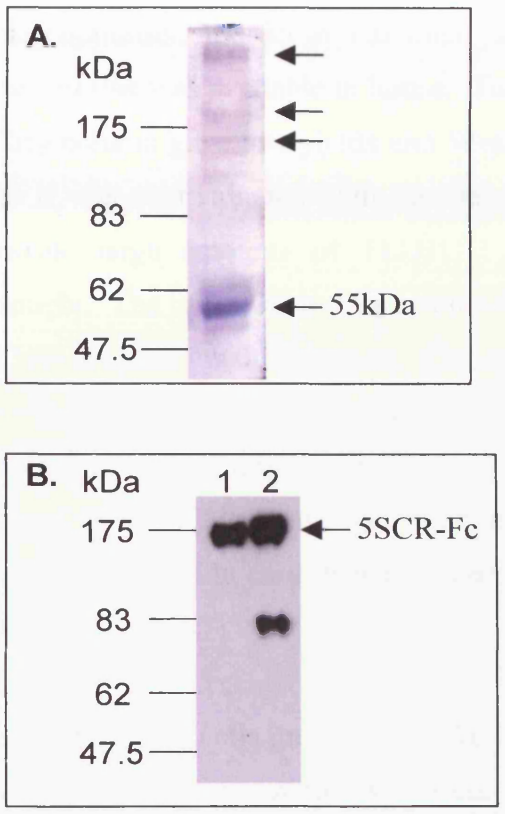


Figure 3.15 SDS PAGE analysis of the cation exchange purification of 5SCR-Fc.

A. The product from the Mono S column was resolved on a 7.5% gel and Coomassie stained. This revealed that the product was not pure, the product contained numerous bands the most prevalent of which was at 55kDa.

B. To ascertain if the product from the cation exchange column contained 5SCR-Fc, the product (lane 1) was Western blotted (7.5% gel) along with 5SCR-Fc cell culture supernatant as a positive control (lane 2). Probing with anti-rat IgG HRPO revealed a band corresponding to 5SCR-Fc in the Mono S product.

3.2.5.6 Optimisation of anti-Crry affinity chromatography

As the only method to give pure 5SCR-Fc was monoclonal anti-Crry affinity chromatography this method was optimised in order to maximise yield. TLD1C11 is a mouse IgG1 antibody and the cell line was available in house. The TLD1C11 used previously to make the anti-Crry column gave low yields and Western blot analysis of the TLD1C11 showed that it was contaminated with bovine antibodies (figure 3.16A). A method to generate large amounts of TLD1C11 with minimised contamination was therefore sought. The use of high expression cell culture systems is dealt with in chapter 5 and was adopted for this purpose. The optimisation of the purification is discussed here. The batch of TLD1C11 used for the previous affinity column had been purified using protein G affinity chromatography (method 2.3.3.2) for which mouse IgG1 has a good affinity. However, bovine antibodies also have a good affinity for protein G and this resulted in co-purification and contamination of the mouse IgG1.

Where supernatant had been collected from cells grown in media supplemented with conventional FBS, protein A affinity chromatography was used. The supernatant was mixed with a binding buffer (1M glycine/NaOH, 0.15M NaCl pH 8.6) and was run over the protein A column. The column elution was optimised by eluting with 0.1M citrate at pH steps 6, 5, 4 and 3 in order to establish which pH to use to elute the antibody. Elutions at pH 6 and 3 resulted in protein being washed off the column, SDS PAGE analysis of these elutions revealed that the pH 6 elution contained bovine antibodies whilst the pH 3 elution contained TLD1C11 as well as bovine antibody contamination (figure 3.16B). A method was therefore established which involved an additional wash of the column with 0.1M citrate pH 6 followed by an elution with 0.1M citrate pH 3. Adoption of this method reduced bovine immunoglobulin contamination but did not completely remove it.

In the high expression systems (Integra Biosciences CL1000 flasks) very little serum containing media is used so it is financially viable to use ultra low bovine immunoglobulin FBS. The problems of bovine immunoglobulin contamination are therefore eliminated and the antibody can be purified using protein G as previously described. This approach gave good yields of pure TLD1C11 (figure 3.16C). 20mg

of TLD1C11, purified by both methods discussed above, was used to make an anti-Crry column. The column was used to purify 5SCR-Fc however the yield was still low and the supernatant remained undepleted revealing that the column had a 1mg capacity.

As the yield from the TLD1C11 column was low it was investigated whether the binding of the antibody to Sepharose via lysine residues resulted in a reduction of the antibody's activity. This was investigated by making a column where covalent binding of the antibody to the matrix occurs via the carbohydrate groups present on the Fc of the antibody. This results in more of the antibody molecules binding to the matrix in the correct conformation for ligand binding and therefore column yields are increased. TLD1C11 (10mg) was coupled to Affi-Gel Hz matrix (Bio Rad) according to method 2.3.4.2. The resulting column was used to purify 5SCR-Fc from cell culture supernatant. SDS PAGE analysis revealed that 5SCR-Fc was a minor constituent of the elution; the major component was a protein with a mass of 55kDa (figure 3.16D). Analysis of antibody that had not coupled to the matrix during the coupling process revealed that this band was a by-product of the coupling process as it was present in the antibody after the coupling but was absent before (figure 3.16D). Use of this column did not result in increased yields and it could not be concluded whether binding via the lysine residues reduced the ligand binding ability of TLD1C11. A possible reduction in function due to binding via the lysine residues was demonstrated using the Biacore® 3000. TLD1C11 was amine coupled to a CM5 chip and 5SCR-Fc was flowed over the chip (method 2.5.7). As the masses of TLD1C11 and 5SCR-Fc are equivalent, a response shift for 5SCR-Fc binding should be equivalent to the amount of TLD1C11 bound. However, the resulting response was much lower than expected indicating that the amine coupling of TLD1C11 through lysine residues had resulted in a reduction of its ligand binding ability (data not shown). Despite this result the decision was taken to make a large capacity column using amine coupling as the alternative column had given an unwanted by-product.

Figure 3.16 SDS PAGE of the optimisation of the anti-Crry method.

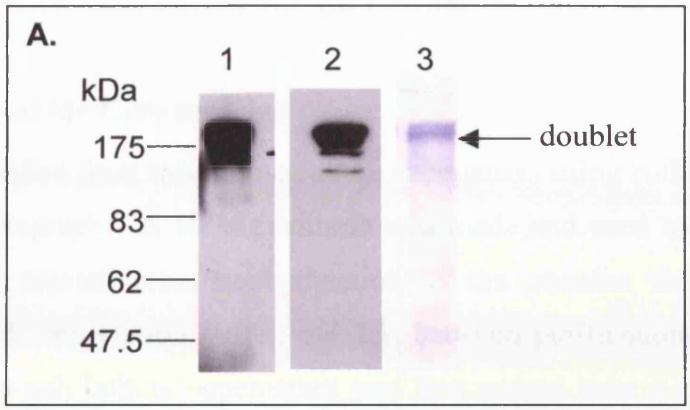
All proteins were analysed on 7.5% gels.

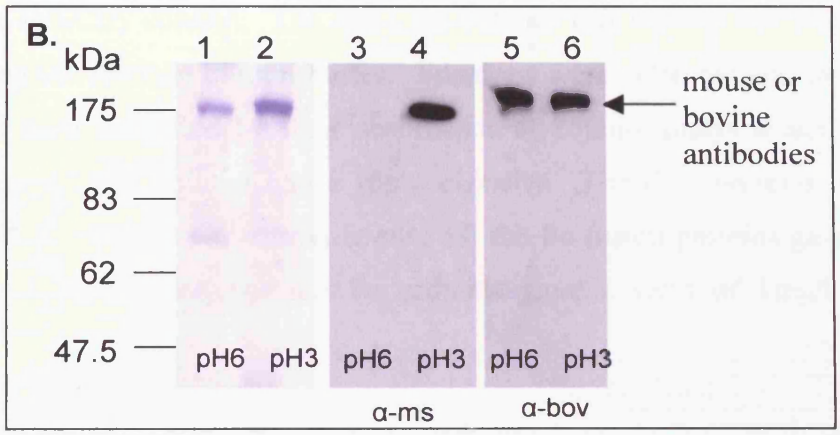
A. A gel loaded with TLD1C11 used to make the previous anti-Crry column (described section 3.2.4.4) was either Coomassie stained (lane 3) or transferred to nitrocellulose where it was probed with anti-bovine IgG HRPO (lane 1) or anti-mouse IgG HRPO (lane 2). The Coomassie stain revealed that the protein consisted of a doublet at 175kDa while the Western blots revealed that the TLD1C11 also contained bovine antibodies.

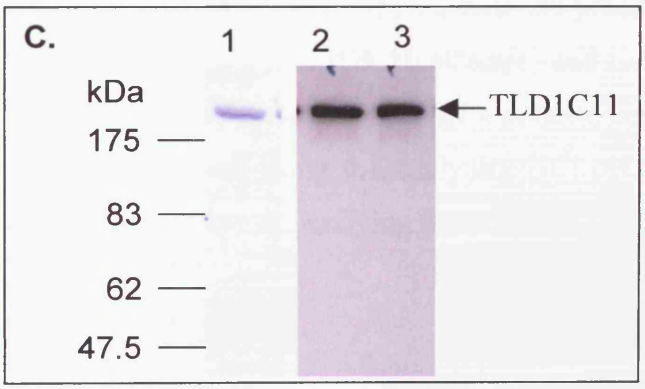
B. The products of the pH 3 and pH 6 elutions of the protein A column were analysed by Coomassie staining (lanes 1 and 2) or by Western blotting (lanes 3-6). Lanes 3 and 4 were probed with anti-mouse IgG HRPO while lanes 5 and 6 were probed with anti-bovine IgG HRPO. Both the pH 6 and pH 3 elutions consisted of proteins that just entered the gel, the Western blots revealed that the pH 6 elution consisted of bovine IgG and not mouse IgG (lanes 5 and 3 respectively) while the pH 3 elution consisted of both mouse IgG (lane 4) and bovine IgG (lane 6).

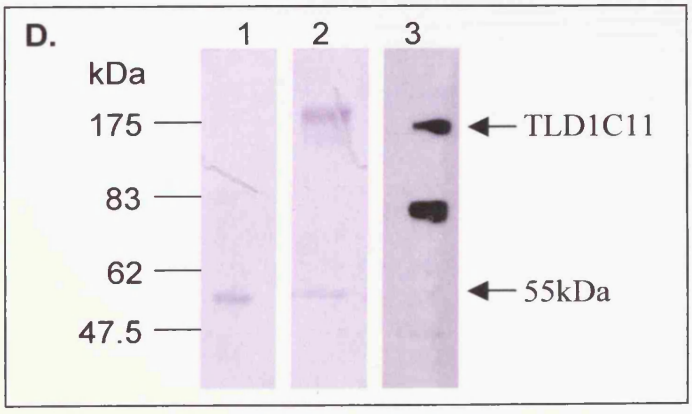
C. The product from the protein G column (lanes 1 and 2) was Coomassie stained (lane 1) or Western blotted and probed with anti-mouse IgG HRPO (lane 2), lane 3 contains TLD1C11 culture supernatant. The Coomassie stain revealed that the product contained a protein with a mass of 175kDa, the Western blot confirmed that this band was TLD1C11.

D. The product (lane 1) from the Affi-Gel Hz anti-Crry column along with the TLD1C11 antibody that had not coupled with the matrix during the coupling procedure (lane 2) was Coomassie stained. The product (lane 3) was transferred to nitrocellulose and probed with anti-rat IgG HRPO. The major band of the product (lane 1) was 55kDa, this band was also in the TLD1C11 that had not coupled to the column (lane 2) along with uncoupled TLD1C11 at 175kDa, suggesting it was a by-product of the coupling procedure. The Western blot of the product revealed that it did contain 5SCR-Fc although this was a minor constituent.


A.
1
2
3
kDa
175
83
62
47.5
doublet


B.
kDa
1
2
3
4
5
6
175
83
62
47.5
mouse or
bovine
antibodies
pH6
pH3
pH6
pH3
pH6
pH3
α-ms
α-bov


C.
1
2
3
kDa
175
83
62
47.5
TLD1C11


D.
1
2
3
kDa
175
83
62
47.5
TLD1C11
55kDa

3.2.6 Purification of the Crry proteins

TLD1C11 was purified from hybridoma culture supernatant using both protein A and G affinity chromatography. A 100mg column was made and used to purify all the Crry proteins, to prevent cross contamination of the proteins the column was thoroughly washed with elution buffer, pH 2.5, between purifications. In order to purify the proteins cell culture supernatant was first passed over a Sepharose pre-column before the anti-Crry column. The anti-Crry column was washed and bound protein eluted using the relevant elution buffer. Fractions were collected and those containing protein were identified by their absorbance at 280nm, dialysed against PBS and concentrated. The column had a 10mg capacity. The Crry proteins had differing yields due to different expression levels. All the Fc fusion proteins gave a yield of 4-5mg/l of supernatant, the non-Fc proteins gave a yield of 1mg/l of supernatant.

In order to further optimise the purification and automate the process, the TLD1C11 column was packed into an XK16 column (GE Healthcare) and run using an AKTA Prime system (GE Healthcare). Each run consisted of a column equilibration step, a supernatant load, a wash and elution using 0.1M glycine/HCl pH 2.5 (figure 3.17). This provided a fast and efficient way of purifying the proteins.

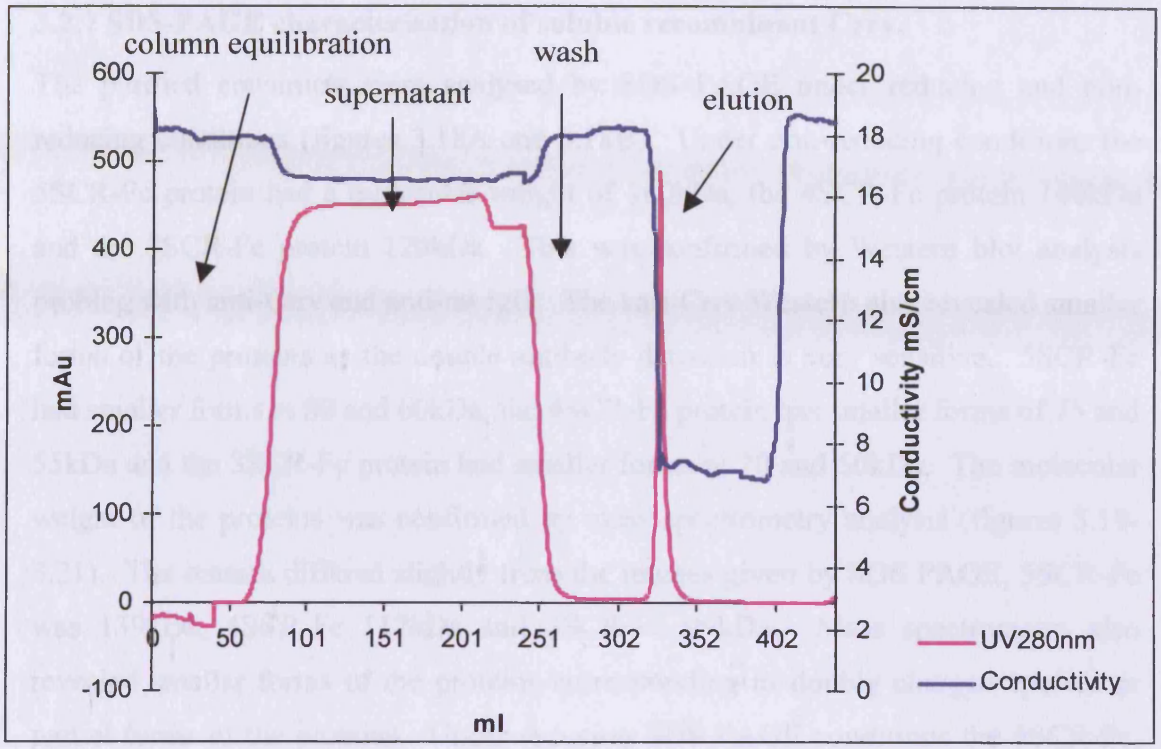


Figure 3.17 Chromatogram of the automated purification of the Crry proteins. TLD1C11 column was loaded with cell culture supernatant containing 4SCR-Fc after an equilibration step. The column was then washed and bound protein was eluted using an appropriate elution buffer. The UV trace is shown in blue while the conductivity is in pink.

3.2.7 SDS-PAGE characterisation of soluble recombinant Crry.

The purified constructs were analysed by SDS PAGE under reducing and non-reducing conditions (figures 3.18A and 3.18B). Under non-reducing conditions the 5SCR-Fc protein had a molecular weight of 160kDa, the 4SCR-Fc protein 140kDa and the 3SCR-Fc protein 120kDa. This was confirmed by Western blot analysis probing with anti-Crry and anti-rat IgG. The anti-Crry Western also revealed smaller forms of the proteins as the double antibody detection is very sensitive. 5SCR-Fc had smaller forms at 80 and 60kDa, the 4SCR-Fc protein has smaller forms of 75 and 55kDa and the 3SCR-Fc protein had smaller forms at 70 and 50kDa. The molecular weight of the proteins was confirmed by mass spectrometry analysis (figures 3.19-3.21). The masses differed slightly from the masses given by SDS PAGE, 5SCR-Fc was 139kDa, 4SCR-Fc 117kDa and 3SCR-Fc 99kDa. Mass spectrometry also revealed smaller forms of the proteins corresponding to doubly charged species or partial forms of the proteins. Under reducing SDS PAGE conditions the 5SCR-Fc, 4SCR-Fc and 3SCR-Fc fusion proteins had molecular weights of 80, 75 and 70kDa respectively. This reduction in molecular weight is due to the disulphide bonds between the constituent arms of the fusion protein being broken. This result was confirmed by Western blot analysis probing with anti-Crry and anti-rat IgG. High molecular weight aggregates of the proteins were visible on the anti-Crry Western.

Under non-reducing conditions, the 4SCR protein gave a diffuse band at 28kDa while the 3SCR protein gave a band of 21kDa (figure 3.18C). This was confirmed by Western blot analysis probing with anti-Crry. Under reducing conditions two bands were visible for the 4SCR protein, the Western blots probed with anti-Crry confirmed both to be 4SCR. The broad band reducing to two single bands is most likely due to different glycosylation of the protein as there is a glycosylation site in SCR4 of rat Crry (ASN 212). The 3SCR protein under reducing conditions had a mass of 25kDa. This slight increase of molecular weight is most likely an affect of its cysteine rich nature of the protein coupled with its low molecular weight as it loses it's conformation through reduction. This increase in molecular weight upon reduction is characteristic of SCR containing proteins. The low molecular weight of these two proteins makes any change in glycosylation or conformation more apparent in this percentage gel. Mass spectrometry was also used to confirm the molecular

weights of 4SCR and 3SCR, as 28kDa and 21kDa respectively (figures 3.22 and 3.23).

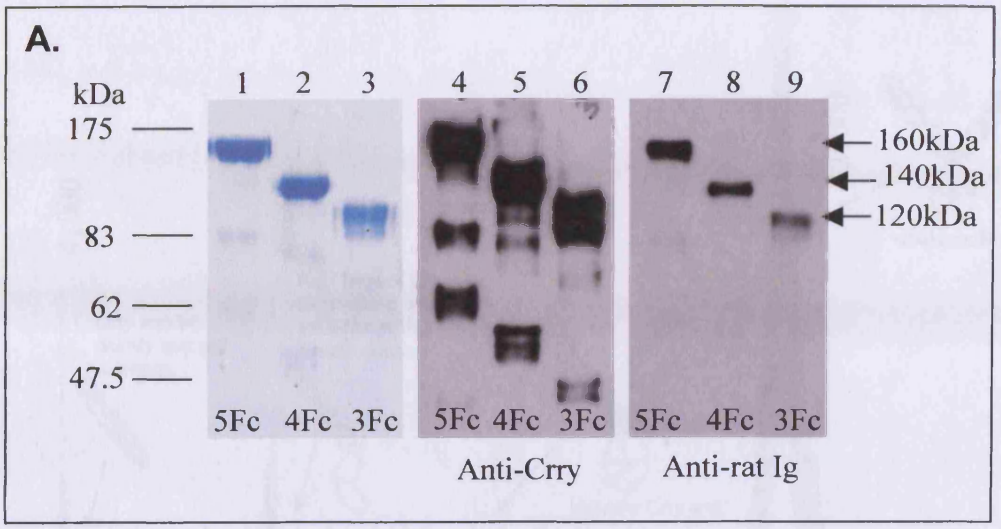
Figure 3.18 SDS PAGE of Crry proteins.

A. 5SCR-Fc, 4SCR-Fc and 3SCR-Fc were resolved on a 7.5% gel using non-reducing conditions. Lanes 1-3 were Coomassie stained whilst lanes 4-9 were transferred to nitrocellulose and probed with TLD1C11 followed by HRPO conjugated anti-mouse IgG (lanes 4-6) or with anti-rat IgG HRPO (lanes 7-9). Coomassie staining revealed bands at 160kDa, 140kDa and 120kDa in lanes 1-3 corresponding to 5SCR-Fc, 4SCR-Fc and 3SCR-Fc respectively. The identity was confirmed by the Western blots, additional bands visible on the anti-Crry Western correspond to partial-forms of the fusion protein.

B. 5SCR-Fc, 4SCR-Fc and 3SCR-Fc were resolved on a 10% gel under reducing conditions. Lanes 1-3 were Coomassie stained whilst lanes 4-9 were Western blotted and probed with TLD1C11 and followed with anti-mouse IgG HRPO (lanes 4-6) or anti-rat IgG HRPO (lanes 7-9). Bands on the Coomassie at 80kDa, 70kDa and 60kDa in lanes 1-3 respectively corresponded to 5SCR-Fc, 4SCR-Fc and 3SCR-Fc. The identity of these bands was confirmed by the Western blots, high molecular weight aggregates were also seen on the anti-Crry blot (lanes 4-6).

C. 4SCR and 3SCR were separated on an 11% gel under non-reducing conditions (lanes 1-4) and reducing conditions (lanes 5-8). Lanes 1, 2, 5 and 6 were Coomassie stained and lanes 3, 4, 7 and 8 were Western blotted and probed with TLD1C11 followed by anti-mouse IgG HRPO. Under non-reducing conditions broad bands at 28kDa and 21kDa, lanes 1 and 2 respectively, were visible corresponding to 4SCR and 3SCR. Under reducing conditions tighter bands at 28kDa and 25kDa were apparent corresponding to 4SCR and 3SCR. The identity of all the bands was confirmed by the Western blots.

Key: 5Fc is 5SCR-Fc, 4Fc is 4SCR-Fc, 3Fc is 3SCR-Fc, 4 is 4SCR and 3 is 3SCR


A.
kDa
175
83
62
47.5
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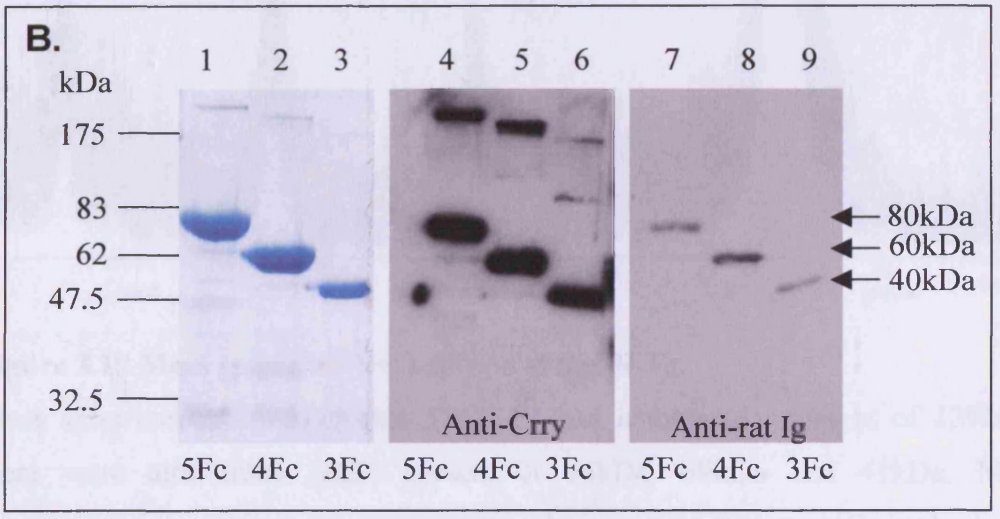
140kDa
120kDa
5Fc 4Fc 3Fc 5Fc 4Fc 3Fc 5Fc 4Fc 3Fc
Anti-Crry
Anti-rat Ig


B.
kDa
175
83
62
47.5
32.5
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80kDa
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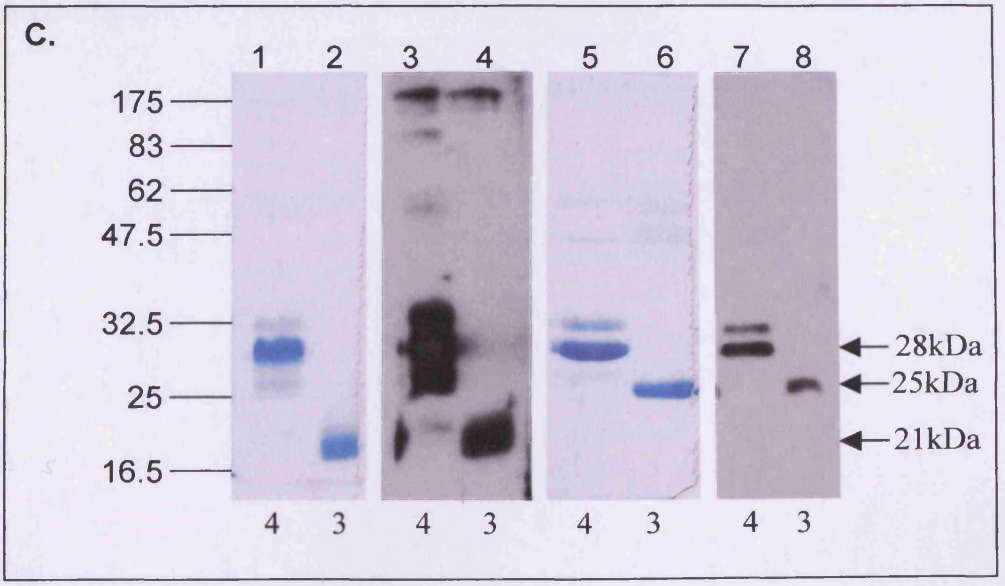
40kDa
Anti-Crry
Anti-rat Ig
5Fc 4Fc 3Fc 5Fc 4Fc 3Fc 5Fc 4Fc 3Fc


C.
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25
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28kDa
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4 3 4 3 4 3 4 3

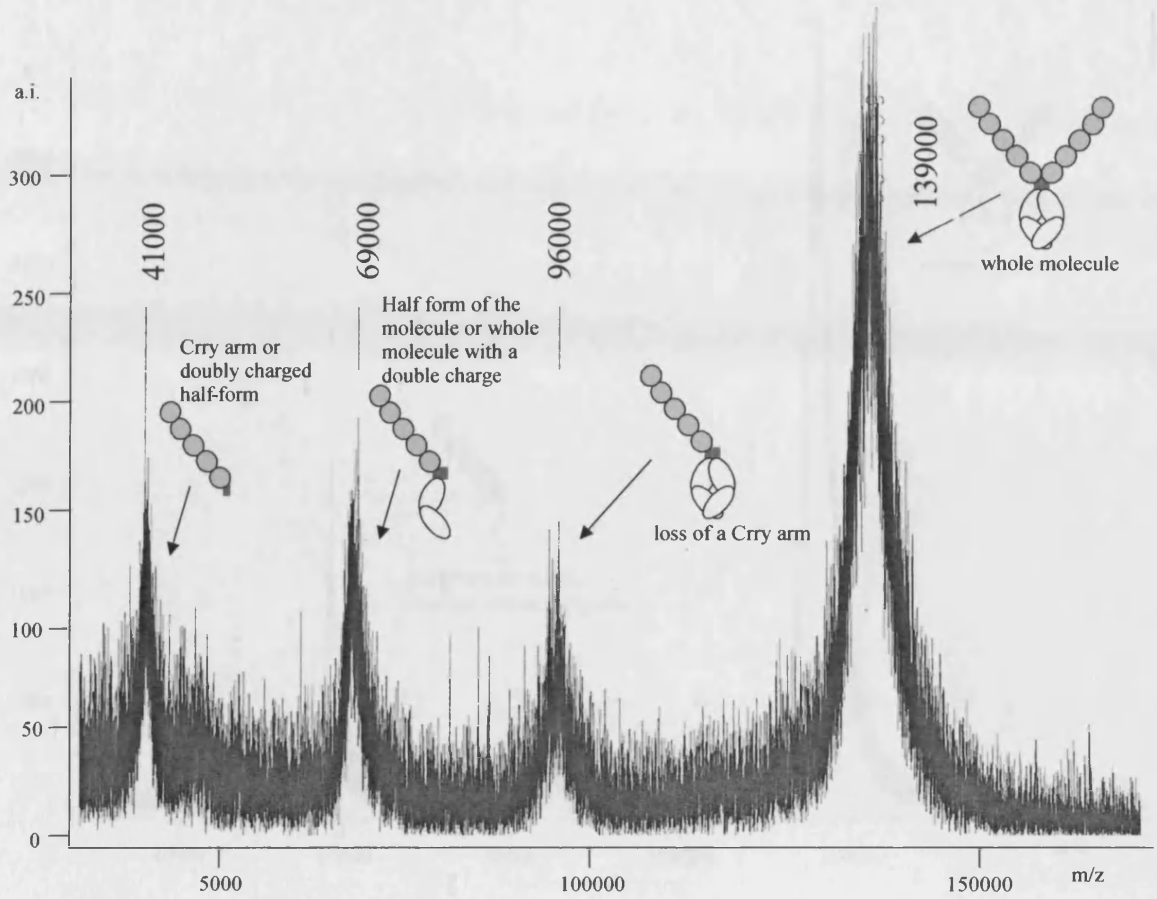


Figure 3.19 Mass spectrometry analysis of 5SCR-Fc.

Mass spectrometry revealed that 5SCR-Fc had a molecular weight of 139kDa; there were also other peaks present at 96kDa, 69kDa and 41kDa. Mass spectrometry trace is shown with the protein corresponding to each peak shown schematically.

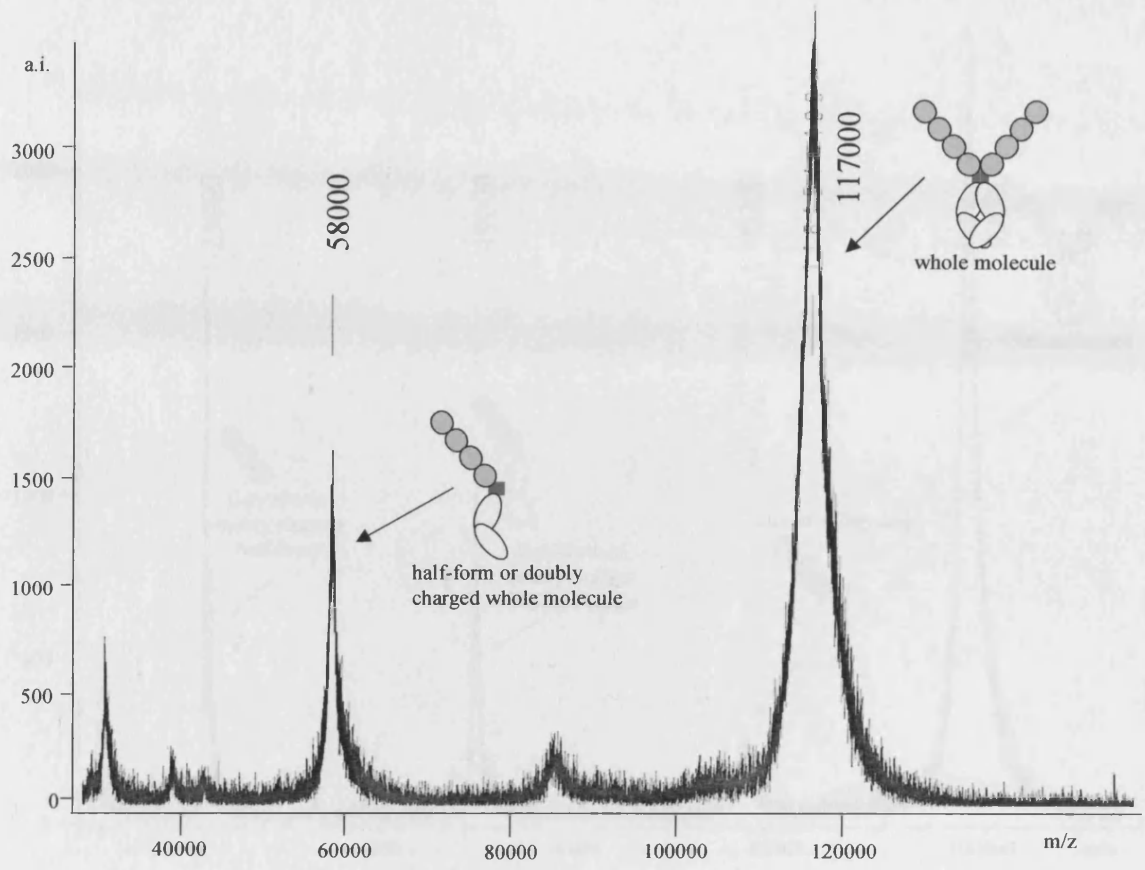


Figure 3.20 Mass spectrometry trace of 4SCR-Fc.

Mass spectrometry revealed that 4SCR-Fc had a mass of 117kDa, a peak at 58kDa was also present corresponding to the half-form or doubly charged whole molecule. The trace is shown with the protein corresponding to each peak represented schematically.

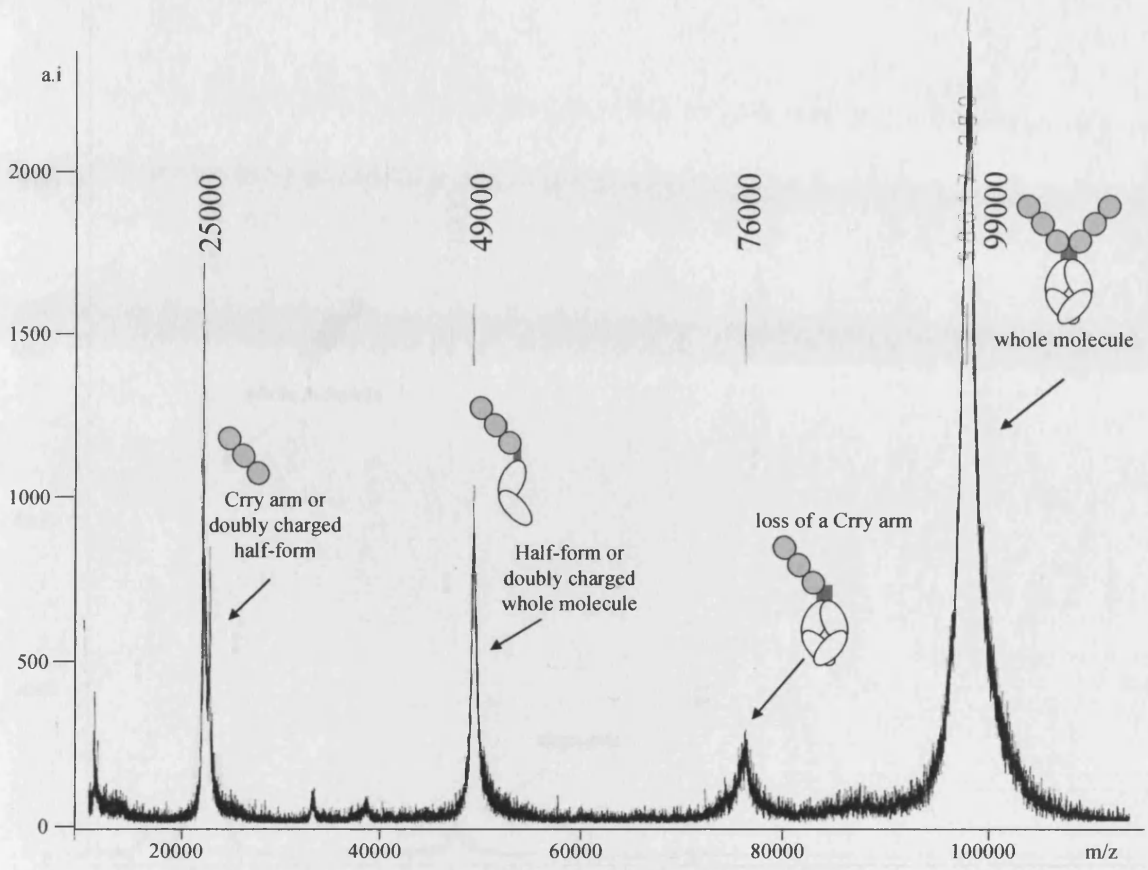


Figure 3.21 Mass spectrometry trace of 3SCR-Fc.

The mass of 3SCR-Fc was 99kDa by mass spectrometry analysis. Additional peaks were also visible at 76kDa, 49kDa and 25kDa corresponding to the proteins schematically represented on the mass spectrometry trace.

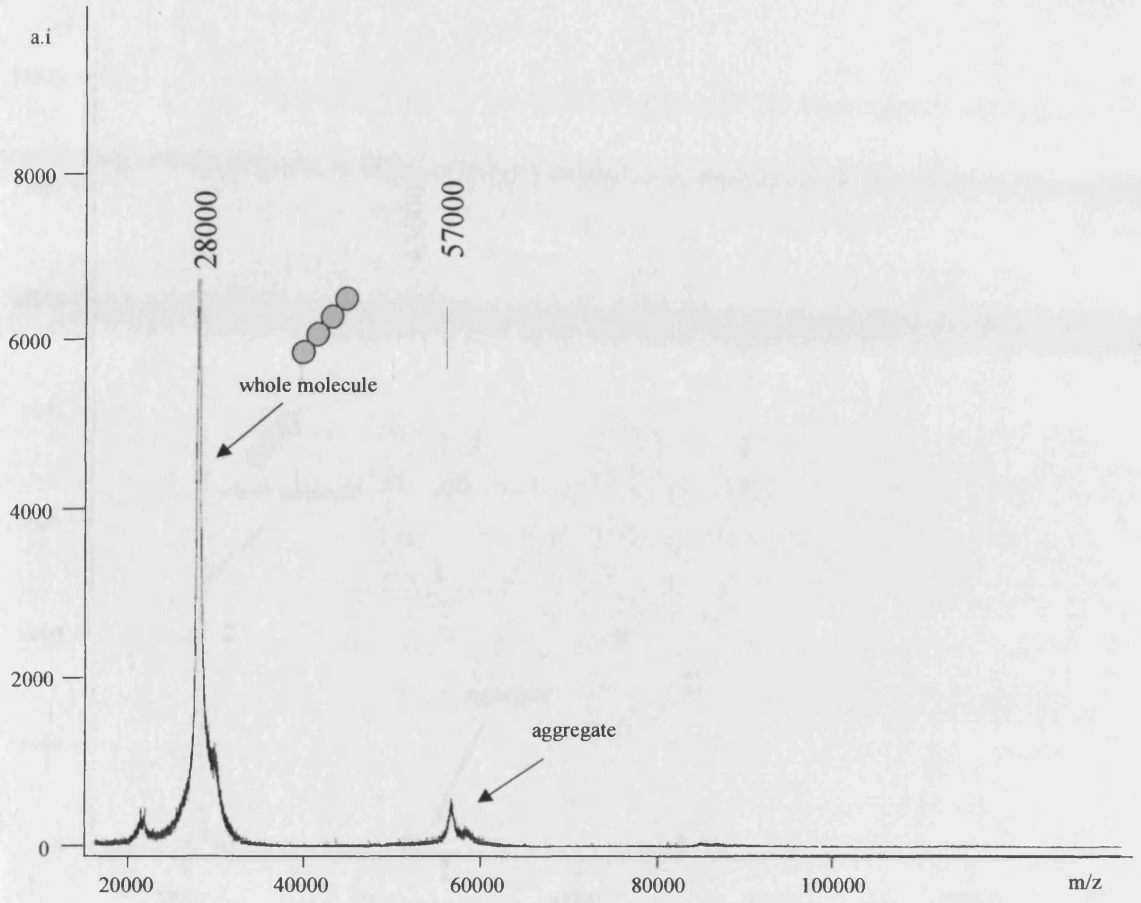


Figure 3.22 Mass spectrometry trace of 4SCR.

Mass spectrometry analysis gave the mass of 4SCR as 28kDa. A peak was also present at 57kDa due to aggregated (dimerised) 4SCR.

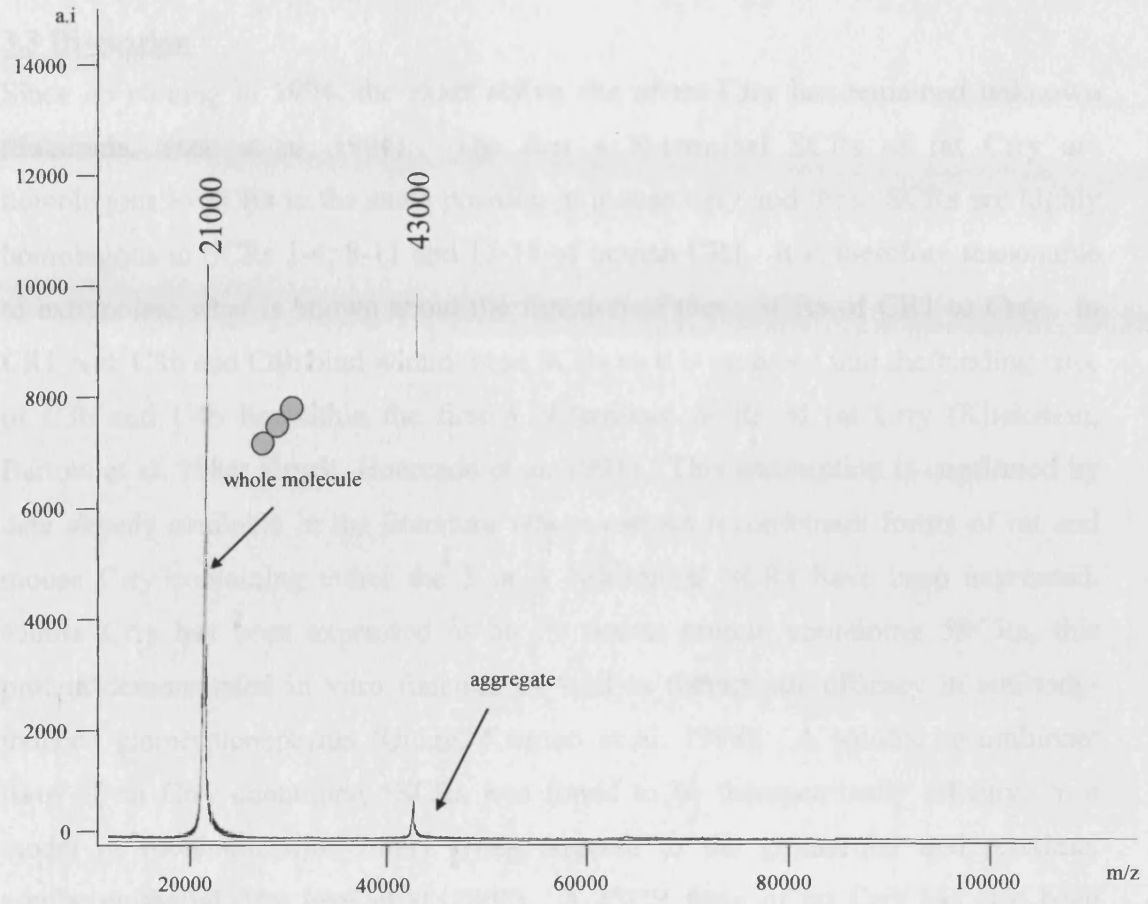


Figure 3.23 Mass spectrometry analysis of 3SCR.

The mass of 3SCR was 21kDa given by mass spectrometry analysis. A peak was also present at 43kDa due to dimerised 3SCR.

3.3 Discussion

Since its cloning in 1994, the exact active site of rat Crry has remained unknown (Sakurada, Seno et al. 1994). The first 4 N-terminal SCRs of rat Crry are homologous to SCRs in the same position in mouse Crry and these SCRs are highly homologous to SCRs 1-4, 8-11 and 15-18 of human CR1. It is therefore reasonable to extrapolate what is known about the function of these SCRs of CR1 to Crry. In CR1 both C3b and C4b bind within these SCRs so it is assumed that the binding sites of C3b and C4b lie within the first 4 N-terminal SCRs of rat Crry (Klickstein, Bartow et al. 1988; Krych, Hourcade et al. 1991). This assumption is confirmed by data already available in the literature where various recombinant forms of rat and mouse Crry containing either the 5 or 4 N-terminal SCRs have been expressed. Mouse Crry has been expressed as an Fc fusion protein containing 5SCRs, this protein demonstrated in vitro function as well as therapeutic efficacy in antibody-induced glomerulonephritis (Quigg, Kozono et al. 1998). A soluble recombinant form of rat Crry containing 5SCRs was found to be therapeutically effective in a model of tubulointerstital injury when targeted to the glomerular and proximal tubular epithelial (He, Imai et al. 2005). A 4SCR form of rat Crry has also been expressed and has demonstrated in vitro function (Fraser, Harris et al. 2002). The generation of the recombinant forms of Crry described in this project, as well as identification of a potential 'prodrug' and active fusion protein, will advance current work in the field by enabling further functional analysis of rat Crry to be carried out. The SCR deletion mutants described in this thesis have been compared for their ability to inhibit complement in lysis assays, cause decay acceleration of the convertases and act as cofactors for factor I (chapter 4).

Here, various soluble recombinant forms of rat Crry have been expressed and purified. These forms contained the 5, 4 or 3 N-terminal SCRs as an Fc fusion protein with the Fc of rat IgG2a, or they contained the 4 or 3 N-terminal SCRs as a non-fusion protein. The cDNA for the 5SCRs of Crry was cloned from a rat oligodendroglioma cDNA. An internal BamH1 site was removed and this vector was used as a template from which to clone the other forms of Crry. The Fc was cloned from a plasmid already expressing the cDNA. Following ligation of the appropriate

cDNA into the plasmid, CHO cells were transfected and expression of the Crry proteins was detected by Western blot analysis.

A number of different methods were investigated to purify 5SCR-Fc and these are summarised in table 3.1. The use of both protein A and G columns resulted in proteins being isolated from the supernatant but these were shown by SDS PAGE analysis not to contain 5SCR-Fc. Antibody affinity methods were also tested, an anti-rat immunoglobulin column failed to purify 5SCR-Fc due to a low antibody-antigen affinity. Two different monoclonal anti-Crry columns resulted in the purification of 5SCR-Fc, although the protein was pure, the yield was low so alternative purification methods were tested. Both cation and anion exchange chromatography resulted in the purification of 5SCR-Fc from supernatant but in both instances 5SCR-Fc was impure and was the minor constituent of the protein yield. In light of this the use of one of the anti-Crry columns (TLD1C11) was optimised and used to purify all the Crry proteins. The method was optimised by altering the hybridoma culture conditions, the purification methodology of the monoclonal antibody as well as by investigating different column matrices and making larger capacity columns.

Method	Outcome
Protein A	Protein was obtained from the column but it did not contain 5SCR-Fc.
Protein G	5SCR-Fc was not present in the column elution.
Anti-rat Ig	No protein was obtained from the column.
Anti-Crry	Gave pure but low yields, method was later optimised.
Anion exchange	5SCR-Fc was a minor product, the major product was albumin.
Cation exchange	Product from the column was impure.

Table 3.1 Summary of the purification methods investigated and their outcomes.

The purified proteins were characterised by SDS PAGE analysis under reducing and non-reducing conditions. 5SCR-Fc gave a major band at 160kDa under non-reducing conditions, which reduced to 80kDa, 4SCR-Fc was 140kDa non-reduced but 70kDa reduced and 3SCR-Fc was 120kDa non-reduced and 60kDa reduced.

These weight reductions under reducing conditions represent the breaking of the disulphide bonds within the protein and its subsequent breakage in half. 4SCR was 28kDa under both reducing and non-reducing conditions while 3SCR was 21kDa non-reduced and 25kDa reduced. The molecular weights are summarised in table 3.2.

Protein	Molecular weight non-reduced (kDa)	Molecular weight reduced (kDa)	Molecular weight from mass spectrometry (kDa)
5SCR-Fc	160	80	139
4SCR-Fc	140	70	117
3SCR-Fc	120	60	99
4SCR	28	28	28
3SCR	21	25	21

Table 3.2 The Crry proteins generated and their molecular weights under non-reducing and reducing conditions.

In summary, this chapter describes the generation and purification of 5SCR-Fc, 4SCR-Fc, 3SCR-Fc, 4SCR and 3SCR as recombinant forms of rat Crry. These reagents have been cloned, expressed in CHO cells and purified from cell culture supernatant.

Chapter 4: Functional characterisation of the Crry proteins.

4.1 Introduction

In the previous chapter, I described the generation, purification and biochemical characterisation of various recombinant forms of rat Crry. The proteins generated contained the 5, 4 or 3 N-terminal SCRs of rat Crry. The proteins generated were termed 5SCR-Fc, 4SCR-Fc, 3SCR-Fc, 4SCR and 3SCR. The aim of generating these SCR deletion mutants of Crry was to enable the identification of an active Crry-Fc reagent for testing in therapy and the identification of a Crry-Fc protein that could be further engineered to create a 'prodrug'. In this chapter, I describe the functional characterisation of the recombinant Crry proteins.

As discussed in the introduction, Crry regulates C by accelerating decay of the convertases as well as acting as a cofactor for the fI mediated cleavage of C3b and C4b. Therefore a variety of functional assays were carried out. Functional analysis of the proteins was carried out using both CP and AP haemolysis assays, fI cofactor assays and by using surface plasmon resonance to investigate the ability of the Crry proteins to decay the alternative pathway C3 convertase. Surface plasmon resonance was also used to investigate the influence SCR deletion had upon the affinity of the proteins for C3b. In both types of haemolysis assay equimolar amounts of Crry were used to compare the ability of the Crry proteins to inhibit the lysis of erythrocytes. This lysis can proceed via the CP or the AP of C depending upon which target cells are used along with the appropriate buffer. In the cofactor assay, the Crry proteins were compared for their ability to assist fI in its cleavage of C3. Surface plasmon resonance was investigated using a Biacore® 3000. This is a system which measures molecular interactions in real time, the readout is directly proportional to the mass of protein bound to the chip surface. The chip consists of a gold covered glass surface coated with dextran and it is on this dextran that the molecular interactions occur. Any mass changes brought about by molecular interactions at the chip surface alter the surface plasmon resonance changing the readout (Malmqvist 1999) (Figure 4.1). This technology was used to assess the ability of the Crry proteins to cause enzyme decay of the AP C3 convertase using a method developed in house (Harris, Abbott et al. 2005). In this experiment the enzyme was built up on the chip surface and

equimolar molar amounts of the Crry proteins compared for their ability to enhance its decay. Surface plasmon resonance was also used to investigate the affinity of the SCR deletion mutants of Crry for rat C3b. This rounded investigation of the function of the Crry proteins will enable identification of a reduction in function due to deletion of a SCR or steric hindrance and also an appreciation of which aspect of the function of Crry has been affected.

The aims of this chapter are:

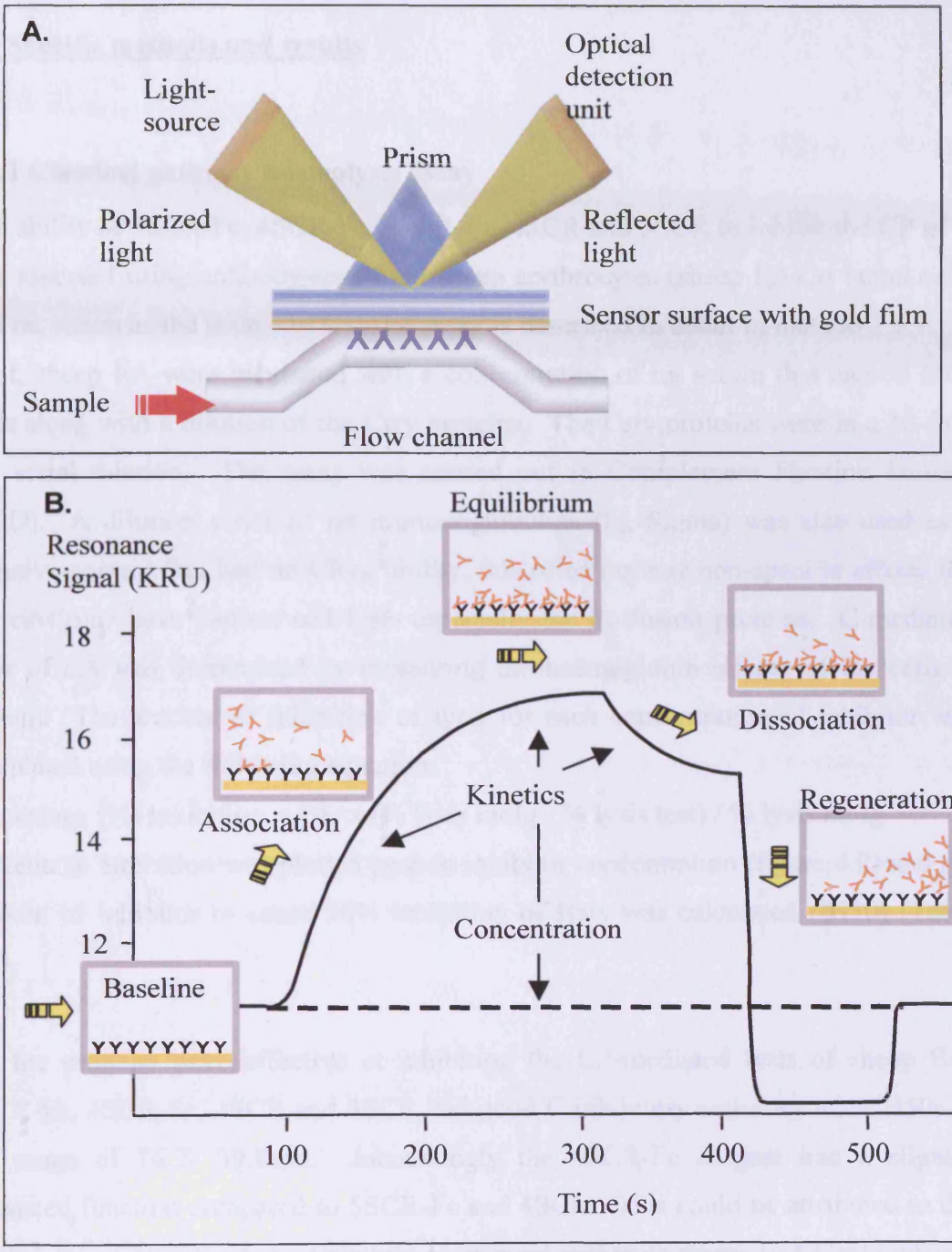
1. To analyse the function of the recombinant Crry proteins by both CP and AP haemolysis assays.
2. To compare the cofactor activities of the Crry proteins.
3. To compare the proteins in their ability to decay the C3 convertase.
4. To investigate the impact of the SCR deletions on the affinity of Crry for rat C3b.
5. To bring together all these methods used for functional analysis to identify a suitable protein to be used in therapy or to be further engineered to form the 'prodrug'.

Figure 4.1 Surface plasmon resonance measurements using the Biacore® 3000.

A. A diagram of the flow cell used for surface plasmon resonance. The molecular interactions occur on a gold covered chip. The change in absorption of polarised light from the chip provides a measurement of molecular interactions. Antibody on the surface illustrated here.

B. A sensogram read out from the Biacore®. A shift upwards in the y-axis represents an association taking place between an immobilised protein i.e. C3b and an analyte i.e. Crry while a shift down towards the x-axis shows dissociation occurring. Regenerating the surface to elute the analyte can increase the dissociation. The sensogram can be used to calculate concentration and kinetic rate constants.

Figures adapted from An introduction to Biacore's surface plasmon reasonance technology available from Biacore.


A.
Optical detection unit
Light-source
Prism
Polarized light
Reflected light
Sensor surface with gold film
Sample
Flow channel
B.
Resonance Signal (KRU)
Equilibrium
Dissociation
Association
Kinetics
Regeneration
Concentration
Baseline
18
16
14
12
100
200
300
400
500
600
Time (s)

4.2 Specific methods and results

4.2.1 Classical pathway haemolysis assay

The ability of 5SCR-Fc, 4SCR-Fc, 3SCR-Fc, 4SCR and 3SCR to inhibit the CP of C was assessed using antibody-sensitised sheep erythrocytes (sheep EA) as target cells and rat serum as the source of C. The assay is described in detail in method 2.5.1. In brief, sheep EA were incubated with a concentration of rat serum that caused 60% lysis along with a dilution of the Crry proteins. The Crry proteins were in a 10-fold 1:2 serial dilution. The assay was carried out in Complement Fixation Diluent (CFD). A dilution series of rat immunoglobulins (Ig, Sigma) was also used as a negative control that had no CReg ability, this ruled out any non-specific effects the proteins may have had on cell lysis especially the Fc fusion proteins. C-mediated lysis of EA was determined by measuring the haemoglobin release of the cells at 415nm. The amount of inhibition of lysis for each concentration of inhibitor was calculated using the following equation:

Percentage (%) inhibition = 100 x (% lysis rat Ig - % lysis test) / % lysis rat Ig

Percentage inhibition was plotted against inhibitor concentration (figure 4.2) and the amount of inhibitor to cause 50% inhibition of lysis was calculated (IH50) (Table 4.1).

All the proteins were effective at inhibiting the CP-mediated lysis of sheep EA. 5SCR-Fc, 4SCR-Fc, 4SCR and 3SCR had good C inhibitory activities with IH50s in the range of 16.7- 39.8nM. Interestingly the 4SCR-Fc reagent had a slightly enhanced function compared to 5SCR-Fc and 4SCR. This could be attributed to the SCR deletion giving rise to a slightly more rigid molecule where two Crry moieties are in close proximity, acting synergistically to bring about more efficient decay than the single Crry in 4SCR or the less rigid Crry moieties in 5SCR-Fc. The 3SCR-Fc reagent had a much reduced function compared to the other proteins; the IH50 was significantly different from the other proteins at 112.8nM, compared to 4SCR-Fc and 3SCR ($p<0.001$). This reduction in function was not seen in the 3SCR reagent so cannot be attributed to deletion of an active site SCR, instead it can be attributed to steric hindrance caused by the deletion of SCR bringing the active site close to the Fc.

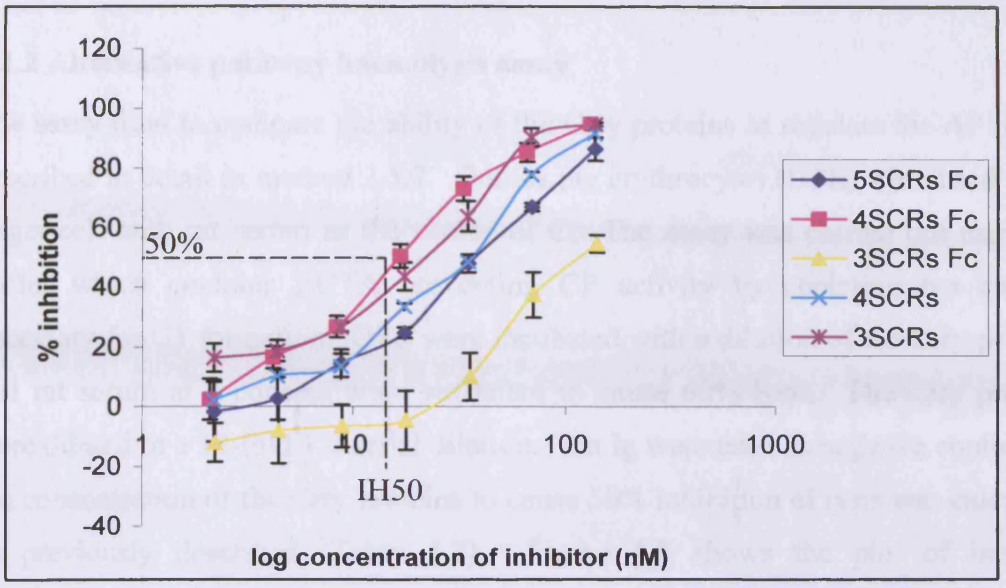


Figure 4.2 Classical pathway haemolysis assay: Graph of % inhibition of lysis against concentration of Crry.

The Crry reagents were compared for their ability to inhibit CP-mediated lysis of antibody coated sheep erythrocytes. All the Crry proteins inhibited lysis. 5SCR-Fc (dark blue), 4SCR-Fc (pink), 4SCR (turquoise) and 3SCR (purple) showed comparable functions. 3SCR-Fc (yellow) showed a reduced function. Each data point represents the mean value +/- 1 SD of a single experiment performed in triplicate. Representative graph from three separate experiments.

	5SCR-Fc	4SCR-Fc	3SCR-Fc	4SCR	3SCR
IH50	39.8	16.7	112.8	31.0	20.3
SD	2.7	0.6	17.7	0.7	1.3

Table 4.1 Classical pathway haemolysis assay: IH50 for each Crry protein.

IH50 is the concentration (nM) of the respective Crry protein that causes 50% inhibition of lysis. SD, standard deviation of three measurements.

4.2.2 Alternative pathway haemolysis assay

The assay used to compare the ability of the Crry proteins to regulate the AP of C is described in detail in method 2.5.2. Guinea pig erythrocytes (GPE) were used as the target cell with rat serum as the source of C. The assay was carried out using AP buffer which contains EGTA preventing CP activity by chelating out calcium necessary for C1 formation. GPE were incubated with a dilution of the Crry proteins and rat serum at a concentration sufficient to cause 60% lysis. The Crry proteins were diluted in a 10-fold 1:2 serial dilution. Rat Ig were used as negative control and the concentration of the Crry proteins to cause 50% inhibition of lysis was calculated as previously described (Table 4.2). Figure 4.3 shows the plot of inhibitor concentration against percentage inhibition of lysis.

In contrast to the CP assay, a significant difference was seen between the activities of 4SCR and 4SCR-Fc ($p<0.05$) as well as between the rest of the proteins and the 3SCR reagents, $p<0.001$ between 3SCR and either 5SCR-Fc, 4SCR-Fc or 4SCR. The 4SCR protein was the most active protein needing the lowest concentration to cause 50% inhibition of lysis, 74.1nM, the 4SCR-Fc protein was less active, the IH50 was 167.8nM. This showed that the AP regulatory activity was sterically hindered even in the 4SCR-Fc, presumably because an active site required for this regulation lies closer to the Fc. The 5SCR-Fc was active but as with the CP assay showed a slightly reduced function compared to 4SCR-Fc. The 3SCR form of Crry showed some activity, IH50 635.3nM, although 50% inhibition of lysis was not achieved with the concentrations used in this assay. This reduction in activity can be attributed to a deletion of an active site revealing that a crucial AP regulatory site lies within the fourth N-terminal SCR of rat Crry. The 3SCR-Fc showed no activity with the concentrations used in this assay as any remaining activity in the 3SCR reagent is sterically hindered by the Fc.

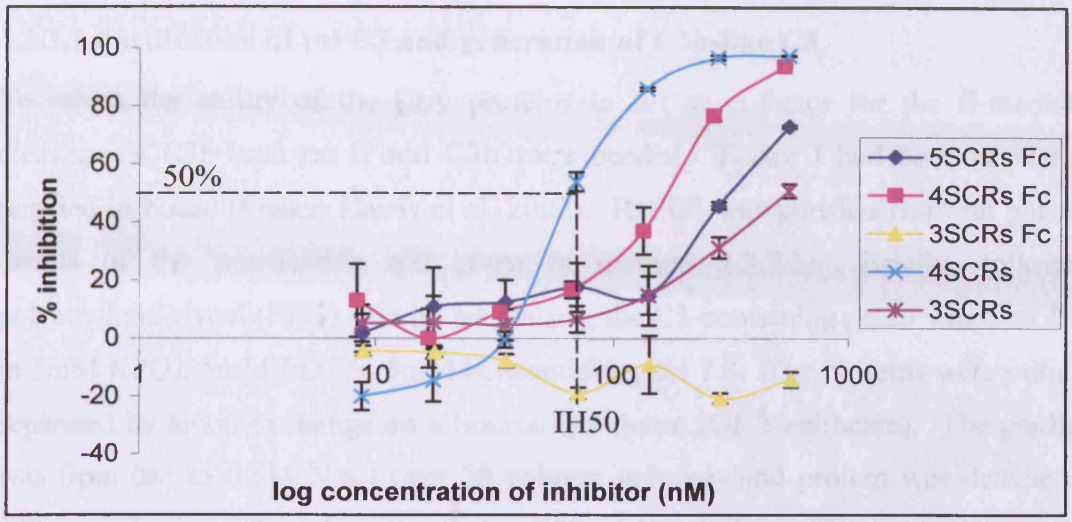


Figure 4.3 Alternative pathway haemolysis assay: Graph of % inhibition of lysis against concentration of Crry protein.

The ability of the Crry reagents to inhibit AP mediated lysis of guinea pig erythrocytes was assessed. 4SCR (turquoise) was most efficient at regulating the AP, followed by 4SCR-Fc (pink) and 5SCR-Fc (dark blue). 3SCR (purple) showed some activity in this assay whilst no activity was detected for 3SCR-Fc (yellow). Each value represents the mean value +/- 1 SD of a single experiment performed in triplicate.

	5SCR-Fc	4SCR-Fc	3SCR-Fc	4SCR	3SCR
IH50	312.9	167.8	Undetected	74.1	545
SD	23.1	17.8	N/A	1.8	51

Table 4.2 Alternative pathway haemolysis assay: IH50 for each Crry protein.

IH50 is the concentration (nM) of the respective Crry protein needed to cause 50% inhibition of lysis. SD, standard deviation of 3 measurements.

4.2.3 Cofactor assay

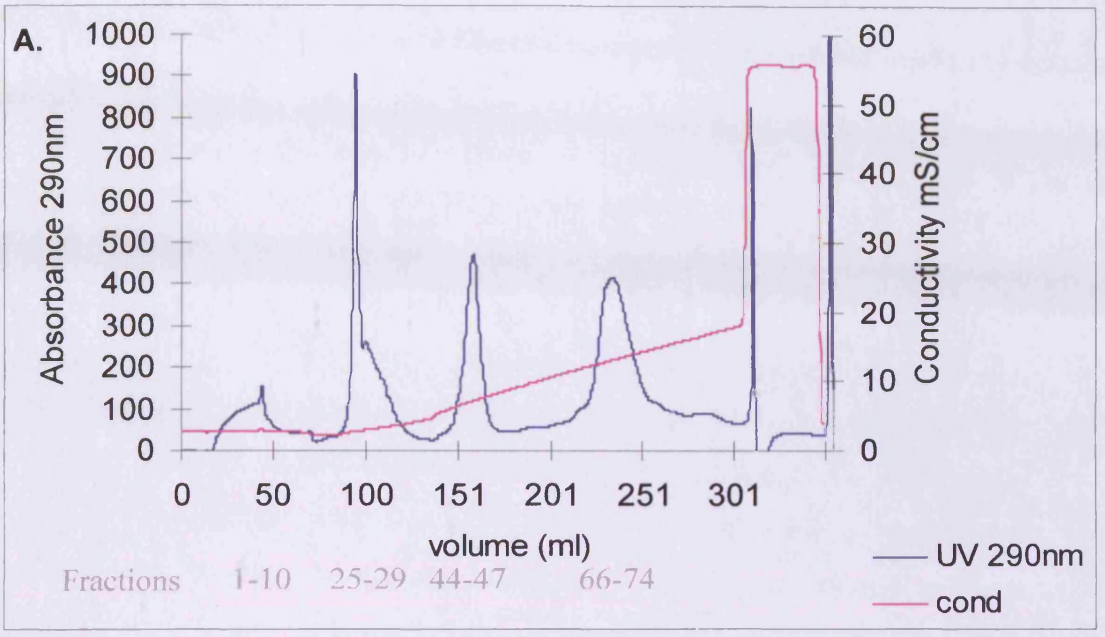
4.2.3.1 Purification of rat C3 and generation of C3b-like C3

To assess the ability of the Crry proteins to act as cofactor for the fI-mediated cleavage of C3b both rat fI and C3b were needed. Factor I had been previously purified in house (Fraser, Harris et al. 2002). Rat C3 was purified from rat plasma; details of the purification are given in method 2.3.7.3. Briefly, following polyethylene glycol (PEG) cuts on rat plasma, the C3-containing pellet was dissolved in 5mM KPO_4, 5mM EDTA, 5mM benzamidine, pH 7.8. The proteins were initially separated by anion exchange on a Source Q column (GE Healthcare). The gradient was from 0M to 0.5M NaCl over 20 column volumes and protein was detected at 290nm as benzamidine also absorbed at 280nm interfering with protein detection at this wavelength (figure 4.4A). The C3 containing peak (fractions 44-47) was identified by dot blot analysis (figure 4.4B) and dialysed into 50mM $NaPO_4$ pH 6. The peak was then subjected to cation exchange on a Mono S column (GE Healthcare) with a gradient of 0-1M NaCl over 20 column volumes (figure 4.5A). The peak containing C3 was identified by dot blot analysis and the fractions 12-16 combined. The C3 was analysed by SDS PAGE on a 10% gel under non-reducing and reducing conditions (figure 4.5B and C). To obtain C3b-like C3, the C3 was methylamine inactivated by incubation with 0.1M methylamine in borate buffered saline, pH 8.3 for 2 hours at 37°C. The inactivated C3 ($C3_{MA}$) was then dialysed into PBS to remove the methylamine.

Figure 4.4 Anion exchange purification of rat C3 from plasma.

A. Chromatogram of the anion exchange of rat C3. The product from the 30% PEG cut of rat plasma was loaded onto the Source Q column. The proteins were separated using an increasing salt gradient, demonstrated by a change in conductivity (pink). This separation resulted in 3 major peaks, the corresponding fraction numbers are shown in grey beneath the peaks.

B. Dot blot of the Source Q fractions. The fractions corresponding to the peaks as well as the initial run through fractions (fractions 1-10) and a sample that had not been loaded onto the column (pre) were dotted onto nitrocellulose (1µl). Following blocking, the dot blot was probed with sheep anti-human C3c (known to cross react with rat C3) followed by HRPO conjugated anti-sheep IgG. The fraction numbers corresponding to each dot are below the dots. Fractions 44-47 had a strong development for rat C3 whilst fractions 66-74 showed low amounts of rat C3. Fractions 44-47 corresponding to the second peak to elute from the Source Q column were processed further.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900
800
700
600
500
400
300
200
100
0
Conductivity mS/cm
60
50
40
30
20
10
0
0
50
100
151
201
251
301
volume (ml)
Fractions
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25-29
44-47
66-74
UV 290nm
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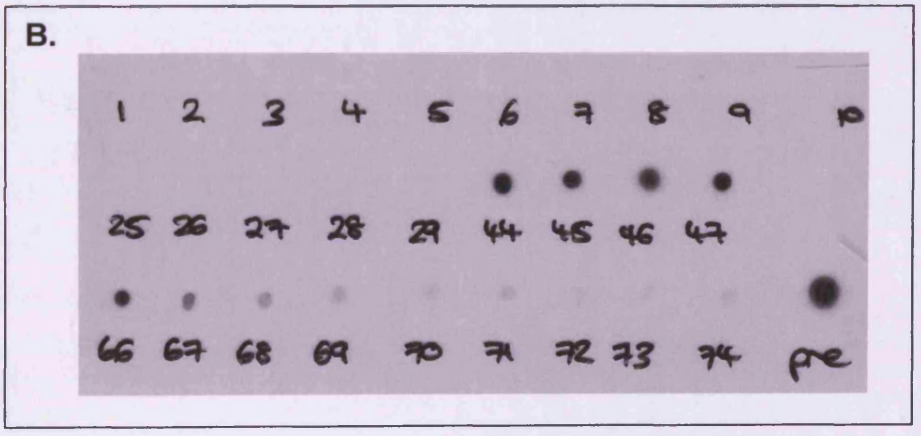
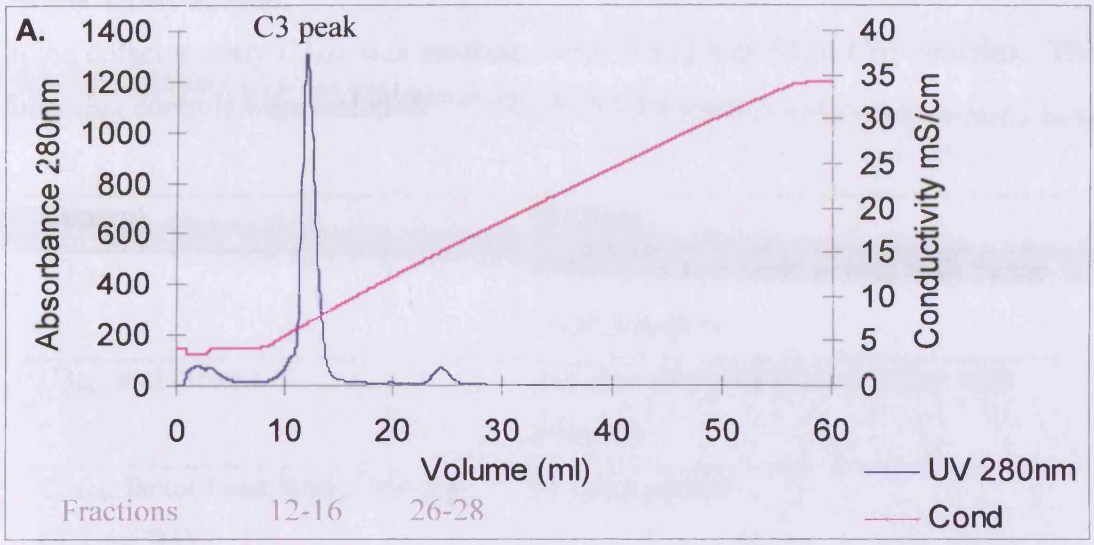

B.
1 2 3 4 5 6 7 8 9 10
25 26 27 28 29 44 45 46 47
66 67 68 69 70 71 72 73 74 pe

Figure 4.5 Cation exchange purification of rat C3.

A. Chromatogram of the cation exchange of rat C3. Fractions 44-47 from the Source Q column were dialysed into 50mM $NaPO_4$, pH 6 and loaded onto a Mono S column. The proteins were separated using an increasing salt gradient, change in conductivity in pink. The gradient resulted in the elution of two peaks, the fraction numbers corresponding to these peaks are shown in grey beneath the respective peak.

B. Dot blot of the Mono S peaks. 1µl of each fraction corresponding to the peaks and pre column sample were dotted onto nitrocellulose and probed with sheep anti-human C3c followed by anti-sheep IgG HRPO. The fraction number for each dot is shown at the bottom right hand corner of each dot. The first peak (fractions 12-16) contained rat C3, these fractions were therefore combined.

C. SDS PAGE analysis of the purified C3. A 10% SDS PAGE gel was loaded with the product from the Mono S column (lanes 1 and 3) along side a sample of protein that had not been loaded onto the column (lanes 2 and 4). The samples were either reduced (lanes 1 and 2) or non-reduced (lanes 3 and 4). The pre column sample contained a mixture of proteins, the major one was C3 identified by its α-chain (112kDa) and β-chain (75kDa) under reducing conditions. The mono S product was rat C3, again identified by its characteristic breakdown under reducing conditions.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C3 peak
Absorbance 280nm
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1000
800
600
400
200
0
Conductivity mS/cm
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35
30
25
20
15
10
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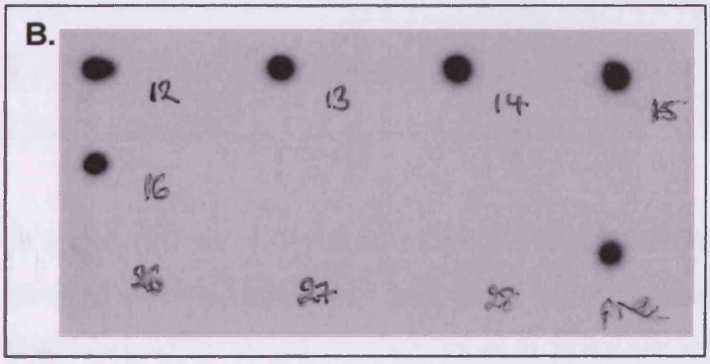
30
40
50
60
Volume (ml)
UV 280nm
Cond
Fractions
12-16
26-28


B.
12
13
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15
16
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27
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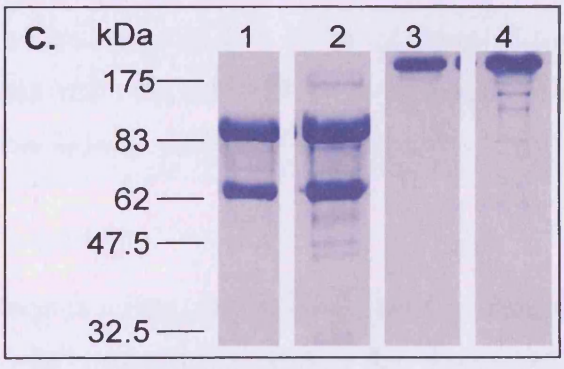
FT


C.
kDa
1
2
3
4
175
83
62
47.5
32.5

4.2.3.2 Cofactor assay

In the cofactor assay $C3_{MA}$ was incubated with fI and individual Crry proteins. The following controls were included:

Control	Purpose
$C3_{MA}$	Rule out contamination with both factor I and cofactors
$C3_{MA}$ and factor I	Rule out contamination of either with cofactors
$C3_{MA}$, factor I and human soluble CR1 (sCR1)	Positive control
$C3_{MA}$ and human sCR1	Rule out contamination of CR1 and $C3_{MA}$ with factor I
Either 5SCR-Fc, 4SCR-Fc, 3SCR-Fc, 4SCR or 3SCR with $C3_{MA}$	Rule out contamination of Crry proteins and $C3_{MA}$ with factor I

The incubations were carried out for 4 hours at 37°C. Following incubation the samples were resolved on 10% SDS PAGE gels under reducing conditions and either Western blotted and probed for rat C3 or silver stained (figure 4.6A and B). All Crry proteins had cofactor activity shown by the presence of a 43kDa band (figure 4.6C). The cleavage product was also seen in the control incubations of the cofactor with $C3_{MA}$ without fI, due to the contamination of the cofactors (Crry and CR1) or $C3_{MA}$ with fI. As the Crry and sCR1 are recombinantly produced it is highly unlikely that these proteins are contaminated, it is more likely that the $C3_{MA}$ has a slight factor I contamination.

This background cleavage provided an interesting and revealing observation as it was most prevalent in the 4SCR incubation, slightly less in the 5SCR-Fc and 4SCR-Fc incubations and barely visible in the 3SCR reagent incubations. This indicated that in the presence of added fI the reactions were driven further so Western blot and silver stain analysis was no longer sensitive to slight changes in the efficiency of cleavage and production of the 43kDa fragment. With less fI, at the level provided

with the contamination, the reactions did not proceed as far and the analysis revealed differences in activity of the proteins. Hence 4SCR appeared to be the most active cofactor followed by 5SCR-Fc and 4SCR-Fc, with the 3SCR reagents having a much reduced function.

Figure 4.6 SDS PAGE analysis of the cofactor assay.

A. Following incubation, 6µl of each test sample was resolved on a 10% SDS PAGE gel and Western blotted. It was probed with sheep anti-human C3c (known to cross react with rat C3) followed by anti-sheep IgG HRPO. No cleavage product was seen in incubations 1 and 2 showing that any cleavage can be attributed to the cofactor. In the presence of fI, all the Crry proteins acted as a cofactor for the cleavage of C3 (lanes 10-14). In the absence of fI (lanes 5-9) cleavage was also seen for 5SCR-Fc, 4SCR-Fc and 4SCR due to a low level fI contamination of the $C3_{MA}$. Cleavage was seen for human CR1 with and without factor I (lanes 3 and 4).

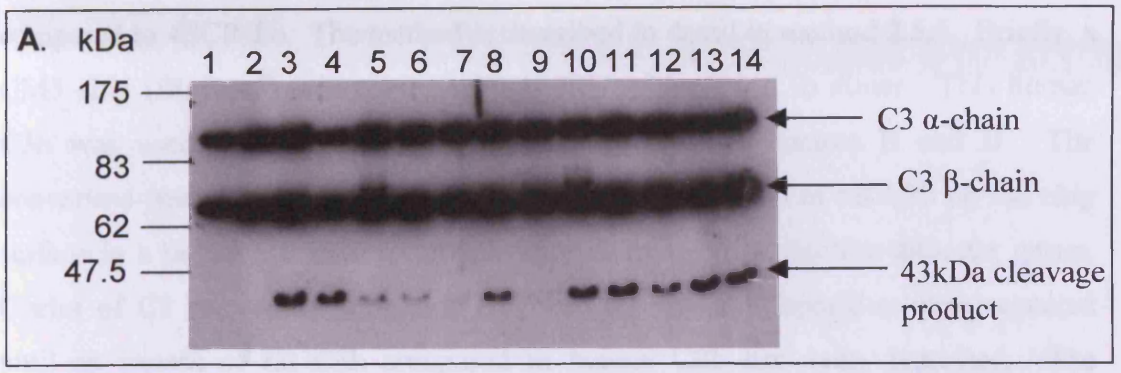
B. Similarly, 6µl samples of the cofactor incubations were resolved on a 10% SDS PAGE gel and silver stained. This result confirms the Western blot but also highlights additional bands resulting from the cleavage at 76kDa just above the β-chain of C3. The Crry-Fc proteins are also apparent at 70kDa, 62kDa and 55kDa for the 5SCR-Fc, 4SCR-Fc and 3SCR-Fc proteins respectively, CR1 is visible at 200kDa. The non-Fc Crry proteins are of lower molecular weights that are not apparent on the gel.

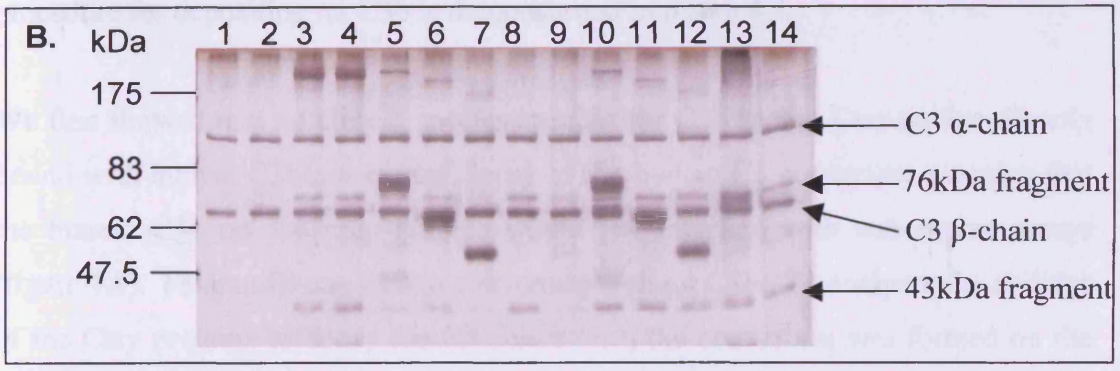
C. Diagram of the C3 chain structure showing the cleavage by fI. The C3 convertase cleaves C3 in the α-chain resulting in the release of C3a (8kDa) and the formation of C3b (189kDa). Factor I in the presence of a cofactor cleaves the α-chain resulting in 68kDa and 43kDa fragments of the α-chain (blue arrows), when $C3_{MA}$ is used as the substrate the resulting fragments are 76 and 43kDa. CR1 can cause an additional cleavage of the α-chain (red arrow). The disulphide bonds are indicated by thin black lines and the C- and N-terminus ends of the peptide chains are marked.

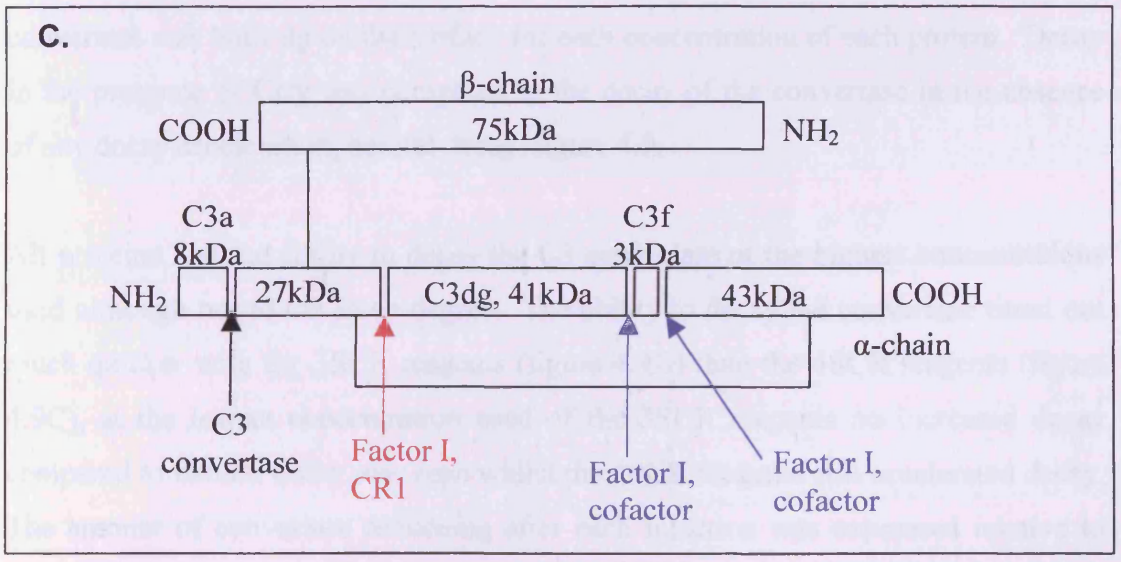
Figure adapted from Complement Methods and Protocols. B. P. Morgan. 2000. New Jersey, USA. Humana Press.

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14
$C3_{MA}$	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Factor I	-	+	+	-	-	-	-	-	-	+	+	+	+	+
Cofactor	-	-	CR1	CR1	5Fc	4Fc	3Fc	4	3	5Fc	4Fc	3Fc	4	3

Key: 5Fc is 5SCR-Fc, 4Fc is 4SCR-Fc, 3Fc is 3SCR-Fc, 4 is 4SCR, 3 is 3SCR and CR1 is human CR1.







4.2.4 Decay accelerating function

The decay accelerating abilities of 4SCR-Fc, 3SCR-Fc, 4SCR and 3SCR were investigated using surface plasmon resonance, 5SCR-Fc was not included in this experiment as previous functional analysis showed that it did not differ in function compared to 4SCR-Fc. The method is described in detail in method 2.5.5. Briefly, a CM5 chip (Biacore®) was coated with 100RU of human C3b dimer. This human C3b was used to form the C3 convertase by injecting factors B and D. The convertase was used to cleave rat C3 resulting in deposition of rat C3b on the chip surface in a native orientation through the generation of a reactive thioester group. Cycles of C3 convertase formation followed by rat C3b deposition were repeated until an excess of rat C3b compared to human C3b had been deposited. The procedure for depositing rat C3b is demonstrated in figure 4.7.

We first showed that rat Crry is species specific for C3b in that Crry neither directly bound with human C3b nor caused decay of the human C3 convertase meaning that the human C3b on the chip surface would not interfere with subsequent assays (figure 4.8). Human fB can form a convertase with rat C3b. To compare the abilities of the Crry proteins to decay the C3 convertase, the convertase was formed on the chip and decayed using the Crry proteins in a 1:2 serial dilutions. A separate convertase was built up on the surface for each concentration of each protein. Decay in the presence of Crry was compared to the decay of the convertase in the absence of any decay acceleration, natural decay, figure 4.9.

All proteins had the ability to decay the C3 convertase at the highest concentrations used although not to the same degree. The ability to decay the convertase titred out much quicker with the 3SCR reagents (figure 4.9D) than the 4SCR reagents (figure 4.9C), at the lowest concentration used of the 3SCR reagents no increased decay compared to natural decay was seen whilst the 4SCR reagents still accelerated decay. The amount of convertase remaining after each injection was expressed relative to natural decay and plotted against concentration (figure 4.10) to quantify the activity of the proteins (table 4.3). The results show the concentration of Crry protein needed to decay 50% of the convertase. The 4SCR reagent had a slightly better activity than

4SCR-Fc, 0.3μM compared to 0.75μM, due to steric restriction of the 4SCRs of Crry in the Fc fusion protein. The 3SCR reagents had a much reduced activity with 2μM of the 3SCR protein and 2.8μM of the 3SCR-Fc needed to cause 50% decay. This reveals that SCR4 of Crry contains an active site necessary for efficient decay acceleration of the AP. This correlates with the AP haemolysis assay where the 3SCR reagents had a much reduced function.

	4SCR-Fc	**3SCR-Fc**	**4SCR**	**3SCR**
Concentration required for 50% removal of convertase (μM)	0.75	2.80	0.30	2.00

Table 4.3 The concentration of the Crry proteins necessary to cause 50% decay of the convertase.

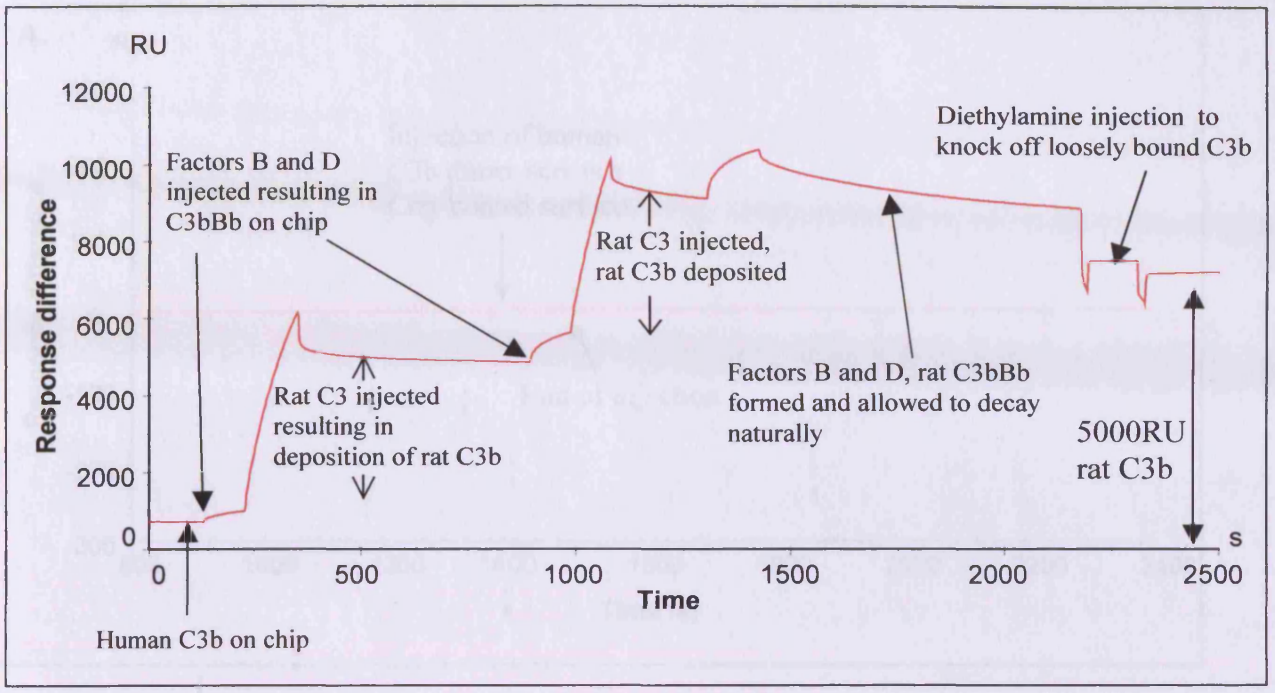


Figure 4.7 The immobilisation of rat C3b on the chip surface.

The chip was coated with 100RU of human C3b dimer and this was used to drive the deposition of rat C3b. Rat C3 was flowed across the chip following formation of C3bBb with factors B and D. Cleavage of rat C3 resulted in the deposition of rat C3b. This cycle of C3bBb formation followed by rat C3b deposition was repeated until the total amount of rat C3b deposited reached 5000RU, far in excess of the human C3b deposited. The nature of the C3b deposited was checked by flowing factors B and D across the surface, the formation of the convertase was observed along with its natural decay. Any loosely bound C3b was removed from the surface using a short injection of 50mM diethylamine/PBS pH 11.

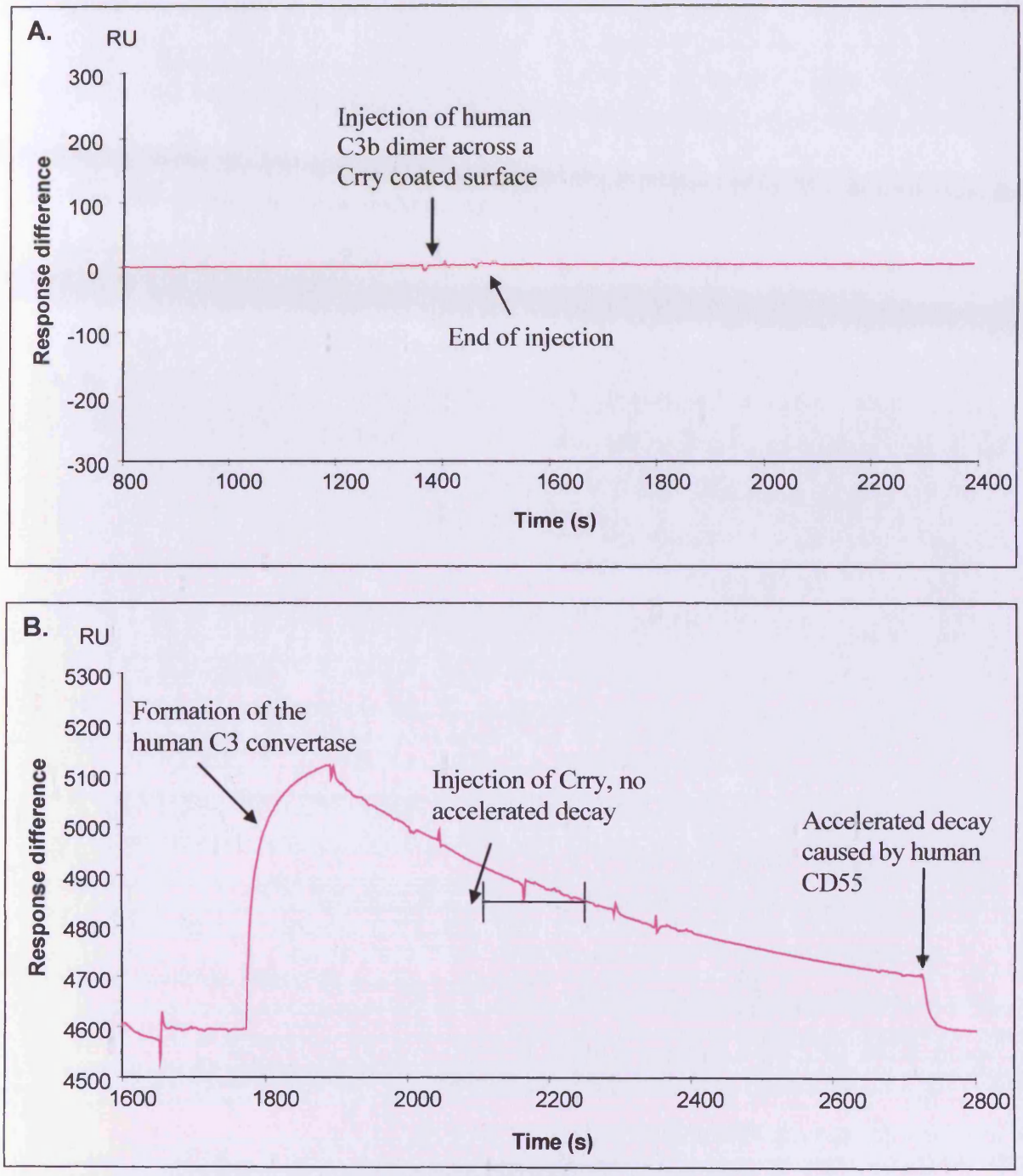


Figure 4.8 The interaction of rat Crry with human C3b.

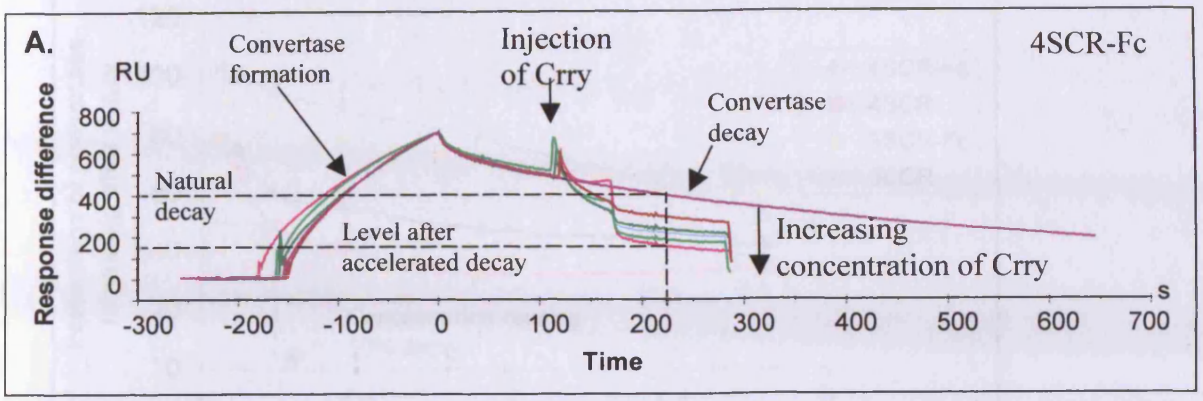
A. 4SCR was deposited on the surface of the chip and human C3b dimer (5mM) was flowed across the surface, no increase in response was seen revealing that rat Crry does not interact with human C3b.

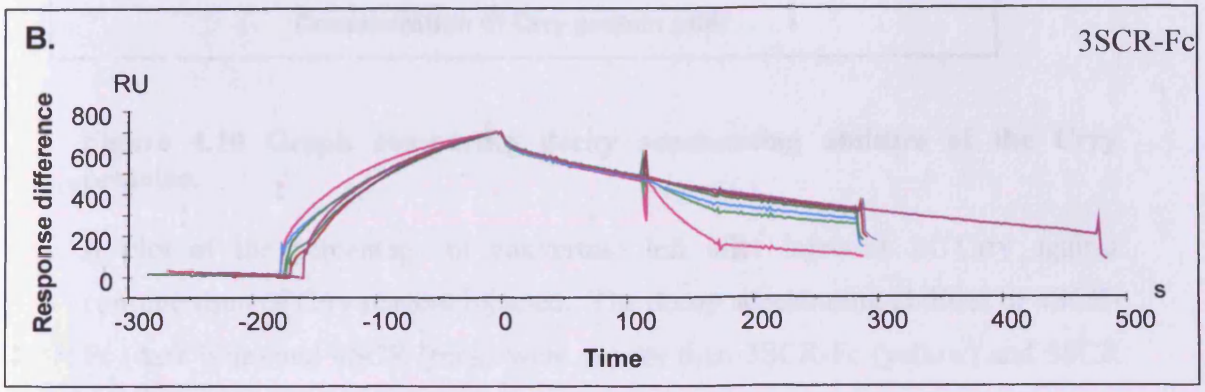
B. Human C3b was deposited on the chip surface and the human C3 convertase, C3bBb, was formed by injection of human factors B and D. Injection of 4SCR caused no accelerated decay of the convertase while human CD55 was able to cause enhanced decay demonstrating that Crry does not interact with the human C3 convertase.

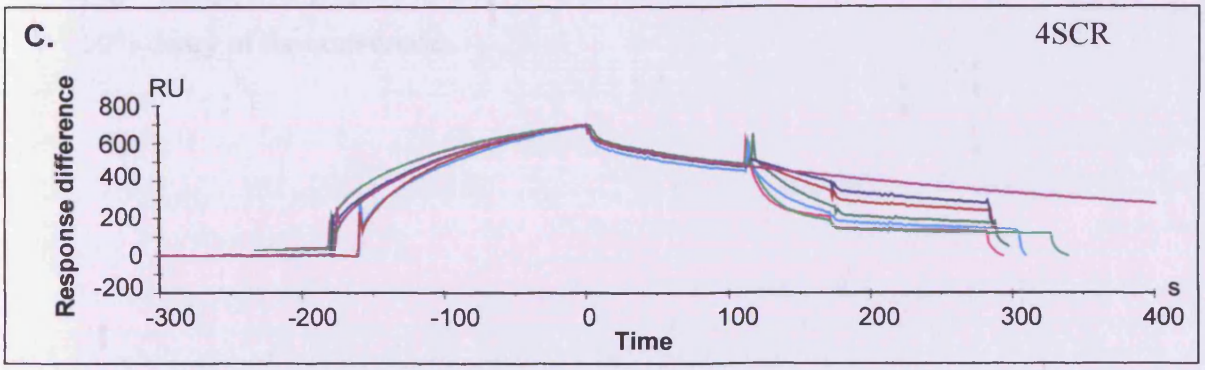
Figure 4.9 Graphs showing the decay of the C3 convertase on the surface of a Biacore® chip.

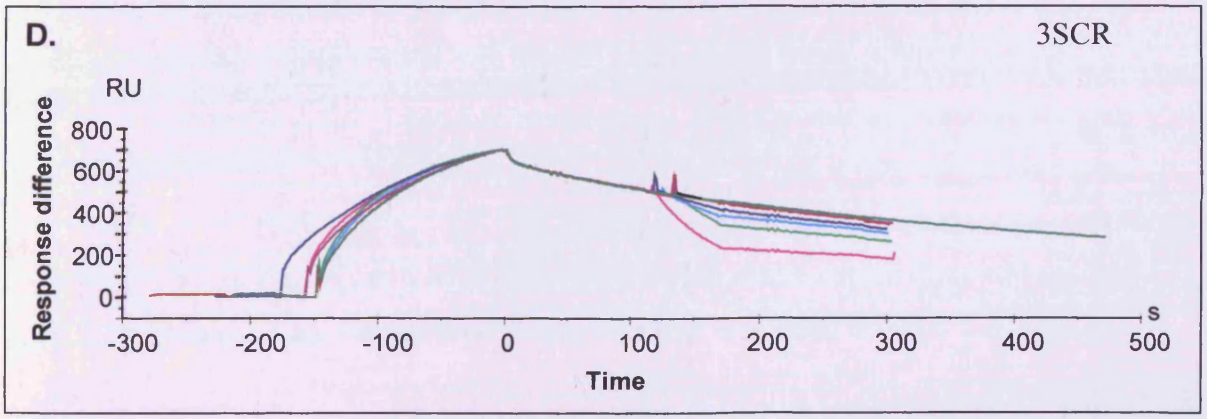
Each graph represents a different protein and the different lines on each graph illustrate the different concentrations used. Each graph shows the formation of the convertase shown by an increase in the y axis and then decay of the convertase either accelerated by a Crry protein or natural decay in the absence of any decay accelerating factor, decay is shown by a decrease in the level of the y axis. The formation and decay of the convertase is marked on figure A. For each graph the pink line represents 1.8μM of the respective Crry protein, light green represents 900nM, light blue 450nM, dark green 225nM, dark red 110nM, dark blue 55nM (absent in the 4SCR-Fc titration). Natural decay is shown in purple.

A. Decay caused by 4SCR-Fc. Following injection of 4SCR-Fc an initial binding event was seen as evidenced by an increase in the level of the y-axis. This was followed by rapid decay of the convertase. Each protein concentration used caused enhanced decay compared to natural decay. The amount of convertase remaining after the accelerated decay caused by the Crry proteins was compared to the amount remaining at the same time point with natural decay to quantify the decay accelerating abilities of the proteins.
B. Decay caused by 3SCR-Fc. Enhanced decay was observed for the 1.8μM, 900nM and 450nM injections. The other concentrations did not significantly enhance decay.
C. Decay caused by 4SCR. All concentrations exhibited an initial binding to the convertase followed by the decay of the convertase.
D. Decay caused by 3SCR. Concentrations between 1.8μM and 225nM caused enhanced decay of the convertase, for the other concentrations used there was little observable difference with natural decay.


A.
4SCR-Fc
RU
Convertase formation
Injection of Crry
Convertase decay
Natural decay
Level after accelerated decay
Increasing concentration of Crry
Response difference
800
600
400
200
0
-300
-200
-100
0
100
200
300
400
500
600
700
s
Time


B.
3SCR-Fc
RU
Response difference
800
600
400
200
0
-300
-200
-100
0
100
200
300
400
500
s
Time


C.
4SCR
RU
Response difference
800
600
400
200
0
-200
-300
-200
-100
0
100
200
300
400
s
Time


D.
3SCR
RU
Response difference
800
600
400
200
0
-300
-200
-100
0
100
200
300
400
500
s
Time

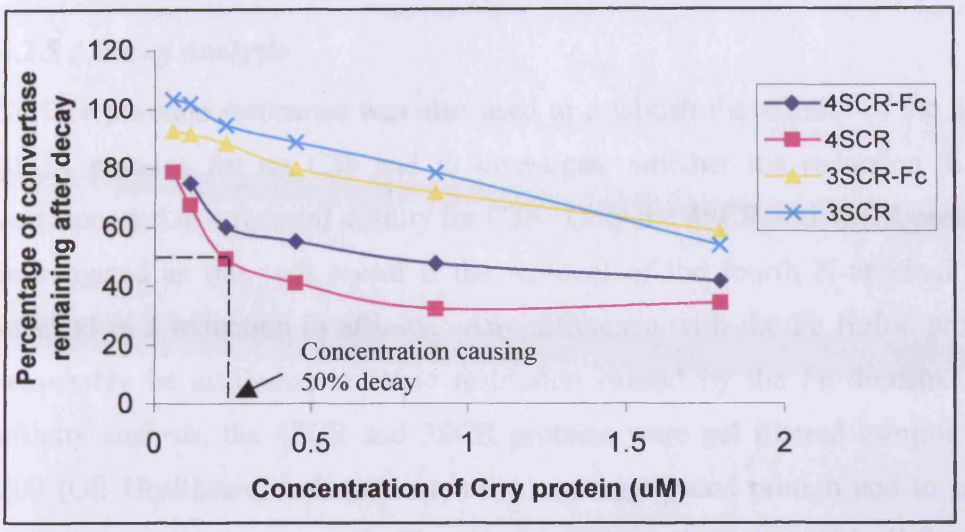


Figure 4.10 Graph comparing decay accelerating abilities of the Crry proteins.

A plot of the percentage of convertase left after injection of Crry against concentration of Crry reagent injected. The decay accelerating abilities of 4SCR-Fc (dark blue) and 4SCR (pink) were greater than 3SCR-Fc (yellow) and 3SCR (light blue). The graph was used to determine the concentration needed to cause 50% decay of the convertase.

4.2.5 Affinity analysis

Surface plasmon resonance was also used to establish the affinity of the 4SCR and 3SCR proteins for rat C3b and to investigate whether the reduction in function corresponded to a reduced affinity for C3b. Only the 4SCR and 3SCR proteins were investigated as this will reveal if the removal of the fourth N-terminal SCR has resulted in a reduction in affinity. Any difference with the Fc fusion proteins can reasonably be attributed to steric restriction caused by the Fc domain. Prior to affinity analysis, the 4SCR and 3SCR proteins were gel filtered using a Superdex 200 (GE Healthcare) column to remove any aggregated protein and to ensure the proteins were in the correct buffer (10mM HEPES, 150mM NaCl pH 7.4) (method 2.3.9). A chip (CM5; Biacore®) was coated with 100RU of human C3b dimer. As previously described, this human C3b was used to form the C3 convertase which in turn cleaved rat C3 causing deposition of C3b on the surface in a native conformation, figure 4.7. For affinity analysis only 500RU of rat C3b was bound to the chip. The chip surface was stabilised by injecting 10µl 50mM diethylamine/PBS pH 11 to elute any loosely bound rat C3b. The 4SCR reagent (12.5µM) was diluted in a 1:2 serial dilution in HPS-P buffer (Biacore®; 10mM HEPES, 150mM NaCl, 0.005% (w/v) surfactant P20, pH 7.4). Each concentration was flowed over the chip in duplicate (figure 4.11A). A 1:2 serial dilution of 3SCR was also created beginning at 33µM and these dilutions were also flowed over the rat C3b coated chip (figure 4.11B; method 2.5.6).

The amount of rat C3b on the chip surface was then increased to 1000RU by injecting factors B and D along with rat C3. The chip surface was stabilised and the affinity analysis was repeated. The higher density of rat C3b enabled more reliable affinity data for the 3SCR reagent to be collected. In the second affinity analysis, the 4SCR concentration series began at 25µM and the 3SCR concentration series began at 33µM. For each protein, the response was plotted against concentration and steady state affinity analysis was used to calculate the affinity of the Crry proteins for rat C3b (figure 4.13). The affinity (KD) of the 4SCR protein with the two different densities of rat C3b was comparable, $5.1x10^{-6}$M and $5.61x10^{-6}$M, and was greater than the affinity of 3SCR (table 4.4). The 3SCR affinity dramatically altered with the different C3b coats, $1.36x10^{-4}$ and $6.65x10^{-5}$ because the interaction has a much

lower affinity. Coating with the lower density resulted in very little response with the 3SCR reagent, the response was close to the noise limit of the machine so the data is not reliable. Coating with 1000RU of rat C3b increased the response seen with the 3SCR interaction. However, for both analyses the concentration of 3SCR used did not reach the KD. Whilst an accurate determination of the 3SCR/C3b affinity cannot be determined, these data illustrate that deletion of the fourth N-terminal SCR of rat Crry reduces the affinity for rat C3b.

	500RU rat C3b coat			**1000RU rat C3b coat**		
	KD (M)	**RMax (RU)**	**Chi^2**	**KD (M)**	**RMax (RU)**	**Chi^2**
4SCR	5.1×10^{-6}	79.9	0.104	5.6×10^{-6}	135	0.4
3SCR	1.4×10^{-4}	70.7	0.114	6.7×10^{-5}	90.8	0.122

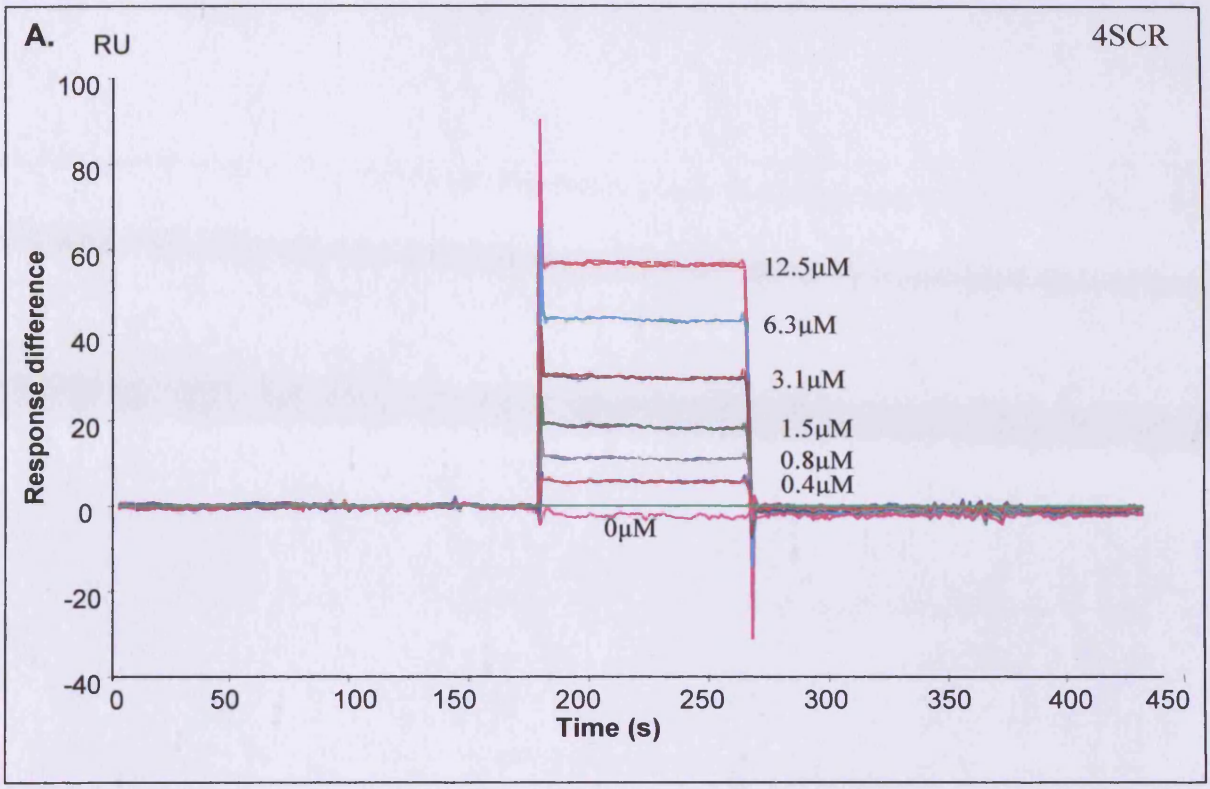
Table 4.4 Affinity results for 4SCR and 3SCR.

KD is the dissociation equilibrium constant, the greater the number the lower the affinity. RMax is the anticipated maximum response for each protein, the point at which response no longer increases with concentration.

Figure 4.11 Sensograms from the affinity analysis of 3SCR and 4SCR binding to 500RU of rat C3b.

Sensograms from the affinity experiment carried out when the chip surface was coated with 500RU of rat C3b. Each protein was injected across the surface at a variety of concentrations as indicated by the concentrations on each sensogram.

A. 4SCR affinity analysis. All concentrations of 4SCR injected had a clear binding.
B. 3SCR affinity analysis. The maximum response difference achieved with the 3SCR proteins was much lower than for the 4SCR protein. Injection of the lower concentrations resulted in very little binding. Both these observations indicate that 3SCR has a reduced affinity compared to 4SCR.


A.
RU
4SCR
Response difference
100
80
60
40
20
0
-20
-40
0
50
100
150
200
250
300
350
400
450
Time (s)
12.5μM
6.3μM
3.1μM
1.5μM
0.8μM
0.4μM
0μM

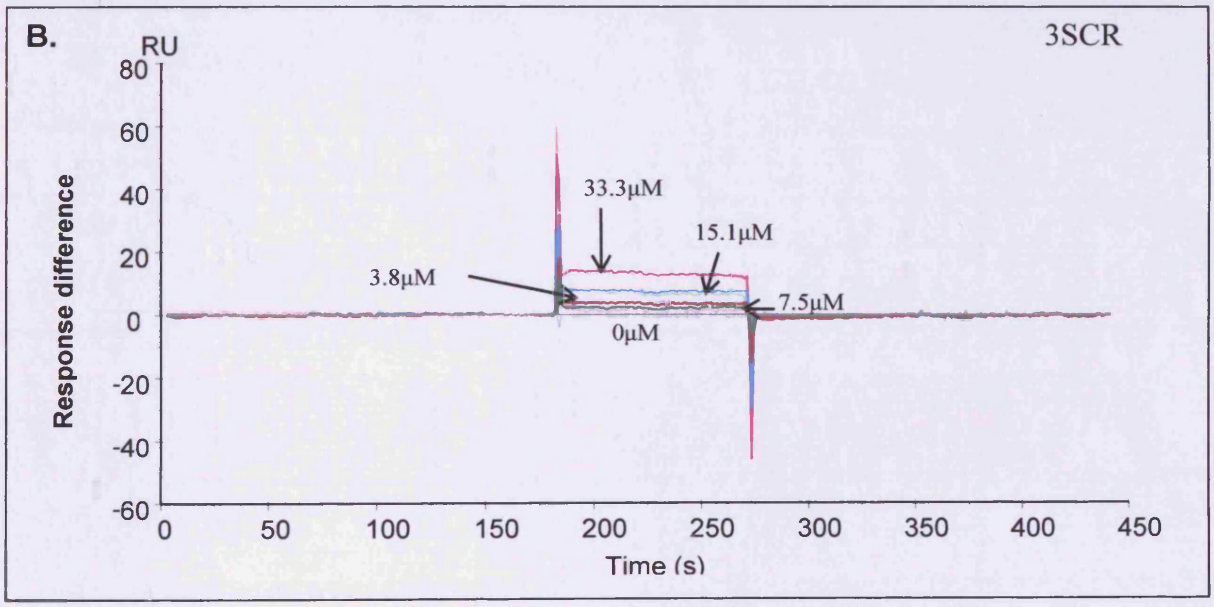
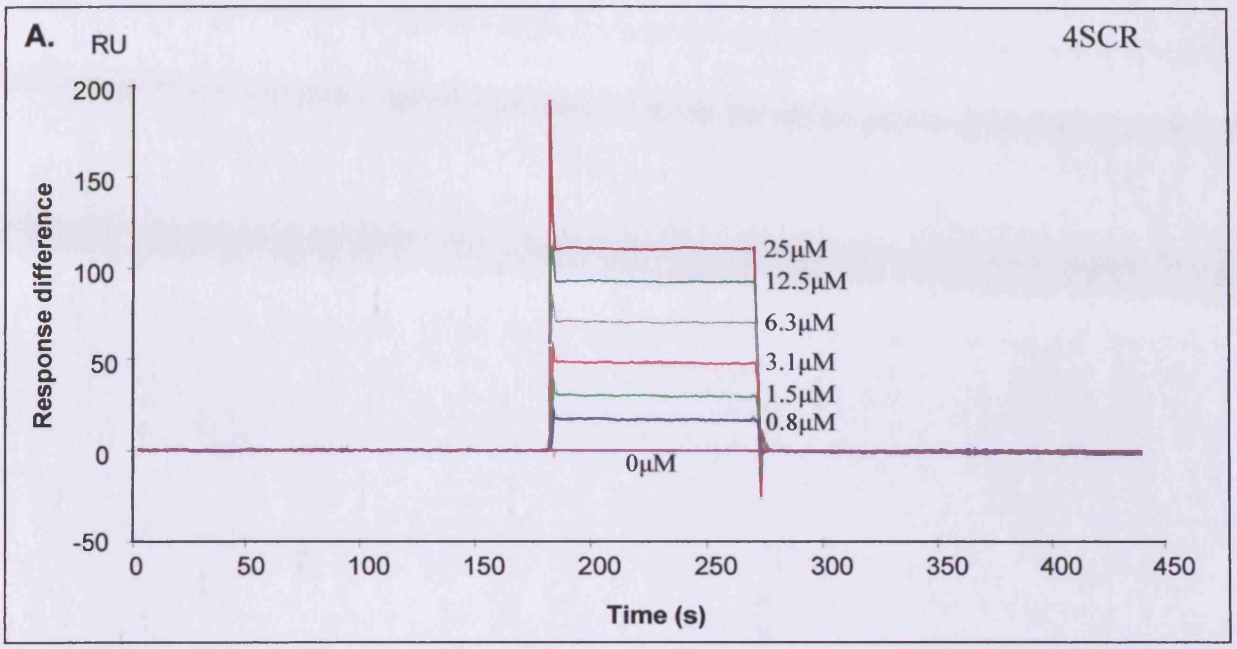

B.
RU
3SCR
Response difference
80
60
40
20
0
-20
-40
-60
0
50
100
150
200
250
300
350
400
450
Time (s)
33.3μM
15.1μM
3.8μM
7.5μM
0μM

Figure 4.12 Sensograms from the affinity analysis of 4SCR and 3SCR with 1000RU rat C3b.

Sensograms from the affinity experiment carried out when the chip surface was coated with 1000RU of rat C3b. Each protein was injected across the surface in a 1:2 serial dilution, concentrations indicated on each sensogram.

A. 4SCR affinity analysis, binding was seen for every concentration of 4SCR used.
B. 3SCR affinity analysis, the response with the 1000RU coat was greater than the response with the 500RU coat.


A.
RU
4SCR
Response difference
200
150
100
50
0
-50
0
50
100
150
200
250
300
350
400
450
Time (s)
25μM
12.5μM
6.3μM
3.1μM
1.5μM
0.8μM
0μM

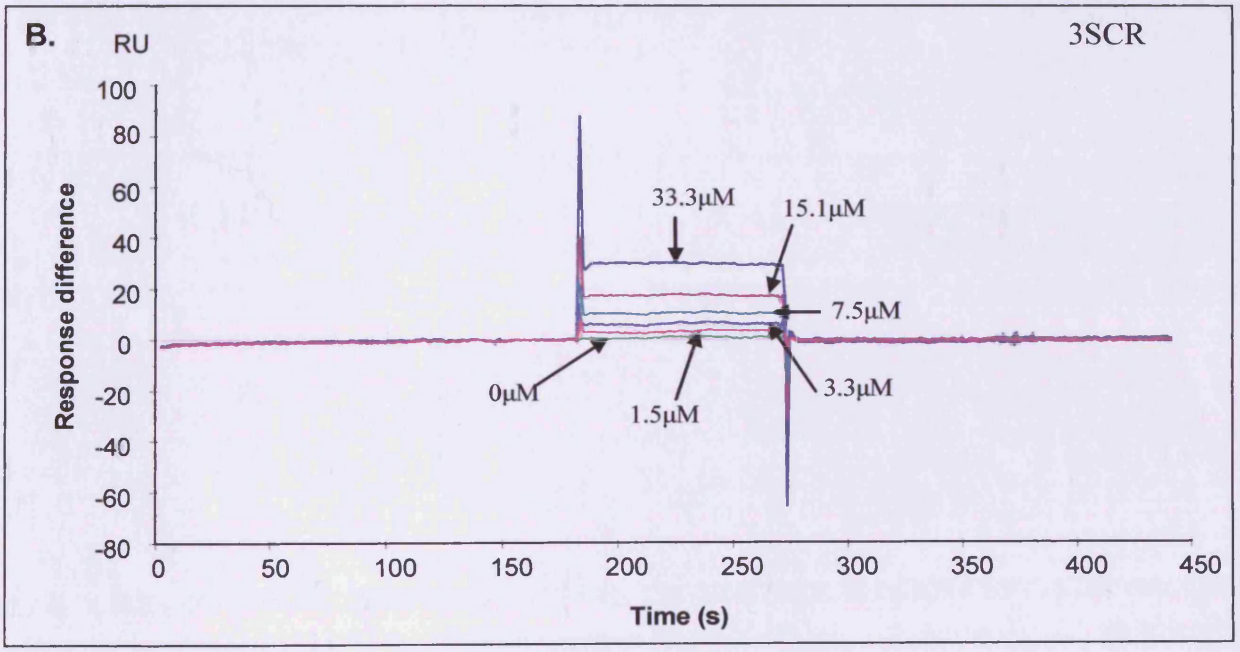
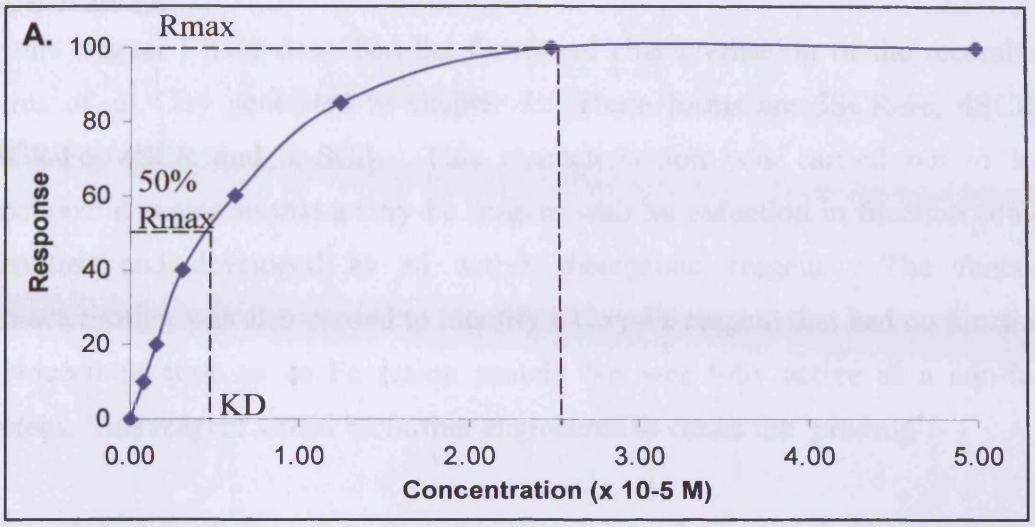
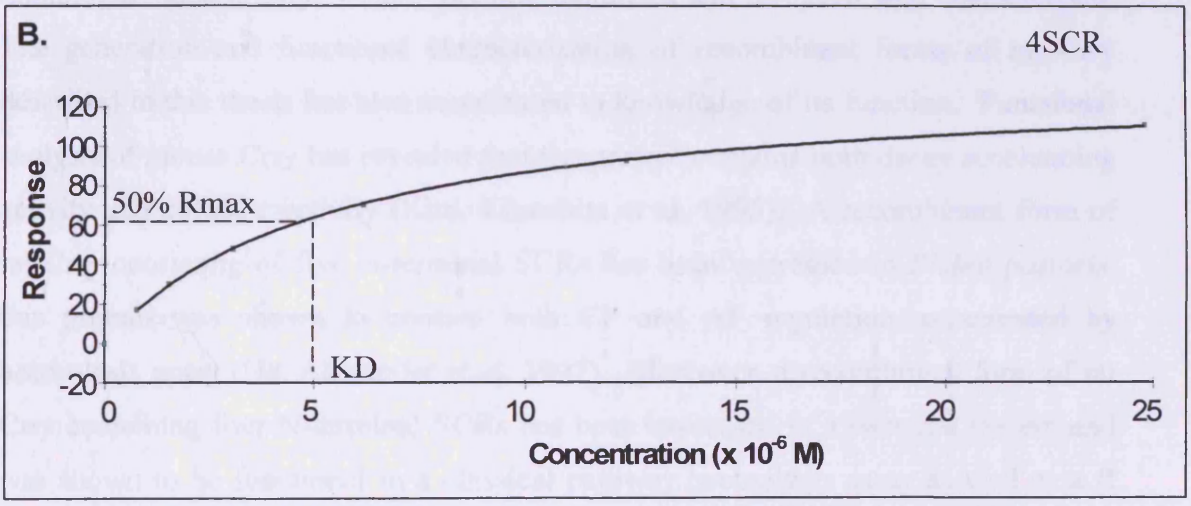

B.
RU
3SCR
Response difference
100
80
60
40
20
0
-20
-40
-60
-80
0
50
100
150
200
250
300
350
400
450
Time (s)
33.3μM
15.1μM
7.5μM
3.3μM
1.5μM
0μM

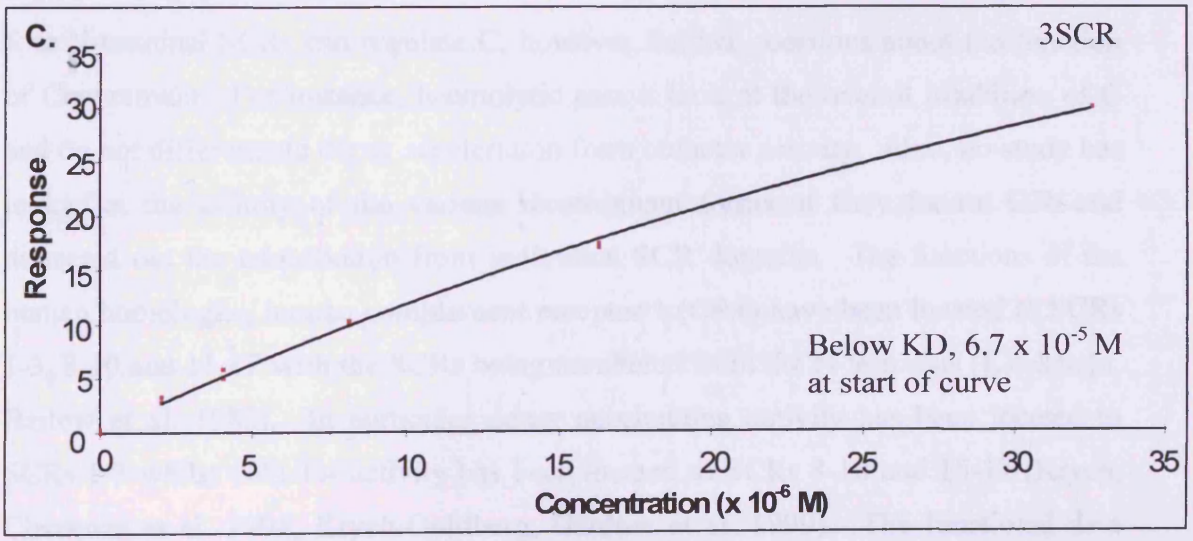
Figure 4.13 Calculation of the steady state affinity constants.

In order to calculate the affinity constants, response was plotted against concentration, shown here for the data achieved with the 1000RU coat of rat C3b.

A. Example curve showing calculation of Rmax, the point at which response no longer increases with concentration. The KD (dissociation equilibrium constant) corresponds to 50% Rmax.
B. 4SCR plot of response against concentration used to calculate the KD, $5.6x10^{-6}$M.
C. 3SCR plot of response against concentration used to calculate the KD, $6.7x10^{-5}$M.


A.
Rmax
100
80
60
40
20
0
50%
Rmax
KD
Response
0.00
1.00
2.00
3.00
4.00
5.00
Concentration (x 10-5 M)


B.
4SCR
120
100
80
60
40
20
0
-20
50% Rmax
KD
Response
0
5
10
15
20
25
Concentration (x 10-6 M)


C.
3SCR
35
30
25
20
15
10
5
0
Response
Below KD, 6.7 x 10-5 M
at start of curve
0
5
10
15
20
25
30
35
Concentration (x 10-6 M)

4.3 Discussion

In this chapter I have described the functional characterisation of the recombinant forms of rat Crry generated in chapter 3. These forms are 5SCR-Fc, 4SCR-Fc, 3SCR-Fc, 4SCR and 3 SCR. This characterisation was carried out to locate functional domains so that a Crry-Fc reagent with no reduction in function could be identified and developed as an active therapeutic reagent. The functional characterisation was also carried to identify a Crry-Fc reagent that had no function or a reduced function as an Fc fusion protein but was fully active as a non-fusion protein. This reagent would be further engineered to create the 'prodrug'.

Most of the knowledge of rat Crry function comes from observations of its mouse homologue, mouse Crry, which was first identified and has been well characterised. The generation and functional characterisation of recombinant forms of rat Crry described in this thesis has also contributed to knowledge of its function. Functional analysis of mouse Crry has revealed that the protein contains both decay accelerating activity and cofactor activity (Kim, Kinoshita et al. 1995). A recombinant form of rat Crry consisting of five N-terminal SCRs has been expressed in *Pichia pastoris,* this protein was shown to contain both CP and AP regulation as assessed by haemolysis assay (He, Alexander et al. 1997). Moreover, a recombinant form of rat Crry containing four N-terminal SCRs has been expressed in a bacterial system and was shown to be functional in a classical pathway haemolysis assay as well as a fI cofactor assay (Fraser, Harris et al. 2002). These studies have revealed that the first four N-terminal SCRs can regulate C, however, further questions about the function of Crry remain. For instance, haemolytic assays look at the overall inhibition of C and do not differentiate decay acceleration from cofactor activity. Also, no study has looked at the affinity of the various recombinant forms of Crry for rat C3b and dissected out the contribution from individual SCR domains. The functions of the human homologue, human complement receptor 1 (CR1) have been located to SCRs 1-3, 8-10 and 15-17 with the SCRs being numbered from the N-terminus (Klickstein, Bartow et al. 1988). In particular decay accelerating activity has been located to SCRs 1-3 whilst cofactor activity has been located to SCRs 8-10 and 15-17 (Krych, Clemenza et al. 1994; Krych-Goldberg, Hauhart et al. 1999). The functional data compiled here contributes to the literature by providing a comparison of different SCR containing proteins enabling the function of rat Crry to be understood in more

depth.

The ability of the Crry proteins to inhibit the lysis of erythrocytes via both the CP and AP was investigated. All the proteins were effective at inhibiting the CP while the 3SCR reagents showed a marked reduction in the ability to inhibit the AP. This suggests that the removal of the fourth SCR of rat Crry removes a site involved in inhibiting the alternative pathway. This is supported by the observation of steric hindrance in the 4SCR-Fc reagent compared to the 4SCR protein showing that an active site lies close to the Fc and hinge. This has an interesting similarity to DAF in which SCRs 1-4 are necessary for AP regulation while SCRs 1-3 are sufficient to regulate the CP (Coyne, Hall et al. 1992). In the classical pathway, differences in function are seen between 3SCR-Fc and 3SCR showing that the Fc sterically hindered the ability to regulate the CP. This implies that sites for the regulation of the CP lie within the third N-terminal SCR close to the Fc as well as in the fourth N-terminal SCR demonstrated by the reduced ability of 3SCR compared to 4SCR to regulate the CP. Differences were also seen between 4SCR-Fc and 5SCR-Fc with the 4SCR-Fc protein being more active. This may be due to the more rigid nature of the 4SCR-Fc protein resulting in the Crry arms can act together causing more efficient regulation. This synergy between two complement regulatory domains has been noted in human CR1. Correct spacing between two active sites within CR1 is needed to bring about efficient decay of the C5 convertase suggesting they act synergistically. Also the decay of the C3 convertase bought about by CR1 is increased if two copies of one active site are present in a recombinant protein (Krych-Goldberg, Hauhart et al. 1999; Krych-Goldberg, Hauhart et al. 2005). All the proteins had cofactor activity although fI titration revealed that the 4SCR was the most active followed by 5SCR-Fc and 4SCR-Fc with the 3SCR reagents being least active.

Surface plasmon resonance assessment of the ability of the Crry proteins to decay the AP C3 convertase confirmed the reduced ability of the 3SCR proteins to regulate the AP, although activity was seen for these proteins at the higher concentrations used within this assay. The 4SCR protein showed a slightly better function than 4SCR-Fc. Activity was seen for the 3SCR proteins at higher concentrations. This suggested that AP function was not entirely removed with the fourth N-terminal SCR but that

the affinity of 3SCR for C3b was less than 4SCR. This was confirmed by steady state affinity analysis. The functional analysis is summarised in table 4.5.

	Classical pathway	Alternative pathway	Decay acceleration	Cofactor activity	C3b affinity
5SCR-Fc	++	+	N/A	++	N/A
4SCR-Fc	++	+ (steric)	+ (steric)	++	N/A
3SCR-Fc	+ (steric)	-	+/-	+	N/A
4SCR	++	++	++	++	++
3SCR	++	+/-	+/-	+	+/-

Table 4.5 Summary of the functional analysis of the recombinant forms of Crry. The functional data for the Crry proteins is summarised by a ranking system. Proteins which were the most functional in the respective assay or had a high affinity are marked ++, proteins showing reduced function or affinity are marked +, proteins showing very little activity are marked +/- whilst proteins showing no function are marked -. N/A means that the protein was not tested in the particular assay.

These data are supported by the literature which report functional homology between CR1 and Crry. As already stated, the function of CR1 lies within SCRs 1-3, 8-10 and 15-17. A study using domain-by-domain phylogenetic analysis classified SCRs into 11 sub-families: a-k (McLure, Dawkins et al. 2004). This study classified the functional SCRs of CR1 a, j, e. The first three N-terminal SCRs of rat and mouse Crry were also classified a, j, e. As there is close homology between these SCRs demonstrated by their classification, it indicates that function may lie in these SCRS in Crry. Interestingly the fourth N-terminal SCR of Crry was classified as f and so was SCR 4, 11 and 18 of CR1. Again, this homology and conservation suggests that this SCR has some functional role.

In conclusion, functional analysis has revealed interesting insights into the function of Crry. The fourth N-terminal SCR contained a C3b binding site and removal of this SCR altered the ability of the proteins to decay the convertase and had a particularly striking effect on the ability to inhibit the AP. Cofactor activity and CP inhibition were also affected. The functional analysis revealed that functional sites

within Crry were deleted before steric hindrance needed to create a 'prodrug' was achieved. This thesis will therefore now focus upon 4SCR-Fc as a promising active therapeutic.

Chapter 5: The bulk generation of 4SCR-Fc and 4SCR

5.1 Introduction

In the previous chapter, I discussed the functional characterisation of the Crry proteins described in chapter 3, the aim was to identify two Crry-Fc reagents with different properties. One Crry-Fc would effectively inhibit C and could be used therapeutically in rat models of disease. The other form would be non-functional as Crry-Fc due to steric hindrance imparted on the Crry by the proximity of the Fc, but fully active as a non fusion protein. This form would be further engineered into a 'prodrug' as been discussed previously (section 1.5.6.4). However, the functional characterisation only identified one type of Crry-Fc, the fully active form that could be used in vivo without further engineering. Development of a 'prodrug' of Crry-Fc was not possible as removal of SCRs reduced the affinity of Crry for C3b and reduced the function of Crry before sufficient steric hindrance was achieved. This thesis will therefore now focus upon 4SCR-Fc as an effective anti-complement reagent and this will be compared with 4SCR to show the effect that the Fc has on the activity, half-life and therapeutic efficacy of the protein.

Sufficient quantities of the proteins for in vivo testing of the reagents need to be generated. As already discussed in chapter 3, mammalian cells including CHO have lower protein expression levels than other expression systems such as bacteria and yeast. We chose to use CHO cells as the expression system, despite their lower expression levels, as they carry out appropriate post-translational modifications of proteins including folding and glycosylation which are necessary for correct function. Correct post-translational modifications of proteins may also reduce immunogenicity as proteins need to resemble their native form so that an immune response is not mounted causing clearance and inhibition of function. A point of consideration in generating proteins for therapeutic use is the contamination of proteins with bacterial toxins. This may be a particular issue when using a bacterial expression system. One way protein yields from mammalian cell cultures can be increased is by using a high density culture system; these have become increasingly common in protein production in recent years with a surge of protein therapeutics entering the market place. These high density culture systems aim to increase the

amount of protein produced per unit of medium by driving the culture until the medium is completely exhausted such as in a fermenter system or by maximising the number of cells cultured in the medium such as the Tecnomouse (Integra Biosciences).

Fermenter systems, that is the culturing of cells within stirred tank reactors, have historically been limited by oxygen supply, waste product accumulation, the need for sophisticated process control, shear sensitivity of animal cells and the challenges of growing adherent cell lines (Glacken, Fleischaker et al. 1983). However, the development of the technology allowing such issues to be resolved has enabled many different therapeutics and diagnostic products to be produced by mammalian cells within these systems. Among the proteins produced in such systems is follicle-stimulating hormone which is produced by CHO cells using fermenter cultures and has been used to treat infertile women (Loumaye, Dreano et al. 1998).

Hollow fibre systems, the culturing of cells on the outside of hollow fibres used to provide nutrients to the cells, were originally described in 1972 but the use of this technology was limited by irregular oxygenation of the cells (Knazek, Gullino et al. 1972). In the early nineties, the Tecnomouse (Integra Biosciences) was launched which enabled optimal combination of nutrient supply via hollow fibres and oxygen supply via silicon membranes. This marked the beginning of the growth of hollow fibre systems and now many membrane based cell culture systems are on the market including the CELLine flasks (Integra Biosciences).

The advent of high density culture systems has been crucial for monoclonal antibody production. Since the introduction of hybridoma technology in 1975 the in vivo production of ascites in mice has been preferred for its cost effectiveness and high yields of antibody (Kohler and Milstein 1975). However, due to growing ethical concern leading to a ban of ascites production in many European countries including the UK, and the risk of product contamination with non-specific immunoglobulins and viruses, an in vitro alternative became necessary. High density culture systems have provided such an alternative. In order to generate large quantities of 4SCR-Fc, 4SCR and TLD1C11 (anti-Crry antibody) high density culture systems were used. The optimisation and comparison of these systems is described here. The following

high density culture systems were used: a B Braun Biotech eukaryotic fermenter system (Bioreactor), Integra Biosciences CELLine flasks (CL1000 flasks) for both adherent and non-adherent cells and an Integra Biosciences Tecnomouse.

The aims of this chapter were:

1. To compare hybridoma high density culture systems and to use these to generate large quantities of TLD1C11 for the purification of 4SCR and 4SCR-Fc.
2. To compare high density culture systems for anchorage dependent cells and use these to generate large quantities of 4SCR and 4SCR-Fc to enable in vivo characterisation of the proteins and to generate sufficient quantities of the reagents for in vivo therapy.

5.2 Specific methods and results

5.2.1 Generation of TLD1C11

The TLD1C11 hybridoma was cultured using the Tecnomouse (Integra Biosciences), a CL1000 flask (Integra Biosciences) or a conventional tissue culture flask (T80, Nunc). These systems were compared for protein production by an ELISA (method 2.4.9). The cell numbers within the Tecnomouse and CL1000 flask were recorded using a haemocytometer and cell viability was assessed using trypan blue staining. SDS PAGE analysis of the product from the Tecnomouse and CL1000 flask was carried out to investigate whether the use of high density culture systems caused aggregation of the protein or breakdown of the product.

5.2.1.1 Tecnomouse

The Tecnomouse (Integra Biosciences) uses hollow fibre technology. In this technology fine hollow fibres are bundled together and cells are cultivated on the outside of these fibres. Oxygenated culture medium is circulated within the fibres. The porous fibre membrane allows nutrients and metabolic waste products to pass across the membrane, while large cellular protein products are retained within the cellular compartment. This technology allows the culture of a large number of cells

in a small volume resulting in a concentrated product. The Tecnomouse is shown diagrammatically in figure 5.1 along with a photograph of the equipment.

The Tecnomouse was prepared and inoculated following method 2.2.5.3. In brief, following preparation of the Tecnomouse system and cassette, 6 x 10^8 cells (total) were harvested from T80 tissue culture flasks in the log phase of growth. The cells were resuspended in 10ml high density hybridoma medium (described 2.2.1) and were placed in the Tecnomouse cassette. Every seven to ten days the bulk culture medium was replaced and the cells were harvested, split 1 in 4 and reseeded. The cell number of the remaining harvest was counted, as well as the number of viable cells using trypan blue staining. The total cell number remained around 10^8 throughout the culture period; it peaked at $2x10^9$ around day 20 and day 60. The cell viability remained on average at about 50%, towards the end of the culture period the viability dropped to 20% for five consecutive harvests. This may have been due deterioration of conditions within the cassette over the culture period, gradual blockage of the hollow fibres may have resulted in impaired gas and nutrient exchange. This decrease in viability also corresponded with a decrease in cell number. It was at this point that the culture was stopped (figure 5.2A).

To assess the yield of TLD1C11, the supernatant harvested was used in an ELISA (method 2.4.9). The rate of production was 0.8mg/ml of harvest which was significantly higher than a T80 flask which was 5μg/ml (table 5.1). SDS PAGE analysis of the medium harvest revealed that the antibody was visible on an SDS PAGE gel at the Coomassie detection level showing how much greater the protein expression was than in a T80 flask where product was not visible at the Coomassie level. The protein was at the correct mass, 175kDa (figure 5.2B). The identification of the protein was confirmed by Western blot analysis. The Western blot showed other TLD1C11 bands that were not visible on the Coomassie stained gel. This shows that some smaller forms of TLD1C11 were produced under the high expression conditions, perhaps half forms or degraded antibody, but the majority had the correct mass.

	T80 flask	Tecnomouse	CELLine flask
Yield	5μg/ml	0.8mg/ml	1mg/ml

Table 5.1 Methods used to generate TLD1C11 and their yields.

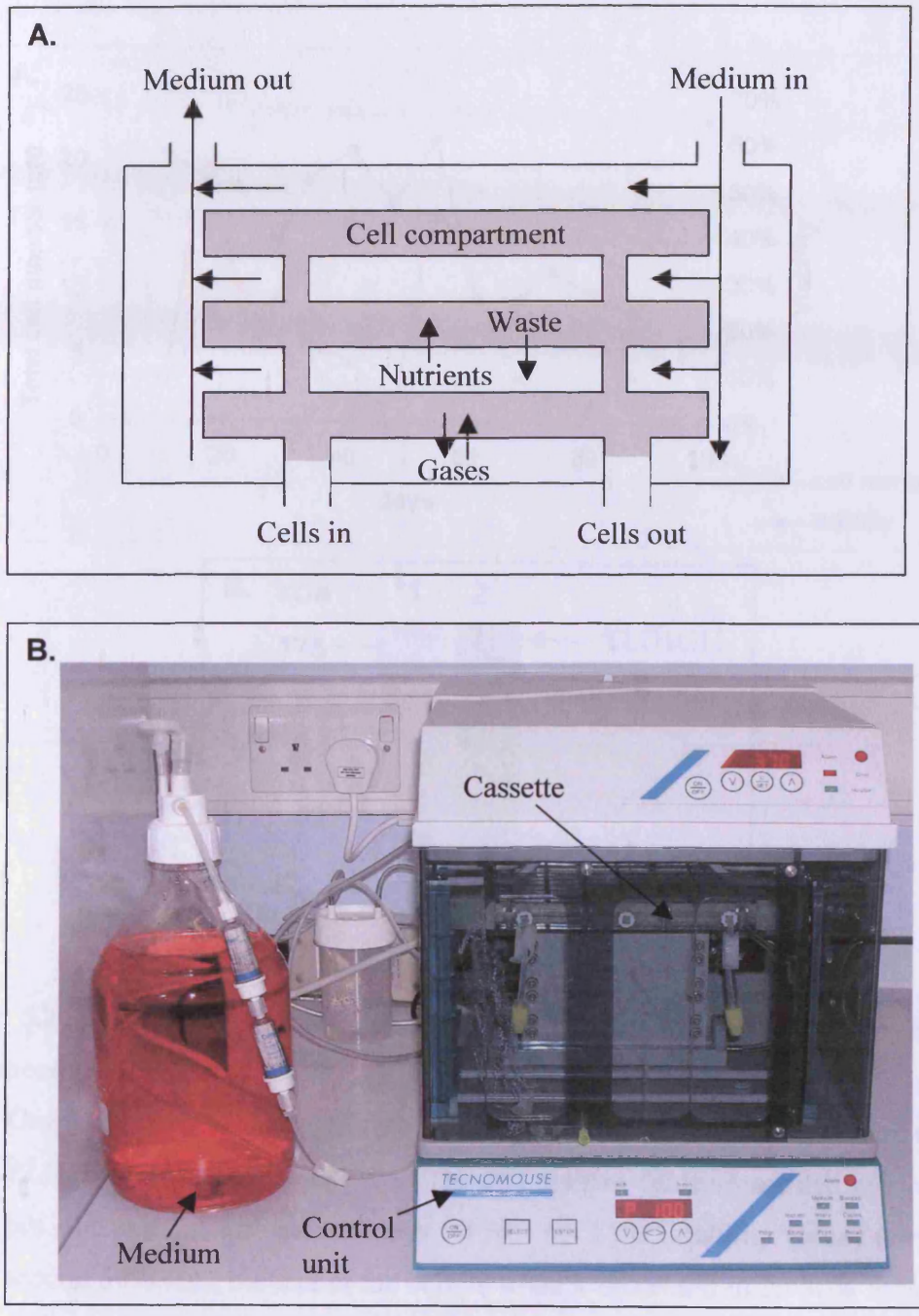


Figure 5.1 Tecnomouse diagram and photograph

A. A diagram of the Tecnomouse cassette. The cell compartments are shown in grey, nutrients move into this compartment whilst metabolic waste products move out. Gases diffuse in and out. Medium is pumped around in the extracellular space (Lukacik, Roversi et al. 2004).

B. A photograph of the Tecnomouse. The cassette sits within the control unit which maintains temperature at 37°C and the cells in 5% CO_2, medium is pumped into the cassette. The large bottle to the left is the medium which is pumped around the extracellular space.

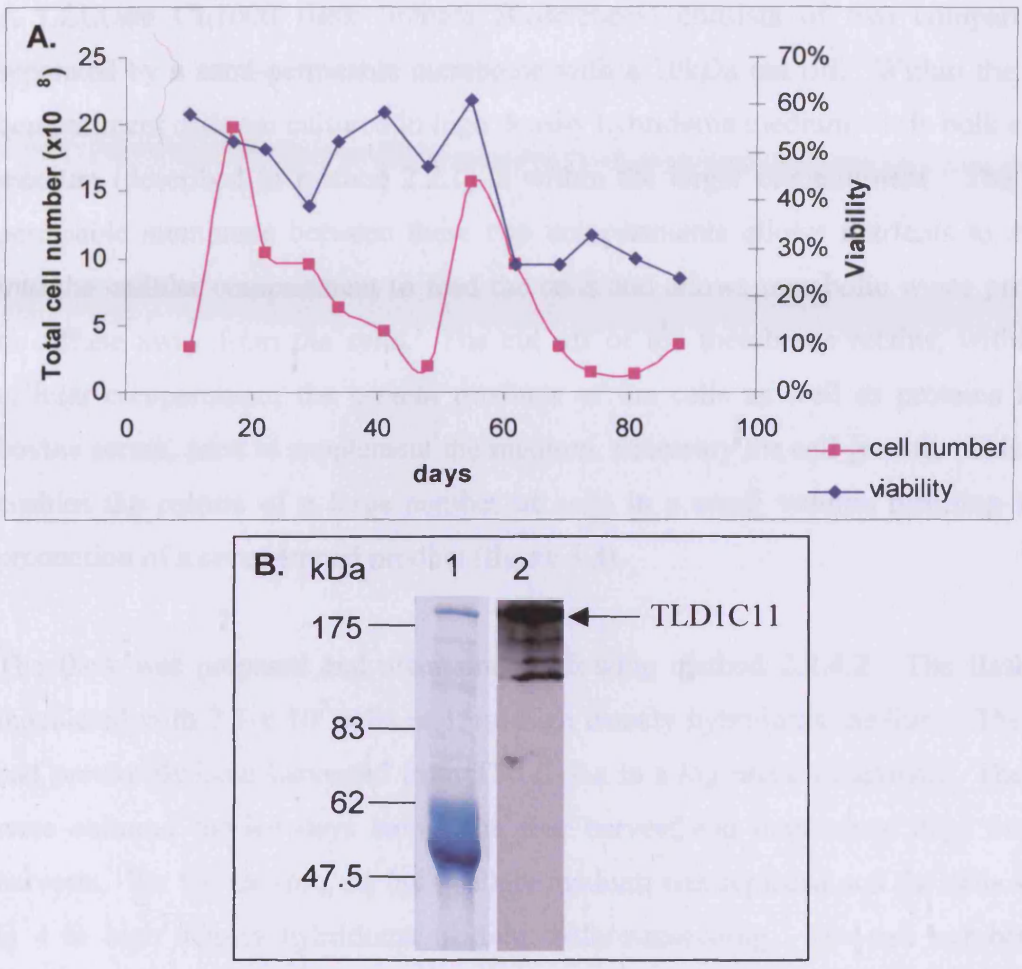


Figure 5.2 Assessment of cell viability, number and product formed in the Tecnomouse culture.

A. Graph showing the change in cell number and viability for the duration of the TLD1C11 Tecnomouse culture. The cell number (pink) remained around 10^8 but did peak at 10^9 around days 20 and 60. The viability (blue) remained around 50% until the end of the culture when it decreased to 20-30% indicating deterioration in culture conditions in the cassette, cell number also decreased during this period.

B. SDS PAGE analysis of the product from the Tecnomouse. A 7.5% gel was loaded with a sample of Tecnomouse supernatant and Coomassie stained after electrophoresis (lane 1) or transferred to nitrocellulose and probed with anti-mouse IgG HRPO (lane 2). On the Coomassie gel the major product was around 50kDa, characteristic of albumin from the bovine serum used to supplement the culture medium. A at 175kDa was also present. Western blot revealed that this 175kDa was TLD1C11, however, a number of smaller products were also identified.

5.2.1.2 CL1000 flasks

A CELLine CL1000 flask (Integra Biosciences) consists of two compartments separated by a semi-permeable membrane with a 10kDa cut off. Within the small compartment cells are cultured in high density hybridoma medium while bulk culture medium (described in method 2.2.1) is within the larger compartment. The semi-permeable membrane between these two compartments allows nutrients to diffuse into the cellular compartment to feed the cells and allows metabolic waste products to diffuse away from the cells. The cut off of the membrane retains, within the cellular compartment, the protein products of the cells as well as proteins in the bovine serum, used to supplement the medium, necessary for cell growth. This flask enables the culture of a large number of cells in a small volume resulting in the production of a concentrated product (figure 5.3).

The flask was prepared and maintained following method 2.2.4.2. The flask was inoculated with 2.5 x 10^7 cells in 15ml high density hybridoma medium. The cells had previously been harvested from T80 flasks in a log phase of growth. The cells were cultured for ten days before the first harvest and then seven days between harvests. For the harvest, the bulk culture medium was replaced and the cells split 1 in 4 in high density hybridoma medium before reseeding. The cell number was established as described previously. The total cell numbers reached a maximum at 5 x 10^8 and remained around 2.5 x 10^8 for the culture period. The viability of the cells decreased to 40% where it stayed for the culture period (figure 5.4A). The yield as calculated by ELISA was 1mg/ml of harvest supernatant, again significantly higher than the T80 flask yield (table 5.1). SDS PAGE analysis revealed that the product was visible with Coomassie staining. As with the Tecnomouse, the product was of the correct mass, 175kDa, showing that it was neither aggregated nor broken down by the culture conditions used (figure 5.4B). The identification of the protein was confirmed by Western blot analysis. The Western blot did show additional bands indicating that some degradation of the antibody may have occurred but the majority of the antibody was the correct mass.

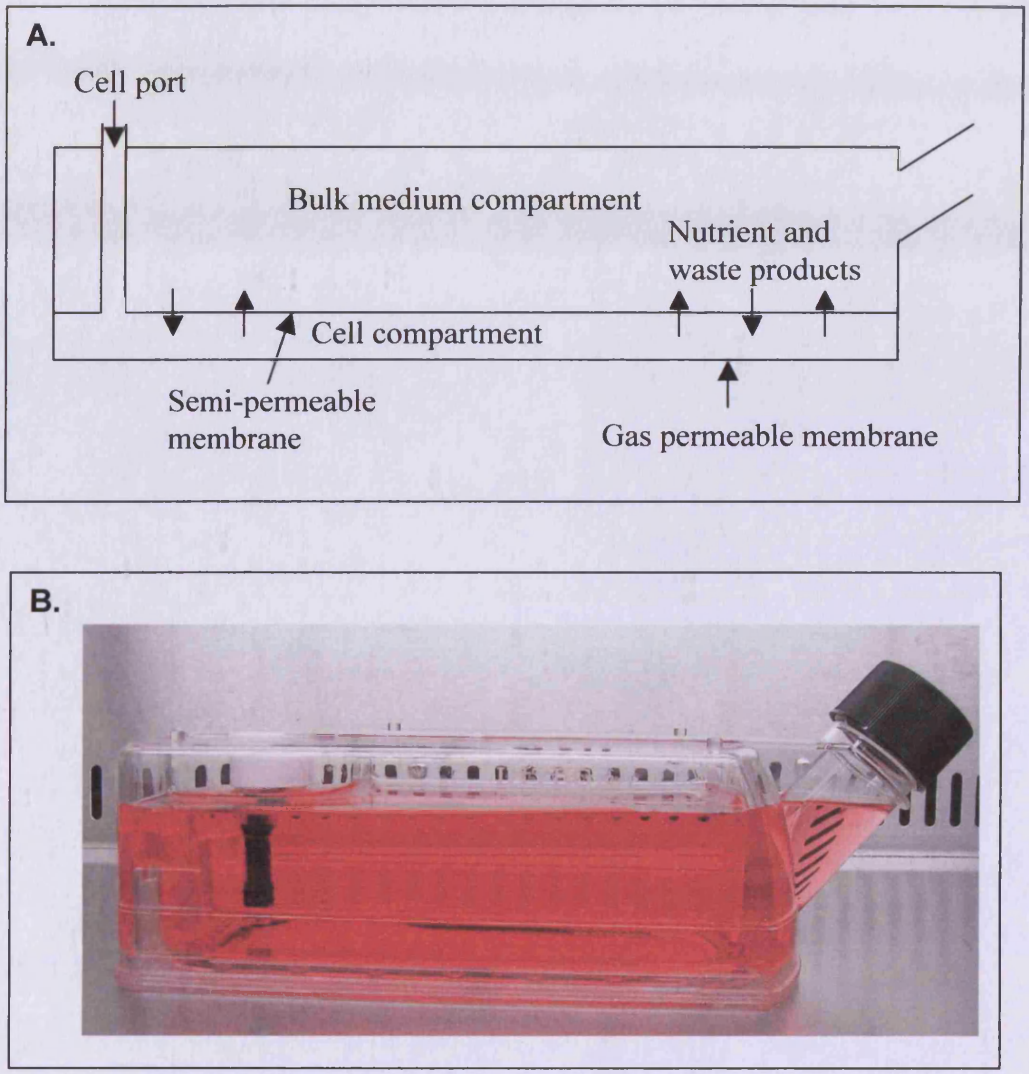


Figure 5.3 CL1000 flask diagram and photograph.

C. Diagram of the CL1000 flask. Cells are placed in the flask through the cell port. Nutrients pass into the cell compartment and waste products out into the bulk medium compartment through the semi-permeable membrane. Gas enters the cell compartment through the gas permeable membrane while the medium is gassed via the lid.

D. A photograph of a CL1000 flask.

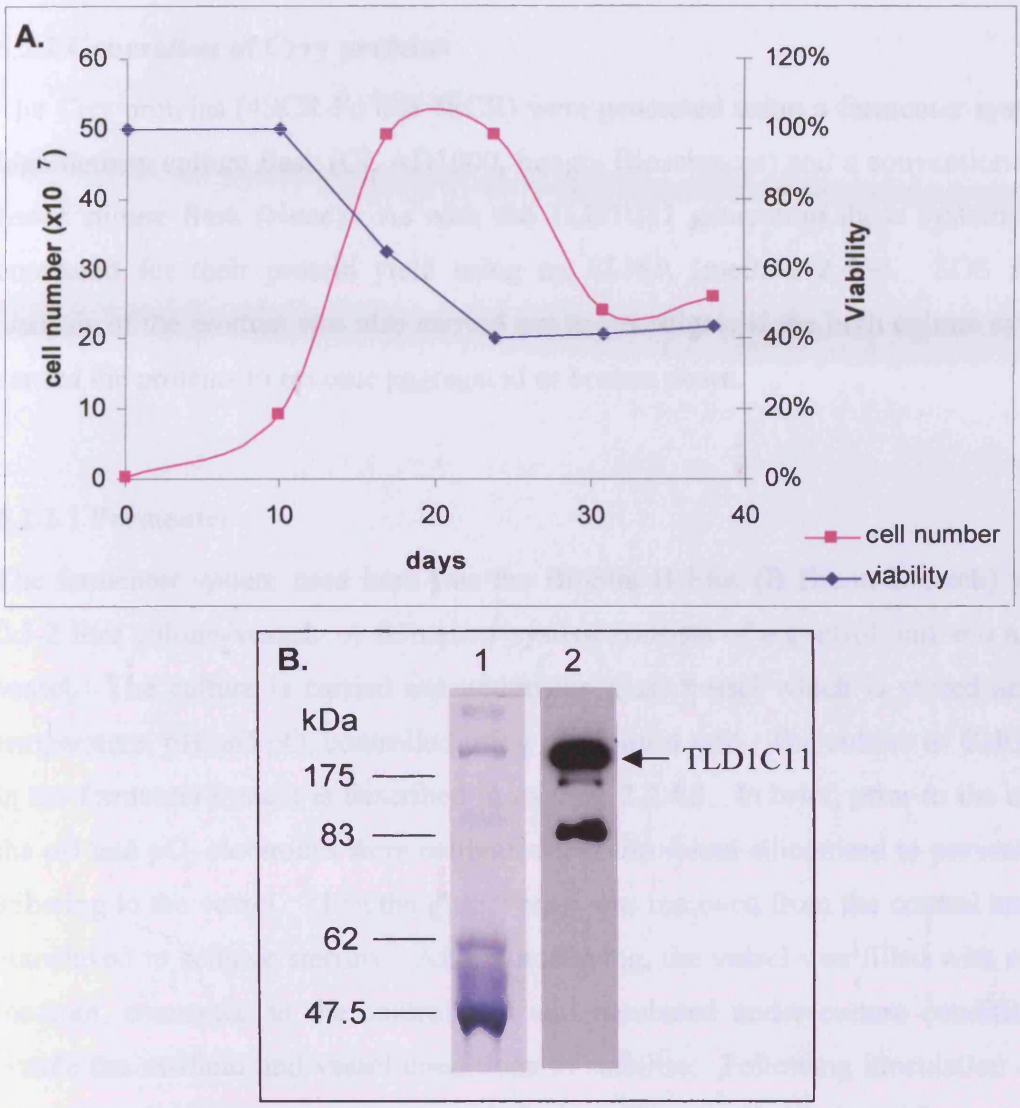


Figure 5.4 CELLine flask cell number, viability and SDS PAGE

A. Graph showing the number (pink) and viability (blue) of the TLD1C11 hybridomas cultured in the CL1000 flask. The cell number peaked at 5 x 10^8 and then dropped to 2.5 x 10^8. The viability began at 100% and dropped to around 40% for the duration of the culture.

B. SDS PAGE analysis of the supernatant from the CL1000 flask. A 7.5% gel was loaded with supernatant and was Coomassie stained (lane 1) or transferred to nitrocellulose and probed with anti-mouse IgG HRPO (lane 2). The major protein in the supernatant was albumin at around 50kDa from the bovine serum used to supplement the media. At 175kDa was also visible and this was identified as TLD1C11 by Western blot analysis. Half forms or degraded forms of the antibody were also visible on the Western.

5.2.2 Generation of Crry proteins

The Crry proteins (4SCR-Fc and 4SCR) were generated using a fermenter system, a high density culture flask (CL AD1000, Integra Biosciences) and a conventional T80 tissue culture flask (Nunc). As with the TLD1C11 generation these systems were compared for their protein yield using an ELISA (method 2.4.9). SDS PAGE analysis of the product was also carried out to investigate if the high culture systems caused the proteins to become aggregated or broken down.

5.2.2.1 Fermenter

The fermenter system used here was the BioStat B Plus (B Braun Biotech) with a 0.5-2 litre culture vessel. A fermenter system consists of a control unit and a glass vessel. The culture is carried out within the glass vessel which is stirred and has temperature, pH and pO_2 controlled using the control unit. The culture of CHO cells in the fermenter system is described in method 2.2.4.3. In brief, prior to the culture the pH and pO_2 electrodes were calibrated and the vessel siliconised to prevent cells adhering to the vessel. Then the glass vessel was removed from the control unit and autoclaved to achieve sterility. After autoclaving, the vessel was filled with culture medium, connected to the control unit and incubated under culture conditions to enable the medium and vessel conditions to stabilise. Following inoculation of the cells, the stirring speed was controlled, the pH controlled using CO_2 and 0.1M Sodium carbonate and the pO_2 controlled using O_2 and N_2. Additional system pumps enabled medium to be removed and added to the culture and a sample port enabled cell numbers and viability to be monitored throughout the fermentation. The fermenter is shown schematically is figure 5.5 along with a photograph of the system.

As CHO cells are adherent, they were attached to microcarrier beads (Cytodex 1, GE Healthcare) for the fermentation. The beads were prepared following the manufacturer's instructions, method 2.2.4.3. For the first fermentation, the beads were autoclaved within the vessel in PBS (phosphate buffered saline), the PBS was decanted off, the beads washed into medium and the cells were inoculated through the injection port once the litre of medium within the vessel had stabilised at the

desired culture conditions. The vessel was stirred for five minutes every hour within the first three hours to enable the cells to attach to the beads. A sample taken from the fermenter at three hours post inoculation revealed that cells were attached to the beads, hence full stirring was commenced. However, at day three post inoculation no cells were visible on the beads so the fermentation was stopped. The addition of the cells into the large volume of medium within the vessel had meant that many cells did not attach to beads. Also commencing full stirring at 3 hours post inoculation may have resulted in weakly attached cells falling off the beads. It was therefore decided to attach the cells to the beads outside the vessel prior to inoculation and to delay the time before full stirring was commenced for future cultures. The beads were therefore prepared in a separate 500ml siliconised glass bottle and autoclaved. The beads were washed into medium and the cells added to the beads within the 500ml glass bottle. The cells were incubated with the beads overnight at 37°C with 5% CO_2 with occasional mixing. The beads with the cells attached were then added to the culture vessel of the fermenter and the fermentation begun. This approach was used to inoculate the 4SCR-Fc fermentation with 1.5 x 10^8 cells. Following inoculation, the cell numbers went through a long lag phase where the cell number was below that of inoculation. Once the culture was established the cell number remained around 10^8, decreasing to 2.8 x 10^7 before a medium change increasing to 8 x 10^8 after a medium change. The viability also altered when the medium was changed, decreasing to 50% before a feed and increasing to 100% after a feed (figure 5.6A).

For the 4SCR fermentation 2 x 10^8 cells were attached to beads in a 500ml glass bottle and then added to the culture vessel as described above. The cell numbers within this culture also went through a long lag phase when the cells were adapting to the new culture conditions; it took 8 days for the cell numbers to recover to their inoculation density. The cell numbers remained around 10^8, but decreased prior to a medium change to 6 x 10^7 and increased after a change to 1 x 10^9. The viability of the cells remained around 80% at the beginning of the culture, but did dip after the medium change to 45%. Both the cell numbers and viability dipped at this point but then recovered without any additional medium being added. This effect may have been caused by unexpected variations in gas pressure during this time period (figure 5.6B).

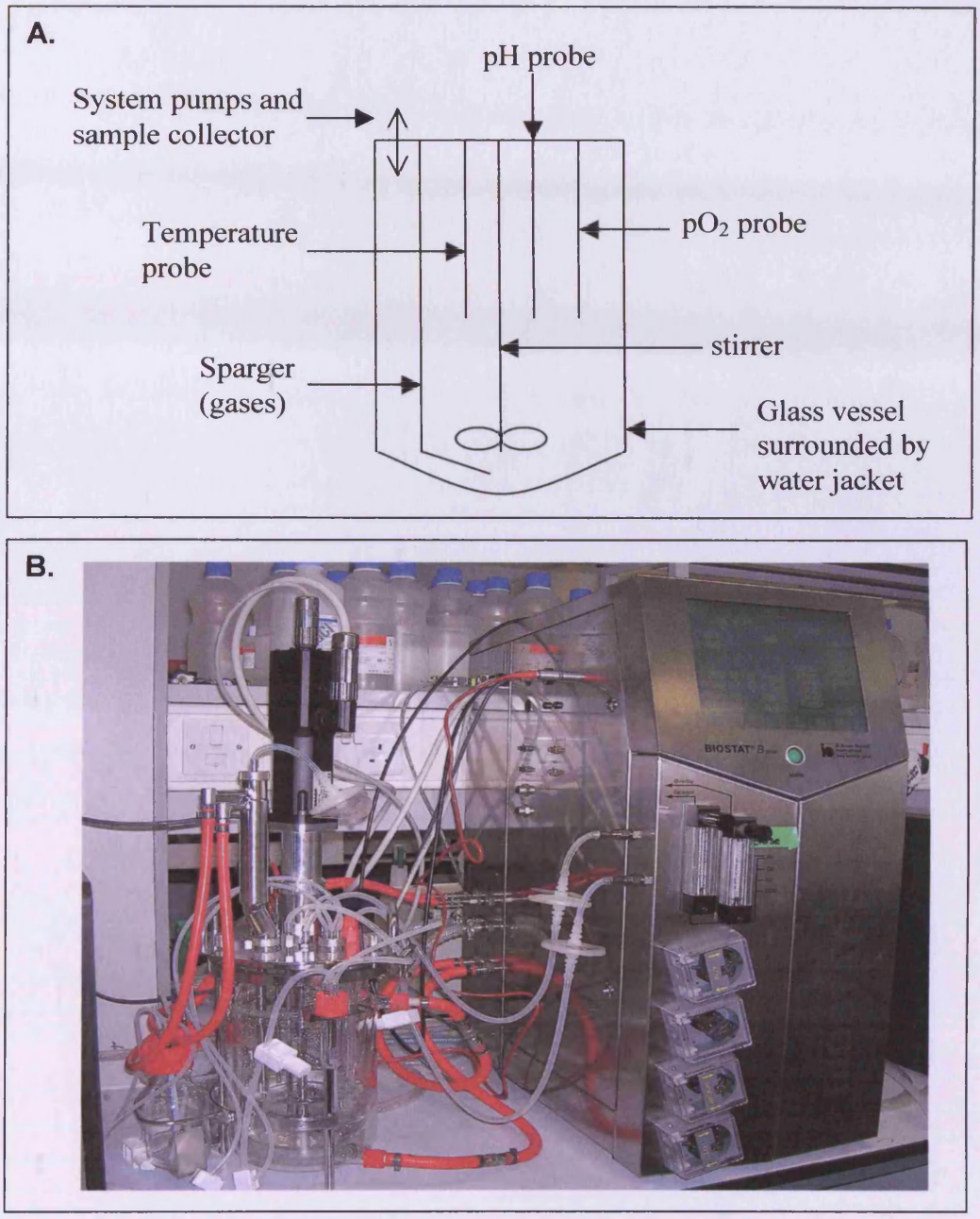


Figure 5.5 Fermenter diagram and photograph.

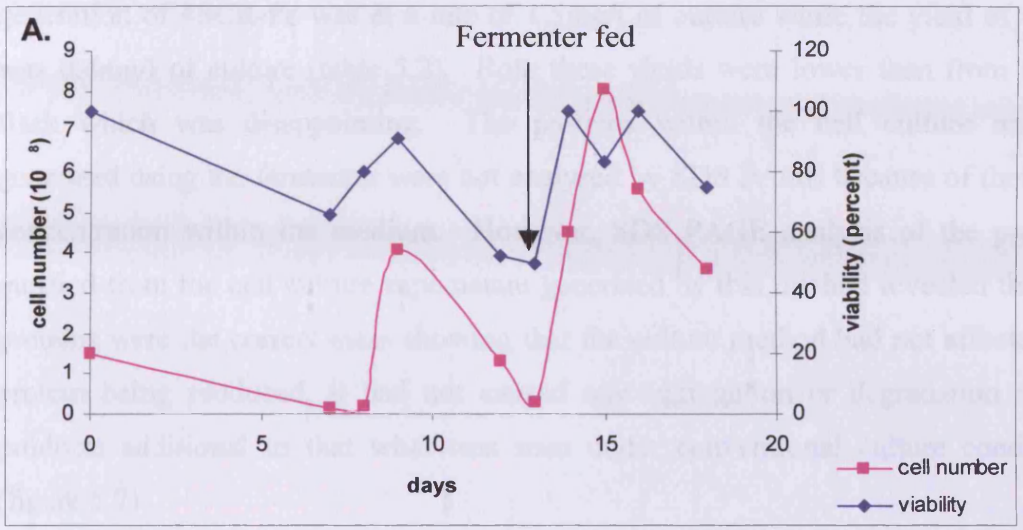
A. A diagram of the fermenter culture vessel. The vessel is surrounded by a water jacket and contains a stirrer, a temperature probe, a pH probe and a pO_2 probe. Gas enters the culture in fine bubbles through the sparger. The lid of the vessel contains inlets and outlets which can be attached to the system pumps to change media and add acids or bases to maintain pH.

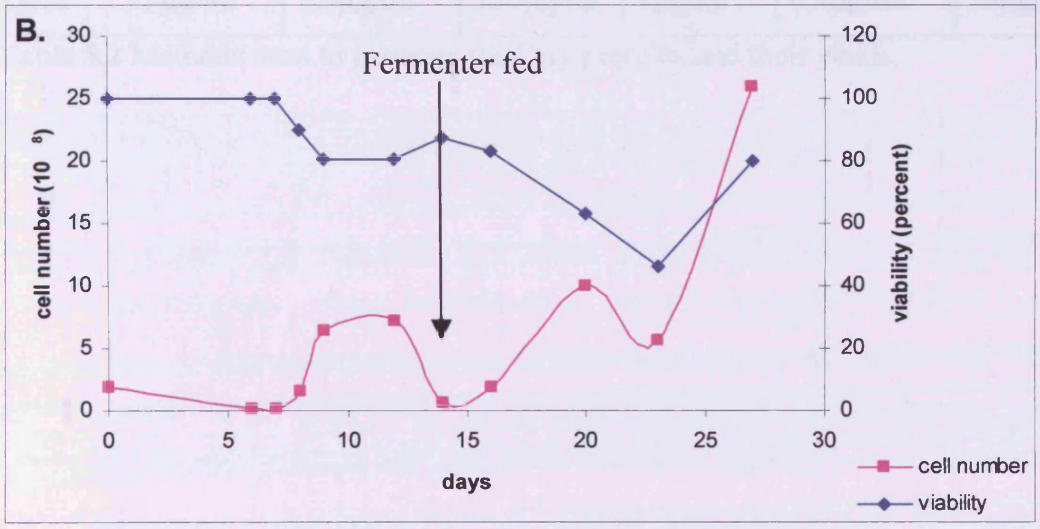
B. A photograph of the fermenter. The culture vessel sits next to the control unit. Each variable such as stirrer, temperature, pH, pO_2 is connected to the control unit, each one in monitored throughout the culture and controlled.

Figure 5.6 Fermenter cell numbers and viability.

A. Cell numbers (pink) and viability (blue) in the 4SCR-Fc fermenter culture. The cell number fell over the initial period and then increased to 7 x 10^8. Prior to a medium change the cell numbers decreased to 4 x 10^8, the cell numbers increased following a medium change. The viability also increased after an initial lag period, but fell prior to a medium change and increased after.

B. Cell numbers (pink) and viability (blue) in the 4SCR fermenter culture. The cell numbers went through an initial lag period and then began to increase. The numbers fell prior to a medium change and then increased. The viability remained steady, dropping slightly through the culture period until a rapid drop at day 24 after which it increased again. This drop and increase did not correspond to a medium change but may have corresponded to an unexpected drop in gas pressure.


A.
Fermenter fed
cell number (10 8)
viability (percent)
days
cell number
viability


B.
Fermenter fed
cell number (10 8)
viability (percent)
days
cell number
viability

The generation of both the proteins was quantified by ELISA (method 2.4.9). The generation of 4SCR-Fc was at a rate of 1.5mg/l of culture while the yield of 4SCR was 0.4mg/l of culture (table 5.2). Both these yields were lower than from a T80 flask which was disappointing. The proteins within the cell culture medium generated using the fermenter were not analysed by SDS PAGE because of their low concentration within the medium. However, SDS PAGE analysis of the proteins purified from the cell culture supernatant generated by this method revealed that the proteins were the correct mass showing that the culture method had not affected the protein being produced, it had not caused any aggregation or degradation of the products additional to that what was seen under conventional culture conditions (figure 5.7).

	4SCR-Fc			**4SCR**		
	T80	**Fermenter**	**CELLine**	**T80**	**Fermenter**	**CELLine**
Yield	5μg/ml	1.5μg/ml	150μg/ml	1μg/ml	0.4μg/ml	20μg/ml

Table 5.2 Methods used to generate the Crry proteins and their yields.

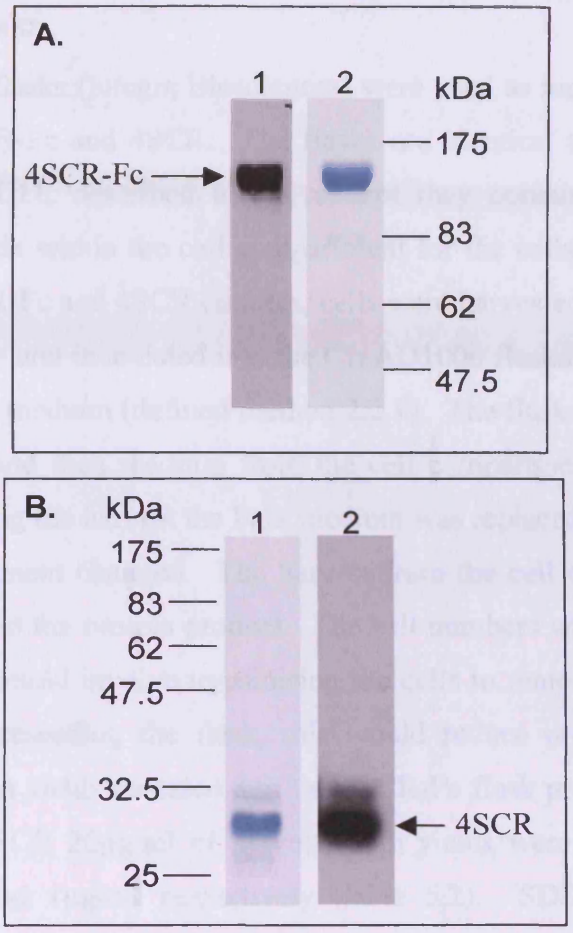


Figure 5.7 SDS PAGE analysis of the fermenter products.

A. SDS PAGE of the 4SCR-Fc fermenter product. The 4SCR-Fc purified from the fermenter supernatant was loaded onto a 7.5% gel and subjected to electrophoresis. After electrophoresis, the gel was either Coomassie stained (lane 2) or transferred to nitrocellulose and probed with anti-rat IgG HRPO (lane 1). The product from the fermenter supernatant contained a band of the correct mass and this was confirmed by Western blot analysis to be 4SCR-Fc. The culture conditions had not altered protein formation or caused its degradation.

B. SDS PAGE of the 4SCR fermenter product. The 4SCR purified from the fermenter culture was resolved on an 11% gel and either Coomassie stained (lane 1) or Western blotted onto nitrocellulose and probed with anti-Crry (TLD1C11) followed by anti-mouse IgG HRPO (lane 2). The product from the fermenter supernatant was the correct mass and it was confirmed as 4SCR by the Western blot. The culture conditions had not caused additional aggregation or degradation of the product.

5.2.2.2 CL AD1000 flasks

CELLine CL AD1000 flasks (Integra Biosciences) were used as high density culture flasks to generate 4SCR-Fc and 4SCR. The flasks are identical to CL1000 flasks used to culture TLD1C11, described above, except they contain a polyethylene terephtalate (PET) matrix within the cell compartment for the cells to attach to. To inoculate both the 4SCR-Fc and 4SCR cultures, cells were harvested from T80 flasks in a log phase of growth and inoculated into the CL AD1000 flasks at 2.5 x 10^7 cells in 15ml transfected cell medium (defined method 2.2.1). The flasks were allowed to establish for ten days and then medium from the cell compartment was harvested every seven days. During the harvest the bulk medium was replaced and the medium within the cell compartment changed. The harvest from the cell compartment was retained as this contained the protein product. The cell numbers within each culture were not taken as this would involve trypsinising the cells to remove them from the PET matrix and then reseeding the flask, this would reduce protein production. Quantification of protein yields revealed that the 4SCR-Fc flask produced 150μg/ml of harvest while the 4SCR 20μg/ml of harvest, both yields were higher than T80 flasks being 5μg/ml and 1μg/ml respectively (table 5.2). SDS PAGE analysis showed that the major protein within the supernatants harvested from the cell compartments was characteristic of albumin. However, bands corresponding to 4SCR-Fc and 4SCR were both visible on the Coomassie stains, although the band corresponding to 4SCR was weak. The identity of these bands was confirmed by Western blot analysis which revealed that the proteins were of the correct mass with no additional bands, showing that the culture conditions had not caused additional aggregation or degradation of the proteins (figure 5.8).

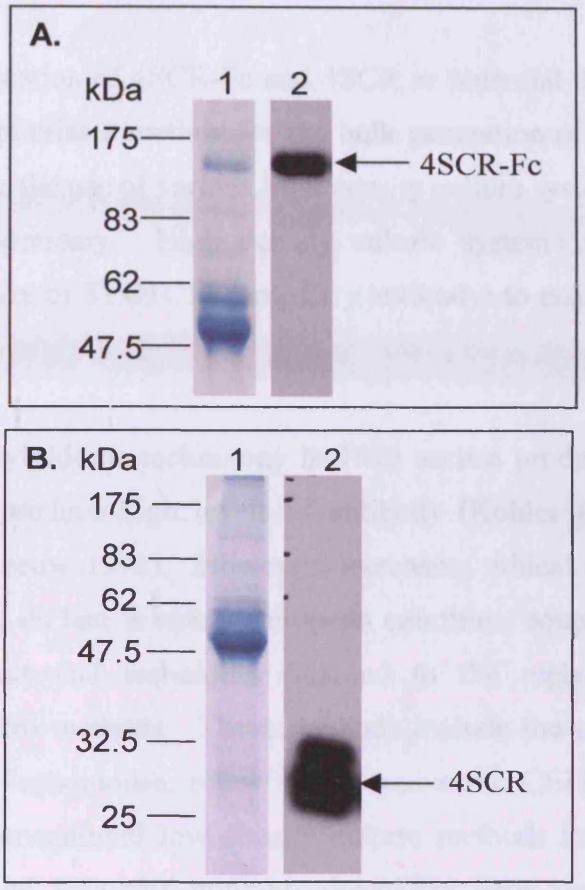


Figure 5.8 SDS PAGE analysis of the products from the CL AD1000 flasks.

A. SDS PAGE analysis of the 4SCR-Fc culture. The supernatant from the cell compartment of the CL AD1000 flask was resolved on a 7.5% gel and either Coomassie stained (lane 1) or transferred to nitrocellulose and probed with anti-rat IgG HRPO (lane 2). The major protein in the supernatant was albumin shown by its characteristic band at 50kDa. A band was visible at 140kDa corresponding to the expected mass of 4SCR-Fc. The identity of this band was confirmed by the Western blot. No additional bands for 4SCR-Fc were seen showing that the culture conditions had not caused aggregation of degradation of the protein.

B. SDS PAGE analysis of the 4SCR culture. The supernatant from the cell compartment was resolved on a 11% gel and either Coomassie stained (lane 1) or Western blotted onto nitrocellulose and probed with anti-Crry (TLD1C11) followed by anti-mouse IgG HRPO (lane 2). On the Coomassie stain the major protein was at 50kDa corresponding to albumin, a faint band was visible at 28kDa corresponding with the mass of 4SCR. The Western blot confirmed the identity of this band as 4SCR, no additional 4SCR bands were seen showing that the culture conditions had not caused aggregation or degradation of the protein.

5.3 Discussion

Following the identification of 4SCR-Fc and 4SCR as potential therapeutic reagents it was necessary to optimise a method for the bulk generation of these reagents. In this chapter, I describe the use of various high density culture systems to generate the amount of protein necessary. High density culture systems were also used to generate large quantities of TLD1C11 (anti-Crry antibody) to enable the purification of the Crry proteins.

Since the advent of hybridoma technology in 1975 ascites production has been the favoured method to produce high levels of antibody (Kohler and Milstein 1975; Hendriksen and de Leeuw 1998). However, increasing ethical concern about this technique resulting in its ban in many European countries, coupled with improved cell culture equipment and techniques has led to the replacement of ascites production with in vitro methods. These methods include the use of hollow fibre systems such as the Tecnomouse, roller bottles and static CELLine culture flasks (CL1000 flasks). Conventional low density culture methods have been shown to produce between 1 and 100μg/ml antibody depending upon the hybridoma being cultured (Falkenberg 1998). High density culture methods have been shown to produce far greater amounts with CELLine flasks able to produce 0.7 – 2.5mg/ml (Trebak, Chong et al. 1999). The Tecnomouse can produce greater than 1mg/ml (Nagel, Koch et al. 1999).

I have used high density culture methods and demonstrated increased production compared to conventional static cultures, in agreement with what is reported in the literature. Supernatant from the Tecnomouse contained 0.8mg/ml of TLD1C11, the CELLine flask 1mg/ml and the T80 flask 5μg/ml. The high density culture methods had a harvest greater than a T80 flask by at least 160 times. There is a different volume yield for each system with the Tecnomouse and CELLine flask generating 15ml/week while the amount generated from the T80 flask depends upon how many are grown. A direct comparison between systems can be achieved by calculating how many T80 flasks would need to be grown to generate the same yield as the Tecnomouse and CELLine flask. The Tecnomouse yields 12mg/week which would be equivalent to 2000ml T80 supernatant per week, harvested from 30 T80 flasks. The CELLine flask yields 15mg/week, equivalent to 3000ml T80 flask supernatant

or 50 flasks. Not only does the culture of this number of flasks represent a significant investment of time, but also a high cost in cell culture media especially foetal bovine serum (FBS). Both high expression systems use very little FBS, 2.5ml of FBS per week as opposed to 300-450ml if T80 flasks were used to generate the equivalent amount of antibody. Purification of the TLD1C11 from a larger volume of supernatant generated using conventional flasks also increases the time taken. The production of monoclonal antibodies in high density systems has significant advantages over the traditional ascites generation in terms of reduced animal use and suffering and also in terms of a reduction in non-specific antibodies, limited viral contamination and the ability to generate antibodies from a wider variety of species with such a system.

Out of the two high density culture systems, the CELLine flasks have the greater yield. This system is also the least cumbersome as the Tecnomouse has a special control unit and can become complicated when many different culture cassettes are being used each with their own feed bottle and tubing. The ease of culturing in the CELLine flask compared to the Tecnomouse is illustrated by the removal from sale of the Tecnomouse by the manufacturers in early 2005 due to decreasing sales as customers prefer the CELLine flask which needs no specialist equipment. The high expression culture conditions caused no aggregation or degradation of the product when investigated by Coomassie staining a polyacrylamide gel but Western blot analysis did show some smaller forms of the antibody presumably degradation but these forms were minor, the majority of antibody generated was of the correct mass.

Since tissue plasminogen activator (tPA) became the first recombinant therapeutic protein produced by mammalian cells to obtain market approval in 1986, the number of recombinant proteins being produced for therapeutic use in mammalian cells has increased. As previously discussed, mammalian cells have certain advantages in recombinant protein production but they are limited by low yields. These low yields can be overcome by using high density culture systems such as a fermenter and adherent CELLine flasks. Protein generation on a fermenter system can reach the gram per litre range (Robinson and Memmert 1991). The expression of proteins within this project using a fermenter system was lower than conventional static cultures for both 4SCR-Fc and 4SCR. This disappointing lower expression reflects

the complexity of fermenter cultures and highlights the need for further optimisation. Both the cell numbers within the 4SCR-Fc and 4SCR fermenter cultures went through a long lag phase as the cells adapted to the culture conditions. Alterations of the conditions such as stirring speed and gassing speed may have shortened this lag phase by reducing shear stress on the cells. Cell numbers, growth and protein production within a fermenter system can be limited by oxygenation, waste accumulation, shear stress and nutrient availability. In order to optimise the fermenter each one would need to be dealt with. Oxygenation would need to be maximised whilst keeping the stress on the cells caused by the gassing process to a minimum. Waste accumulation and shear stress need to be minimised, for shear stress this would be done by decreasing stirrer speeds and gassing speeds. Nutrient availability would need to be maximised by increasing both glucose and glutamine content of the medium. As good results were obtained from CELLine flasks (below), the fermenter was not fully optimised for these proteins.

The adherent CELLine flasks (CL AD1000 flasks) have been shown to increase protein production by BHK (Baby Hamster Kidney) cells by 25 times compared to T80 flasks (Mittermaier and Zang-Gandor). In this project the 4SCR-Fc yield was 150μg/ml, 30 times greater than the T80 flask and the 4SCR yield was 20μg/ml, 20 times greater than a T80 flask. This increase in production using CELLine flasks represents a saving in time and space needed for the culture and subsequent purification of proteins if compared to using conventional cultures. The expression may be boosted further by increasing the glucose and glutamine content of the medium. SDS PAGE analysis revealed that neither the fermenter nor CELLine flasks caused additional aggregation or degradation of the proteins.

Production of proteins using these high density culture systems also resulted in minimal contamination of the products after purification. The contamination of the proteins with bovine antibodies in particular can be minimised as the ratio of desired protein to bovine antibodies is greater using these systems than normal methods making the contaminant a minor rather than a major constituent. For instance 15mg of TLD1C11 was produced per 2.5ml of bovine serum in the CELLine flasks as opposed to 450ml of bovine serum using conventional cultures.

In conclusion, various high density culture systems were compared for the generation of antibodies and recombinant proteins. For generating antibodies from hybridomas and recombinant proteins from anchorage dependent cells, CELLine flasks were favoured as they resulted in the generation of high levels of the desired proteins and required no specialist equipment or optimisation to use. The CELLine flasks are economical and minimally time consuming. Sufficient quantities of the Crry proteins for in vivo characterisation have been generated. Following generation all proteins were purified from cell culture supernatant as described previously (method 3.2.5).

Chapter 6: The *in vivo* characterisation of 4SCR-Fc and 4SCR

6.1 Introduction

Due to the implication of C in various pathologies (discussed in chapter 1, table 1.2) C has itself become a therapeutic target. The concept of developing C inhibitors began when hereditary angiodema (HAE) was found to be associated with a C1-inh deficiency (Donaldson and Evans 1963). This disorder was first treated with ε-aminocaproic acid, an anti-fibrinolytic agent that also inhibits C (Frank, Sergent et al. 1972). It was later treated with C1-inh purified from plasma. With the advent of molecular biology techniques it was possible to design C inhibitors, furthermore hybridoma technology meant that monoclonal antibodies specific for C components and with inhibitory function could be used as therapeutics. This has resulted in a large number of inhibitors being tested each with their own effects, advantages and disadvantages. First generation anti-C therapeutics are discussed in chapter 1 (section 1.5). Many of these reagents were limited by their short half-life in vivo resulting in rapid clearance from the circulation, their immunogenicity in animal models preventing testing in models of chronic disease, their immunogenicity in humans resulting in limited treatment times or their tendency to systemically inhibit C which could render individuals susceptible to bacterial infections. For example, treatment of mice with a Crry-Ig fusion protein resulted in mice having an increased susceptibility to acute septic peritonitis compared with mice treated with a targeted anti-C reagent, CR2-Crry (Atkinson, Song et al. 2005).

The aim of this project was to overcome some of the limitations of the first generation therapeutics. The aim was to generate a long lived reagent by generating an Fc fusion protein of the CReg Crry. This approach has previously been demonstrated to increase the half-life of the CReg DAF (Harris, Williams et al. 2002). In order to confirm that this approach lengthened the half-life of Crry and to establish by how much, the half-life of 4SCR-Fc was compared to 4SCR. By generating an Fc fusion protein containing both a rat regulator and a rat Fc domain the aim was to produce a non-immunogenic reagent that would enable the testing of the reagent in rat models of chronic disease. Previous CReg-Fc proteins have contained a human Fc or a mouse Fc (Quigg, Kozono et al. 1998; Harris, Williams et al. 2002). The human Fc reagent was limited to acute therapeutic studies in the rat

whilst the mouse Fc reagent was used in murine models. Also, the human therapeutic sCR1 based on complement receptor 1 has been limited to acute therapeutic studies because neutralising antibodies are generated when the reagent is injected into non-human species (Piddlesden, Storch et al. 1994). Therefore in this study the immunogenicity of Crry-Fc was investigated, in particular the generation of neutralising antibodies. A further aim of this project was to generate a 'prodrug' of Crry that could be activated at the disease site thereby eliminating the problem of systemic C inhibition. However, as discussed in chapter 4, this approach proved to be non-viable for Crry.

The therapeutic efficacy of the active Crry-Fc (4SCR-Fc) was compared with 4SCR in the rat model experimental autoimmune myasthenia gravis (EAMG). EAMG can be induced by immunisation of purified AChR (AChR) or by passive transfer of a monoclonal anti-AChR antibody (mAb35). In this project mAb35 was used to induce the disease which has a pathogenesis very similar to myasthenia gravis (MG). MG is a well defined antibody-mediated autoimmune disease of the neuromuscular junction characterised by muscle weakness and fatigue. Anti-AChR antibodies bind to the motor end plate, reducing the number of functional AChR either by direct blockade or by causing accelerated internalisation and degradation of the receptor (Drachman, de Silva et al. 1987; Lindstrom, Shelton et al. 1988; Loutrari, Kokla et al. 1992). This results in neuromuscular transmission failure (Kaminski, Li et al. 2003). C has been implicated in EAMG as C activation products have been found at the motor end plate and C depletion or deficiency abrogates disease (Lennon, Seybold et al. 1978; Engel, Sakakibara et al. 1979; Lennon and Lambert 1980; Sahashi, Engel et al. 1980; Fazekas, Komoly et al. 1986; Christadoss 1988; Nakano and Engel 1993). Anti-C therapy has proved effective in EAMG as sCR1 suppresses disease in the model by reducing muscle weakness and associated weight loss (Piddlesden, Jiang et al. 1996). C may mediate damage in EAMG by causing lysis of the membrane at the motor end plate or through the proinflammatory effects of the early C components C3a and C5a (Engel and Arahata 1987).

The aims of the work presented in this chapter were:

1. To establish the circulating half-life of 4SCR-Fc in rats and compare this with the half-life of 4SCR.

2. To establish if the Crry-Fc was immunogenic, in particular if antibodies were generated to the reagent.
3. To establish the therapeutic efficacy of 4SCR-Fc in EAMG compared to 4SCR and look at the ability of these reagents to inhibit C within this model.

6.2 Specific methods and results

6.2.1 In vivo half-life

To determine the in vivo half-life of the 4SCR-Fc and 4SCR proteins, these proteins were iodinated with I^{125} and injected intravenously into rats following method 2.6.2. This work was carried out in collaboration with Dr Anwen Williams, Dept of Rheumatology who performed the i.v. injections and harvested blood samples. The amount of the proteins remaining in the circulation of the rats was calculated from the counts of blood samples taken at various time points. The half-life that is calculated here is the β-phase half-life; this refers to the rate of elimination of the proteins from the body as opposed to their distribution throughout the body. Calculations used here are based upon the assumption that the clearance of the protein is biphasic as the body is viewed as two compartments; a central compartment containing the blood and well-perfused organs such as the heart, lungs, kidney and liver, and a peripheral compartment consisting of less well-perfused tissues such as muscle, fat and skin. Following administration of the proteins into the circulation they immediately equilibrate amongst the central and peripheral compartments, i.e. the protein will be distributed throughout the body and this is referred to as the α-phase half-life. The elimination of the protein occurs immediately after administration alongside this distribution and is termed the β-phase half-life. The two half-lives can be distinguished if the β-phase half-life is longer than the distribution phase. The rate of elimination is assumed to be proportional to protein concentration in the system and so can be described by first order kinetics. This two-compartment model is explained diagrammatically in figure 6.1.

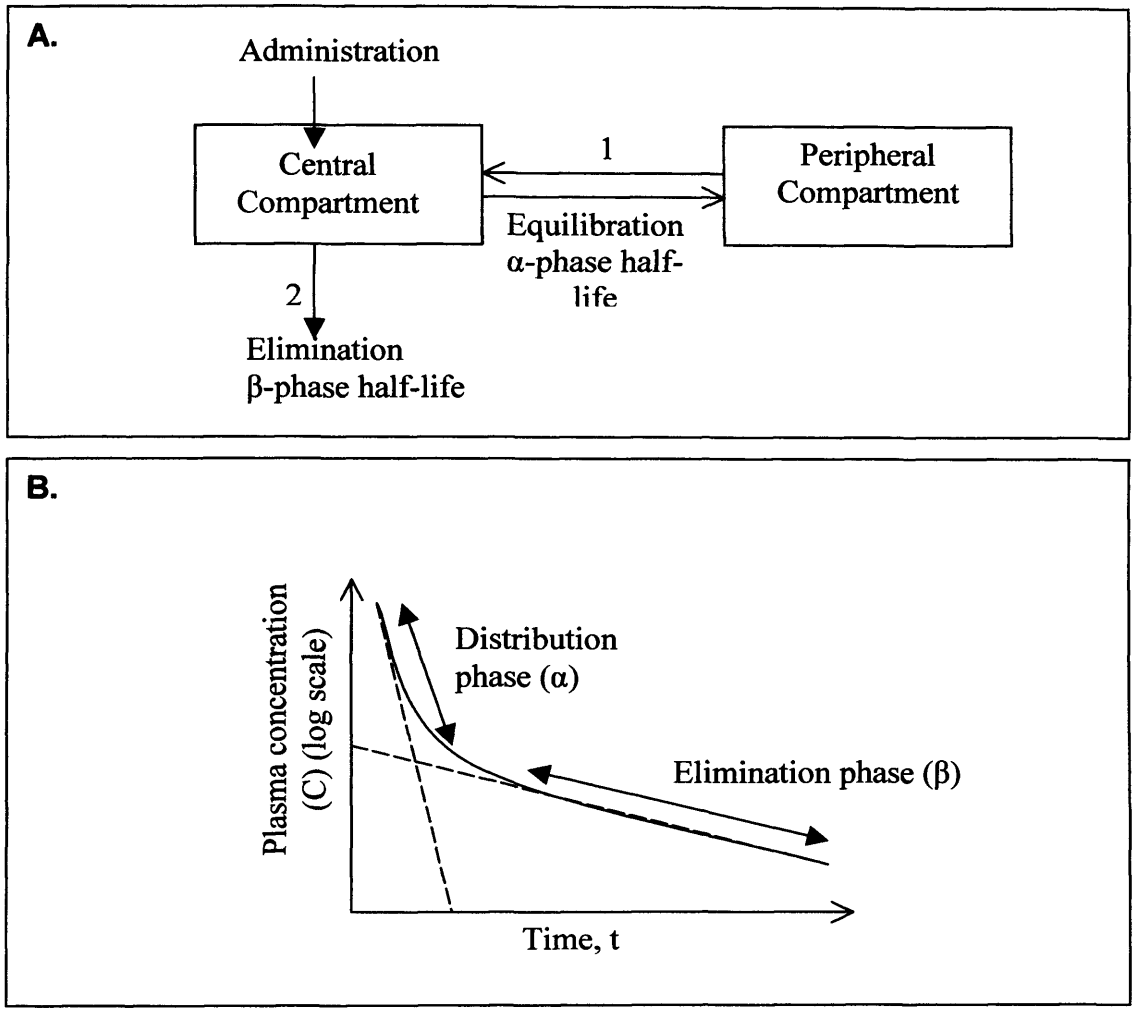


Figure 6.1 The two-compartment model.

A. A schematic of the two compartment model. The proteins enter the central compartment where they reach instantaneous equilibrium before equilibrating with the peripheral compartment (1). Elimination begins immediately after administration (2).

B. Graph showing the phases of the two-compartment model. Plasma concentration plotted against time, the protein shows an initial rapid decrease in concentration corresponding to the distribution phase or α-phase. There is then a more lengthy elimination phase, the β-phase.

Both figures are adapted from Clinical Pharmacology, 3rd Edition. J.M. Ritter, L.D. Lewis and T.G.K. Mant. 1995. London, UK. Ernold Arnold. pp26.

The half-life of the proteins was calculated using the following equations. In the two compartment model the exponential decline of the elimination phase is represented by:

$C_t = C_0 e^{-kt}$ or $\ln C_t = \ln C_0 - kt$

Where C_t is the plasma concentration of the protein at time, t. C_0 is the theoretical initial concentration when time = 0 if the α-phase is discounted and k is the rate constant. A plot of ln C against t is linear once equilibrium is reached, the y intercept corresponds to C_0 and the slope –k. The half-life is independent of concentration and can therefore be related to rate constant, k, by substituting C_0 as 2 and C as 1 (Waller 1994). From this,

β-phase half-life = 0.693 / k

To take into account the initial distribution of the proteins through the body, protein-bound counts were expressed as a percentage of the counts at 5 minutes. The percentage counts were plotted against time in hours (figure 6.2). The slope of the graph during the elimination phase was calculated from the linear region. From the slope of the graph the β-phase half-life of both the proteins was calculated using the above equation. The 4SCR protein was eliminated rapidly from the circulation, the distribution throughout the body and the elimination from the body were indistinguishable. After the clearance of most of the protein from the body, the line began to flatten off corresponding to low levels of radioactivity remaining within the rats. The β phase half-life of 4SCR was 7.2 minutes (standard deviation +/- 2.1 minutes). The 4SCR-Fc had a significant increase in half-life compared to the 4SCR reagent, the β-phase half-life of 4SCR-Fc was 52.9 hours ($p<0.001$ determined by an unpaired t-test, standard deviation +/- 16 hours).

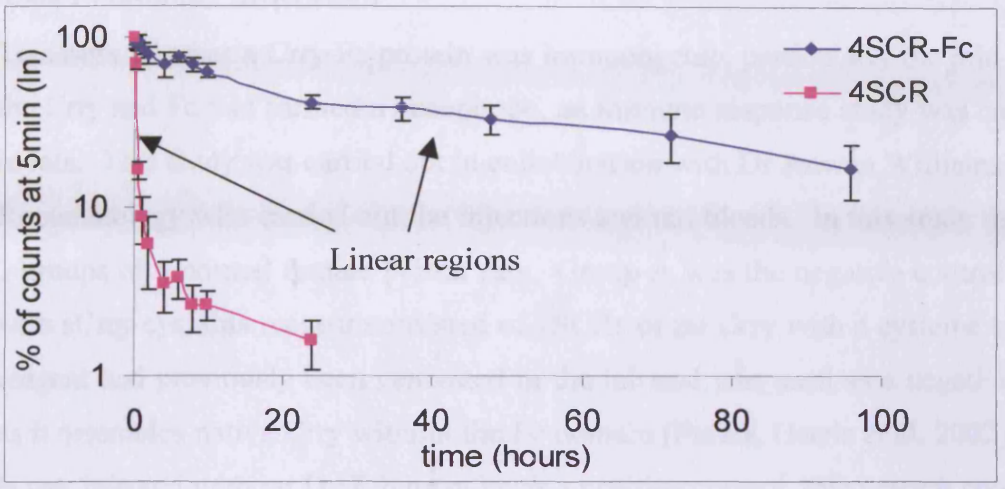


Figure 6.2 β-phase in vivo half-life of 4SCR-Fc and 4SCR.

Time in hours was plotted against the natural log (ln) of protein bound counts as a percentage of the counts at 5 minutes to eliminate the initial distribution phase of the proteins. The elimination of 4SCR-Fc is shown in blue while the elimination of 4SCR is shown in pink. Data points represent the mean counts +/- 1 SD from 5 rats. Analysis of the linear slopes indicated that 4SCR-Fc had a β-phase half-life of 52.9 hours while 4SCR had a β-phase half-life of 7.2 minutes.

6.2.2 Immunogenicity of Crry-Fc

6.2.2.1 Antibody generation

To assess whether a Crry-Fc protein was immunogenic, particularly the join between the Crry and Fc that formed a neoepitope, an immune response study was carried out in rats. This study was carried out in collaboration with Dr Anwen Williams, Dept of Rheumatology who carried out the injections and tail bleeds. In this study there were 3 groups of 5 normal female Wistar rats. Group A was the negative control injected with sCrry-cys, this reagent consisted of 4SCRs of rat Crry with a cysteine tail. This reagent had previously been generated in the lab and was used as a negative control as it resembles native Crry without the Fc domain (Fraser, Harris et al. 2002). Group B was injected with rat DAF-human Fc as a positive control, this reagent consisted of 4SCRs of rat DAF fused to the Fc of human IgG1. This reagent had also been generated in the lab previously and was used as a positive control to prove that this dosing regime could be used to mount an immune response to an immunogenic protein, namely the human Fc present in the molecule (Harris, Williams et al. 2002). Group C was injected with a Crry-Fc reagent, 5SCR-Fc was used here to determine if fusing the Crry to the Fc caused the protein to be immunogenic. The experiment was carried out following method 2.6.3.1 which involved administering the proteins intravenously and collecting tail bleeds at different time points. The serum was harvested from the bleeds and screened for antibodies to the injected proteins by ELISA.

The DAF-Fc group (group B) mounted an immune response to the protein, proving that this injection regime could be used to generate an immune response to an immunogenic protein (figure 6.3A). Screening the antibodies produced by ELISA revealed that all the animals mounted an IgM response which began class switching to IgG1 from about day 28 (figure 6.3B), this class switching was seen strongly in animals B1, B2 and B3 and more weakly in animal B0. Animal B2 showed the weakest IgM response but also showed a switch to IgG2b.

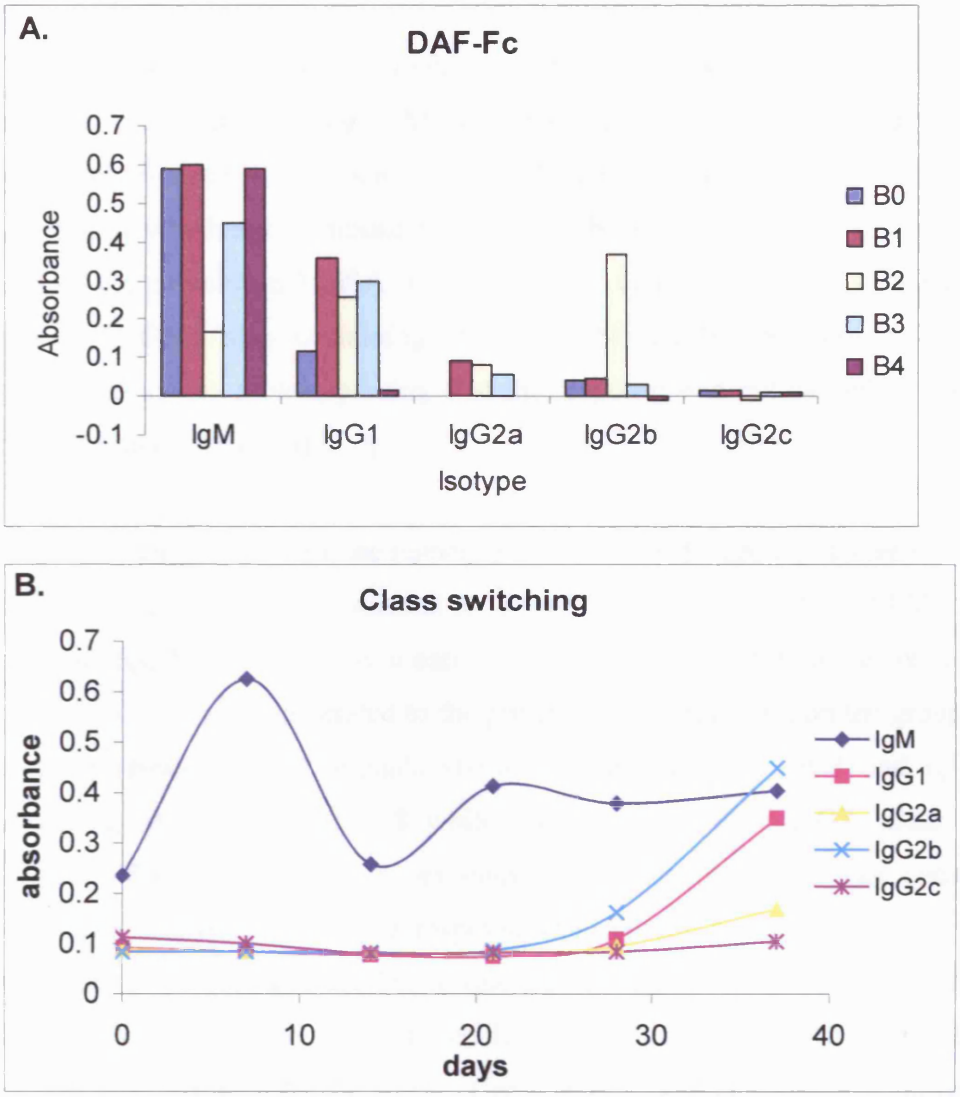


Figure 6.3 Immune response generated in group B (DAF-Fc).

A. ELISA result showing the antibodies generated to DAF-Fc as detected in the day 35 serum samples. All animals (B0-B4) mounted an IgM response, class switching was predominantly seen to the IgG1 isotype but also to IgG2a and IgG2b.

B. Representative ELISA result (animal B2) showing the class switching of the antibody response in DAF-Fc animals. The IgM response peaked at day 7 while class switching was seen from day 28.

Group A (sCrry-cys) also mounted an immune response, figure 6.4A. All the animals mounted a strong IgM response, A0 showed IgG2a and IgG2c class switching, figure 6.4B. This response in the negative control group was unexpected. To explore whether the immune response had been mounted to the cysteine tag at the end of the protein an ELISA was carried out coating the plate with an unrelated protein (CD59) also containing this tag. No antibodies were detected which responded to this protein proving that the response was not caused by the cysteine residue (data not shown).

Group C mounted an immune response to the 5SCR-Fc protein, figure 6.5A. In this group the IgG2a response was not monitored as the coat for the ELISA, 5SCR-Fc, contains IgG2a so the detection antibody would cross react with the coat rather than detect any antibodies generated to the protein. All animals within the group mounted an IgM response. All animals showed some degree of IgG1 and IgG2b class switching from around day 28 while C1-C4 also showed IgG2c class switching (figure 6.5B). Generation of an immune response raised further questions, we wished to address whether the response was specific for the join in the molecule between the 5SCRs and the Fc, to one particular part of the molecule such as the Crry or the Fc or to the whole molecule. To address these questions an ELISA was carried out coating with 5SCR-Fc, sCrry-cys or CD59-Fc (same Fc; rat IgG2a) and incubated with the immune serum from one of the animals (method 2.6.3.2). Development was seen in the ELISA in response to all three coats revealing that the response was to the whole molecule (figure 6.5C). If the response had been to a neoepitope formed at the join of the two proteins only the 5SCR-Fc coat would have developed. Similarly if the response had been to one part of the molecule either the Crry or the Fc, either the sCrry-cys coat or CD59-Fc coat would have also developed. Instead the antibodies generated during the immune response recognised all three proteins equally.

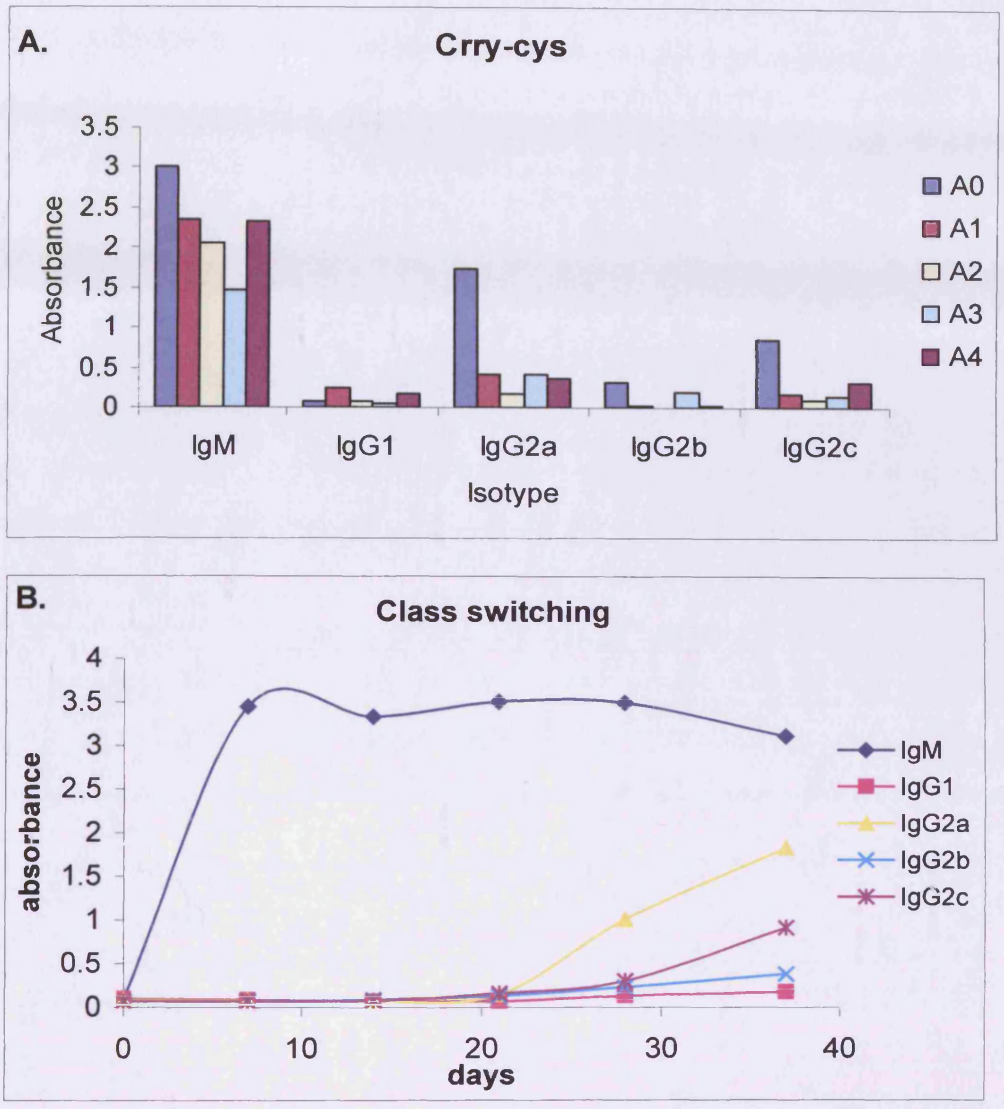


Figure 6.4 Immune response in group A (Crry-cys).

A. ELISA results for the Crry-cys group (group A) showing response at day 35 for all animals (A0-A4). All animals mounted an IgM response which mainly class switched to IgG2a and IgG2c, some IgG1 and IgG2b class switching was seen.

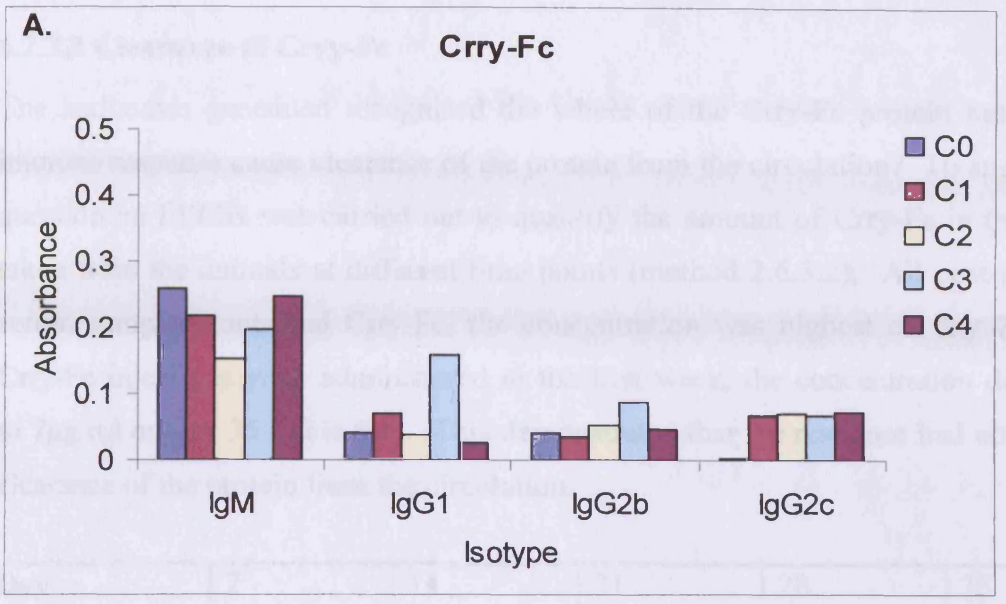
B. Representative ELISA result (animal A0) showing class switching of the antibodies generated by group A animals. The IgM response was the strongest, peaking at day 7 and remaining constant. Class switching was evident at day 28 when IgG2a antibodies were generated. An IgG2c response was evident at day 35.

Figure 6.5 Immune response generated in group C (Crry-Fc).

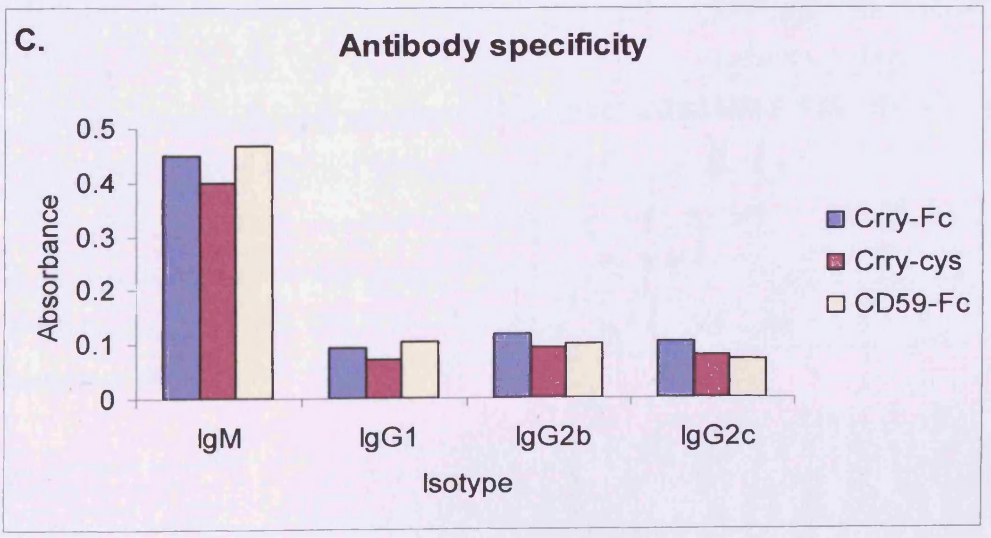
A. ELISA result showing the antibodies generated to Crry-Fc in the day 35 serum samples. All animals (C0-C4) mounted an IgM response, class switching was seen to all isotypes. IgG2a was not included in the ELISA due to cross reactivity of the detection antibody with the coat.

B. Representative ELISA result (animal C3) showing the antibody class switching within the Crry-Fc group. The IgM response increased over the 35 days of the experiment. Class switching to IgG2b was evident at day 28, this response decreased by day 35. IgG1 and IgG2c responses increased from day 28.

C. ELISA result showing the specificity of the antibodies in the day 35 serum samples from one of the group C animals. The antibodies equally recognised Crry-Fc, Crry-cys and CD59-Fc revealing that the response was to the whole molecule rather than the join between the Crry and Fc or one particular part of the molecule.


A.
Crry-Fc
0.5
0.4
0.3
0.2
0.1
0
Absorbance
C0
C1
C2
C3
C4
IgM
IgG1
IgG2b
IgG2c
Isotype


B.
Class switching
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
absorbance
IgM
IgG1
IgG2b
IgG2c
0
10
20
30
40
days


C.
Antibody specificity
0.5
0.4
0.3
0.2
0.1
0
Absorbance
Crry-Fc
Crry-cys
CD59-Fc
IgM
IgG1
IgG2b
IgG2c
Isotype

6.2.2.2 Clearance of Crry-Fc

The antibodies generated recognised the whole of the Crry-Fc protein but did the immune response cause clearance of the protein from the circulation? To answer this question an ELISA was carried out to quantify the amount of Crry-Fc in the serum taken from the animals at different time points (method 2.6.3.2). All post-injection serum samples contained Crry-Fc, the concentration was highest on day 7 as two Crry-Fc injections were administered in the first week, the concentration decreased to 7μg/ml on day 35 (table 6.1). This demonstrated that the response had not caused clearance of the protein from the circulation.

Day	7	14	21	28	35
Conc. (μg/ml)	10.4	7.3	4.3	4.6	6.8

Table 6.1 Serum concentrations of Crry-Fc

Concentrations of Crry-Fc within the serum taken from group C animals during the immune response study. Animals were injected day 0, 3, 7, 14, 21 and 28. Blood was collected prior to administration of reagent.

6.2.2.3 Function-blocking study

To assess whether the antibodies inhibited the function of Crry-Fc, CHO cells expressing rat Crry on their cell surface were utilised in a series of flow cytometry experiments (method 2.6.3.2). First, deposition of the antibodies from the immune sera on the CHO cells was measured. All the immune sera caused a shift compared to the secondary only control and incubation with normal rat serum revealing that anti-Crry antibodies were deposited on the CHO cells (figure 6.6).

Secondly, the ability of these deposited antibodies to inhibit the function of Crry was assessed following method 2.6.3.2. Crry expressing CHO cells were initially pre-incubated with heat inactivated rat or rabbit serum prior to C deposition using rat serum to reveal if the positive control, rabbit polyclonal anti-Crry antiserum, could be directly compared to the immune sera (rat) in the subsequent assay. Pre-incubation with the rat or rabbit serum did not alter C3b deposition compared to pre-incubation in buffer only revealing that antibodies or CReg present in the sera would not interfere with the subsequent assay and that the two sera types could be directly compared (figure 6.7A). Following incubation with anti-CHO antiserum, the CHO cells were incubated with the immune sera or rabbit polyclonal anti-Crry antiserum to deposit the anti-Crry antibodies. This was followed by incubation in rat serum to deposit C3b. The immune sera all showed equivalent C3b deposition which was not enhanced compared to incubation with buffer only, the antibodies did not inhibit the function of Crry. On the other hand, incubation with the rabbit polyclonal anti-Crry antiserum did cause enhanced C3b deposition as the function of Crry was inhibited resulting in less regulation of C and more C3b deposition (figure 6.7B). The difference in C3b deposition between CHO cells incubated with the polyclonal anti-Crry antiserum and the immune sera was further evident when the CHO cells were not incubated with the anti-CHO antiserum. In this assay C activation proceeds via the AP resulting in a lower level of C activation and revealing a greater difference in C deposition between the Crry inhibiting polyclonal antiserum and the non-Crry inhibiting immune sera (figure 6.7C)

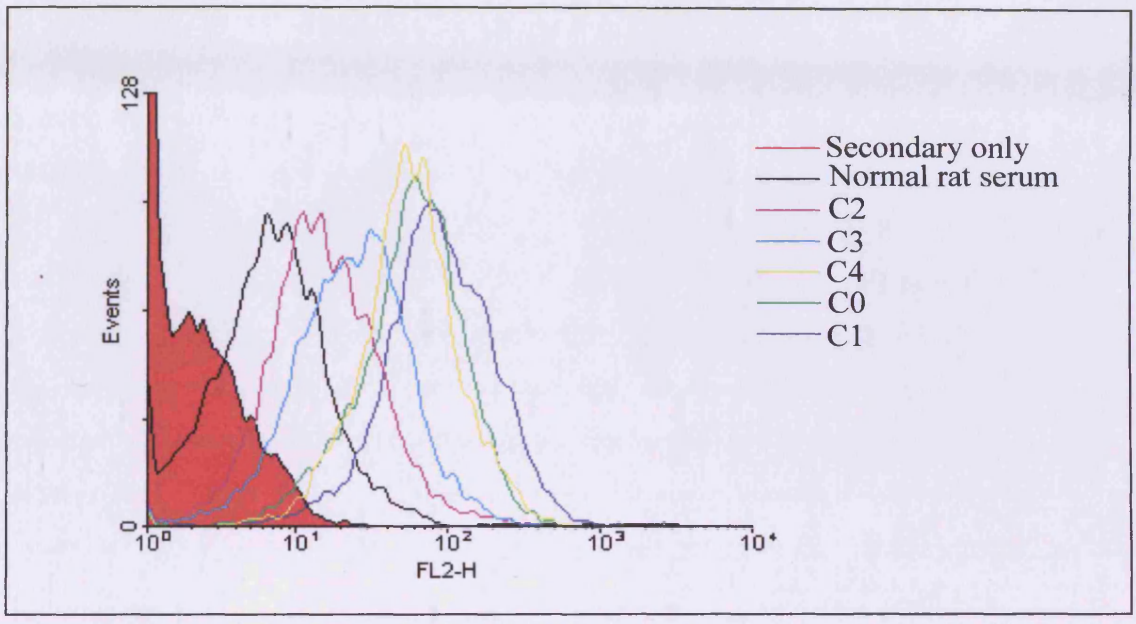


Figure 6.6 Deposition of anti-Crry antibodies from immune sera onto Crry expressing CHO cells.

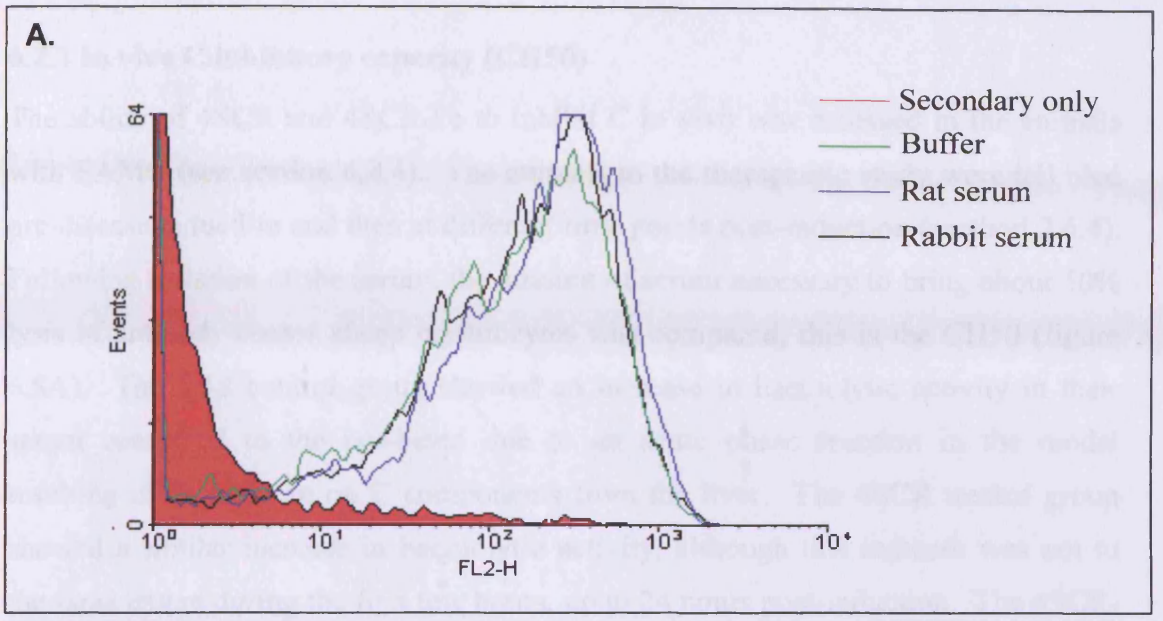
CHO cells expressing rat Crry on their surface were incubated with normal rat serum or with the immune serum from each animal. The deposition of anti-Crry antibodies was detected using anti-mouse IgG RPE (cross reactive with rat antibodies) by flow cytometry. Normal rat serum (black) caused a shift in comparison to the secondary only control; however, all immune sera (purple, light blue, green, yellow and dark blue) produced a greater shift showing that anti-Crry antibodies were deposited on the CHO cells. The amount of deposition varied between animals but all produced a shift in comparison to normal rat serum.

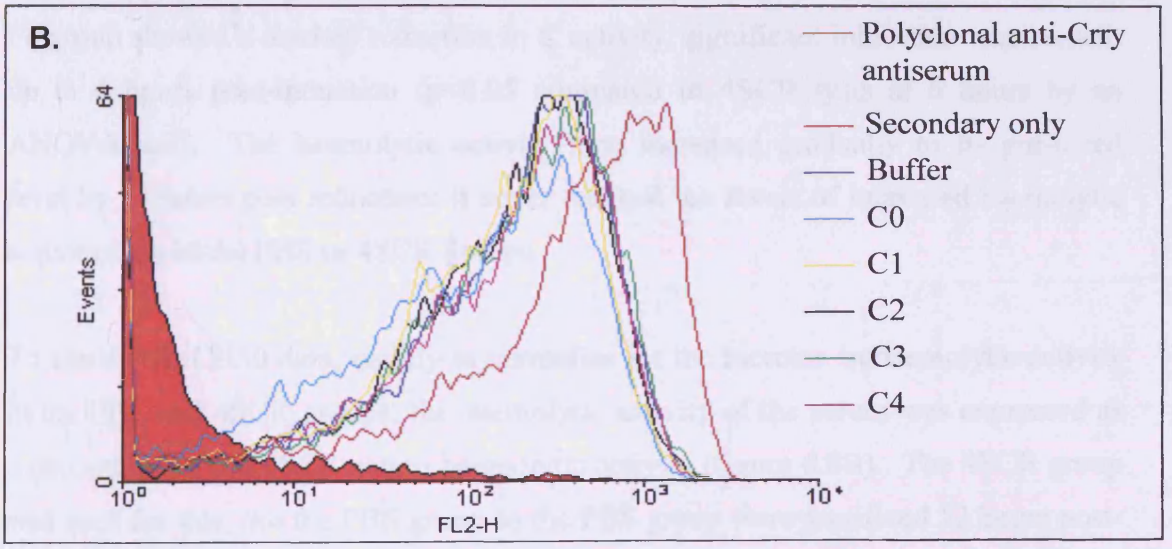
Figure 6.7 Inhibition of Crry function by anti-Crry antibodies.

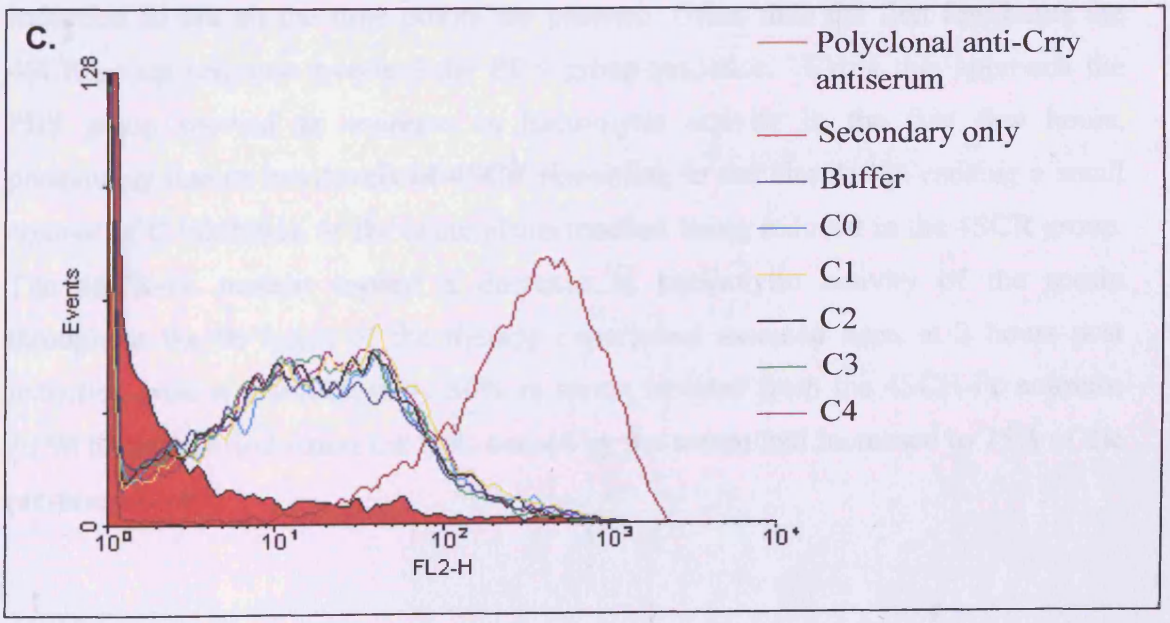
A. Deposition of C3b on rat Crry CHO cells pre-incubated with rat serum (heat inactivated), rabbit serum (heat inactivated) and buffer prior to incubation with rat serum as a source of C. Incubation with rat and rabbit serum before C deposition did not cause any difference in C3b deposition on the CHO cells compared to pre-incubation with buffer. Hence, incubation with the immune sera could be directly compared to incubation with the rabbit polyclonal antiserum in figure B.

B. Deposition of C3b on rat Crry CHO cells incubated with anti-Crry antibodies following sensitisation to C activation with anti-CHO antiserum. Prior to C activation the cells were incubated with immune sera, buffer or rabbit polyclonal anti-Crry antiserum. No difference in C3b deposition was seen for the cells incubated with the immune sera or buffer only. Enhanced C3b deposition was seen for the cells incubated with the polyclonal antiserum as the function of Crry had been inhibited.

C. Deposition of C3b on rat Crry CHO cells proceeding via the alternative pathway. CHO cells that had not been incubated with anti-CHO antiserum were incubated with the immune sera, buffer or rabbit polyclonal anti-Crry antiserum followed by deposition of C using rat serum. As above, the immune sera did not result in enhanced C3b deposition while the polyclonal antiserum inhibited Crry resulting in enhanced C3b deposition.


A.
64
Events
0
10^0
10^1
10^2
10^3
10^4
FL2-H
Secondary only
Buffer
Rat serum
Rabbit serum


B.
64
Events
0
10^0
10^1
10^2
10^3
10^4
FL2-H
Polyclonal anti-Crry antiserum
Secondary only
Buffer
C0
C1
C2
C3
C4


C.
128
Events
0
10^0
10^1
10^2
10^3
10^4
FL2-H
Polyclonal anti-Crry antiserum
Secondary only
Buffer
C0
C1
C2
C3
C4

6.2.3 In vivo C inhibitory capacity (CH50)

The ability of 4SCR and 4SCR-Fc to inhibit C in vivo was assessed in the animals with EAMG (see section 6.2.4). The animals in the therapeutic study were tail bled pre-disease induction and then at different time points post-induction (method 2.6.4). Following isolation of the serum, the amount of serum necessary to bring about 50% lysis of antibody-coated sheep erythrocytes was compared, this is the CH50 (figure 6.8A). The PBS control group showed an increase in haemolytic activity in their serum compared to the pre-bleed due to an acute phase reaction in the model resulting in an increase on C components from the liver. The 4SCR treated group showed a similar increase in haemolytic activity, although this increase was not to the same extent during the first few hours, up to 24 hours post-induction. The 4SCR-Fc group showed a marked reduction in C activity, significant inhibition was evident up to 6 hours post-induction ($p<0.05$ compared to 4SCR lysis at 6 hours by an ANOVA test). The haemolytic activity then increased gradually to its pre-bleed level by 24 hours post induction; it never reached the levels of increased haemolytic activity seen in the PBS or 4SCR groups.

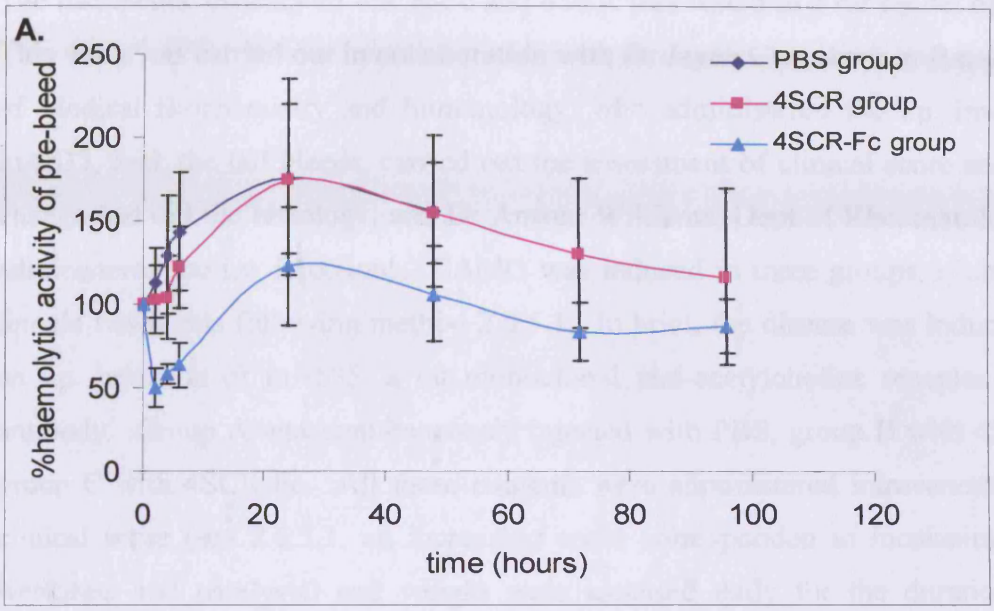
To clarify the CH50 data, mainly to normalise for the increase in haemolytic activity in the PBS and 4SCR groups, the haemolytic activity of the serum was expressed as a percentage of the 4SCR serum haemolytic activity (figure 6.8B). The 4SCR group was used for this, not the PBS group as the PBS group were sacrificed 52 hours post-induction so not all the time points are present. Other than the first few hours the 4SCR group response overlaid the PBS group response. Using this approach the PBS group showed an increase in haemolytic activity in the first few hours, presumably due to low levels of 4SCR remaining in the circulation causing a small amount of C inhibition or the acute phase reaction being reduced in the 4SCR group. The 4SCR-Fc protein caused a decrease in haemolytic activity of the serum throughout the 96 hours of the therapy experiment assessed here, at 2 hours post induction lysis was inhibited by 50% in serum isolated from the 4SCR-Fc animals. At 96 hours post-induction the lysis caused by the serum had increased to 75% of the pre-bleed level.

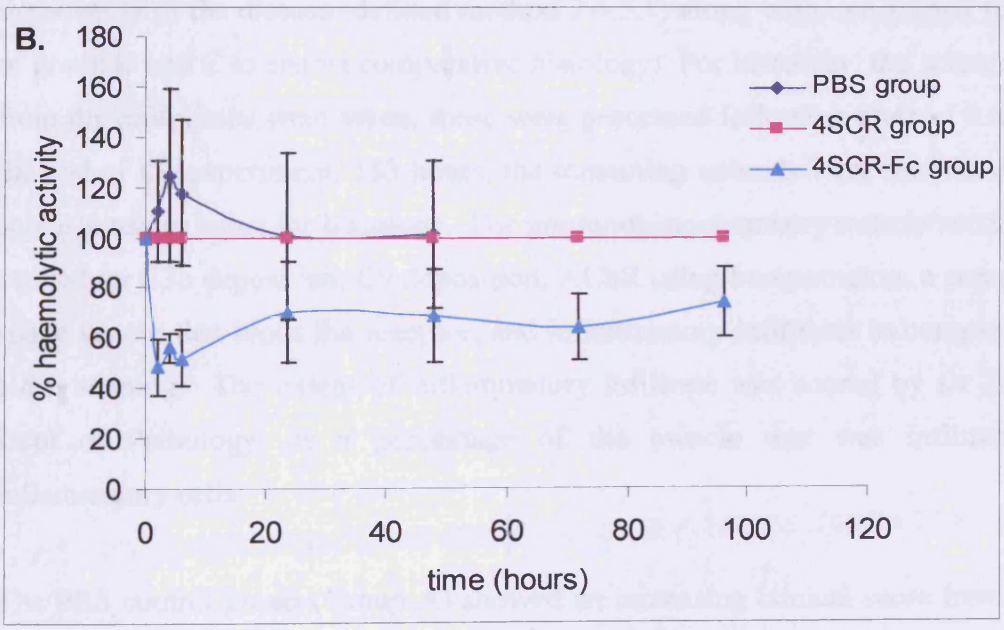
Figure 6.8 CH50 of 4SCR and 4SCR-Fc.

A. Graph showing the CH50 from the PBS group (blue), 4SCR group (pink) and 4SCR-Fc group (aqua) in diseased animals. The haemolytic activity of the serum from the PBS group increased, the 4SCR group also showed an increase in haemolytic activity although this was to a lesser extent during the first few hours. The 4SCR-Fc group had a marked reduction in haemolytic activity of the serum compared to the PBS and 4SCR groups.

B. The haemolytic activities of the serum from the diseased animals were expressed as a percentage of the 4SCR level and plotted against time. The PBS group (blue) had an increase in haemolytic activity compared to the 4SCR group (pink) during the first few hours. The 4SCR-Fc group (aqua) had reduced haemolytic activity.

Data points on both graphs are the average for the animals in each group, +/- 1 SD.


A.
%haemolytic activity of pre-bleed
250
200
150
100
50
0
0
20
40
60
80
100
120
time (hours)
PBS group
4SCR group
4SCR-Fc group


B.
% haemolytic activity
180
160
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
time (hours)
PBS group
4SCR group
4SCR-Fc group

6.2.4 Therapeutic efficacy of 4SCR-Fc and 4SCR

The therapeutic efficacy of 4SCR-Fc and 4SCR was tested in a rat model of EAMG. This work was carried out in collaboration with Dr Jayne Chamberlain-Banoub, Dept of Medical Biochemistry and Immunology, who administered the i.p. injection of mAb35, took the tail bleeds, carried out the assessment of clinical score and weight change and did the histology, and Dr Anwen Williams, Dept of Rheumatology, who administered the i.v. injections. EAMG was induced in three groups of six normal female Lewis rats following method 2.6.5.1. In brief, the disease was induced using an i.p. injection of mAb35, a rat monoclonal anti-acetylcholine receptor (AChR) antibody. Group A was simultaneously injected with PBS, group B with 4SCR and group C with 4SCR-Fc. All these reagents were administered intravenously. The clinical score (see 2.6.5.1, an increasing score corresponded to increasing muscle weakness and paralysis) and weight were assessed daily for the duration of the experiment. At 52 hours post induction group A (PBS control) was sacrificed due to the severity of the disease (defined method 2.6.5.1) along with one animal from each of group B and C to enable comparative histology. For histology, the soleus muscles from the hind limbs were taken, these were processed following method 2.6.5.2. At the end of the experiment, 183 hours, the remaining animals were sacrificed and the soleus muscles taken for histology. For immunohistochemistry muscle sections were stained for C3b deposition, C9 deposition, AChR using bungarotoxin, a compound in snake venom that binds the receptor, and inflammatory infiltrates to complement the H&E staining. The extent of inflammatory infiltrate was scored by Dr Jim Neal, Dept of Pathology, as a percentage of the muscle that was infiltrated with inflammatory cells.

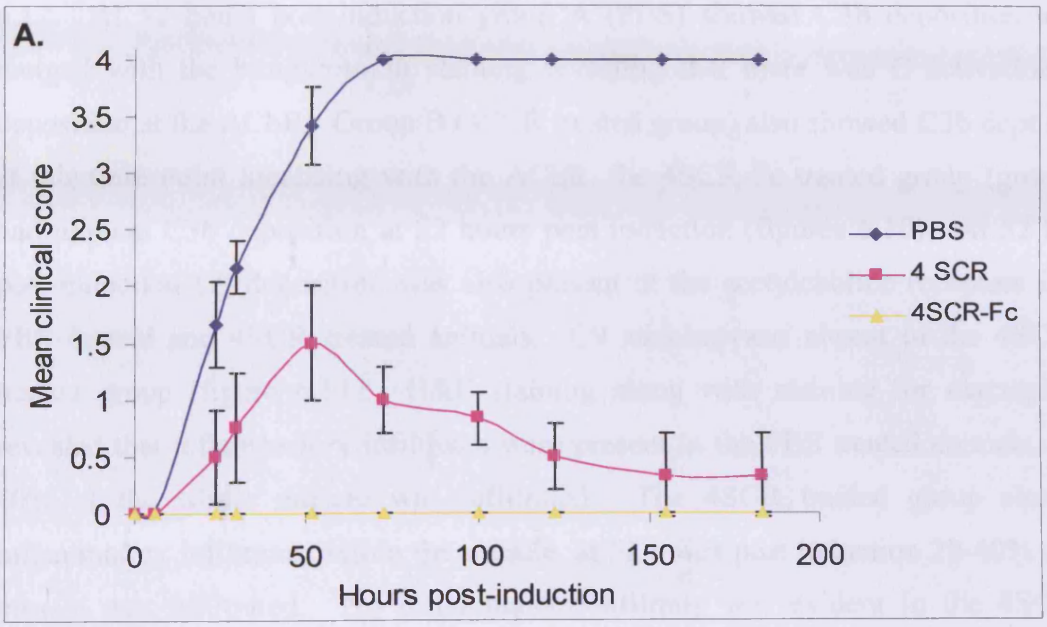
The PBS control group (Group A) showed an increasing clinical score from 6 hours post-induction corresponding to increasing muscle weakness and paralysis. The score increased to 3.5 at 52 hours post-induction when they were sacrificed due to disease severity, from this point on the score was taken as 4. The 4SCR group (group B) had a clinical score which also increased from 6 hours post-induction, although the severity of disease was not equivalent to that in the PBS group. The maximum clinical score was 1.5 at 52 hours post-induction. The animals then began to recover from the disease. The 4SCR-Fc group showed no clinical signs of the

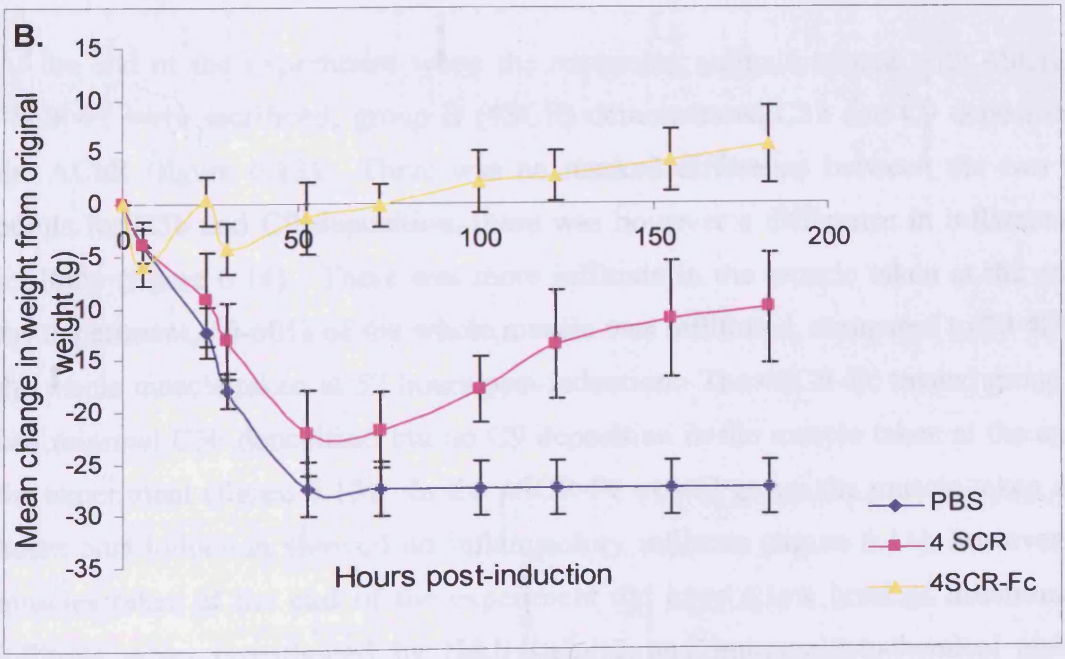
disease throughout the experiment, figure 6.9A. These clinical signs of the disease were also manifest in the change in weight seen in the animals throughout the experiment. The PBS group had a marked reduction in weight from 6 hours post induction; throughout the experiment the animals lost an average of 28g, around 15% of their starting body weight. These animals were sacrificed at 52 hours post-induction so from that point on the weights of these animals were regarded as being unchanged. The 4SCR group also lost weight in a manner which mirrored their clinical scores, the animals progressively lost weight from 6 hours post-induction until 52 hours post-induction when they began to recover and gain weight. The 4SCR-Fc group on the other hand showed no weight loss during the experiment, they progressively gained weight, figure 6.9B.

Figure 6.9 Clinical score and weight assessment of 4SCR-Fc, 4SCR and PBS-treated animals in the EAMG model.

A. Graph showing clinical scores of the animals. The clinical score corresponding to muscle weakness and paralysis increased in the PBS group (blue) from 6 hours post induction until it reached 3.5 at 52 hours post induction. At this point the animals were sacrificed. The 4SCR group (pink) showed an increasing clinical score from 6 hours post-induction to 1.5 at 52 hours post-induction at which point the animals began to recover and the clinical score decreased. The 4SCR-Fc group (yellow) showed no clinical score throughout the experiment. Each data point represents the average of 6 animals +/- 1 SD.

B. Graph showing weight change throughout the therapy experiment. The PBS group (blue) lost weight through the experiment in line with their increase in clinical score. The 4SCR group (pink) lost weight until 52 hours post-induction when they began to recover and progressively gained weight. The 4SCR-Fc group (yellow) gained weight throughout the experiment. Each data point represents the average of 6 animals +/- 1 SD.


A.
Mean clinical score
4
3.5
3
2.5
2
1.5
1
0.5
0
0
50
100
150
200
Hours post-induction
PBS
4 SCR
4SCR-Fc


B.
Mean change in weight from original weight (g)
15
10
5
0
-5
-10
-15
-20
-25
-30
-35
0
50
100
150
200
Hours post-induction
PBS
4 SCR
4SCR-Fc

The histology from one animal from the PBS group and a representative animal from the other two groups sacrificed at 52 hours post-induction is shown in figures 6.10-6.12. At 52 hours post-induction group A (PBS) showed C3b deposition which merged with the bungarotoxin staining revealing that there was C activation and deposition at the AChR. Group B (4SCR treated group) also showed C3b deposition at this time point localising with the AChR, the 4SCR-Fc treated group (group C) had minmal C3b deposition at 52 hours post induction (figures 6.10). At 52 hours post-induction C9 deposition was also present at the acetylcholine receptors in the PBS treated and 4SCR treated animals. C9 staining was absent in the 4SCR-Fc treated group (figure 6.11). H&E staining along with staining for macrophages revealed that inflammatory infiltrates were present in the PBS treated animals, up to 40% of the whole muscle was infiltrated. The 4SCR treated group also had inflammatory infiltrates within the muscle, at 52 hours post induction 20-40% of the muscle was infiltrated. No inflammatory infiltrate was evident in the 4SCR-Fc treated group (figure 6.12).

At the end of the experiment when the remaining animals treated with 4SCR and 4SCR-Fc were sacrificed, group B (4SCR) demonstrated C3b and C9 deposition at the AChR (figure 6.13). There was no marked difference between the two time points for C3b and C9 deposition, there was however a difference in inflammatory infiltrate (figure 6.14). There was more infiltrate in the muscle taken at the end of the experiment, 40-60% of the whole muscle was infiltrated, compared to 20-40% of the whole muscle taken at 52 hours post-induction. The 4SCR-Fc treated group still had minimal C3b deposition but no C9 deposition in the muscle taken at the end of the experiment (figure 6.13). In the 4SCR-Fc treated group the muscle taken at 52 hours post induction showed no inflammatory infiltrate (figure 6.11), however, the muscles taken at the end of the experiment did have a low level of inflammatory infiltrate when investigated by H&E staining and immunohistochemical analysis staining for macrophages (figure 6.14).

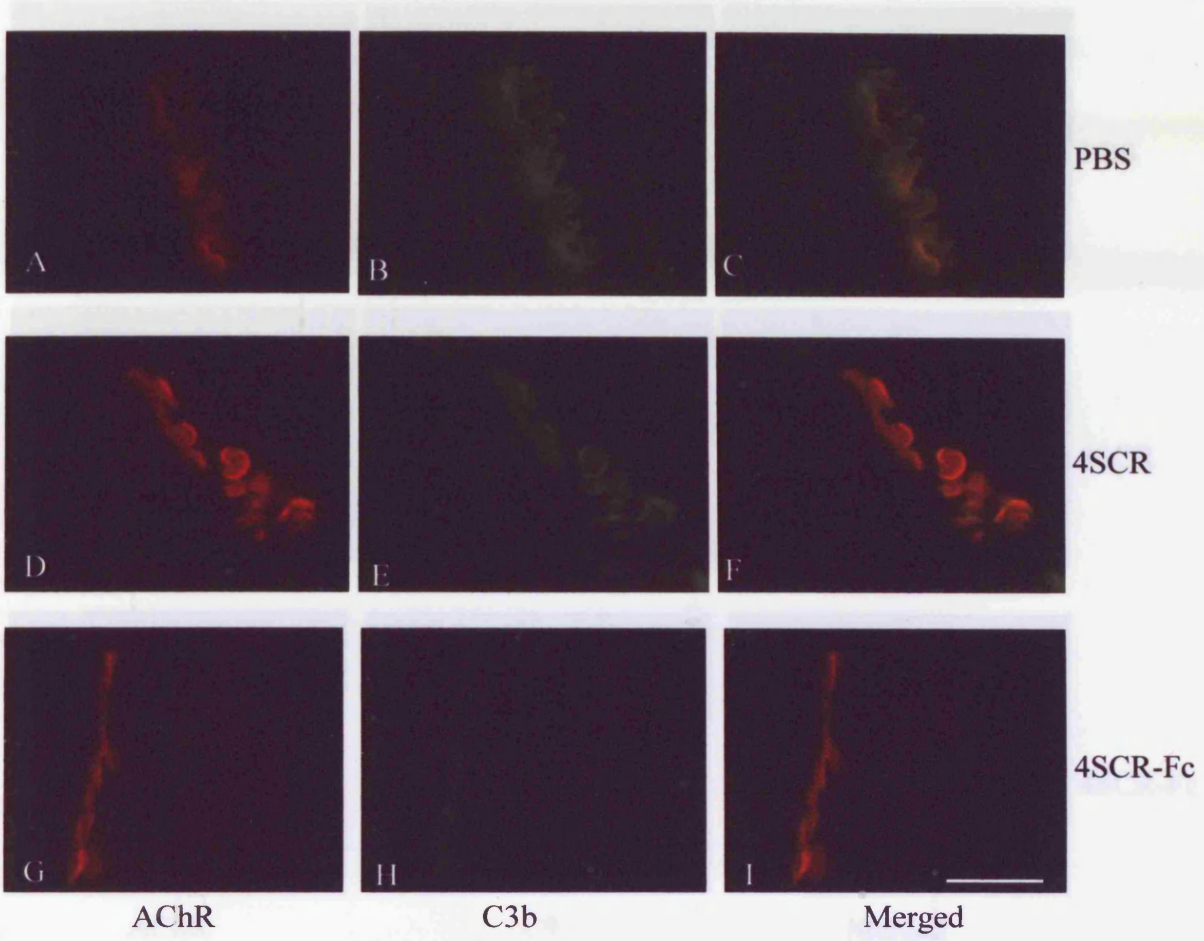


Figure 6.10 Immunohistochemical analysis of C3b deposition in all groups at 52 hours post-induction.

A, D and G are bungarotoxin stained sections; B, E and H are stained for C3b deposition while C, F and I are the merged bungarotoxin and anti-C3b stained sections. In the PBS treated group (A, B and C) C3b deposition was evident at the AChR. In the 4SCR treated group (D, E and F) C3b deposition was also found at the AChR. In the 4SCR-Fc treated group (G, H and I) minimal C3b deposition was seen at the AchR, it was limited compared to the 4SCR and PBS treated animals.

All images were taken at 400x magnification and cropped to focus on an area containing acetylcholine receptors. Bar in I represents 100μm. Representative images are shown here.

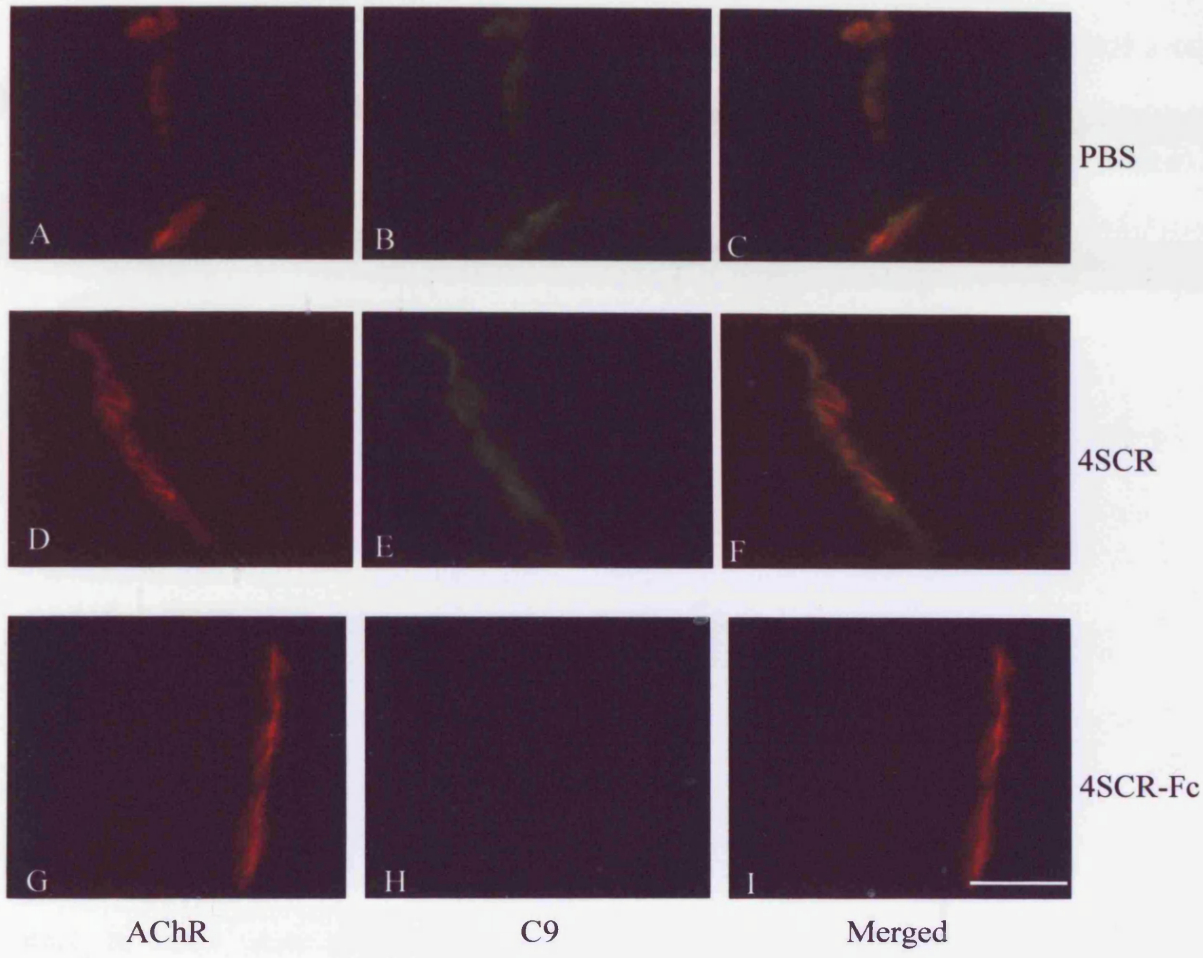


Figure 6.11 Immunohistochemical analysis of C9 deposition in the muscles taken at 52 hours post-induction.

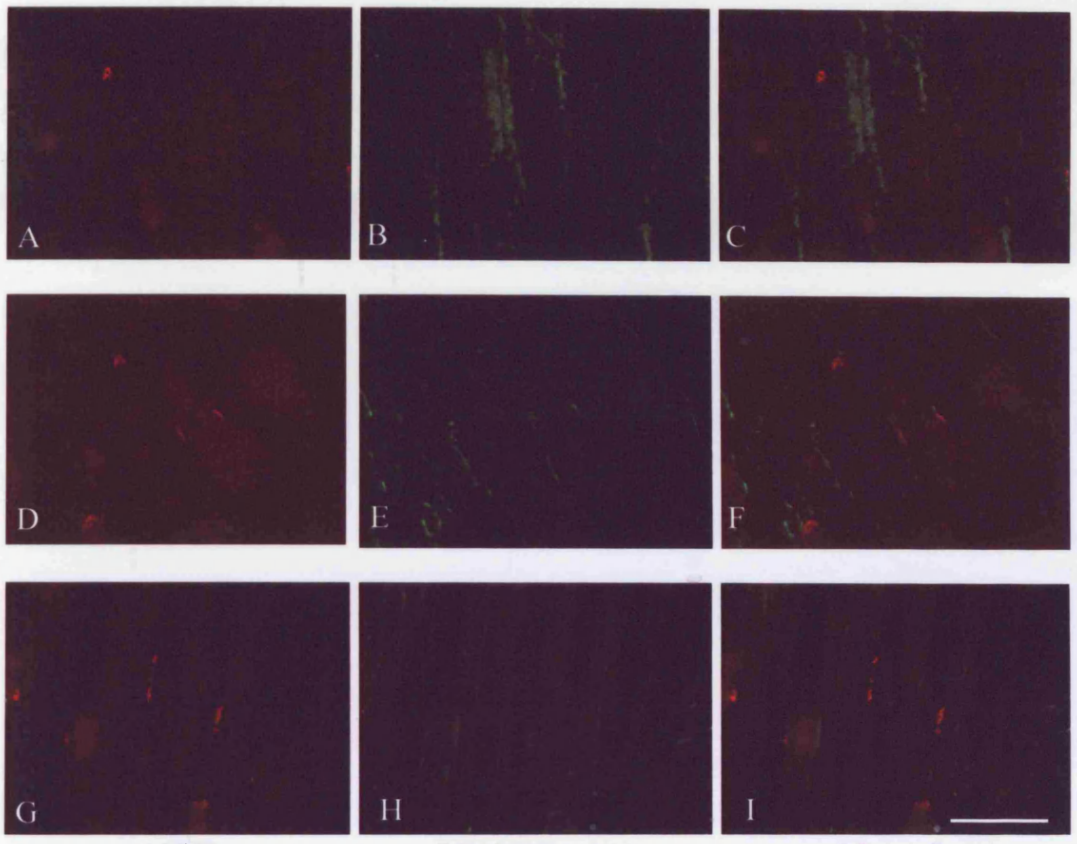
A, D and G are bungarotoxin stained sections, highlighting the acetylcholine receptors present in the tissue. B, E and H are stained for C9. C, F and I are the merged images of the bungerotoxin and anti-C9 stained sections. The PBS-treated group (A, B and C) showed C9 deposition merging with the bungarotoxin stain revealing that C9 deposition was at the AChR. The 4SCR-treated group (D, E and F) also had C9 deposition at the AChR. The 4SCR-Fc treated group (G, H and I) had no C9 deposition at the AChR or elsewhere in the tissue.

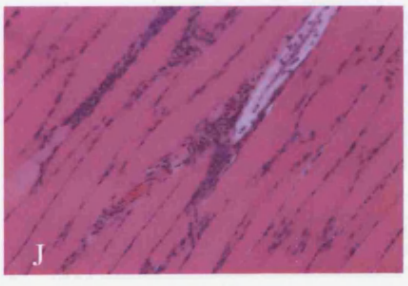
All images were taken at 400x magnification and cropped to focus on an area containing acetylcholine receptors. Representative images are shown here. Bar in I represents 100μm.

Figure 6.12 Histological analyses of inflammatory infiltrates within the muscles taken at 52 hours post-induction.

The immunohistochemical analysis is shown in A-I. A, D and G show the bungarotoxin stained sections. B, E and H show the sections stained for macrophages using anti-CD68. C, F and I are the merged images. Group A (PBS treated) show marked inflammatory infiltrates within the sections, this staining was evident throughout the muscle, not just at the AChR (A, B and C). Group B (4SCR treated) showed some inflammatory infiltrate throughout the muscle, 20-40% of the muscle was infiltrated (D, E and F). Group C, those treated with 4SCR-Fc, showed no inflammatory infiltrate (G, H and I). The immunohistochemical analysis was supported by H&E staining (J, K and L). The PBS-treated group (J) showed a high level of inflammatory infiltrate within the muscle. The 4SCR-treated group (K) showed some inflammatory infiltrates while there was none present in the muscle of the 4SCR-Fc treated group (L).

All immunohistochemical images were taken at 400x magnification. Bar in figure I represents 400μm. H&E stained images were taken at 100x magnification.


A
B
C
PBS
D
E
F
4SCR
G
H
I
4SCR-Fc
AChR
Macrophages
Merged


J
PBS

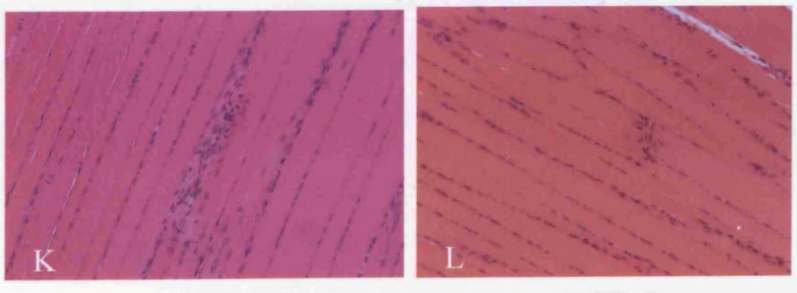
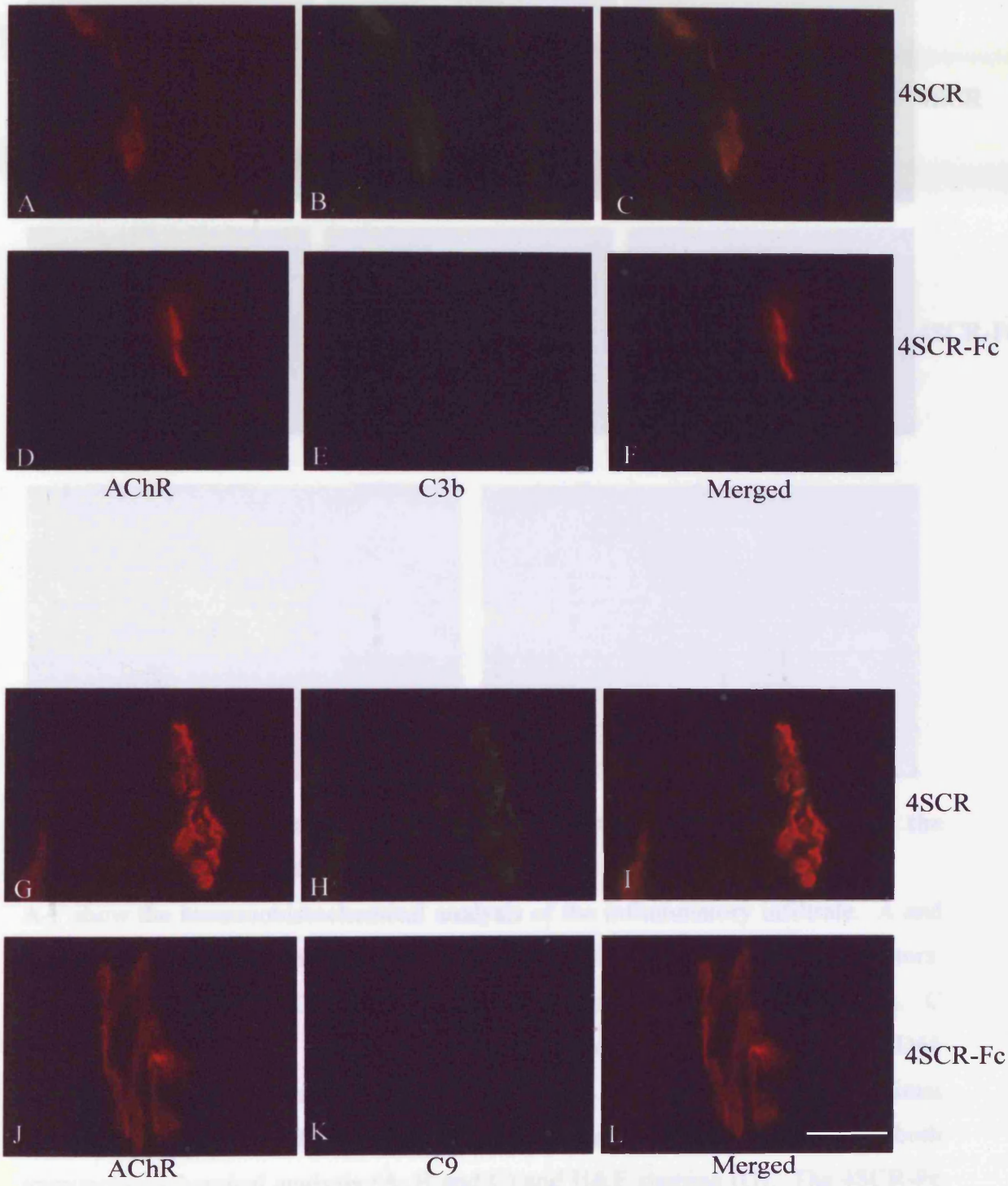

K
L
4SCR
4SCR-Fc

Figure 6.13 Immunohistochemistry of C deposition within the muscles taken at the end of the experiment.

A, D, G and J are the bungarotoxin stained sections. B and E are stained for C3b deposition while H and K are stained for C9 deposition. C, F, I and L are the merged images of the anti-AChR stain and the respective anti-C component stain. The 4SCR treated animals sacrificed at the end of the experiment demonstrated both C3 (A, B and C) and C9 (G, H and I) deposition which merged with the AChR. On the other hand, the 4SCR-Fc treated animals had minimal C3b deposition (D, E and F) and no C9 deposition (J, K and L) at the AChR or present elsewhere in the tissues.

All images were taken at 400x magnification and cropped to focus on an area containing acetylcholine receptors. Bar in L represents 100μm.


A
B
C
4SCR
D
E
F
4SCR-Fc
AChR
C3b
Merged
G
H
I
4SCR
J
K
L
4SCR-Fc
AChR
C9
Merged

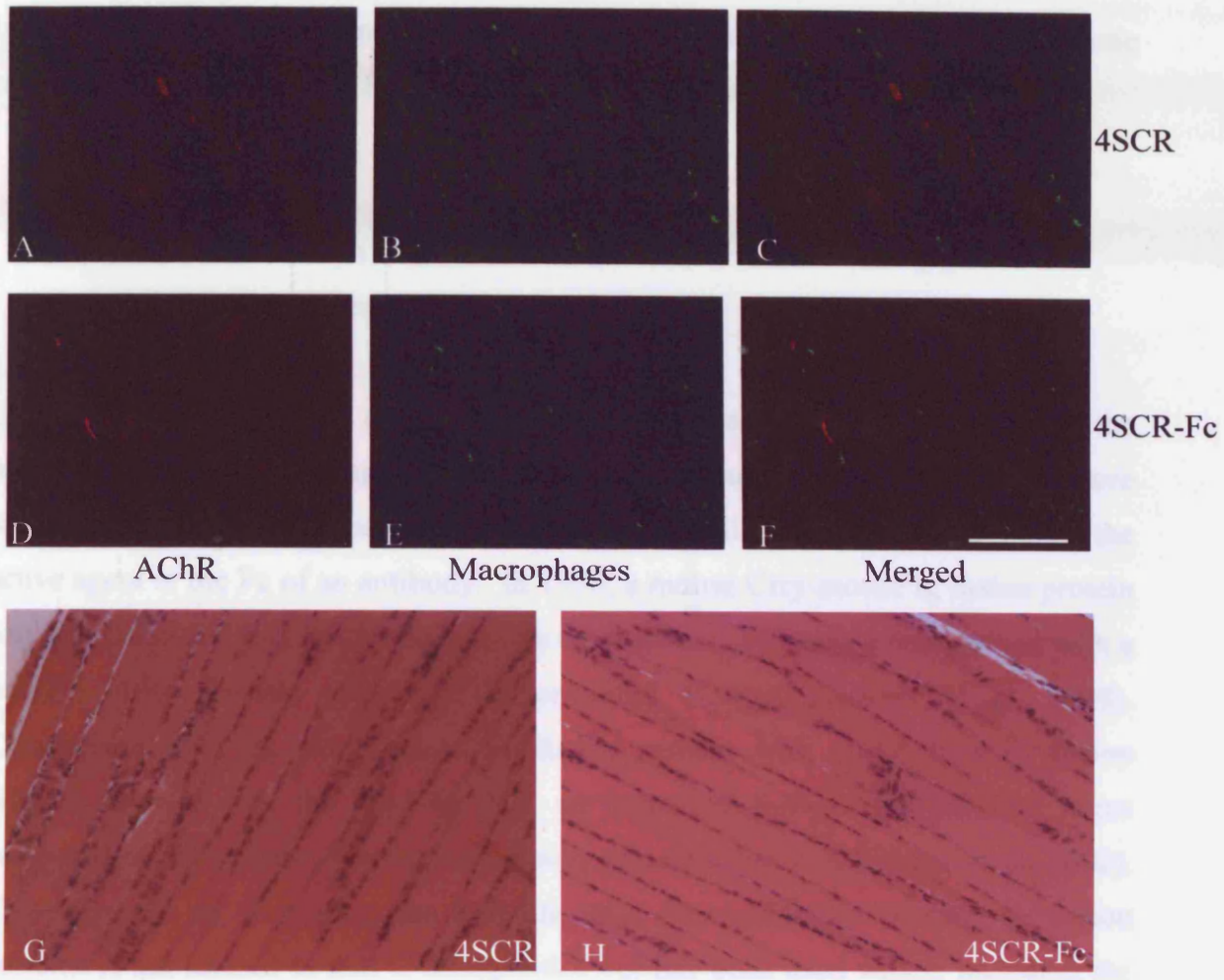


Figure 6.14 Histochemical analyses of inflammatory infiltrates within the muscles taken at the end of the experiment.

A-F show the immunohistochemical analysis of the inflammatory infiltrate. A and D show the bungarotoxin stained sections highlighting the acetylcholine receptors. B and E show the anti-CD68 staining demonstrating macrophage infiltration. C and F show the merged images of the bungarotoxin staining and the anti-CD68 staining. The H&E stained sections are in G and H. The 4SCR treated animal showed inflammatory infiltrate throughout the muscle when investigated by both immunohistochemical analysis (A, B and C) and H&E staining (G). The 4SCR-Fc treated animal showed an increased amount of inflammatory infiltration compared to the muscles at 52 hours post induction but the amount was still low when analysed by immunohistochemistry (D, E and F) and H&E staining (H).

All immunohistochemical images were captured at 400x magnification while all H&E sections were captured at 100x magnification. Bar in F represents 400μm.

6.3 Discussion

In this chapter I have described the in vivo characterisation of the therapeutic reagents, 4SCR-Fc and 4SCR. The proteins were characterised in terms of their

- in vivo half-life,
- immunogenicity,
- ability to inhibit C in vivo
- and therapeutic efficacy.

One of the limitations of anti-C therapeutics has been the short half-life of the reagents resulting in their rapid clearance from the circulation. Previous studies have documented a strategy to increase the half-lives of anti-C therapeutics by fusing the active agent to the Fc of an antibody. In 1998, a mouse Crry-mouse Ig fusion protein was generated that had a β-phase half-life of 40 hours, although a comparison with a non-Fc fusion protein was not demonstrated (Quigg, Kozono et al. 1998). Comparison of a rat DAF-human Fc fusion protein with sDAF (non-Fc fusion protein) showed that the DAF-Fc had an increased β-phase half-life, 33 hours compared to 20 minutes for the non-fusion protein (Harris, Williams et al. 2002). This approach of increasing the half-life of a therapeutic by creating Fc fusion proteins is not limited to anti-C therapeutics but has been used widely including the generation of IFN-γ receptor, CTLA-4 and TNF-α receptor fusion proteins (Kurschner, Ozmen et al. 1992; Linsley, Wallace et al. 1992; Pugsley 2001).

The Crry-Fc fusion protein generated here had an increased β-phase half-life of 53 hours compared to 7 minutes for the non-Fc fusion protein, 4SCR. This increase in half-life was due to several factors including the increase in size of the protein taking the protein above the filtration limit of the kidney. Fc fusion proteins are also actively retained in the circulation due to the interaction of the Fc with Fc receptors such as the Brambell receptor which acts to retain immunoglobulins in the circulation by binding to the Fc domain (Telleman and Junghans 2000). Brambell receptors (FcRn) are widely expressed on vascular endothelial cells and can prevent IgG catabolism by directing the release of IgG into extracellular fluid following endocytosis (Telleman and Junghans 2000).

Another limitation of anti-C therapeutics has been their immunogenicity in animals. A number of anti-C reagents have been based upon human regulators, for example sCR1 is based upon human CR1. Use of this reagent is limited in animal models as long-term administration results in neutralising antibodies being generated (Piddlesden, Storch et al. 1994). To enable the long-term C inhibition in chronic disease models to be investigated the fusion proteins generated here consisted of a rat regulator, Crry, fused to a rat Fc domain. To investigate if this chimeric molecule was immunogenic an immune response study was carried out. Antibodies were generated to the Crry-Fc although further investigation revealed that these antibodies caused neither the Crry-Fc to be cleared from the circulation nor its function to be inhibited. This immune response may have been caused by altered glycosylation, folding or other post-translational modifications of the protein. Some CHO cell lines do not carry out N-linked glycosylation, although the cell line used here does carry out this glycosylation (Raju, Briggs et al. 2000). It is still possible that the protein differed from its native conformation in some way as CHO cells carry out altered glycosylation under high ammonium ion concentrations (Chen and Harcum 2005). While the high expression systems used to generate these proteins (see chapter 5) appeared not to alter the proteins, perhaps high ammonium ion content within the cell culture may have altered glycosylation. Recombinant follicle-stimulating hormone (FSH) expressed in CHO cells has been shown to differ from native FSH, recombinant FSH contained less heavily sialylated carbohydrates as CHO cells differ from native cells in their ability to sialyte carbohydrates (Loumaye, Dreano et al. 1998). Species specific glycosylation of immunoglobulins has also been demonstrated, the glycosylation of Fc and proteins in general may differ in chinese hamsters from the glycosylation of proteins in rats (Raju, Briggs et al. 2000). Similar immune responses have been noted to other therapeutic proteins. Patients on sCR1 have been noted to produce antibodies to the reagent, although similarly the antibodies neither enhanced clearance nor reduced the protein's function (personal communication Prof. B. P. Morgan). In the clinical trials of etanercept, a TNF receptor Fc fusion protein, 2% of patients produced antibodies to the reagent, these antibodies were non-neutralising (Anderson 2005). Although the immune response study was carried out using 5SCR-Fc, it is reasonable to assume that an equivalent response would be generated to 4SCR-Fc, which was used in the therapy experiment. The antibodies were generated to the whole molecule and not a neoepitope at the

join, which would differ between 5SCR-Fc and 4SCR-Fc. Both 5SCR-Fc and 4SCR-Fc were produced using the same cell line under the same conditions and therefore have the same potential to be immunogenic due to altered glycosylation, folding or other post-translational modifications.

While the immune response has not altered the function and clearance of the protein within the time period of this experiment (35 days), there is no guarantee that long term treatment with this reagent will not drive the immune response to cause enhanced clearance or function inhibition. This result highlights the need to select an appropriate cell line in which to produce recombinant proteins. Perhaps generation of this protein in a rat or mouse cell line would have resulted in a non-immunogenic protein being produced. The immune response within the negative control group, those treated with sCrry-cys, was surprising. The response was not due to the cysteine residue but the Crry itself. The generation of antibodies may have been driven by lipopolysaccharide contamination of the protein as this was a bacterially expressed protein. Also, as discussed previously bacteria do not fold and carry out glycosylation of proteins, while this protein has been refolded and shown to have no change in function, slight changes in folding and differences in glycosylation may have resulted in this native protein becoming immunogenic.

Anti-C therapeutics have been tested in a wide range of animal models including antigen and collagen induced arthritis, glomerulonephritis and ischaemia-reperfusion injuries (for more information see section 1.5). In particular, administration of Crry has proved to be a viable therapeutic strategy in glomerulonephritis where administration of mouse Crry-Ig in a murine model substantially reduced renal injury (Quigg, Kozono et al. 1998). Crry-Ig has also been used in a model of airway hyper-responsiveness where it was proven to be protective (Taube, Rha et al. 2003), in a mouse model of lupus where it decreased inflammation (Alexander, Bao et al. 2003) and in mesenteric ischaemia-reperfusion where it decreased intestinal mucosal injury (Rehrig, Fleming et al. 2001). Studies of transgenic mice over expressing soluble Crry have also shown the therapeutic potential of Crry. These animals are protected from antibody-induced acute renal failure (Schiller, Cunningham et al. 2001), experimental allergic encephalomyelitis (Davoust, Nataf et al. 1999), membrane

proliferative glomerulonephritis (Muhlfeld, Segerer et al. 2004) and collagen-induced arthritis (Banda, Kraus et al. 2003).

Here, Crry was proven to be protective against EAMG. Two recombinant forms of Crry, 4SCR-Fc and 4SCR, were used to treat EAMG in rats. Both reagents reduced the clinical severity of disease with the long-lived 4SCR-Fc reagent having the most profound effect. Animals treated with 4SCR-Fc did not develop a clinical score or have the weight loss associated with the disease. Histological analyses revealed that these animals had low levels of C3b deposition but no C9 deposition at the AChR or elsewhere on the motor end plate. Low levels of inflammatory infiltrate within the muscle were also noted; less than 20% of the muscle was infiltrated. These beneficial clinical effects of 4SCR-Fc were due to its ability to inhibit C in vivo as seen by the measurement of haemolytic activity in the serum from these animals. The haemolytic activity of the serum collected from the 4SCR-Fc group was reduced by 50%. This 50% reduction was sufficient to prevent disease as the level of C was taken below the threshold required to bring about the damage associated with EAMG. The detection of C3b deposition by immunohistochemistry demonstrates that the C system was still active but the level of C3b deposition required for C to cause damage to the motor end plate was never achieved. It is possible that the C3b detected has been inactivated by Crry acting as a cofactor for factor I. Therefore the C3b staining identified may actually be iC3b staining, iC3b no longer participates in the C cascade.

The 4SCR reagent also reduced clinical signs although not to the same extent as 4SCR-Fc, the clinical scores peaked at 52 hours post-induction. The reduction in clinical score after this point may be due to the 4SCR reagent inhibiting C enough so that only minor damage occurred at the motor end plate. Clearance of the anti-AChR antibody from the circulation prevented continued C activation so the clinical score decreased. The weight loss of the animals also peaked at 52 hours post-induction after which the animals gained weight. Histological analyses revealed C3b and C9 deposition at the AChR and inflammatory infiltrate. The haemolytic activity of the serum from the animals treated with 4SCR was the same as the PBS treated group except for the first few hours where the serum from the 4SCR treated animals had a reduced haemolytic activity compared to the PBS group. In both the PBS treated

animals and 4SCR treated animals an increase in haemolytic activity was observed presumably due to an acute phase response caused by EAMG resulting in increased synthesis of a number of proteins in the liver including many C components. During the first few hours, the haemolytic activity of the serum from the 4SCR treated animals did not increase to the same extent as the PBS treated animals; this may be due to C inhibition caused by the 4SCR reagent present in the serum although given the β-phase half-life of the protein this is unlikely. It is more likely that the presence of the 4SCR reagent during the initial C activation, immediately following mAb35 administration, lessened the initial injury delaying the acute phase response. The presence of 4SCR at this early time point preventing massive C attack following the initial activation may also be the reason for the decreased disease profile in terms of histology, clinical scores and weight loss seen in these animals. As previously discussed, both 4SCR-Fc and 4SCR inhibited C in vivo when assessed in diseased animals. This assessment of in vivo activity was complicated by the acute phase response in the disease resulting in enhanced production of many C components. Assessment of the in vivo C inhibitory activity of these proteins in non-diseased animals would clarify the ability of the proteins to inhibit C in vivo.

In conclusion, 4SCR-Fc and 4SCR are therapeutically active in EAMG with the 4SCR-Fc reagent being most efficient at inhibiting the clinical signs of disease. This enhanced therapeutic effect of 4SCR-Fc is a consequence of its increased β-phase half-life compared to the 4SCR reagent and therefore its ability to inhibit C in vivo for a longer period than the 4SCR reagent.

Chapter 7: Final discussion.

The overall aim of this study was to design and develop better inhibitors of C for use in rats. The specific aims of this study were:

1. To generate a totally rat CReg-Fc fusion protein using rat Crry and rat Fc,
2. To carry out functional analysis of rat Crry to locate functional domains within Crry and enable the generation of a rat Crry 'prodrug',
3. To test both the Crry-Fc and 'prodrug' in rat models of disease.

In this study I have generated a variety of recombinant forms of rat Crry, the following proteins were made: 5SCR-Fc, 4SCR-Fc, 3SCR-Fc, 4SCR and 3SCR Functional characterisation of these recombinant proteins identified a functional Crry-Fc, 4SCR-Fc, but a 'prodrug' was not identified as functional domains within Crry were deleted before steric hindrance sufficient for the 'prodrug' was achieved. 4SCR-Fc was compared with 4SCR (non-Fc fusion protein) in a rat model of EAMG where it reduced clinical severity and C deposition in vivo.

7.1 Current anti-C therapeutics and their limitations

The C system has been heavily implicated in the pathogenesis of a number of inflammatory diseases. Among these diseases is rheumatoid arthritis where C activation products have been found in the synovial fluid and on the synovial membrane (Brodeur, Ruddy et al. 1991; Kemp, Spragg et al. 1992). In glomerulonephritis, C activation products are deposited in the glomeruli and in MG, C activation products are detected on the post-synaptic membrane (Davis and Cavallo 1976; Engel, Lambert et al. 1977; Adler, Baker et al. 1984; Engel and Arahata 1987). Due to the implication of C in such diseases, C became a therapeutic target and anti-C therapy was born. Anti-C therapy began with cobra venom factor. This reagent was a useful proof-of-principle demonstrating that anti-C therapy could successfully reduce disease severity in a number of animal models (Morgan and Harris 2003). However, its in vivo use was limited as it is strongly immunogenic resulting in the generation of neutralising antibodies. Over the years, many different anti-C therapeutics have been tested, these include a number of peptides. FUT-175, one such peptide is a broad spectrum serine protease inhibitor that acts as an inhibitor

of C1s, factor D and the C3/C5 convertases. FUT-175 was effective at treating glomerulonephritis patients (Fujita, Inoue et al. 1993). Compstatin a synthetic peptide that prevents the cleavage of C3 inhibits C activation in extracorpeal circuits (Sahu, Kay et al. 1996; Nilsson, Larsson et al. 1998). Several antibodies that block the activity of C components have been used in therapeutic models. Anti-C5 monoclonal antibodies have been tested in models of immune complex nephritis and collagen-induced arthritis, and has reached clinical trials in myocardial infarction patients and patients undergoing cardiopulmonary bypass (Wang, Rollins et al. 1995; Wang, Hu et al. 1996; Granger, Mahaffey et al. 2003; Shernan, Fitch et al. 2004). Finally, recombinant forms of the membrane regulators have been used therapeutically. Soluble CR1, the most studied, consists of the extracellular domains of human CR1 and has been tested in various animal models including models of MG, multiple sclerosis, rheumatoid arthritis and I/R injures (Weisman, Bartow et al. 1990; Piddlesden, Storch et al. 1994; Piddlesden, Jiang et al. 1996; Goodfellow, Williams et al. 1997; Goodfellow, Williams et al. 2000). This reagent is still undergoing development for clinical use, having been tested in adults with ARDS and in cardiopulmonary bypass where it effectively inhibited C activation (Zimmerman, Dellinger et al. 2000; Rioux 2001).

Despite the success of many of the anti-C therapeutics described above in animal models, their uses in humans have been limited. Many of the peptides have broad-spectrum activities and as a result act non-specifically. They are also small molecules which are rapidly cleared in vivo meaning that effective anti-C therapy requires regular administration. Soluble recombinant forms of the membrane regulators are also limited by rapid in vivo clearance. A number of different approaches have been employed to extend the in vivo half-life of recombinant CReg. Soluble CR1 has been expressed as a form containing the albumin binding site from streptococcal protein G at the C-terminus. This resulted in an increased half-life, as the reagent bound albumin in the circulation, whilst not inhibiting function (Makrides, Nygren et al. 1996). Addition of targeting domains has also been used to increase the in vivo efficacy of sCR1. Addition of Sialyl Lewis X tetrasaccharide groups to sCR1 created a molecule that localised to sites of inflammation by binding P-, E- and L- selectins. Through this interaction the molecule also inhibited selectin-dependent leukocyte adhesion (Mulligan, Warner et al. 1999; Rittershaus, Thomas et

al. 1999). Recombinant CReg have also been targeted to membranes. In this approach a lipid targeting peptide was attached to the C-terminus. The peptide contained positively charged amino acids that bound the phospholipids in the membrane and a terminal myristoyl group that inserted into the membrane bilayer (Smith and Smith 2001). This technology has been applied to a truncated form of sCR1, rat Crry and rat CD59. It extended the circulating half-life of rat Crry from 15 minutes to 50 minutes although the true half-life is likely to be much greater than this as the targeted Crry would no longer be in the plasma but bound to cell surfaces (Fraser, Harris et al. 2002). The final approach used to extend the circulating half-life of recombinant CReg is the generation of CReg-Fc fusion proteins. The CReg replaces the Fab arms of an antibody while the Fc increases the plasma half-life by increasing the protein size and by interacting with Fc receptors retaining the proteins in the circulation. This interaction with Fc receptors serves to increase the plasma half-life as binding to Fc receptors prevents protein catabolism (Telleman and Junghans 2000). Comparison of a DAF-Fc fusion protein with sDAF (non-Fc) revealed that utilising the Fc increased the circulating half-life from 20 minutes to 33 hours (Harris, Williams et al. 2002).

A further disadvantage of anti-C therapy is the tendency of the therapeutics to systemically inhibit C, that is they inhibit all the roles of C rendering an individual susceptible to bacterial infections. Targeting C therapeutics to sites of inflammation has been used to overcome the problems of systemic C inhibition. As discussed above, sCR1 was targeted to sites of inflammation by adding Sialyl Lewis X tetrasaccharide groups. Complement receptor 2 (CR2) binds C3 activation fragments present at sites of C activation and has been used to target CReg to sites of C activation and inflammation. A DAF-CR2 fusion protein targeted to sites of C activation in a model of lupus nephritis and a Crry-CR2 fusion protein significantly reduced C-mediated injury in an intestinal I/R injury model whilst also reducing susceptibility to infection compared to an untargeted Crry-Fc protein (Song, He et al. 2003; Atkinson, Song et al. 2005). Further engineering of CReg-Fc to bring the active site of a CReg in to close proximity to the Fc had resulted in steric hindrance of the CReg by the Fc and the creation of the 'prodrug'. Insertion of a short enzyme target site, for enzymes at high levels at sites of inflammation, between the CReg and Fc resulted in the generation of a CReg-Fc that could be activated at sites of

inflammation. Activation of this CReg-Fc at sites of inflammation unleashed powerful anti-C activity from a reagent that otherwise had negligible activity (Harris, Hughes et al. 2003).

A final limitation of anti-C therapeutics is the fact that many reagents created to date are based on human regulators so their use is limited in rat models of disease by their immunogenicity. The testing of sCR1 in rat models has been limited to short term therapy due to the generation of neutralising antibodies (Piddlesden, Jiang et al. 1996). To date the CReg-Fc proteins generated in our laboratory contain a human Fc domain so their use is limited to acute studies in the rat. As rat models of chronic diseases offer a way of testing the potential of anti-C therapeutics, particularly CReg-Fc fusion proteins, in chronic diseases efficient rat anti-C therapeutics are needed.

In this study various recombinant forms of rat Crry were generated in order to identify two different Crry-Fc fusion proteins both with long circulating half-lives but different functional characteristics. One would be fully active as a fusion protein so could enable the testing of rat CReg-Fc in models of chronic disease. The other would be functionally inactive as a fusion protein but could be further engineered into a 'prodrug' to overcome the problems of systemic C inhibition and enable this novel anti-C therapeutic to be tested in vivo.

7.2 Effective C inhibition by Crry-Fc

Recombinant forms of rat Crry were generated by amplifying and ligating DNA encoding the 5, 4 or 3 N-terminal SCRs into an appropriate vector. To create the Fc fusion proteins the Crry-encoding DNA was ligated in frame with DNA encoding the Fc of rat IgG2a. Transfection of CHO cells with the generated vectors resulted in production of five different proteins: 5SCR-Fc, 4SCR-Fc, 3SCR-Fc, 4SCR and 3SCR. These proteins were purified from cell culture supernatant using a monoclonal anti-Crry affinity column.

To identify the two different Crry-Fc required for this project the function of the purified proteins was compared. All the proteins inhibited CP-mediated lysis with the 3SCR-Fc protein being the least effective comparison with 3SCR and 4SCR revealed that this was due to steric hindrance imparted by the Fc on the 3SCRs

within this protein. Both 3SCR and 3SCR-Fc had reduced abilities to inhibit AP-mediated lysis due to the removal of an active site in SCR4. The reduced ability of the 3SCR reagents to inhibit the AP was also seen using surface plasmon resonance to compare the ability of the proteins to decay the AP C3 convertase. Steady state affinity analysis revealed that the reduced ability of the 3SCR reagents to inhibit C was partly due to a drastically reduced affinity for C3b. A decrease in binding to Bb was not investigated in this project and cannot be ruled out. Further work could be carried out looking at the affinity of the recombinant forms of Crry for Bb as well as for CP components such as C4b and C2a. All the proteins had cofactor activity, although this function was reduced in the 3SCR proteins. The functional analysis revealed that it was not possible to generate a Crry 'prodrug' as active domains in Crry were deleted before steric hindrance sufficient for the 'prodrug' was achieved. 4SCR-Fc was fully active as a fusion protein and was therefore the focus of the project as an active Crry-Fc reagent.

In addition to identifying two categories of Crry-Fc fusion protein, the functional characterisation shed additional light on the function of the SCRs within Crry. Previous knowledge of the function of rat Crry came from observation of the mouse homologue, mouse Crry, and the related human protein, CR1, as well as the use of recombinant forms of rat Crry. The five N-terminal SCRs of rat Crry have been expressed recombinantly and were shown to have the ability to regulate both the CP and AP (He, Alexander et al. 1997). A later study where the four N-terminal SCRs were expressed recombinantly revealed that CP regulation and cofactor activity lay within these four SCRs (Fraser, Harris et al. 2002). The functional characterisation carried out in this project revealed that the ability to regulate the CP lay within the SCRs1-3. Steric hindrance of the 3SCR-Fc reagent suggested the site or sites required for function lay close to the Fc. Removal of SCR4 decreased the ability of Crry to regulate the AP although removal of this SCR did not entirely remove this function. In particular removal of this SCR reduced the ability of Crry to accelerate the decay of the AP convertase. Removal of the SCR4 also reduced cofactor activity and the affinity for C3b. Through removal of SCR4 and a reduced binding of Crry to C3b, Crry had a weaker interaction with the convertase and is therefore less efficient at decaying the convertase. The reduction of Crry binding to C3b also reduced the ability of fI to cleave C3b. Figure 7.1 summarises the functional data. The function

of the related human protein, CR1, has been located to SCRs 1-3, 8-10 and 15-17 (Klickstein, Bartow et al. 1988; Krych, Clemenza et al. 1994; Krych-Goldberg, Hauhart et al. 1999). These SCRs in CR1 are homologous to SCRs 1-3 in rat Crry, indeed a study which carried out phylogenetic analysis of SCRs and classified them into 11 sub-families classified the first four SCRS of CR1 and Crry into the same sub-families (McLure, Dawkins et al. 2004). This homology and conservation between different regulators in different species suggests that these SCRs may be crucial for function. Crry also shows similarities to DAF as the AP regulation carried out by DAF has been mapped to the SCRs1-4 while the SCRs1-3 are sufficient to regulate the CP (Coyne, Hall et al. 1992).

The attachment of the Fc domain to the 4SCRs of rat Crry significantly increased the half-life of the reagent. The 4SCR-Fc reagent had a β-phase half-life of 53 hours compared to 7 minutes for the 4SCR reagent. This approach has been used to increase the in vivo half-life of a number of therapeutics, not solely anti-C therapeutics, including CTLA-4 and a TNF receptor (Linsley, Wallace et al. 1992; Pugsley 2001). Within the field of anti-C therapeutics a mouse Crry-Fc fusion protein and a rat DAF- human Fc fusion protein have been made (Quigg, Kozono et al. 1998; Harris, Williams et al. 2002). Both these reagents also had long β-phase half-lives of over 30 hours. Generation of Fc fusion proteins is not the only approach used to increase the half-life of anti-C therapeutics, discussed previously. The generation of Fc fusion proteins has the advantage over other methods to extend circulating half-lives as a native molecule is used to increase the half-life reducing the potential immunogenicity of the therapeutic.

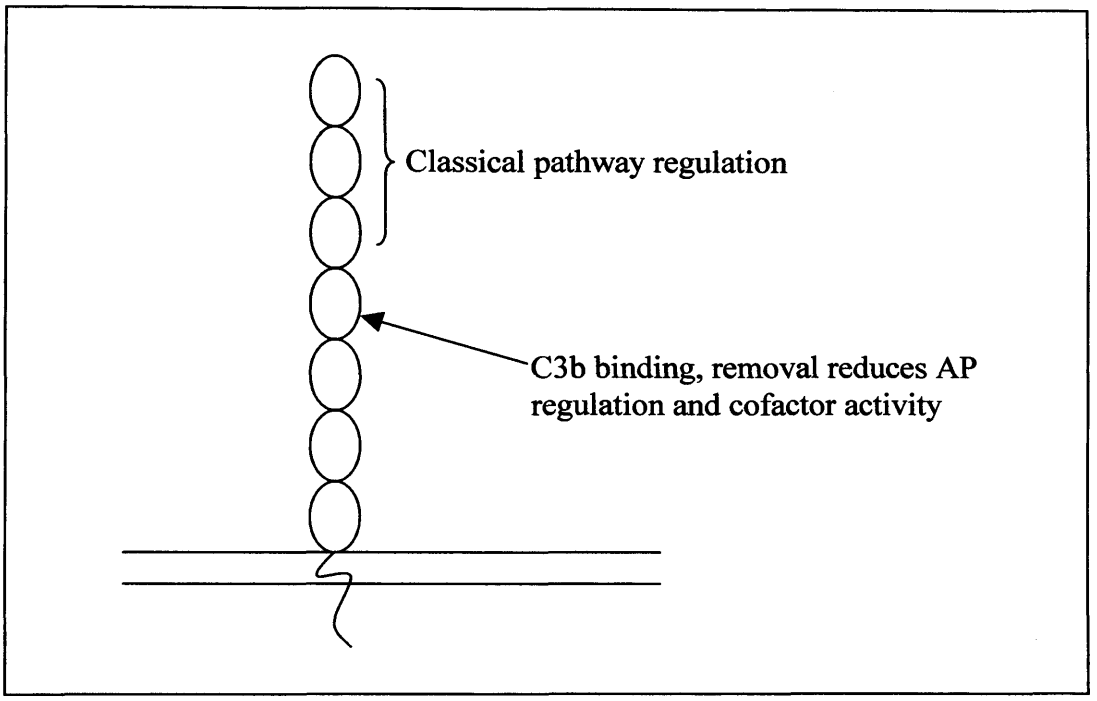


Figure 7.1 Functional domains of rat Crry

Diagram summarising the functional characterisation carried out within this project. CP regulation lies within the three N-terminal SCRs while SCR4 binds C3b and is involved in the regulation of the AP, cofactor activity.

I have shown that a Crry-Fc reagent did cause the generation of an immune response in vivo as detected by antibody generation. The antibodies were to the entire protein and not just to the join in between the Crry and Fc domains. Further investigation of the antibody response revealed that the antibodies did not enhance the clearance of the protein or inhibit its function. The CHO cells, used to produce the proteins, carrying out different post-translational modifications compared to native proteins may have caused the immune response. The immune response may be reduced or prevented altogether by using an alternative cell line, such as a rat or mouse cell line. Immune responses to other anti-C therapeutics have been noted, in particular, antibodies against sCR1 have been produced in human trials although similarly these antibodies did not result in clearance of the protein or inhibition of function (personal communication, Prof. B P Morgan). Non-neutralising antibodies have also been detected against other therapeutic Fc fusion proteins. In clinical trials of etanercept, a TNF receptor Fc fusion protein, 2% of patients produced antibodies against the therapeutic although these were not neutralising and did not effect therapeutic efficacy (Anderson 2005).

In a rat model of EAMG both 4SCR-Fc and 4SCR reduced the clinical severity of the disease. 4SCR-Fc prevented any clinical signs developing, animals did not lose weight and C deposition and inflammatory infiltrate within the muscle was reduced. The short-lived 4SCR reagent inhibited C sufficiently during the initial disease induction phase such that the clinical severity and weight loss were reduced. Soluble CR1 has also been shown to reduce muscle weakness and weight loss in EAMG (Piddlesden, Jiang et al. 1996).

7.3 Future work

The aim of this project was to generate two different Crry-Fc. One that would be fully active and could be tested in models on chronic disease and the other would be further engineered to form a 'prodrug'. The functional analyses revealed that it was not possible to generate the 'prodrug' as active sites within Crry were deleted before sufficient steric hindrance was achieved. The flexible nature of the SCRs within Crry meant that it was difficult to sterically hinder its function. Perhaps a better candidate for a 'prodrug' is CD59. CD59 is a small regulator, 20-28 kDa, with a large multimolecular substrate, the MAC, suggesting that steric hindrance would be

easier to achieve. A rat CD59- human Fc fusion protein has been made and shown to have drastically decreased function compared to the non-Fc fusion protein (Harris, Williams et al. 2002). As CD59 only inhibits the terminal pathway, a CD59-Fc 'prodrug' also has the advantage of still allowing C to opsonise pathogens and immune complexes with C3b and thereby further limiting systemic C inhibition. Disruption of the terminal pathway of C has been shown to be effective against disease in studies using animals deficient in certain terminal pathway components. For instance, C5 deficient mice are protected from EAMG and C6 deficiency protects against glomerulonephritis and graft rejection (Christadoss 1988; Brauer, Baldwin et al. 1995; Brandt, Pippin et al. 1996). Inhibition of the terminal pathway with anti-C5 monoclonal antibodies protects against rheumatoid arthritis and aids recovery following myocardial infarction (Wang, Rollins et al. 1995; Vakeva, Agah et al. 1998). Finally, administration of CD59 has proved successful in a model of rheumatoid arthritis where membrane targeted CD59 decreased disease severity and in experimental nephritic syndrome antigen-targeting of CD59 to membranes proved protective (Fraser, Harris et al. 2003; He, Imai et al. 2005).

The fully active Crry-Fc reagent was made and tested in EAMG. The reagent was effective at protecting rats from this disease and 4SCR-Fc now needs to be tested in a chronic model of disease such as collagen-induced arthritis. Mice transgenic for Crry were protected from disease as were mice treated with an anti-C5 monoclonal antibody (Wang, Rollins et al. 1995; Banda, Kraus et al. 2003). Soluble CR1 was also protective against collagen-induced arthritis although the treatment was temporally limited by the immunogenicity of the reagent (Goodfellow, Williams et al. 2000). The use of Crry-Fc reagent in collagen-induced arthritis will address whether the reagent is effective in a chronic model of disease.

Longer term, human analogues of this reagent have to be considered. Crry is such a potent regulator of C as it possesses both decay acceleration activity and cofactor activity but it is not present in humans. The only human regulator to possess both decay accelerating and cofactor activities is CR1. The attachment of functional SCRs from CR1 to a human Fc may result in a human analogue of equivalent function. Another alternative is the generation of a DAF-MCP hybrid Fc fusion protein. Previously sDAF and sMCP have been produced but their function is

reduced compared to sCR1 (Christiansen, Milland et al. 1996). Generation of a hybrid DAF-MCP molecule with the reagents arranged C-terminus to C-terminus resulted in an increase of function as both decay acceleration and cofactor activity were present (Higgins, Ko et al. 1997). Previously a hybrid Fc fusion protein has been created with DAF on one arm and CD59 on the other, such technology could be employed to generate an Fc fusion protein containing DAF on one arm and MCP on the other as a human analogue of Crry-Fc (Harris, Williams et al. 2002).

7.4 Final conclusion

The role of C in a number of inflammatory diseases has been well established. As a result C has become a therapeutic target. A number of different anti-C therapeutics have been tested in in vivo models as well as in clinical trials. Amongst these approaches are small peptides, however, these are limited by their broad spectrum activities and short in vivo half-lives. While the problems of their broad spectrum activities can be overcome by further engineering to generate peptides with specific activities, their short circulating half-lives limit their use to acute conditions such as I/R injuries, haemodialysis and cardiopulmonary bypass. The most promising way of treating chronic conditions is with therapeutic antibodies, such as the anti-C5 monoclonal antibody, or with soluble recombinant forms of the membrane regulators. Recent disappointing results in the treatment of myocardial infarction with the anti-C5 antibody have raised questions about its potential therapeutic efficacy in chronic conditions (Granger, Mahaffey et al. 2003; Mahaffey, Granger et al. 2003; Shernan, Fitch et al. 2004). The approach used in this study to engineering soluble recombinant membrane regulators to contain Fc domains or targeting domains offers the most promising method for treating chronic conditions. Here, one such reagent has demonstrated therapeutic efficacy in an acute disease model. Further studies to investigate the efficacy of such reagents in chronic disease models, as well as identifying the ideal regulator and targeting strategy will enable such therapeutics to reach the clinic.

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