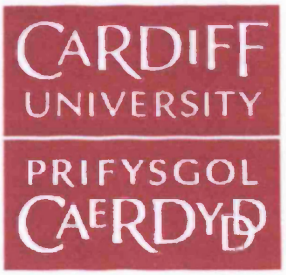

Characterisation of the Human γ-Secretase Complex Using an *E. coli* System

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PhD Thesis

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ABSTRACT

Alzheimer's disease (AD) is a form of progressive dementia which affects many of the world's population. AD patients show a decrease in cognitive function, loss of memory and at a later stage, decreasing physical activity. So far, the only definite diagnosis of AD is still based on post-mortem demonstration of extensive cell loss, and the presence of amyloid plaques and neurofibrillary tangles in the brains of sufferers. Cleavage of the amyloid precursor protein (APP) by β-secretase results in the production of the C-terminal fragment (CTF) of APP, C99, which is in turn cleaved by the γ-secretase. This cleavage event produces the Aβ peptides, which aggregate to form the amyloid deposits seen in the brains of AD patients. γ-Secretase has eluded identification for many years, however it has been shown in various eukaryotic systems that it is a highly stable protein complex of high relative molecular mass consisting of the membrane proteins PS-1, Nicastrin (NicA), Pen-2 and Aph-1.

This thesis shows that *E. coli* represents a simple and efficient system that can be used to analyse human membrane proteins, and in this case to verify that PS-1, NicA, Pen-2 and Aph-1 are the minimal components required for γ-secretase activity. Components of the γ-secretase complex were successfully cloned and expressed in *E. coli* and their topologies in the bacterial membrane were studied *in vivo* by a genetic approach which involves generating hybrids of the target membrane proteins to alkaline phosphatase (PhoA). In addition, initial experiments carried out with the aim of reconstituting γ-secretase activity in *E. coli* are described. Furthermore, the *E. coli* two hybrid system was successfully utilised to unveil interactions between the γ-secretase components and to isolate candidate interaction partners of PS-1 and its gain of function mutant PS-1Δexon9. Thus *E. coli* can be used as a model system to aid our understanding of the processes involved in AD.

ABBREVIATIONS

Aβ	Amyloid-β-peptide
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease
Amp	Ampicillin
ApoE	Apolipoprotein E allele
APP	Amyloid precursor protein
APS	Ammonium persulphate
Ara	Arabinose
A-tet	A-tetracycline
BACE	β-site APP cleaving enzyme
bla	β-Lactamase
BCIP/XP	5-bromo-4-chloro-3-indolylphosphate
BSA	Bovine Serum Albumin
C83	83 residue C-terminal fragment of APP
C99	99 residue C-terminal fragment of APP
C. elegans	*Caenorhabditis elegans*
Cm	Cholamphenicol
D. melanogaster	*Drosophila melanogaster*
DMF	Dimethylformamide
E. coli	*Escherichia coli*
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic reticulum
EtOH	Ethanol
FAD	Familial Alzheimer's disease

F.C.	Final Concentration
His-tag	6X histidine tag
IPTG	Isopropyl-β-D-thiogalactoside
kb	Kilo basepair
kDa	Kilo dalton
LB	*Luria Bertani* medium
NBT	4-nitroblue-tetrazoliumchloride
NicA	Nicastrin
NZA	NZ Amine A medium
OD_X	Optical Density at wavelength X
PAGE	Polyacrylamide Gel Electrophoresis
PhoA/AP	Alkaline Phosphatase
PKC	Protein kinase C
pNPP	p-nitrophenyl phosphate
PS	Presenilin
RIP	Intramembraneous proteolysis
Rpm	Revolutions per minute
Psi	Pound force per square inch
PS	Presenilin
RT	Room temperature
sAPP	Soluble N-terminal APP fragment
S. cerevisiae	*Saccharomyces Cerevisiae*
SDS	Sodium dodecyl sulphate
TACE	TNF-α converting enzyme
TBS-T	Tris Buffered Saline with Tween-20

TCA	Trichloroacetic acid
Tet	Tetracycline
TM	Transmembrane
TNF-α	Tumour necrosis factor α
wt	Wildtype
w/v	Weight per volume

CHAPTER (1)

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

1.1 Alzheimer's disease

1.1.1 Introduction

Alzheimer's disease (AD) is the most widespread form of progressive dementia worldwide as it affects about 17-25 million people. In western countries AD is regarded as the fourth leading cause of death after heart diseases, cancer and stroke. The incidence of AD increases with age, and it doubles every five years beyond the age of 65. However, diagnosis of AD is limited to the exclusion of other diseases and the only definite diagnosis to date is still based on post mortem histopathological demonstration of amyloid plaques and neurofibrillary tangles (Figure 1.1) (Czech et al., 2000).

AD is a brain disease which especially affects the temporal and parietal cortex, hippocampus, and amygdala. AD patients suffer from loss of memory, deterioration in cognitive function, and eventually decreasing physical abilities (Czech et al., 2000). The neuropathology of AD is defined by extensive neuronal cell loss and by the presence of neurofibrillary tangles and amyloid plaques in the brains of sufferers. Neurofibrillary tangles are intracellular protein aggregates in the form of paired helical filaments, mostly consisting of the microtubule associated protein tau (Goedert *et al.*, 1992). Amyloid plaques are extracellular protein aggregates composed in the majority of the β-sheet structure, amyloid β-peptide (Aβ) (Glenner and Wong, 1984; Masters et al., 1985b). Aβ is deposited in the brain parenchyma and in the majority of AD cases, in the wall of cerebral blood vessels (Masters et al., 1985a).

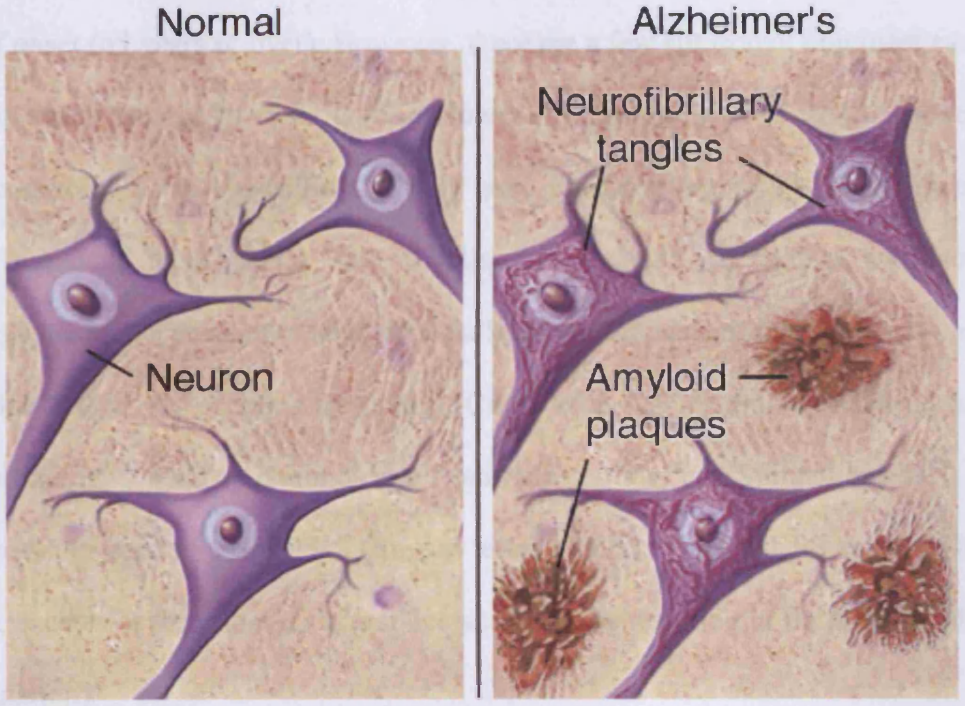


Figure (1.1) Diagramatic representation comparing neural tissue from a healthy individual, to that of one suffering from AD.

Neurofibrillary tangles are shown as intracellular protein aggregates in the form of helical filaments and amyloid plaques are extracellular protein aggregates. Taken from: http://www.ahaf.org/alzdis/about/plaques_tangles.jpg

1.1.2 The genetics of AD

There are two types of AD. Most cases of AD are sporadic with no obvious inheritance pattern and late age of onset (65 years or over). However, there are a few autosomal dominant cases of familial AD (FAD) where the age of onset is much lower (St George-Hyslop, 1998). The major cause of FAD is mutations in the presenilin-1 (PS-1) gene on chromosome 14. There are more than 50 missense mutations identified to date, localised throughout the protein with hot spots more predisposed for pathogenic mutations, especially in the transmembrane segments and the region from residue 260 to 290 encoded by exon 8 (Czech et al., 2000). Some mutations have also been localised to the PS-2 gene on chromosome 1, and the majority of PS mutations have been found to occur in residues conserved in PS-1 (Rogaev et al., 1995). Thus, all PS mutations are missence mutations, except for the mutation of a splice acceptor site resulting in the deletion of exon 9 (delta exon 9 mutation).

Down's syndrome patients often develop AD by the age of 50. The APP gene was mapped to chromosome 21 (Goate et al., 1991) and data that shows increased expression of APP in the brains of Down's syndrome patients is available (Czech et al., 2000). This led to the discovery of several rare missense mutations within the APP gene which are located within or close to the Aβ sequence. Examples are the 'Dutch' mutation (E693Q) which causes a structural change in Aβ, thus accelerating the rate of fibril formation (Levy et al., 1990; Wisniewski et al., 1991), the 'Flemish' mutation (A692G) which alters γ-secretase activity resulting in an increase of Aβ secretion (Haass et al., 1994) and the 'Swedish' mutation (KM670-671NL) which promotes APP cleavage by β-secretase (Vassar et al., 1999) also resulting in higher levels of Aβ produced (See section 1.3.2).

The apolipoprotein E4 allele (*apoE4*) was found to considerably increase susceptibility for sporadic and familial AD (Strittmatter et al., 1993). Apolipoprotein is a plasma membrane protein involved

in cholesterol transport (Mahley, 1988). It is produced and secreted in the central nervous system by astrocytes (Boyles et al., 1985; Ignatius et al., 1986; Pitas et al., 1987). Its synthesis is increased following injury and it is implicated in the growth and repair of the nervous system during development or after injury (Boyles et al., 1989; Ignatius et al., 1986).

The risk of AD increases with the dose of the ApoE4 allele, but the ApoE2 allele appears to have protective properties (Corder et al., 1994; Corder et al., 1993). The ApoE genotype influences the onset of AD in patients with Down-syndrome and in those with APP mutations but not in families with presenilin mutations (Bales et al., 1997; Lendon et al., 1997). ApoE is thought to have a role in the aggregation and/or clearance of Aβ in the brain (Bales et al., 1997).

1.2 The amyloid precursor protein (APP)

APP is a type I transmembrane glycoprotein, proteolytic cleavage of which, produces the amyloid peptide (Aβ), which forms the major component of the extracellular amyloid deposits in AD (Kang et al., 1987). The Aβ region of APP consists of 40-42 amino acid residues, located partly within the ectodomain and partly within the transmembrane domain of APP (Figure 1.2) (Kang et al., 1987). Proteolytic cleavage of APP is carried out by α-, β- and γ-secretases.

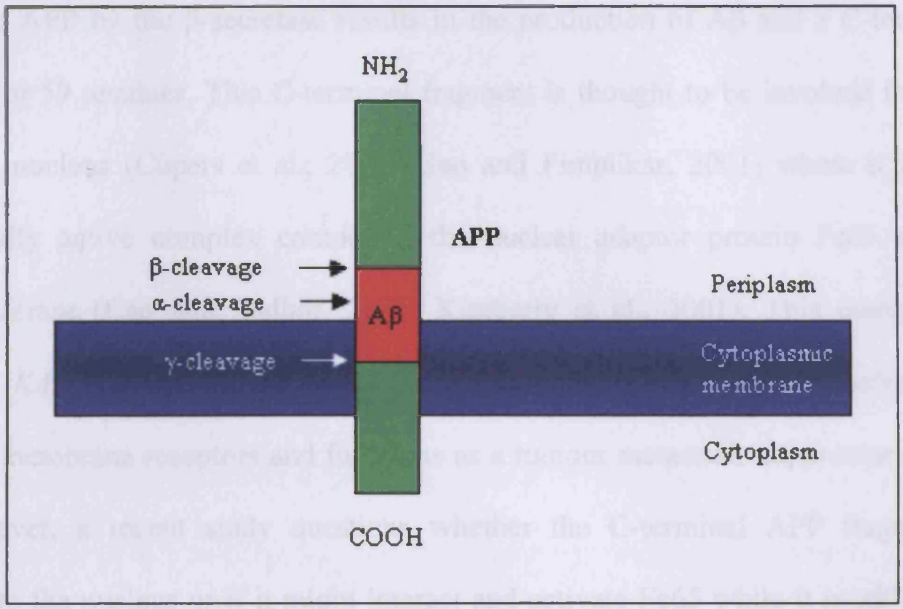


Figure (1.2) Schematic representation of APP.

The figure shows the APP membrane protein, including the position of Aβ sequence within the protein and the α-, β- and γ-secretase cleavage sites.

APP comprises a group of polypeptides whose heterogeneity arises from both alternative splicing and posttranslational processing, including the addition of N- and O- linked sugars, phosphate and sulphate to the protein. 751- and 770-amino acid splice forms of APP are expressed in non-neuronal cells, however neurons express a more abundant 695-residue isoform. The difference between the 751/770- and 695-residue forms is the presence in the former of a 56 amino acid exon that encodes a region homologous to the Kunitz-type serine protease inhibitor motif, suggesting one of the functions of the longer APP isoforms. Certainly, 751/770 forms of APP present in human platelets inhibit the factor XIa serine protease in the coagulation cascade (Smith et al., 1990). APP is expressed in cells and tissues throughout the body, which raises the question of why substantial, clinically important Aβ deposition occurs only in the brain. The APP sequence has been highly conserved during evolution in mammals and close homologues were found in *D. melanogaster* and *C. elegans*, although those lack the Aβ sequence (Selkoe, 1998).

Processing of APP by the β-secretase results in the production of Aβ and a C-terminal fragment (CTF) of 57 or 59 residues. This C-terminal fragment is thought to be involved in signaling as is found in the nucleus (Cupers et al., 2001; Gao and Pimplikar, 2001) where it interacts with a transcriptionally active complex containing the nuclear adaptor protein Fe65 and the histone deacetyltransferase (Cao and Sudhof, 2001; Kimberly et al., 2001). This complex targets, for example, the *KAI1* promoter (Baek et al., 2002). As *KAI1* is a cell surface molecule, it interacts with plasma membrane receptors and functions as a tumour metastasis suppressor (Maecker et al., 1997). However, a recent study questions whether the C-terminal APP fragment has to be translocated to the nucleus or if it might interact and activate Fe65 while it is still associated with the membrane (Cao and Sudhof, 2004). This highlights the need for more work to fully understand how APP fragments activate transcription.

It was shown that the axonal transport of APP in neurons is mediated by direct binding of APP to the kinesin light chain subunit of kinesin-I, a microtubule motor protein (Kamal et al., 2000). Recent experiments by Kamal and co-workers (Kamal et al., 2001) have indicated that APP functions as a kinesin-I membrane receptor, mediating the axonal transport of γ-secretase and presenilin 1, and that the processing of APP to Aβ by secretases can occur in an axonal membrane compartment transported by kinesin-I.

1.3 The secretases and APP cleavage

1.3.1 The α-secretase pathway

α-Secretase cleaves APP on the C-terminal side of residue 16 of the Aβ sequence, which generates an 83-residue C-terminal fragment, C83 (Esch et al., 1990). Cleavage of APP by α-secretase destroys the Aβ sequence, and therefore it is generally thought that the α-secretase pathway reduces amyloid plaque formation, however this has not been indisputably proven (Nunan and

Small, 2000). Furthermore, the C-terminally truncated form of APP released by α-secretase may have trophic actions which could oppose the neurotoxic effects of aggregated Aβ (Mok et al., 2000).

The precise subcellular localisation of the α-secretase is unknown, but the *trans*-Golgi has been suggested as one of the sites of α-secretase cleavage (Nunan and Small, 2000). An explanation for the uncertainty about the localisation of α-secretase is that there may be more than one enzyme (Nunan and Small, 2000). α-Secretase activity has constitutive and regulated components. The constitutive activity has not yet been identified, but the regulated activity seems to be under the control of protein kinase C (PKC) (Sinha et al., 1999).

Two members of the ADAM (a disintegrin and metalloprotease) family, tumour necrosis factor-α (TNFα)-converting enzyme (TACE or ADAM-17) and ADAM-10, are candidate α-secretases (Nunan and Small, 2000). TACE cleaves pro-TNFα, releasing the extracellular domain (TNFα) in a similar manner to APP cleavage (Nunan and Small, 2000). The inhibition or knockout of TACE decreases the release of the α-secretase cleaved product αAPP (Buxbaum et al., 1998b). However, cells deficient in TACE still have a residual α-secretase activity (Buxbaum et al., 1998) and therefore, TACE may play a role in regulated PKC-dependent α-secretion. Over-expression of ADAM-10 increased α-secretase cleavage of APP, while a dominant negative form of ADAM-10 with a point mutation in the zinc-binding site was found to inhibit basal and stimulated α-secretase activity, but did not totally abolish αAPP production (Lammich et al., 1999). ADAM-10 exists only in a proenzyme (inactive) form in the Golgi, but becomes activated at the plasma membrane (Lammich et al., 1999). Therefore, TACE and ADAM-10 were both thought to be α-secretases.

It is likely that several proteases contribute to α-secretase activity, and therefore it may be difficult to regulate APP processing pharmacologically through this pathway. However, most studies aimed at developing inhibitors of Aβ production are focused on the two enzymes directly responsible for cleaving Aβ from APP, that is β- and γ-secretase (Nunan and Small, 2000).

1.3.2 The β-secretase pathway

BACE (β site APP cleaving enzyme) or Asp2 has been identified as the β-secretase (Hussain et al., 1999; Lin et al., 2000; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). It is an unusual member of the pepsin family of aspartyl proteases, which has an N-terminal catalytic domain containing two important aspartate residues, a transmembrane domain (17 residues) and a short C-terminal cytoplasmic tail (Vassar et al., 1999). BACE contains four potential N-linked glycosylation sites and a pro-peptide sequence at the N-terminus. In the cell, BACE is expressed initially as a pre-proprotein, and then processed to its mature form in the Golgi apparatus (Haniu et al., 2000). BACE possesses many of the features of β-secretase including full-length cleavage of APP at Asp1 of the Aβ sequence and also at Glu11, which is an alternative cleavage site (Vassar et al., 1999). Additionally, the swedish mutation is known to promote cleavage of APP at Asp1 by BACE. Furthermore, BACE is co-expressed with APP in many regions of the brain, particularly in neurons, and has a subcellular distribution similar to that of the observed activity for β-secretase (Vassar et al., 1999).

1.3.3 The γ-secretase pathway

The final step in the production of Aβ is the cleavage of the APP C99 fragment by γ-secretase. The exact position of cleavage by γ-secretase is critical for the development of AD (Nunan and Small, 2000). Production of the more amyloidogenic long Aβ species by cleavage adjacent to the residues

42 or 43 is strongly associated with disease pathogenesis (Scheuner et al., 1996; Small and McLean, 1999).

Before the γ-secretase complex was identified, PS-1 and PS-2 were two candidate γ-secretases (Nunan and Small, 2000). A knockout of both PS-1 and PS-2 completely inhibited all γ-secretase activity (Herreman et al., 2000) which led to the conclusion that presenilins are required for γ-secretase activity. Furthermore, presenilins are localised to subcellular compartments known to be the sites of γ-secretase processing (Hartmann et al., 1997). Subcellular and fractionation experiments have shown that PS and γ-secretase co-purify as a high molecular weight complex (Li et al., 2000a). In addition, γ-secretase inhibitors can affinity-label PS subunits (Esler et al., 2000; Li et al., 2000b). Thus, it was thought that even if the γ-secretase is not identical to the presenilins, then the protease activity is at least intimately associated with presenilins *in vivo* because both presenilins have been reported to bind to APP (Weidemann et al., 1997; Xia et al., 1997).

As a result of inhibitor studies, γ-secretase was thought to be an aspartyl protease (Wolfe et al., 1999a). Wolfe and co-workers (Wolfe et al., 1999b), found that mutation of Asp257 and Asp385 in the transmembrane domain of PS-1 inhibits γ-secretase activity. They suggested that the two Asp residues in PS-1 form part of an active catalytic site. However in contrast to this idea, Capell and co-workers (Capell et al., 2000), found that PS-1 mutants lacking Asp257 secrete significant amounts of Aβ. However, PS was never shown to have protease activity, so it was suggested that it could simply be a regulatory subunit of γ-secretase, or a protein that is somehow involved in the trafficking of proteins targeted to γ-secretase (Nunan and Small, 2000).

Recently, regulated intamembaneous proteolysis (RIP) has been described which is a mechanism very similar to the γ-secretase cleavage of APP. RIP involves the cleavage of transmembrane

proteins within the plane of the membrane to liberate cytosolic fragments which can then enter the nucleus to control gene expression. This mechanism can influence many processes in the cell including differentiation, lipid metabolism and the unfolded protein response (Brown et al., 2000). RIP has been shown to occur in animals and also in bacterial cells, using proteases that are evolutionary related to the ones used in animals (DeBose-Boyd et al., 1999; Haze et al., 1999; Katayama et al., 1999; Mumm et al., 2000; Niwa et al., 1999; Ray et al., 1999).

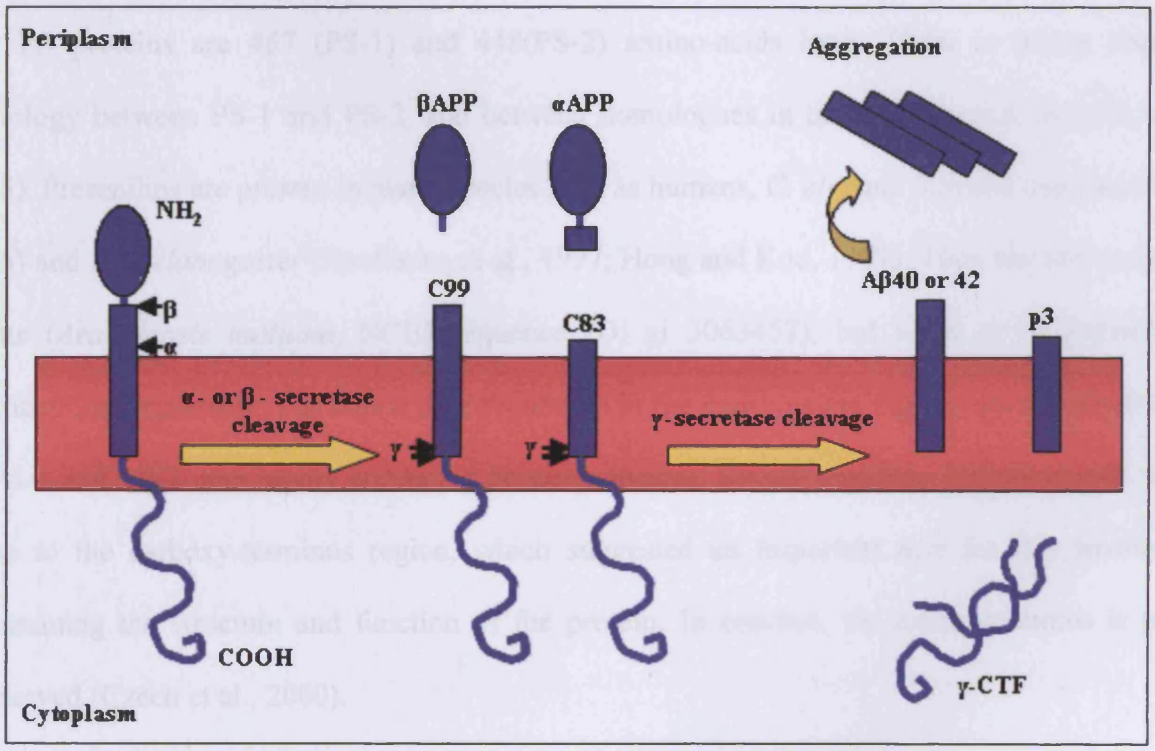


Figure (1.3) APP processing by α-, β- and γ-secretases.

Cleavage by α-secretase (PC7, TACE or ADAM-10) produces αAPP and the C-terminal fragment, C83. Both TACE and ADAM-10 can be activated by protein kinase C (PKC) which is regulated by the muscarinic acetylcholine (Ach) receptor. C83 is cleaved by γ-secretase to produce p3. Cleavage of APP by β-secretase (BACE) produces βAPP and C99. γ-secretase cleaves C99 to release Aβ, which is the major component of the amyloid plaques of AD.

1.4 Presenilin-1 (PS-1) and Presenilin-2 (PS-2)

PS-1 and PS-2 (Figure 1.4) are composed of a hydrophilic amino-terminus followed by eight putative transmembrane domains. Most of these regions are connected by small hydrophilic loops, except one longer stretch of mostly hydrophilic residues between transmembrane 6 and 7, called the 'large loop' (Czech et al., 2000).

1.4.1 Conservation among species

The PS proteins are 467 (PS-1) and 448(PS-2) amino-acids long. There is strong sequence homology between PS-1 and PS-2, and between homologues in different species (Rogaev et al., 1995). Presenilins are present in many species such as humans, *C. elegans* (Levitan and Greenwald, 1995) and *D. melanogaster* (Boulianne et al., 1997; Hong and Koo, 1997). They are also present in plants (*Arabidopsis thaliana*, NCBI sequence ID: gi 3063457), but seem to be restricted to multicellular organisms. The amino acid sequences in the hydrophobic regions are almost identical in PS-1 and PS-2, and highly conserved between species. Strong sequence homology was shown close to the carboxy-terminus region, which suggested an important role for this terminus in maintaining the structure and function of the protein. In contrast, the amino-terminus is poorly conserved (Czech et al., 2000).

1.4.2 Expression and post-translational modifications of the presenilins

The PS-1 and PS-2 genes are encoded by 12 exons giving rise to two major mRNA transcripts (7 and 2.7 kb for PS-1, 2.3 and 2.6 kb for PS-2) (Rogaev et al., 1995; Sherrington et al., 1995), which undergo alternative splicing (Czech et al., 2000). Presenilin transcripts were detected in the brain and most peripheral tissue (Rogaev et al., 1995; Sherringdon et al., 1995). Both PS-1 and PS-2 co-localise in a large number of neuronal cell bodies, and PS-1 is also present in the processes of neurons (Blanchard et al., 1997).

At the subcellular level, presenilins co-localise in the endoplasmic reticulum (ER) and Golgi apparatus (Cook et al., 1996; Culvenor et al., 1997; Kovacs et al., 1996; Takashima et al., 1996). They can be detected in synaptic terminals (Lah et al., 1997) and in somatic ER-Golgi intermediate compartment structures (Czech et al., 2000). They were also shown to be present on the inner nuclear envelope, the associated kinetochores and centrosomes which suggests a role in chromosomal segregation (Li et al., 1997). Finally, presenilins were found in the plasma membrane where they could be involved in cell-cell interaction (Dewji and Singer, 1997).

Presenilins are neither glycosylated nor modified by sulphation, acylation or the addition of glycosaminoglycans (De Strooper et al., 1997), but they are phosphorylated on serine residues (Seeger et al., 1997; Walter et al., 1997). The most important posttranslational modification of presenilins is proteolytic cleavage (Podlisny et al., 1997; Thinakaran et al., 1996). PS-1 is cleaved into a 27-28 kDa amino terminal and an 18-20kDa carboxy-terminal fragment, PS-2 into two polypeptides of 34 kDa and 20 kDa respectively. The presenilin cleavage products remain closely associated in a large non-covalent protein complex of 100-150 kDa (Capell et al., 1998). The cleavage of PS-1 occurs at amino acid position 298 encoded by exon 9 (Podlisny et al., 1997), a region where several PS mutations are clustered. Due to a splicing error, the PS-1Δexon9 mutation results in the deletion of the domain encoded by exon 9 (Perez-Tur et al., 1995) and therefore PS1Δexon9 accumulates as an uncleaved protein (Thinakaran et al., 1996). Since proteolytic processing is highly regulated, this mutation was expected to be responsible for the protein's pathological activity (Steiner et al., 1999). However, it was demonstrated that the pathological function of PS1Δexon9 as well as its reduced biological activity is independent of its lack to undergo proteolytic processing, but rather due to a point mutation (S290) that is the result of the abnormal exon 8/10 splice junction (Steiner et al., 1999).

The presenilins are also processed by the proteases of the Caspase superfamily. Caspases are a conserved family of cytoplasmic cysteinyl aspartate-specific proteases, which are crucial for the physiological pathway to cell death, apoptosis(Cohen, 1997; Porter et al., 1997). According to amino acid sequence homology, caspases can be divided into 3 sub-families (Humke et al., 1998; Porter et al., 1997; Van de Craen et al., 1998; Van de Craen et al., 1997). During induced apoptosis, PS-1 and -2 are cleaved by a protease of the caspase-3 family (Loetscher et al., 1997), and FAD mutations do not seem to alter the sensitivity of PS-1 to caspase mediated cleavage (Van de Craen et al., 1999).

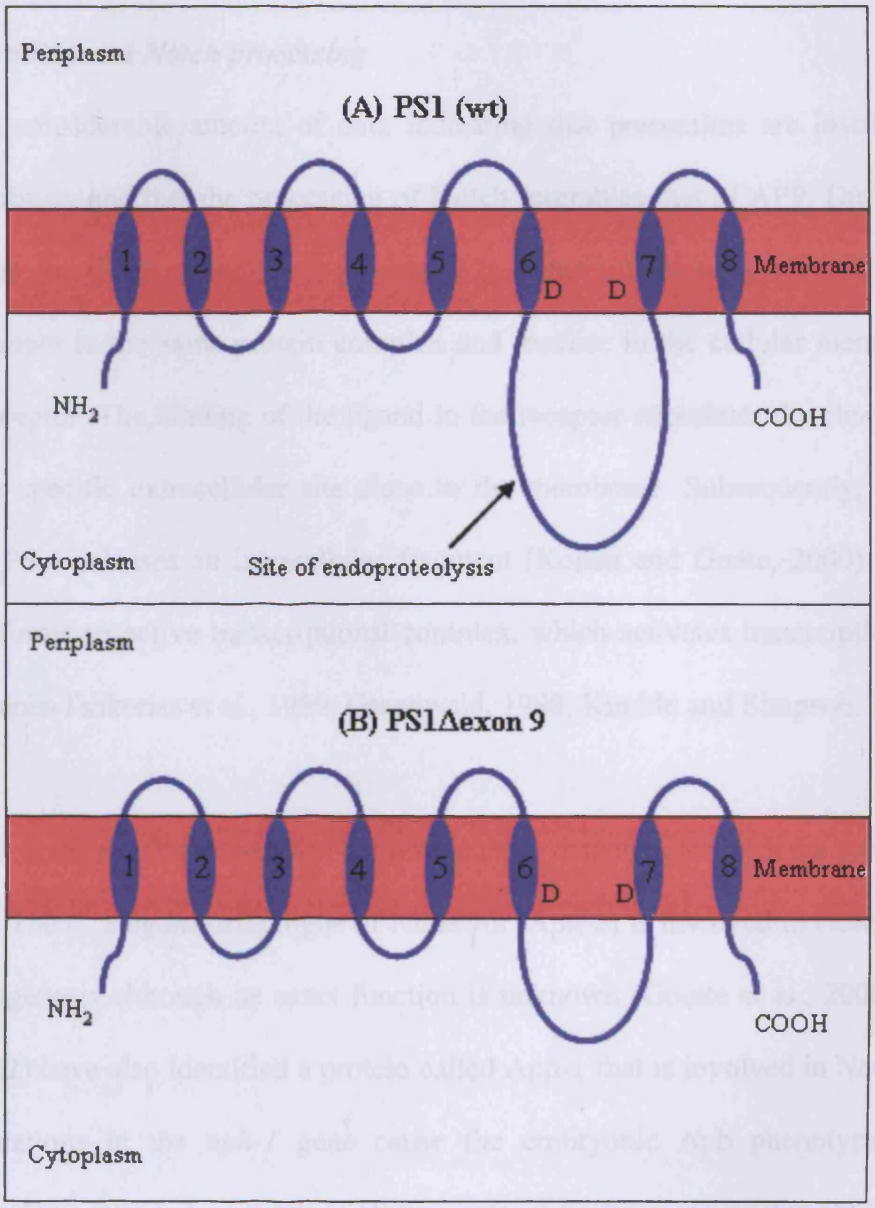


Figure (1.4) Schematic representation of the most established structure of wild type PS-1 and PS-1Δexon9

(A) PS1wt, including the "large loop" which is the known site for proteolytic cleavage of the protein. (B) PS1Δexon9 produced as a result of a splicing error of the mRNA. The mutation deletes exon 9 and results in lack of proteolytic processing and accumulation of the full-length protein.

1.4.3 Functions of the presenilins

1.4.3.1 Presenilins and Notch processing

There was a considerable amount of data indicating that presenilins are involved in the Notch signalling pathway and that the processing of Notch resembles that of APP. During the passage of Notch through the Golgi system, it is processed by a furin-mediated cleavage. The resulting two fragments remain in the same protein complex and localise in the cellular membrane to form the functional receptor. The binding of the ligand to the receptor stimulates the cleavage of one of the subunits at a specific extracellular site close to the membrane. Subsequently, intra-membranous cleavage by PS-1 releases an intracellular fragment (Kopan and Goate, 2000) that travels to the nucleus and forms an active transcriptional complex, which activates transcription of Notch target genes (Artavanis-Tsakonas et al., 1999; Greenwald, 1998; Kimble and Simpson, 1997)

Recently, it was shown that presenilins in humans exist in a complex with the protein Nicastrin (Yu et al., 2000). The *C. elegans* orthologue of Nicastrin (Aph-2) is involved in Notch signaling during early embryogenesis although its exact function is unknown (Goutte et al., 2000). Goutte and co-workers (2002) have also identified a protein called Aph-1 that is involved in Notch signaling in *C. elegans*. Mutations in the *aph-1* gene cause the embryonic Aph phenotype which indicates defective Notch signaling. As a result of all these recent findings, it was thought that presenilins are part of a larger γ-secretase complex which also includes Nicastrin, Aph-1 and possibly Notch (Goutte, 2002).

1.3.4.2 Other interaction partners of the presenilins

Presenilins have been found to interact with a variety of other proteins. A neuronal calcium-binding protein, Calsenilin (Buxbaum et al., 1998a) was found to interact with both PS-1 and PS-2, by using the yeast two-hybrid system. The brain G-protein G_0 was shown to interact with the carboxy-

terminus of PS-1 which hinted towards a role of PS-1 as regulator of G-protein activation (Smine et al., 1998). Several proteins have been shown to interact with the 'large loop' of the presenilins including the cytoskeletal protein filamin and a filamin homologue (Zhang et al., 1998), μ-calpain (Shinozaki et al., 1998) and rab11 a small GTPase belonging to the p21 ras related superfamily, which indicates that presenilins might be involved in vesicular routing (Dumanchin et al., 1999). Presenilins were also shown to bind to members of the catenin protein family (Levesque et al., 1999; Murayama et al., 1998; Tesco et al., 1998; Zhou et al., 1997). The catenins form a gene family related to the Drosophila Armadillo gene and are characterised by a series of imperfect repeats of an amino acid motif (Peifer et al., 1994) These repeats in β- and Δ-catenin interact with the large loop of PS-1 (Levesque et al., 1999; Murayama et al., 1998). Catenins interact with cadherin adhesion molecules and they are thought to be essential for the wingless/wnt signaling cascade which directs many vital developmental decisions in *Drosophila* and vertebrates (Czech et al., 2000).

1.5 The γ-secretase complex

γ-Secretase has eluded identification for many years though evidence suggested that presenilin (PS) is the active site of the protease (Wolfe and Selkoe, 2002). Presenilin is cleaved into N- and C-terminal fragments (NTF and CTF) that remain associated, and these heterodimers seem to be the biologically active form of the protein (Thinakaran et al., 1996). However, it appears that PS does not act alone, as the levels of PS heterodimers are tightly regulated by other limiting factors (Thinakaran et al., 1997), and overexpression of PS does not increase γ-secretase activity and produce more product. It seems that PS carries out secretase function by forming a highly stable protein complex of high relative molecular mass with a number of cofactor proteins (Ratovitski et al., 1997; Takasugi et al., 2002). Other components of the γ-secretase complex are now thought to be nicastrin (NicA), Pen-2 and Aph-1 (figure 1.5).

1.5.1 Nicastrin (NicA)

Nicastrin (NicA), a type I transmembrane glycoprotein, was discovered via its association with presenilin (Yu et al., 2000) and it is thought to be one of the putative limiting factors for γ-secretase activity. Deletion of NicA in *C. elegans* and *D. melanogaster* results in a phenotype similar to the deletion of presenilin (Chung and Struhl, 2001; Hu et al., 2002; Levitan et al., 2001; Lopez-Schier and St Johnston, 2002; Yu et al., 2000), and recent evidence has shown that NicA maturation depends on the presence of presenilin (Edbauer et al., 2002; Kimberly et al., 2002; Leem et al., 2002; Tomita et al., 2002) and is directly associated with γ-secretase (Esler et al., 2002; Kimberly et al., 2002). Nevertheless, over-expression of both PS and NicA was found to be insufficient to generate more γ-secretase activity (Kimberley et al., 2002), which suggested that there were other limiting factors.

1.5.2 Pen-2 and Aph-1

Genetic screens designed to modify a PS-deficient phenotype in *C. elegans* have uncovered two novel genes, *aph-1* (Goutte et al., 2002) and *pen-2* (Francis *et al.*, 2002). Aph-1 is a multi-transmembrane protein encoded by a gene whose deletion leads to hypoplasia of the anterior pharynx in *C. elegans* and it was found to be a Notch pathway member possibly involved in PS function (Goutte et al., 2002) ; *aph-1* was also identified as one of the PS enhancer genes (*pen-1*) together with *pen-2* which codes for a double membrane spanning protein (Francis et al., 2002). Genetic analyses demonstrated that these proteins are needed for PS endoproteolysis and γ-secretase activity (Francis et al., 2002), but their biochemical role remained unknown. Kimberley and co-workers (Kimberly et al., 2003) have shown that Aph-1 and Pen-2 directly associate with PS and NicA in the active protein complex. Co-expression of all four proteins leads to marked increases in PS heterodimers, full glycosylation of NicA, and enhanced γ-secretase activity. These

findings suggest that the four membrane proteins comprise the limiting components of γ-secretase and co-assemble to form the active enzyme in mammalian cells.

Takasugi and co-workers (Takasugi et al., 2003) have shown that *D. melanogaster* Aph-1 increases the stability of Drosophila PS holoprotein in the complex. Depletion of Pen-2 by RNA interference (RNAi) prevents endoproteolysis of PS and promotes stabilisation of the holoprotein in both Drosophila and mammalian cells, including primary neurons. Co-expression of Drosophila Pen-2 with Aph-1 and NicA increases the formation of PS fragments as well as γ-secretase activity. Therefore, Aph-1 stabilises PS holoprotein in the complex, whereas Pen-2 is required for PS endoproteolysis and conferring γ-secretase activity to the complex. So, these studies seem to provide strong evidence that γ-secretase activity requires the formation of a complex between PS, NicA, Aph-1 and Pen-2, but it was unclear if the four components are sufficient for γ-secretase activity. In higher eukaryotic cells, which all contain γ-secretase activity this is impossible to prove as these cells may contain additional so-far unknown γ-secretase components. Therefore co-expression of the four known γ-secretase complex components in such cells will not reveal the minimal composition of the biologically active γ-secretase.

To define the minimal set of components required for γ-secretase activity, Edbauer and co-workers (Edbauer et al., 2003) co-expressed PS, NicA, Aph-1 and Pen-2 in the yeast *S. cerevisiae*. No homologues have been identified in yeast and no endogenous γ-secretase activity was detected. Although it is not possible to formally rule out the possibility that yeast may contain endogenous proteins required for this activity, it is considered improbable, given the complete absence of orthologous γ-secretase complex components from the yeast genome. Therefore yeast may not contain other γ-secretase binding proteins or cofactors of similar functional importance as PS,

NicA, Aph-1 and Pen-2. Edbauer and co-workers also found that the biological activity of γ-secretase is reconstituted by the co-expression of those four membrane proteins.

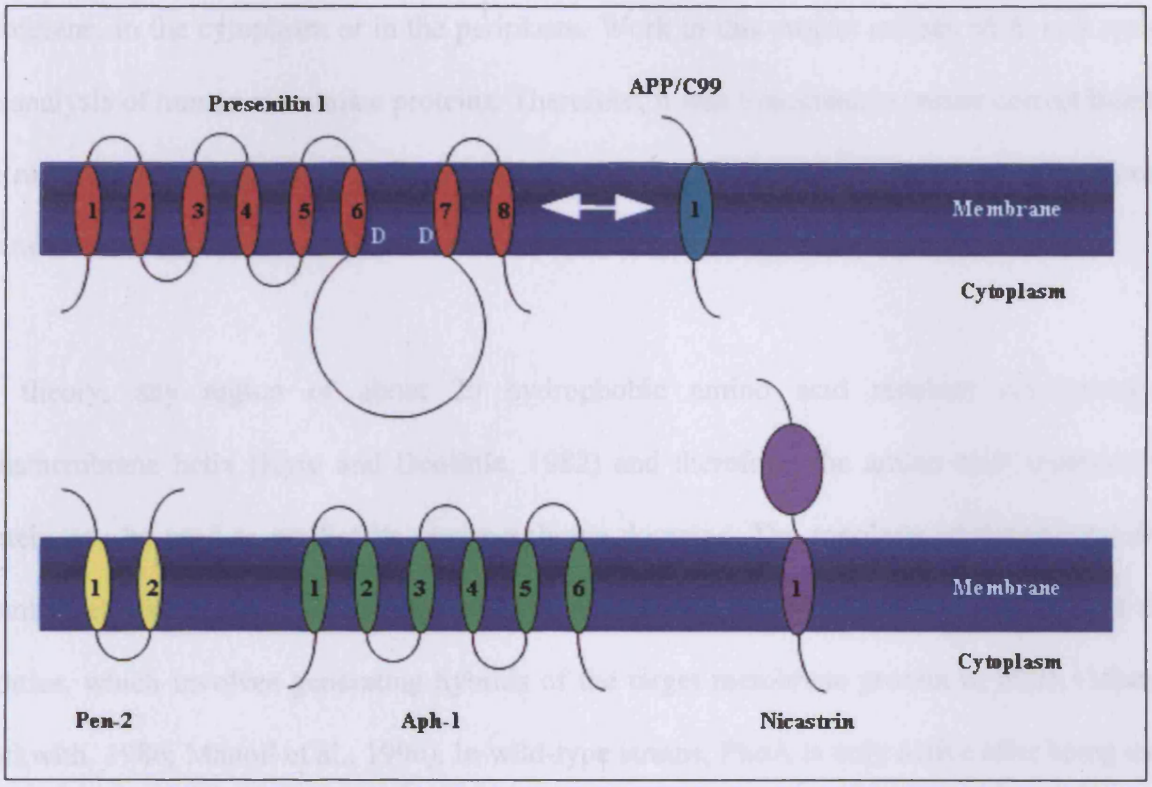


Figure (1.5) A diagramatic representation of the membrane proteins thought to form part of the γ-secretase complex, in addition to the substrate for the protease, C99.

1.6 Topological studies using Alkaline Phosphatase (pho-A) fusions

A fundamental element of the structure of a membrane protein and therefore its function is its transmembrane topology i.e. the location of segments of the polypeptide chain within the membrane, in the cytoplasm or in the periplasm. Work in this project utilises an *E. coli* system for the analysis of human membrane proteins. Therefore, it was important to ensure correct insertion of the recombinant proteins into the bacterial cytoplasmic membrane, so that they can perform their intended function.

In theory, any region of about 20 hydrophobic amino acid residues corresponds to a transmembrane helix (Kyte and Doolittle, 1982) and therefore, the amino acid sequence of any protein can be used to predict its transmembrane domains. The topology of integral cytoplasmic membrane proteins in *E. coli* can be studied *in vivo* by a genetic approach developed in the late eighties, which involves generating hybrids of the target membrane protein to PhoA (Manoil and Beckwith, 1986; Manoil et al., 1990). In wild-type strains, PhoA is only active after being exported to the periplasm where its disulphide bonds can be formed as a result of the oxidising environment in the periplasm (Derman and Beckwith, 1991).

Periplasmic domains of integral membrane proteins can be fused to signal sequenceless PhoA, subsequently the activity of PhoA can be investigated (Manoil and Beckwith, 1986). When PhoA is fused to a cytoplasmic domain it is enzymatically inactive. Therefore, the activity of phoA fusions is used to predict the cellular localisation of the fused membrane protein domain (Boyd et al., 1993b; Ehrmann et al., 1990) (Figure 1.6). In this work, we constructed Pen-2-PhoA and Aph-1-PhoA fusions, to help us identify the topology of human Pen-2 and Aph-1 in the bacterial membrane. Each individual fusion joint was placed at the C-terminus of each hydrophilic region of the membrane protein. Boyd and co-workers introduced this experimental strategy in order to

minimise the disruption of topological signals (Boyd et al., 1993a). Additionally, we describe previous work performed to analyse the topological structure of human PS-1 in *E. coli*.

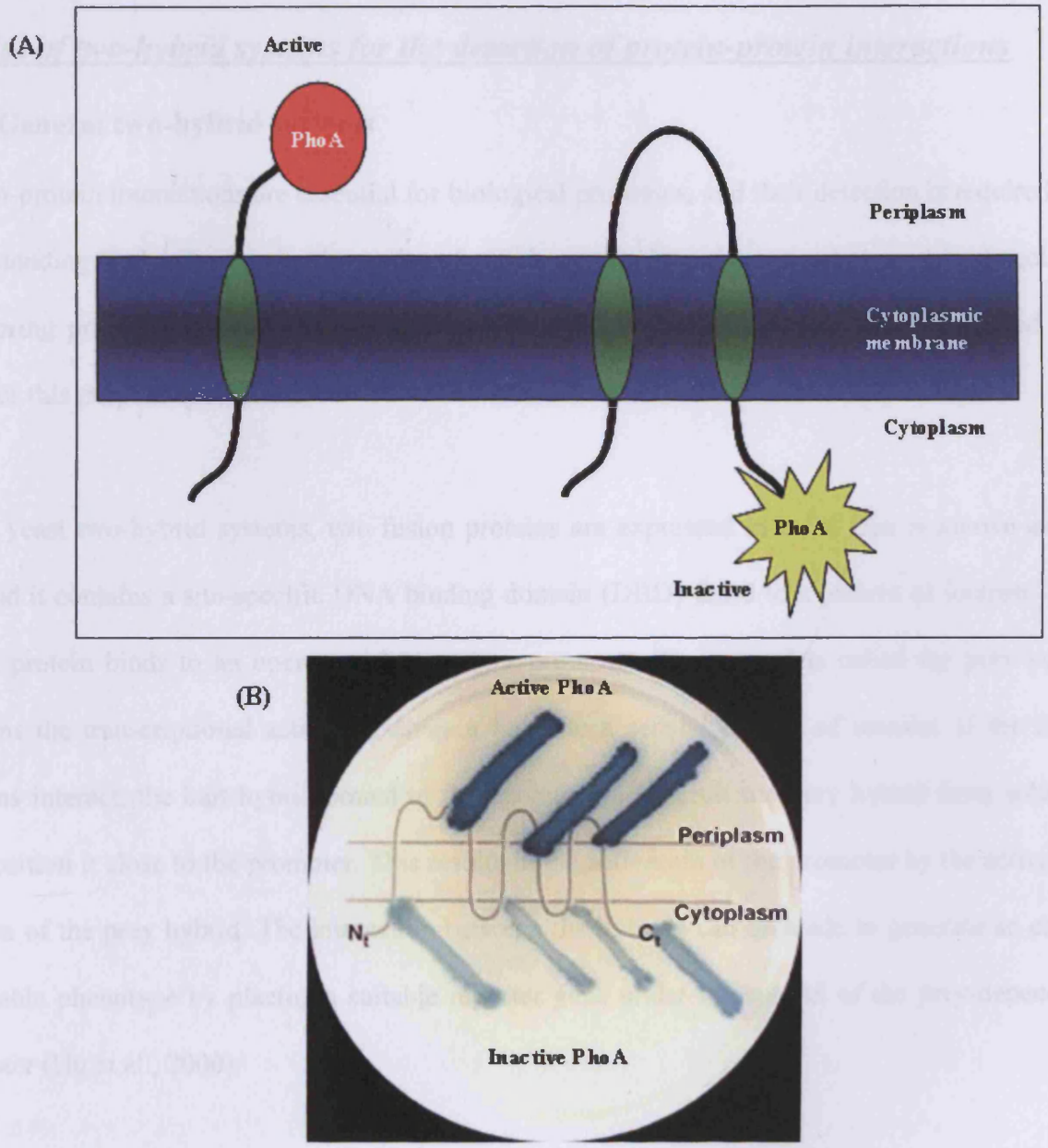


Figure (1.6) A genetic system to study the topology of membrane proteins using *pho-A* fusions.

Bacterial alkaline phosphatase (PhoA) is only active when it is translocated to the periplasm where its disulphide bonds can form as a result of the oxidising environment. (A) The activity of PhoA can be used to predict the position of the fusion joint and therefore the position of the transmembrane domains of the investigated protein. (B) The activity of PhoA can be detected on indicator plates containing Arabinose and the PhoA substrate. When PhoA is in the Periplasm and therefore active, a dark blue colour change is seen. Picture in (B) is taken from: beck2.med.harvard.edu/ Pictures/Dana_Boyd_gram.jpg

1.7 Use of two-hybrid systems for the detection of protein-protein interactions

1.7.1 General two-hybrid systems

Protein-protein interactions are essential for biological processes, and their detection is required for understanding molecular mechanisms. Two hybrid systems have been used as a general tool for monitoring protein-protein interactions, and yeast two-hybrid systems have been developed and used for this purpose successfully.

In the yeast two-hybrid systems, two fusion proteins are expressed in cells. One is known as the bait and it contains a site-specific DNA binding domain (DBD) fused to a protein of interest. This fusion protein binds to an operator adjacent to a promoter. The second is called the prey and it contains the transcriptional activation domain fused to a second protein of interest. If the fused proteins interact, the bait hybrid bound to the operator will recruit the prey hybrid from solution and position it close to the promoter. This results in the activation of the promoter by the activation domain of the prey hybrid. The interaction between the hybrids can be made to generate an easily detectable phenotype by placing a suitable reporter gene under the control of the prey-dependent promoter (Hu et al., 2000).

Designing a two-hybrid method that utilises *E. coli* instead of yeast is valuable for many reasons. One of the advantages of using an *E. coli* two-hybrid system is the speed with which large numbers of interactions can be tested. *E. coli* grows much faster than yeast and can be transformed with higher efficiency, allowing better coverage in library-based screens. In addition, since the cloning steps in yeast often involve passing libraries through *E. coli* hosts, using *E. coli* removes a step from each cycle in a screening program. Furthermore, eukaryotic regulatory proteins such as cell cycle components may be toxic in yeast by interfering with the functions of their yeast homologues. This problem is unlikely to occur in *E. coli* although heterologous proteins may be toxic for other

reasons that are unrelated to function. The smaller genome complexity of *E. coli* and greater evolutionary distance from higher eukaryotes mean that *E. coli* two-hybrid systems should not generate as many false positives and negatives due to interactions between endogenous proteins and eukaryotic bait and prey. Finally, *E. coli* may be a better host for drug screening because its cell envelope seems to be more permeable to small molecules than the cell wall of yeast (Hu et al., 2000).

1.7.2 *E. coli* two-hybrid systems

RNA polymerase (RNAP) in *E. coli* consists of an enzymatic core composed of subunits α, β and β' in the stoichiometry $\alpha_2\beta\beta'$, and one of several σ factors that is necessary for the enzyme to be able to recognise specific promoters (Helmann and Chamberlin, 1988). It has been shown that any protein-protein contact can activate transcription in *E. coli* provided one of the interacting fusion proteins is bound to the DNA via a DBD and the other is bound to an RNAP subunit (Dove et al., 1997).

The generality of this system was tested using two eukaryotic protein domains known to interact specifically (Hu et al., 2000). The dimerisation domain of the yeast transcription factor Gal4 and a part of the yeast $Gal11^P$ protein. Gal11 is a component of the RNAP II holoenzyme and $Gal11^P$ is a mutant form that interacts with the dimerisation domain of Gal4. Gal4 was fused to full-length lambda repressor protein (λcI) at its C-terminus, and $Gal11^P$ was fused to the N-terminal domain of the α-subunit of RNAP. The λcI-Gal4 fusion protein was found to stimulate transcription from the test promoter, but only in the presence of the α- $Gal11^P$ fusion (Hu et al., 2000) (Figure 1.7).

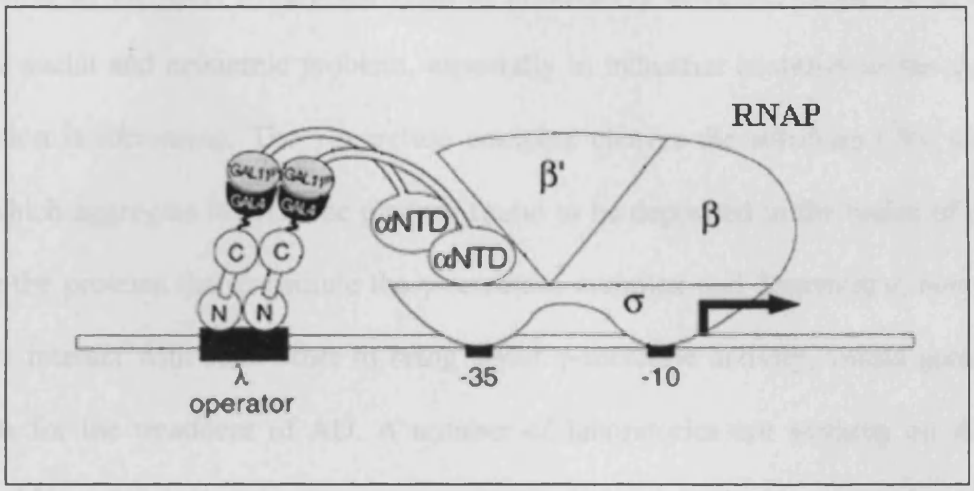


Figure (1.7) Transcription by *E. coli* RNA polymerase (RNAP).

A promoter consisting of a –10 and a –35 element is shown together with the subunit composition of RNAP holoenzyme. The α-Gal11^P fusion protein interacts with the Gal4 dimerisation domain of the λcI-Gal4 fusion protein. The diagram shows the test promoter placO$_R$2-62, which bears the λ operator O$_R$2 centred 62 bp upstream of the transcriptional start site of the lac promoter. Taken from: Dove and Hochschild (1998).

An additional protein, omega (ω), has been called a subunit of RNAP on the basis of its co-purification with RNAP core and holoenzyme (Burgess, 1969). The function of ω is unknown and it was not found to be required for transcription either *in vitro* (Heil and Zillig, 1970) or *in vivo* (Gentry et al., 1991; Gentry and Burgess, 1989), unlike other RNAP subunits. Cells deleted for the gene encoding ω (*rpoZ*) have no observable mutant phenotypes (Gentry and Burgess 1989; Gentry et al., 1991). Dove and Hochschild (Dove and Hochschild, 1998) showed that ω is another one of the RNAP subunits that could be used to generate a two-hybrid system. They fused the Gal11^P fragment to ω and demonstrated that the λcI-Gal4 fusion protein activated transcription in cells containing the ω-Gal11^P fusion protein.

1.8 Aims of the project

AD remains to be the most widespread form of progressive dementia in the world and it is now becoming a social and economic problem, especially in industrial countries as the average age of the population is increasing. The γ-secretase complex cleaves the substrate C99, to produce Aβ peptides, which aggregate to form the plaques found to be deposited in the brains of AD sufferers. Identifying the proteins that constitute the γ-secretase complex and determining how the different components interact with each other to bring about γ-secretase activity, would generate possible drug targets for the treatment of AD. A number of laboratories are working on the γ-secretase complex and have shown that the γ-secretase complex is comprised of Pen-2, Aph-1, NicA and PS-1 (Edbauer, et al., 2003). However, in eukaryotic cells, other so-far unknown γ-secretase components may exist. Therefore, bacteria was thought to be a more suitable organism for the reconstitution of γ-secretase activity. In addition to the lack of endogenous proteins, bacteria have a very simple genome, are easy to cultivate and there are numerous bacterial expression vectors available, which are suitable for recombinant protein expression.

The first aim of the project was to produce the γ-secretase complex components in *E. coli* by cloning the genes encoding them into suitable vectors and subsequently expressing them. Once a good expression level was reached, the second aim was to verify that the components had the correct topology in the bacterial membrane. Finally, the *E. coli* two hybrid system was used to analyse interactions between the different components, and to screen for interaction partners of PS-1 which was thought to be the active site of the complex, and the PS-1 gain of function mutant PS-1Δexon9 which was shown to be involved in FAD.

CHAPTER (2)

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Laboratory chemicals, enzymes and their suppliers

Acrylamide	ICN (Basingstoke, Hampshire, UK)
Ampicillin	AppliChem (Darmstadt, Germany)
Arabinose	Sigma (Poole, Dorset, England)
BCIP	Sigma (Poole, Dorset, England)
Big Dye terminator V3.0 kit	ABI, Applied Biosystems (Warrington, UK)
Chloramphenicol	Sigma (Poole, Dorset, England)
DMSO	AppliChem (Darmstadt, Germany)
DNA standard (γ-DNA BstEII Digested, 1 kb and 100bp)	New England Biolabs (Beverly, MA, USA)
IPTG	Promega (Madison, WI, USA)
NBT	AppliChem (Darmstadt, Germany)
Ni NTA superflow	QIAGEN (Crawley, West Sussex, UK)
Pfu polymerase	Stratagene (Cambridge, UK)
Plasmid preparation kit	QIAGEN (Crawley, West Sussex, UK)
Protein standards	Biorad (Hertfordshire, UK)
Oligonucleotides	QIAGEN (Crawley, West Sussex, UK)
Qiaquick kit	QIAGEN (Crawley, West Sussex, UK)
Restriction enzymes	New England Biolabs (Beverly, MA, USA)
Tetracycline	Fluka (Buchs, Switzerland)
T4 ligase	Promega (Madison, WI, USA)
Vent polymerase	New England Biolabs (Beverly, MA, USA)

Further chemicals not listed above were purchased from Merck (Dorset, England), Sigma (Poole, Dorset, England), Boehringer Mannheim (Lewes, East Sussex, UK), ICN (Basingstoke, Hampshire, UK), Peqlab (Ehrlangen, Germany) or Roche (Lewes, East Sussex, UK).

2.2 Microbiological methods

2.2.1 *E. coli* Strains

Strain	Genotype	Reference
CB4	MC4100 F' OR2-62SD*bla lacZ* with XL1 MRF'Kan	Christian Behrends Diplom Thesis (2002)
DHB4	Δ*malF*3 Δ*phoA* (PvuII) *phoR recA::cat* is *araD*139 Δ*(ara-leu)*7697 Δ*lacX*74 *galE galK thi rpsL* F'*lacI*^{Q} *pro*	Ehrmann Laboratory Collection
KS272	KS272:*F-raD139Δ(ara-leu)7697ΔlacYZI74 galE galK rpsL*	Ehrmann Laboratory Collection
KU98	*degP::kan treA::spec*	Ehrmann Laboratory Collection
MC1000	*F-araD139Δ(ara-leu) 7697ΔlacYZ174 galE galK rpsL*	Ehrmann Laboratory Collection
MC4100	*araD139 deoC1 flbB5301 ptsF25 rbsR relA1 rpsL 150 Δ(argF-lac)U169*	Ehrmann Laboratory Collection
XL1-Blue MRF' Kan	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lacI*^{Q}*ZΔM15* Tn*5* (Kan^{r})]	Stratagene
XL1-Blue MRF' Reporter strain	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 la* [F' *lacI*^{Q} *bla lacZ* (Kan^{r})]	Stratagene

Table (2.1) Description of used *E.coli* strains

2.2.2 Media

2.2.2.1 Sterilisation

Media, solutions and glassware were sterilised by autoclaving (20 min. 121°C, 1 bar). Solutions sensitive to heat such as antibiotics or sugar solutions were filter sterilised by passing through a Millipore filter (diameter 0.2 μm).

2.2.2.2 Liquid and solid media (per 1 l distilled water)

LB *(Luria Bertani)*	10 g Bacto Tryptone
	5 g Bacto Yeast Extract
	7.5 g NaCl
	(5 g Bacto Agar for Top Agar)
NZA (NZ Amine A)	10 g NZA
	5 g Bacto Yeast Extract
	5 g NaCl
	(17 g Bacto Agar for plates)
SOB	20 g Bacto Tryptone
	10 g Bacto Yeast Extract
	5 g NaCl
	2.5 g K_2HPO_4
MacConkey plates	50 g MacConkey lactose agar

M9 10X

76.54 g Na_2HPO_4 x $2H_2O$

30 g KH_2PO_2

5 g NaCl

10 g NH_4Cl

For minimal agar plates, 17 g extra pure agar was used. Subsequently, 100 ml M9 10X were added to the agar, followed by $MgSO_4$ (1 mM F.C.), $CaCl_2$ (0.1 mM F.C.), 1 ml 20% Casamino acids, X-Gal (10 µg/ml F.C.) and the desired antibiotics.

2.2.2.3 Media supplements

Supplement	Stock solution	Final concentration	Storage temperature
Amp	200 mg/ml in sterile H_2O_{MQ}	200 µg/ml	4°C- 8°C
Arabinose	10% in distilled H_2O	0.1%	-20°C
A-tetracycline	1 mg/ml in EtOH	100 ng/ml	-20°C
BCIP/XP	30 mg/ml in DMF	0.03 mg/ml	4°C- 8°C
$CaCl_2$	100 mM in distilled H_2O	0.1 mM	Room temperature
Casamino acids	20% (w/v) in distilled H_2O	0.02%	Room temperature
Cm	30 mg/ml in 100% EtOH.	30 µg/ml	4°C- 8°C
IPTG	1 M in distilled H_2O	100 µg/ml	-20°C
Kan	100 mg/ml in sterile H_2O_{MQ}	100 µg/ml	4°C- 8°C
$MgSO_4$	1 M in distilled H_2O	1 mM	Room temperature
Tet	5-20 mg/ml in 100% EtOH	5-20 µg/ml	-20°C
X-Gal	10 mg/ml in 100% DMF	10-40 µg/ml	-20°C

Table (2.2) Media supplements

2.2.3 Growth conditions and storage of *E.coli*

Liquid cultures (5 ml) were grown in 20 ml glass reaction tubes, in a culture roller drum (about 50 rpm), at 37°C or 28°C. Liquid cultures over 5 ml were grown in Erlenmeyer flasks, with a volume about 4-5 times bigger than the culture volume, at 37°C or 28°C in incubator chests, shaking at 220 rpm. Cultures on agar plates were grown at 37°C or 28°C for 10 to 15 hours. DMSO cultures were prepared for storage of bacterial strains at -80°C, by mixing 1.7 ml of an overnight culture grown in NZA medium with 126 µl DMSO (F.C. 7% DMSO) and quickly freezing them. Stab cultures were used for storage of bacterial strains at room temperature. 1 ml Top-Agar without antibiotics is inoculated with a single colony from an agar plate and incubated for 10 to 15 hours at 37°C or 28°C.

2.2.4 Plate assay system for two-hybrid interactions

A simple plate assay system was developed for the detection of protein-protein interactions. The system involves the use of indicator plates such as MacConkey lactose agar plates and NZA or minimal plates containing 10 µg/ml X-Gal to detect positive two-hybrid interactions. If bait and trait interact, transcription of *lacZ* is elevated up to 50 fold over background and thus a colour change is seen on the indicator plates. On MacConkey plates, background is seen as white/pale red, and a positive interaction is red/dark red. On X-Gal plates, background is white/pale blue and a positive interaction is blue/dark blue.

2.3 Molecular biological methods

2.3.1 Plasmids

Plasmid	Genotype	Antibiotic Resistance	Reference
pCB1	*ps1wt* via *NotI/BglII*	Cm	Christian Behrends, Diplom Thesis (2002)
pCB13	*ps1Δexon9* via *NotI/BglII*	Cm	Christian Behrends, Diplom Thesis (2002)
pCB6	*ps1wt* and C99ω	Cm	Christian Behrends, Diplom Thesis (2002)
pACλcI3.2	Lambda Repressor	Cm	Hochschild *et al.*, 1998
pACLGF2	Lambda Repressor-Gal4	Cm	Hochschild *et al.*, 1998
pBRstar α	α subunit of RNA polymerase	Tet	Hochschild *et al.*, 1998
PBRomega Gal11P	ω-Gal11P	Amp	Hochschild *et al.*, 1998
pBRstar αGal11P	α-Gal11P	Tet	Hochschild *et al.*, 1998
pTRG XR-cDNA	cDNA library	Tet	Stratagene
PRW50/417	Reporter *lacZ* gene	Tet	Hochschild Laboratory Collection
pGDR11	pQE31 derivative $lacI^Q$	Amp	Ehrmann Laboratory Collection
pCS19	pQE-60 derivative $lacI^Q$	Amp	Christoph Spiess, PhD Thesis (1999)
pSA1	*pen2* in pGDR11 via *KpnI/BamHI*	Amp	This Work
pSA2	*aph1* splice variant in pGDR11 via *KpnI/HindIII*	Amp	This Work
pSA3	*pen2* fragment 1 in pEDIE3	Amp	This Work
pSA4	*pen2* fragment 2 in pEDIE3	Amp	This Work
pSA5	*aph1* fragment 1 in pEDIE3	Amp	This Work
pSA6	*aph1* fragment 2 in pEDIE3	Amp	This Work
pSA7	*aph1* fragment 3 in pEDIE3	Amp	This Work
pSA8	*aph1* fragment 4 in pEDIE3	Amp	This Work
pSA9	*aph1* fragment 5 in pEDIE3	Amp	This Work
pSA10	*aph1* fragment 6 in pEDIE3	Amp	This Work
pSA11	*pen2* in pCS19 via *NcoI* and *HindIII*	Amp	This Work

pSA12	*aph1* in pGDR11 via *KpnI/HindIII*	Amp	This Work
pSA13	*aph1* amplified from pSA2 and cloned into pSA1 via *SalI* and *HindIII*	Amp	This Work
pSA14	*aph1* amplified from pSA12 and cloned into pSA1 via *SalI* and *HindIII*	Amp	This work
pSA16	*pen-2* amplified from pSA11 and cloned into pSA14 via *SalI* and *XhoI*	Amp	This work
pSA18	*aph-1*amplified from pSA12 and cloned into pSA11 via *HindIII* and *NheI*	Amp	This work
pSA19	*aph-1* amplified from pcDNA (zeo) Aph1a251 and cloned into pBRω via *SalI* and *NotI*	Amp	This work
pSA20	*aph-1* amplified from pcDNA (zeo) Aph1a251 and cloned into pCB1 via *BglII* and *NotI*	Cm	This work
pSA21	*nicA* amplified from pMH42 and cloned into pBRω via *NdeI* and *NotI*	Amp	This work
pEDIE3	Cloning vector for *phoA* fusions	Amp	Ehrmann Laboratory Collection
pSG33	*ps1*wt and *c99*	Cm	Sandra Grau, PhD Thesis (2004)
pSG34	*ps1Δexon9*	Cm	Sandra Grau, PhD Thesis (2004)
pBRω	ω-subunit of RNAP	Amp	Ehrmann Laboratory Collection
pSG6	*Δssc99* via *NcoI* and *BglII*	Amp	Sandra Grau, PhD Thesis (2004)
pMH1	*ps1* via *NcoI* and *BglII*	Amp	Mona Harnasch, PhD Thesis (2003)
pMH11	*ps1Δexon9* via *NcoI* and *BglII*	Amp	Mona Harnasch, PhD Thesis (2003)
pMH13	*ps1Δexon9* via *BamH1* and *HindIII*	Amp	Mona Harnasch, PhD Thesis (2003)
pMH42	*nicA* via *KpnI* and *SalI*	Amp	Mona Harnasch, PhD Thesis (2003)
pSG36	*nicA* via *BamHI* and *NcoI*	Kan	Sandra Grau, Ehrmann Laboratory
pCDNA (zeo) Aph1a251	Full length Aph1	Amp	Haass Laboratory, Munich

Table (2.3) List of plasmids used

2.3.2 Oligonucleotides

Primer Name	Sequence 5'-3'	Used For
Coding strand of cDNA library	CGTCAGCCTGAAGTGAAAGAAG	cDNA library *E.*coli two-hybrid screen
pen2-5'(*BamHI*)	CGGGATCCGAACCTGGAGCGAGTGTC CAATGAGGAG	pSA1 cloning
pen2-3' (*KpnI*)	GGGGTACCtCAGGGGGTGCCAGGGGTA TGGTGAAGG	pSA1 cloning
AphI/*KpnI*/pGDR11	GGGGTACCTGCTGCGGTGTTTTTCGGC TGCACTTTCGTCG	pSA2 and pSA12 cloning
AphI/*HindIII*/pGDR11	GGTGAAGCTTTCAGTCCAGGTAGTCAG TCCTTACACAAGAGC	pSA2 and pSA12 cloning
pen2-5' (*NcoI*)	CTTCCCATGGCAAACCTGGAGCGAGTG TCCAATGAGGAG	pSA11 cloning
pen2-3' (*HindIII*)	GGTGAAGCTTTCAGGGGGTGCCCAGG GGTATGGTGAAGG	pSA11 cloning
SDpGDR11/*SalI*	GTGGGTCGACAAAGAGGAGAAATTAA CTATGAGAGG	pSA13 and pSA14 cloning
AphI/*HindIII*/pGDR11 noncs	GGTGAAGCTTTCAGTCCAGGTAGTCAG TCCTTACACAAGAGC	pSA13 and pSA14 cloning
universal-fw-*XmaI* (Pen-2)	CCCCCCGGGAACCTGGAGCGAGTGTCC AATGAGG	pSA3 and pSA4 cloning
60-XbaI-rev	GCTCTAGAGAGCGCCAGACATAGCCTT TGATTTGG	pSA3 and pSA4 cloning
101-*XbaI*-rev	GcTCTAGAGGGGTGCCCAGGGGTATGG TGAAGG	pSA3 and pSA4 cloning
universal-fw-*XmaI* (Aph-1)	CCCCCCGGGGCTGCGGTGTTTTTCGGC TGCACTTTCG	pSA5-pSA10 cloning
30-*XbaI*-rev	GCTCTAGAAGCGGGTCCCCAGCCACA GTGATCAAG	pSA5-pSA10 cloning
116-*XbaI*-rev	GCTCTAGATGGCGGATGGAGATGGGT GATCTTCCG	pSA5-pSA10 cloning
154-*XbaI*-rev	GCTCTAGAGGTGAGTCTCCATGGATCC CAACCAC	pSA5-pSA10 cloning
186-*XbaI*-rev	GCTCTAGACGTCTCCTCTCACAGGCAT CAAAGAAC	pSA5-pSA10 cloning
213-*XbaI*-rev	GCTCTAGACTGGCCTCATACCAGGGGT TCAGGAATG	pSA5-pSA10 cloning
251-*XbaI*-rev	GCTCTAGATCCAGGTAGTCAGTCCTTA CACAAGAGC	pSA5-pSA10 cloning
Pen2-3'(*SalI*)	GTGGGTCGACTCAGGGGGTGCCCAGG GGTATGGTGAAGG	pSA16 cloning
(internal *XhoI* site)	GCATTTATCAGGGTTATTGTCTCATGA GCGG	pSA16 cloning
SDAph1*Hind*	GGTGAAGCTTGAGGAGAAATTAACTA	pSA18 cloning

III	TGGGGGCTGCGGTGTTTTTCGGC	
3' *NheI*	CGTGCTAGCTCAGTCCAGGTAGTCAGT CCTTACACAAGAGC	pSA18 cloning
w-AphI/*SalI*	TGGGTCGACTCAGTCCAGGTAGTCAGT CCTTACAC	pSA19 cloning
w-AphI/*NotI*	AGAGCGGCCGCTGCGGTGTTTTTCGGC TGCACTTTCG	pSA19 cloning
cI-AphI/*NotI*	AGAGCGGCCGCAGTGTTTTTCGGCTGC ACTTTCGTCG	pSA20 cloning
cI-AphI/*BglII*	TGAGATCTTCAGTCCAGGTAGTCAGTC CTTACAC	pSA20 cloning
w-NicA/*NdeI*	GGAATTCCATATGGAGGGCAAAAAAT GAAAAAGACAGCTATCG	pSA21 cloning
w-NicA/*NotI*	AGAGCGGCCGCGTATGACACAGCTCCT GGCTCCCG	pSA21 cloning

Table (2.4) List of primers used

2.3.3 Preparation of double stranded plasmid DNA

The plasmid preparation kits from Qiagen were used for double-stranded DNA preparation. The Qiagen Mini/Maxi preparation kits were used for minipreparations from a culture volume of 5 ml and midipreparations from a culture volume of 100-200 ml.

2.3.4 Restriction digestion of double stranded plasmid DNA

Restriction digests were mainly used for genotypic checks of constructed plasmids and for cloning purposes. The enzymes were used in their recommended buffers to achieve an optimal result. A typical reaction was as follows:

X µl DNA (0.5-1 µg for plasmid checks and 8-15 µg for cloning)l

1/10 µl restriction buffer 10x

X µl restriction enzyme (5-10 u/µg DNA)

Made up to 20 µl with H_2O_{MQ} for plasmid checks and 100 µl for cloning.

Incubation time was 1-3 hours, or for enzymes with very low activity O/N at the recommended temperature. If possible, the enzyme was heat-inactivated by incubation for 10 minutes at 65°C or by adding 1/10 volume of agarose gel loading buffer.

2.3.5 Separation of DNA fragments on agarose gels

Plasmid DNA was separated on 0.7-1.7% (w/v) agarose gels in 1X TAE buffer (40 mM Tris, 1 µM EDTA pH 8.0, 0.1% Acetic Acid) with a constant voltage of 100 V. The DNA was then stained in an ethidium bromide bath (1 µg/ml in TAE buffer) thereby the bands became visible in UV light. To identify the size of the DNA fragments they were compared to a number of base-pair standards (λ-DNA *Bst*E II digested, 100 bp ladder and 1 kb ladder, New England Biolabs).

2.3.6 Isolation of DNA fragments from agarose gels

After staining of the DNA fragment with ethidium bromide, it was cut out of the gel with a scalpel. Subsequently the fragment was purified and eluted using a QIAQuick Gel Extraction Kit from Qiagen in accordance with the manufacturer's instructions.

2.3.7 Ligation of compatible ends of vector and insert

After extraction and elution of vector and insert fragments from the agrose gel, they were mixed in a 1:5 and 1:8 ratio (vector: insert) . 1 µl of T4 DNA Ligase (Promega) and 2 µl 10x Ligase buffer were added to each assay and the volume was made up to 20 µl with sterile H_2O_{MQ}. A sample with vector without insert served as a control for the ligation.

For ligation of sticky ends, ligation reactions were incubated in a beaker filled with water at room temperature for a few hours, and then overnight at 4°C in a closed styrofoam box. Subsequently, the

DNA ligase was inactivated by incubation at 65°C for 10 minutes. 1 µl of the ligation reactions was transformed into competent cells by electroporation (described below section 2.3.8.2).

2.3.8 Transformation

2.3.8.1 TSS transformation

This method was used for low-yield transformations. All transformations were performed using this method and all incubations and growing steps were done at the appropriate temperature of 37°C or 28°C, unless stated otherwise.

NZA or LB liquid media (5 ml), containing the appropriate antibiotics, were inoculated with a single *E.coli* colony and grown O/N. The O/N culture was diluted 1/50 in NZA and antibiotics, and grown to an optical density of about 0.5 at 600 nm (OD_{600} = 0.5). The culture at log phase was mixed with ice-cold 2x TSS (20% (w/v) PEG-6000, 10% (v/v) DMSO, 100 mM $MgSO_4$. Dissolved in LB/NZA medium), and plasmid DNA (1-2 µl) was added to 100 µl TSS/culture mixture and incubated on ice for 20-30 minutes. Subsequently, 1 ml NZA was added and a phenotypic expression for 1 hour was performed at the appropriate temperature. The cells were pelleted and resuspended in approximately 200 µl medium and then plated on NZA agar plates with the appropriate antibiotics and incubated O/N.

2.3.8.2 Electrotransformation

This method was used for high yield transformations and was performed according to the BioRad Electroporation Application Guide (Catalogue number: 165-2100).

Preparation of electro-competent cells

An overnight culture was diluted 1/100 in NZA (+/- antibiotics) and was grown at the appropriate temperature to an OD_{600} of 0.5-1.0. The culture was chilled on ice for 15-30 minutes and centrifuged for 10 minutes at 5000 rpm in a GSA rotor. The supernatant was discarded and the pellet was resuspended in 1 volume of ice-cold sterile H_2O_{MQ}. The cells were then sedimented by centrifugation for 10 minutes at 5000 rpm in a GSA rotor and the pellet resuspended in 1/2 volume of ice-cold sterile H_2O_{MQ}. A third centifugation step, analogous to the steps before, was performed and the sedimented cells were resuspended in 1/50 volume of 10% ice-cold sterile glycerol. After sedimenting the cells by centrifugation for 10 minutes at 5000 rpm, the pellets were resuspended in 1/500 volume of 10 % ice-cold glycerol and aliquoted into 40 µl fractions. The aliquots were quickly frozen and stored at –80°C.

Electroporation

Competent cells were thawed on ice. Aliquots (1-3 µl) of the DNA were added to a 40 µl cell aliquot and transferred to a 0.2 cm electroporation cuvette. An electric pulse of 2.5 kV (setting EC2 on the micropulser) was used and the cells were immediately transferred into 1 ml NZA medium and incubated at 37°C or 28°C for 1 hour in an incubator. The cells were pelleted and resuspended in approximately 200 µl medium and were plated on agar plates containing the required antibiotics.

2.3.9 The Polymerase chain reaction (PCR)

PCR was used to amplify specific DNA fragments, for example for use in cloning. A typical reaction is shown below:

DNA (plasmid prep)	2 µl
DNA polymerase	3 u/µl
Polymerase buffer	10 µl 10X
ATP/GTP/CTP/TTP	2.5 mM each
DMSO	10 µl
BSA	1 µl 10X
Primers	2 µl each (0.5 µg/ml)

The reaction volume was made up to 100 µl with sterile H_2O_{MQ}

The reaction was then placed in a Thermo-cycler (Biometra), and the protocol was adjusted to the melting temperature of the primers, the speed of the DNA polymerase used and the length of the DNA fragment to be amplified. A typical cycle is described below:

1) 94°C	3 min
2) 94°C	30 seconds
3) 5°C below Tm of primer	1 min
4) 72°C	1-2 min per kb
5) 72°C	7 min

Steps 2-4 were repeated about 35 times. The reactions were analysed by agarose gel electrophoresis.

2.3.10 DNA sequencing of genes encoding PS-1 and PS-1Δexon9 interacting partners

2.3.10.1 The Sequencing Reaction

Reagent	Volume Required
Big Dye Terminator V3.0 ready reaction mix (ABI)	1 µl
Better Buffer	5 µl
DNA (miniprep)	3 µl
Primer (coding strand)	1 µl
H_2O	7 µl
Total	17 µl

Table (2.5) Reagents used for the sequencing reaction

2.3.10.2 The Polymerase Chain Reaction (PCR) for Sequencing

This PCR was used to amplify DNA fragments for sequencing purposes. The reaction used is described below:

25 cycles

Step 1: Rapid ramp (1°C/sec) to 96°C for 10 sec

Step 2: Rapid ramp (1°C/sec) to 50°C for 5 sec

Step 3: Rapid ramp (1°C/sec) to 60°C for 4 min

Step5: Hold at 4°C

2.3.10.3 Purifying Extension Products

To remove unincorporated dye-labelled terminators, the PCR products were precipitated in 60% isopropanol and rinsed afterwards with 75% isopropanol:

85 µl 70% isopropanol was added to each sample (in a 0.5 ml tube; F.C. 60%), vortexed briefly and the samples were left at room temperature for 10 minutes to precipitate the extension products. The samples were then spun at maximum speed for 20-30 minutes, and the supernatant was removed. 150 µl 75% isopropanol was added, the samples spun at maximum speed for 10 minutes and the supernatant removed. The pellet was then dried in air or gentle heat, and either analysed immediately or stored at room temperature.

2.4 Biochemical methods

2.4.1 Antibodies

Name Of Antibody	Host	Source
Anti Penta His	Mouse	Qiagen (Crawley, West Sussex, UK). Catalogue number: 3466
Anti N-PS-1	Rabbit	Santa Cruz Biotechnology, Inc; USA. Catalogue number: 5c-7860
Anti C99	Rabbit	Zymed laboratories, USA. Catalogue number: 51-2700
Anti PhoA	Mouse	Caltag laboratories, Burlingame, Canada. Catalogue number: ME6200
Anti Aβ	Rabbit	Santa Cruz Biotechnology, Inc; USA. Catalogue number: sc-9129
Anti Pen-2	Rabbit	Christian Haass, Munich
Anti Aph-1	Rabbit	Christian Haass, Munich
Anti NicA	Rabbit	Sigma-Aldrich, UK
Anti mouse secondary antibody, AP coupled	Rabbit	Sigma-Aldrich, UK. Catalogue number: A-4312
Anti rabbit secondary antibody, AP coupled	Goat	Sigma-Aldrich, UK. Catalogue number: A-8025

Table (2.6) List of antibodies used

2.4.2 TCA (Trichloroacetic acid) precipitation of proteins

This method was occasionally performed to concentrate protein samples. Ice cold 20% TCA was added to the protein sample (7% F.C.). The mixture was vortexed and incubated on ice for 30 minutes. The precipitated proteins were then sedimented by centrifugation in a cold table top centrifuge at 13,000 rpm for 10 minutes. The pellet was washed with 100% acetone and sedimented again as described. The protein pellet was then resuspended in sample buffer and analysed by SDS PAGE.

2.4.3 SDS PAGE

SDS PAGE is the electrophoretic separation of proteins in an SDS gel. Depending on the molecular weight of the investigated protein, different percentages of SDS gels were used (Table 2.7). The gels were prepared in a BioRad apparatus and 10-20 µl of samples were loaded carefully. The gels were run at 0.25A per gel for about an hour. Afterwards the gels were either blotted (Section 2.4.6, Materials and Methods), or stained with Coomassie Brilliant Blue from AppliChem, Germany (Section 2.4.4, Materials and Methods). Schaegger gels were used for the separation of proteins with low molecular weights (Table 2.8).

Gel Concentration	7%	10%	12%	15%	Stacking Gel
Reagents					
Buffer A (1.5 M Tris-HCl buffer pH 8.8, 0.4 % SDS)	2.5 ml	2.5 ml	2.5 ml	2.5 ml	*
Buffer B (0.5 M Tris-HCl buffer pH 6.8, 0.4% SDS)	*	*	*	*	2 ml
Acrylamide stock (30%)	1.75 ml	2.5 ml	3 ml	3.75 ml	0.9 ml
H_2O	5.75 ml	5 ml	4.5 ml	3.75 ml	5.1 ml
TEMED	10 µl	10 µl	10 µl	25 µl	20 µl
APS (stock 1%)	25 µl	25 µl	25 µl	25 µl	40 µl

Table (2.7) Composition of SDS minigels

	16.5% separation gel	10% spacer gel	4% stacking gel
Reagents			
Acrylamide (49.5% = 48 g / 100 ml acrylamide; 3% = 1.5 g / 100 ml bisacrylamide)	3.5 ml	1.5 ml	0.5 ml
Gel buffer (3 M Tris-HCl buffer pH 8.45; 0.3% SDS)	3.5 ml	2.5 ml	1.55 ml
H_2O	*	3.5 ml	4.2 ml
32% glycerol	3.5 ml	*	*
APS (10%)	32.5 µl	35 µl	25 µl
TEMED	10 µl	10 µl	10 µl

Table (2.8) Composition of Schaegger gels

2.4.4 Rapid staining procedure with coomassie blue (Wong et al., 2000)

This is a modification of the staining procedure of Fairbanks (Fairbanks et al., 1971), which involves heating the polyacrylamide gels in staining buffers using a microwave. A total processing time of 20 minutes using this rapid procedure allows visualisation of as little as 5 ng of protein.

The gel was incubated in approximately 20 ml of Buffer A (25% 2-propanol, 10% acetic acid, 0.05% Coomassie Blue) and heated in a microwave at high power for 30 seconds. Subsequently it was shaken for at least 10 minutes at RT. Buffer A was exchanged for Buffer B (10% 2-propanol, 10% acetic acid, 0.005% Coomassie Blue) and the gel was heated again for 30 seconds in a microwave. Thereafter, no shaking was required and Buffer B was immediately exchanged by Buffer C (10% acetic acid, 0.002% Coomassie Blue). After additional heating as before, the gel was placed in Destaining Solution (10% acetic acid) O/N.

2.4.5 Drying of SDS Gels

After destaining of the SDS gel, it was dried using the GelAir Drying Frame from BioRad. The gel was incubated in Gel-Drying Buffer (5% EtOH, 10% glycerol, 1 l distilled H_2O) for about 30 minutes and placed between two sheets of cellophane as recommended by the manufacturer. The gel was then dried at 28°C O/N.

2.4.6 Immunoblot analysis (Western blotting)

The Western blot was developed by Towbin and co-workers in 1979 (Towbin et al., 1979). It is the electrophoretic transfer of SDS-gel separated proteins onto a PVDF membrane (FluoroTrans, Pall, Ireland) where they can be detected by antibodies. A primary antibody is specific for the investigated protein, whereas the secondary antibody is specific for the primary antibody. An enzyme (in this case alkaline phospatase, PhoA) is fused to the secondary antibody, and when its substrate (BCIP/XP) is added, a colour change is detected which makes the desired protein band(s) visible.

The membrane was activated in 100% methanol for a few seconds and the blotting apparatus (BioRad Mini Trans-Blot® Cell) was assembled as recommended by the manufacturer. The electro-transfer was carried out for 1 hour at 100 V in Transfer Buffer (15 mM TRIS, 120 mM glycine, 20% methanol, 0.02% SDS). After the transfer, the membrane was stored between two Whatmanpapers (GB 003, Schleicher & Schuell, Germany) at RT.

For detection of the protein band(s) with specific antibodies, the membrane was blocked for at least 1 hour at RT on a table-top shaker (or O/N at 4°C). Blocking was performed with 2% dry milk (Tesco, UK) or BSA (Fisher Scientific, UK) in TBST. Some antibodies, e.g. the mouse anti His-tag antibody require BSA as blocking reagent. Supsequently, the membrane was incubated in 10 ml TBS-T (20 mM TRIS pH 7.5, 150 mM NaCl, 0.05% TWEEN-20) with the appropriate

concentration of primary antibody and placed on a table-top shaker for 1 hour. After the binding of the primary antibody to the investigated protein, the membrane was washed for 5 minutes (3 times) in 10 ml TBS-T. The membrane was then incubated in 10 ml TBS-T with the desired concentration of the secondary antibody for 30-60 minutes and the washing procedure was repeated as before. To detect the protein bands investigated, the membrane was incubated in 10 ml of Staining Solution (10 ml AP buffer [100 mM TRIS pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$], 66 µl NBT (50 mg/ml in 70% DMF), and 33 µl BCIP (50 mg/ml in 100% DMF). To avoid further staining, the membrane was rinsed with tap water or it was incubated in 10 ml 5 mM EDTA for a few minutes.

2.4.6 PhoA (alkaline phosphatase) assays

To determine the PhoA activity of the Pen2-PhoA or the Aph1-PhoA hybrid proteins, an overnight culture of MC4100 containing the relevant plasmid was diluted 1/50 in NZA liquid medium containing 200 µg/ml Amp for about two hours. Subsequently, protein expression was induced by the addition of 0.1% Arabinose and the diluted culture was grown until $O.D_{600}$ was about 0.3-0.6.

Of these cells, 0.2 ml was added to 0.8 ml washing buffer (10 mM Tris pH 8.0, 150 mM NaCl). The cells were gently vortexed and spun down in a table-top centrifuge. This washing step was repeated twice. 0.75 ml of the washed cells was used to determine $O.D_{600}$. To perform the PhoA assay, 100 µl washed cells were added to 900 µl assay buffer (1 M Tris pH 8.0, 1 mM ZnCl), 25 µl 0.1% SDS, 25 µl chloroform and were gently mixed and incubated at RT for 5 minutes. After adding 100 µl PhoA substrate buffer (1 M Tris pH 8.0, 0.4% pNPP [p-nitrophenyl phosphate]), the reaction was gently mixed and incubated at 28°C until colour was visible. The addition of 120 µl K_2HPO_4 stopped the reaction from proceeding further. Cells were spun down for 5 minutes in a table-top centrifuge and $O.D_{420}$ of the supernatants was measured. The following formula was used to determine the PhoA activity of each hybrid protein:

$$\text{PhoA activity (units)} = \frac{OD_{420} \times 1000}{\text{Time (min)} \times OD_{600} \times \text{cell volume (ml)}}$$

2.4.7 β-Galactosidase (β-Gal) assays

To determine the level of interaction between different components of the γ-secretase complex, β-Galactosidase Assays were carried out. MC4100 cells containing the relevant plasmids were grown O/N and then diluted 1/30 and grown for a further 1-2 hours. IPTG was used to induce protein expression and cells were grown for a further 2-3 hours until OD_{600} reached 0.3-0.7. Cells were washed twice in Z-Buffer (for 1 l Z-buffer: 10.7 g $Na_2HPO_4.2H_2O$, 5.5 g $NaH_2PO_4.H_2O$, 0.75 g KCl pH 7.0 and 0.246 g $MgSO_4.7H_2O$. 1 ml of cells in Z-Buffer was taken to determine OD_{600}, and another 1 ml in Z-Buffer was added to 50 µl 0.1% SDS and 50 µl $CHCl_3$, vortexed gently and incubated at room temperature for 5 minutes. To that, 0.2 ml ONPG (4 mg/ml in Z-buffer) was added and incubated at 37°C or 28°C depending on the strain used in the assay. When colour developed, the reaction was stopped by the addition of 0.5 ml 1 M Na_2CO_3. The samples were then spun at high speed for 5 minutes to remove the cells. The $O.D_{420}$ of the supernatants could be read immediately, or the supernatants could be stored at 4°C overnight. As a reference, the following was used: 1 ml Z-buffer, 50 µl 0.1% SDS, 50 µl $CHCl_3$, 0.2 ml ONPG and 0.5 ml 1 M Na_2CO_3. The following equation was then used to calculate β-Gal activity:

$$\text{ß-Gal-activity } \frac{\mu mol}{mg \times min} = \frac{\Delta E405 \times Vt}{OD578 \times cP \times VB \times e}$$

$$= 3.269 \times \frac{OD_{420}}{OD_{578} \times t}$$

t: reaction time

cP: protein conc. (107 µg protein / ml OD_{578})

Vt: final volume of the assay (1.7 ml)

VB: volume of cells added to the assay

e: extinction coefficient of ortho-Nitrophenol at 405 nm (4860 M^{-1})

2.4.8 Protein purification by FPLC (Fast Protein Liquid Chromatography)

FPLC was used to separate a protein from a protein mixture in solution. This method of chromatography takes advantage of the affinity of certain parts of a protein to a stationary phase e.g. the binding of a His-tag to a metal chelating column.The His-tag binds to positive metal ions including Ni^{2+}, Co^{2+} and Cu^{2+}. In this work, Ni NTA (Ni NTA superflow, Qiagen, Germany) was used as a stationary phase and packed into an empty column (XK 16, Amersham Pharmacia Biotech). Elution of His tagged proteins was performed by increased concentrations of imidazole, which competes with the His tags for the binding sites to the positive metal ions.

2.4.8.1 Growth of cells

For expression, double NZA containing the appropriate antibiotics was inoculated with a single colony and cells (200 ml) were grown to saturation overnight. The overnight culture was diluted 1/20 in the same medium (4 l) and growth was maintained at 28°C until the $O.D_{500}$ reached approximately 0.5. At this point, IPTG was added to a final concentration of 100. The culture was grown further at 20°C for 4 hours or overnight with shaking at 220 rpm.

2.4.8.2 Harvest of cells

Cells from a 4 l culture were harvested by centrifugation at 13,000 rpm for 10 minutes in a Beckman GSA centrifuge. The pellet containing the cells can be frozen at -20°C until required, or the cells can be resuspended in 200 ml of lysis buffer (50 mM Tris-HCl pH 8.0 300 mM NaCl).

2.4.8.3 Preparation of a cell lysate

This is performed either prior to membrane preparation, in the case of C99, or following the solubilisation of membrane proteins from whole cell extracts in the case of Pen-2. Cells in lysis buffer were disrupted by sonication for a maximum of 10 minutes or in a French pressure cell 3 times at 15,000 psi. The cell lysate was cleared by centrifugation at 13,000 rpm for 10-30 minutes to remove unbroken cells and aggregates which should be present in the pellet. The supernatant is then used directly for purification of proteins by FPLC, or is used to prepare membranes.

2.4.8.4 Preparation of membranes

The supernatant was ultracentrifuged for 45 min at 45,000 rpm to sediment membranes. To wash, the membranes were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl) by homogenisation, and collected again by ultracentrifugation as described. The membranes were subsequently either used immediately or stored at - 20°C until protein solubilisation was performed.

2.4.8.5 Determination of protein concentration with bicinchoninicacid according to the sigma procedure TPRO-562

5 µl of pure protein, whole cell extract or membranes in a small volume of solubilisation buffer (20 mM Tris-HCl pH 8.5, 300 mM NaCl) were added to 95 µl of solubilisation buffer. This 100 µl was then made up to 1 ml with BCA solution (1/50 buffer B/buffer A, as recommended by the manufacturer). As a control, solubilisation buffer in BCA solution (no protein) was used.

Calibration was performed as recommended by the supplier and the $O.D_{568}$ of each sample was read in a spectrophotometer.

Subsequently, the following equation was used to determine protein concentrations in µg/ml:

$$\frac{OD - 0.003703}{0.013841}$$

2.4.8.6 Solubilisation of membrane proteins

For the solubilisation of membrane proteins like C99 and Pen-2, whole cell extracts or membrane suspensions were adjusted to a protein concentration of 2 to 2.5 mg/ml with the appropriate solubilisation buffer. Detergents like DDM, CHAPSO and SDS were used in a 1% concentration. The protein/detergent mixture was stirred on ice (or at RT for SDS) for 1 to 2 hours and subsequently ultracentrifuged for 45 minutes at 45,000 rpm. The supernatant, containing the solubilised proteins was then loaded on the Ni NTA column for chromatography.

2.4.8.7 Purification with a Ni NTA superflow column

The Ni NTA superflow was obtained from Qiagen, Germany. For a column volume of about 5 ml, 10 ml Ni NTA was loaded onto an empty column, which was then connected to the FPLC according to the manufacturer's instructions. The column was rinsed with distilled water with a flow rate of 3 ml/min to allow the Ni NTA material to settle. Afterwards, the column plunge was pushed down so it fitted directly above the level of material in the column. The column was washed with 5 to 10 column volumes (CV) of equilibration buffer (50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl). The protein solution was loaded on the column with a flow rate of 0.5 to 1 ml/min. Subsequently, the column was washed with 10 CV equilibration buffer to remove any excess proteins that did not bind to the column, and then washed with 10 CV equilibration buffer containing 10 to 30 mM imidazole to remove contaminants. The protein was eluted with ten CV elution buffer (50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl and 500 mM imidazole).

The flow rate for washing steps and elution was 2 ml/min.The Ni NTA was stored in 20% EtOH, and stripping and reloading was performed according to the manufacturer's instructions.

2.4.9 γ-Secretase assays

The following bacterial plasmids containing the individual human γ-secretase components were transformed into the DegP minus strain KU98 (*degP::kan)* pSA1 (Pen-2), pSA12 (Aph-1), pSG6 (C99), pMH13 (PS-1Δexon9) and pMH42 (NicA). Additionally the plasmids containing γ-secretase components (pSA14 (Pen-2 and Aph-1), pSG33 (PS-1) or pSG34 (PS-1Δexon9), and pSG36 (NicA) were transformed into the strain KS272. Cells were grown, harvested and membranes prepared as described previously, and as a control, the cells KU98 or KS272 containing no plasmids were treated similarly.

Membranes of KU98 or KS272 containing the γ-secretase components Pen-2, Aph-1, PS-1Δexon9 and NicA were re-suspended in a small volume of lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl) and the protein concentration of the membrane suspensions was measured using a BCA (bicinchoninic acid) protein determination kit. In the case of KU98 membranes, equal concentrations of each membrane suspension producing a different γ-secretase component were mixed to a total concentration of 400 μg and a total volume of about 5 ml. The mixture was incubated at 37 °C for 24 h, and then the substrate C99 (produced in KU98 and purified by FPLC) was added in different concentrations. In the case of the KS272 membranes, 400 μg of membrane suspension was added to different concentrations of C99 and the mixture was incubated at 37 °C. As a control, membrane suspensions with no added substrate or with missing γ-secretase components were used. 1 ml samples of the assay were removed at 0 hours, 6 hours, 24 hours, 36 hours and 48 hours after the addition of substrate and the reaction was

stopped by freezing the samples at -20°C. The samples were then analysed using an Aβ ELISA kit from the Genetics Company and by Western blotting, to detect levels of Aβ produced.

CHAPTER (3)

CLONING, EXPRESSION & TOPOLOGY

(3) CLONING, EXPRESSION AND TOPOLOGICAL STUDIES OF THE HUMAN γ-SECRETASE COMPONENTS IN *E. COLI*

3.1 Introduction

The γ-secretase pathway, as illustrated in chapter (1), represents the proteolytic cascade that leads to the development of Alzheimer's disease in the human brain. The γ-secretase complex is now thought to be comprised of four cytoplasmic membrane proteins, including Presenilin-1 (PS-1) which is postulated to form the active site of the complex. The other components are Nicastrin (NicA), Aph-1 and Pen-2. Following the cleavage of APP by β-secretase, the C-terminal fragment of APP (C99) which contains Aβ, remains in the membrane. C99 is then processed by the γ-secretase complex in a final cleavage step, producing the highly fibrillogenic $A\beta_{40}$ or $A\beta_{42}$ peptides. PS-1, NicA, Pen-2 and Aph-1 have been shown to constitute the minimum components required for γ-secretase activity in a number of different cell types including *C. elegans* and *D. melanogaster*, but these data do not rule out the involvement of other factors. Therefore, we suggest that a simpler system is required for verification and we propose using *E. coli* as a model system. *E. coli* have a simple genome which is evolutionary very different from that of higher eukaryotes. This suggests that *E. coli* are unlikely to contain co-factors for the γ-secretase, and that they could represent the ideal system for studying the γ-secretase components' functions and interactions. Overproducing the γ-secretase in *E. coli* could allow future analysis and purifications of the complex members, including the possibility of performing controlled enzyme assays.

The production of recombinant human proteins in *E. coli* can be problematic, especially membrane proteins. A common reason for limited overproduction of membrane proteins is degradation by

cellular proteases. For this reason, the strain KU98 (Section 2.2.1, Materials and Methods), which lacks *degP* was chosen for most overproduction studies. Deletion of such proteases was thought of as beneficial as it may result in increased stability of the recombinant proteins. In addition, a suitable vector such as pGRD11 and pCS19 (Section 2.3.1, Materials and Methods) was chosen for cloning the genes encoding the γ-secretase components. In these vectors, expression is under control of the *tac* promoter which is inducible by IPTG or lactose, therefore all *E. coli* strains containing these plasmids were grown in lactose-free media such as NZA. The vectors pGDR11 and pCS19 contain an optimal Shine Dalgarno sequence for improved translation, a multiple cloning site, the *bla* sequence which encodes the Ampicillin (Amp) resistance gene and the lac repressor ($lacI^{Q}$) is over-expressed to ensure tight promoter control. The presence of an N- or a C-terminal His-tag is useful as it will allow detection of protein expression levels by using a monoclonal α-poly histidine antibody, and future purifications via nickel or copper chromatography.

As we are dealing with human membrane proteins in an *E. coli* system, it was important to ensure correct insertion of the recombinant proteins into the bacterial cytoplasmic membrane. In theory, any region of about 20 hydrophobic amino acid residues corresponds to a transmembrane helix (Kyte and Doolittle, 1982) and therefore, the amino acid sequence of any protein can be used to predict its transmembrane segments. The topology of integral cytoplasmic membrane proteins in *E. coli* can be studied *in vivo* by a genetic approach developed in the late eighties, which involves generating hybrids of the target membrane protein to PhoA (Manoil and Beckwith, 1986; Manoil et al., 1990). In wild-type strains, PhoA is only active after being exported to the periplasm where its disulphide bonds can be formed as a result of the oxidising environment in the periplasm (Derman and Beckwith, 1991). Periplasmic domains of integral membrane proteins can be fused to signal sequenceless PhoA that is then properly translocated (Manoil and Beckwith, 1986). When PhoA is

fused to a cytoplasmic domain it is enzymatically inactive. Therefore, the activity of *phoA* fusions is used to predict the cellular localisation of the fused membrane protein domain (Boyd *et al.*, 1993; Ehrmann, et al., 1990). Here, we constructed Pen-2-PhoA, Aph-1-PhoA hybrids and described previous construction of PS-1-PhoA hybrids (Harnasch, 2003). Individual fusion joints were placed at the C-termini of hydrophilic regions of the membrane proteins. Boyd and co-workers introduced this experimental strategy in order to minimise the disruption of topological signals (Boyd et al., 1993).

3.2 Cloning and expression of the human γ-secretase components, and the γ-secretase substrate, c99, in E. coli

3.2.1 Cloning and expression of *pen-2 and aph-1* in different combinations

3.2.1.1 Cloning pSA1 containing pen-2 and pSA2/12 containing aph-1

pen-2 and *aph-1* were amplified by PCR from a lambda-zap-cDNA library (Klostermann et al., 2000) from neuronal cell cultures. In order to produce the proteins, each gene was cloned into a suitable vector for expression studies. To aid detection of the produced proteins, it was thought useful to clone the genes with a His-tag that can be detected with a monoclonal α-penta histidine antibody (Qiagen, Germany). Moreover, the His-tag allows future purification of the proteins via a nickel or copper chromatography column. Human *pen-2* and *aph-1* were cloned into the *E. coli* expression vector pGDR11 where an N-terminal His-tag is encoded by the 5' end of the multiple cloning site.

The gene *pen-2* was amplified from the cDNA library using the oligonucleotides "pen2-5'(BamHI)" and "pen2-3'(*KpnI*)", while *aph-1* was amplified using the oligonucleotides "AphI/*KpnI*/pGDR11" and "AphI/*HindIII*/pGDR11" (Section 2.3.2, Materials and Methods) which resulted in PCR products with flanked restriction sites. The vector pGDR11 was prepared via restriction digestion, and the PCR products were ligated into the multiple cloning site of the vector via *BamHI/KpnI* and *HindIII/KpnI* for *pen-2* and *aph-1* respectively (Figure 3.1). The ligation reactions were then transformed into electrocompetent MC4100 cells (Section 2.2.1, Materials and Methods) and transformants were used to prepare plasmid DNA of the candidates for pSA1 and pSA2. Candidates were then confirmed by restriction digestion and analysed by agarose gel electrophoresis. Sequencing of both inserts excluded the presence of mutations in the *pen-2* sequence of pSA1, but revealed a frameshift mutation in the *aph-1* sequence in pSA2 as a result of

an extra Cytosine at the 3' end of the gene (C728). It was thought that this may affect protein structure and function, therefore a new plasmid was constructed containing wild-type *aph*-1. In this case, *aph*-1 was amplified in the same way (Figure 3.1), but from the plasmid pCDNA (zeo) Aph1a251, obtained from the Haass laboratory, Munich. This resulted in the construction of the plasmid pSA12, which contains wild-type *aph-1* with an N-terminal his tag.

It was not until later on it was revealed that the *aph-1* gene in the cDNA library obtained from Klostermann and co-workers, was in fact a naturally occurring splice variant of *aph-1* (See Swiss-prot).

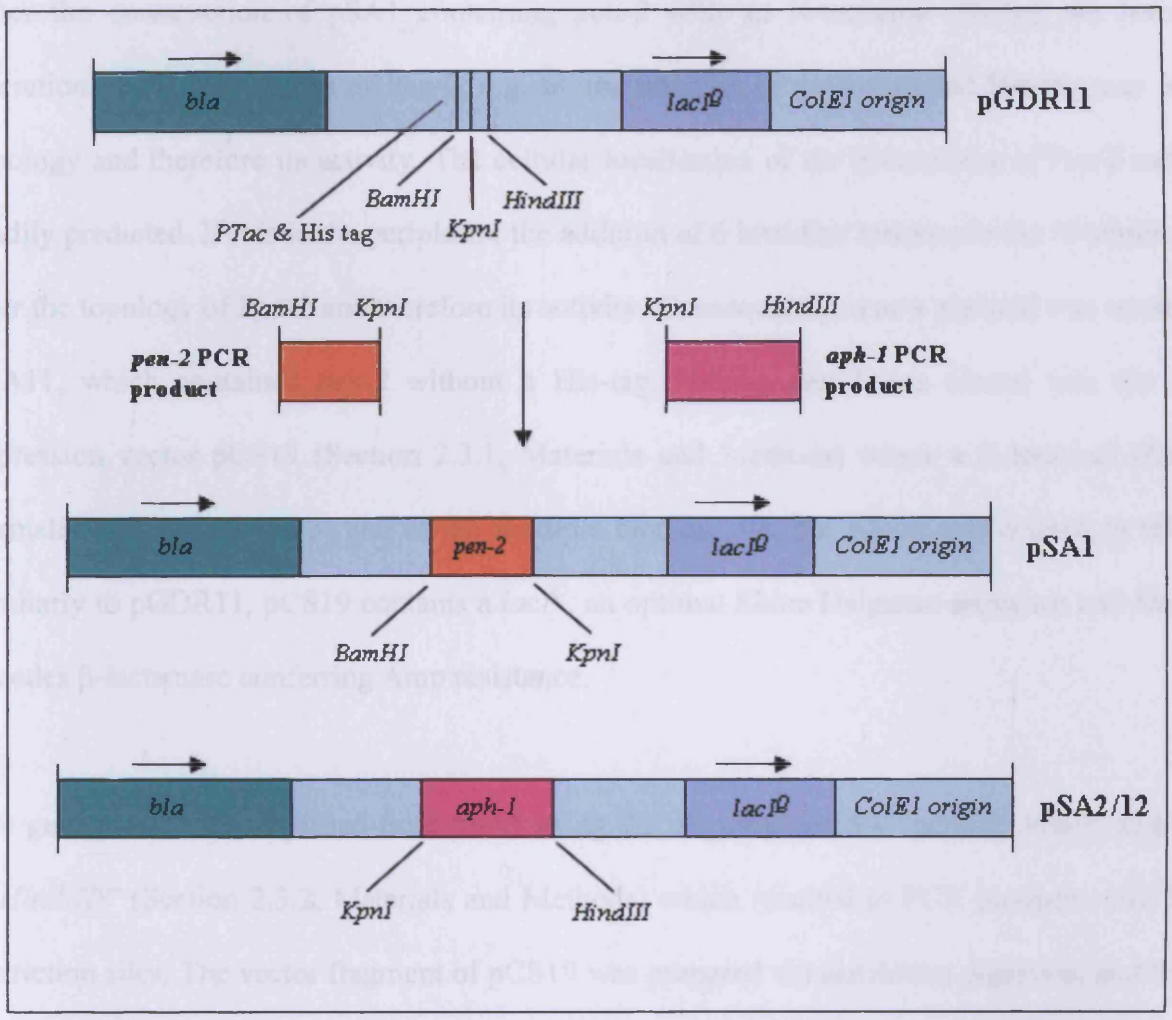


Figure (3.1) Schematic representation of the construction of pSA1 and pSA12 containing human *pen-2* and *aph-1* respectively, both with N-terminal His tags.

The genes were amplified from a lambda-zap-cDNA library from neuronal cell cultures, and in the case of pSA12, from the plasmid pCDNA (zeo) Aph1a251. The genes were cloned into the multiple cloning site of pGDR11 via *BamHI* and *KpnI* for *pen-2*, and *KpnI* and *HindIII* for *aph-1*. This resulted in the fusion of the genes to an N-terminal His-tag. The genes are IPTG inducible, the Lac repressor (*lacI^{Q}*) ensures a tight promoter, and *bla* encodes β-lactamase conferring Amp resistance.

3.2.1.2 Cloning pSA11, containing tag-less pen-2

After the construction of pSA1 containing *pen-2* with an N-terminal His-tag, we learnt that alteration of the N-terminus of Pen-2, e.g. by the addition of an N-terminal His-tag may alter its topology and therefore its activity. The cellular localisation of the N-terminus of Pen-2 cannot be readily predicted. If it is in the periplasm, the addition of 6 histidine residues to the N-terminus may alter the topology of Pen-2 and therefore its activity. Consequently, a new plasmid was constructed, pSA11, which contained *pen-2* without a His-tag. Human *pen-2* was cloned into the *E. coli* expression vector pCS19 (Section 2.3.1, Materials and Methods) where a C-terminal His-tag is normally encoded by the 3' end of the multiple cloning site, but which was deleted in this case. Similarly to pGDR11, pCS19 contains a $lacI^{Q}$, an optimal Shine Dalgarno sequence and *bla* which encodes β-lactamase conferring Amp resistance.

The gene *pen-2* was amplified from pSA1 using the oligonucleotides "pen2-5'(*NcoI*)" and "pen2-3'(*HindIII*)" (Section 2.3.2, Materials and Methods) which resulted in PCR products with flanked restriction sites. The vector fragment of pCS19 was prepared via restriction digestion, and the PCR products were ligated into the multiple cloning site of the vector via *NcoI*/*HindIII* (Figure 3.2). The ligation reactions were then transformed into electrocompetent MC4100 cells, and transformants were used to prepare plasmid DNA of the candidates for pSA11. Candidates were then confirmed by restriction digestion and analysed by agarose gel electrophoresis. Sequencing of the insert excluded the presence of mutations in the *pen-2* sequence.

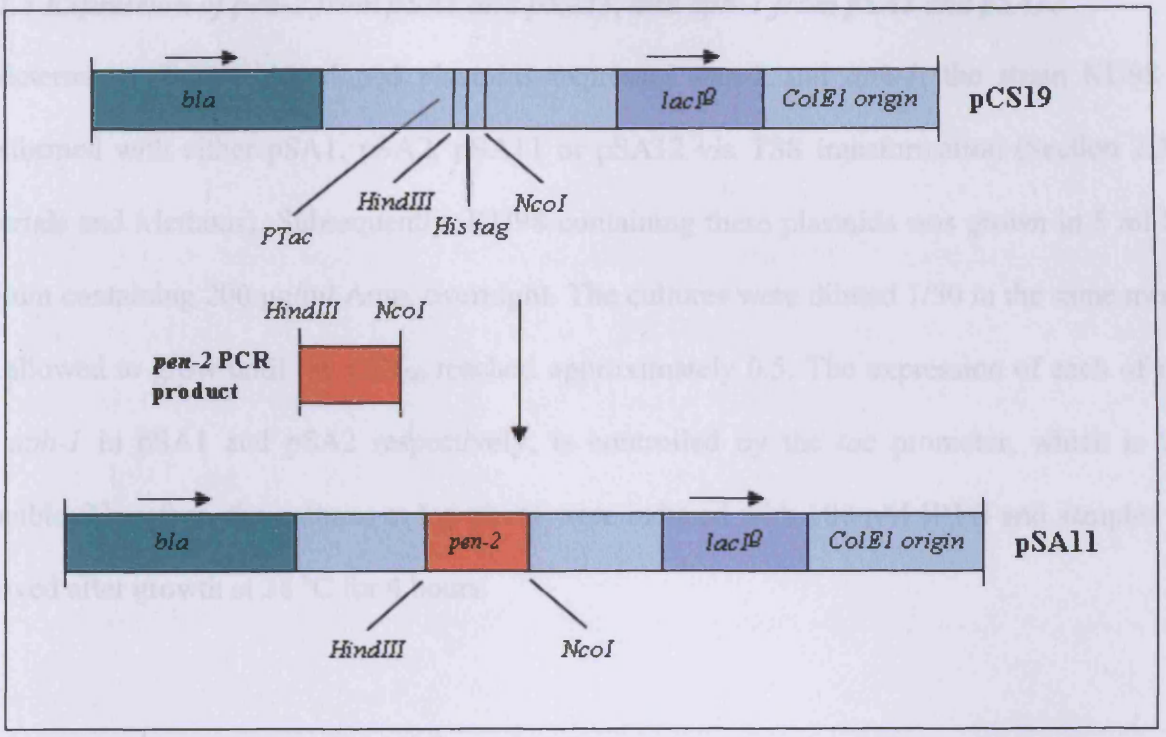


Figure (3.2) Schematic representation of the construction of pSA11 containing tag-less *pen-2*.

The gene was amplified from pSA1, and ligated into the multiple cloning site of pCS19 via *HindIII/NcoI* deleting the C-terminal his tag and resulting in tag-less *pen-2*. The gene is IPTG inducible, the Lac repressor ($lacI^Q$) ensures a tight promoter, and *bla* encodes β-lactamase conferring Amp resistance.

3.2.1.3 Expression of pen-2 from pSA1 and pSA11, and aph-1 from pSA2 and pSA12

To determine whether the cloned plasmids expressed *pen-2* and *aph-1*, the strain KU98 was transformed with either pSA1, pSA2, pSA11 or pSA12 via TSS transformation (Section 2.3.8.1, Materials and Methods). Subsequently, KU98 containing these plasmids was grown in 5 ml NZA medium containing 200 μg/ml Amp, overnight. The cultures were diluted 1/50 in the same medium and allowed to grow until the OD_{600} reached approximately 0.5. The expression of each of *pen-2* and *aph-1* in pSA1 and pSA2 respectively, is controlled by the *tac* promoter, which is IPTG inducible. Therefore, the cultures at log phase were induced with 100 μM IPTG and samples were removed after growth at 28 °C for 4 hours.

To investigate if the presence of other γ-secretase components like PS-1, and the γ-secretase substrate C99 will have any effect on the expression levels of *pen-2* and *aph-1*, the strain KU98 containing one of pSA1, pSA11, pSA2 or pSA12 in addition to pSG33 (containing *ps-1* and *c99* in a pACYC derivative) was also grown as above. Expression in pSG33 is also under the control of the *tac* promoter and can therefore be induced by IPTG. Gene expression was induced by the addition of 100 μM IPTG and samples were removed after growth at 28°C for 4 hours. As a negative control, each strain containing the different combination of plasmids was also grown as before, but no IPTG was added to induce protein expression, and therefore little or no protein should be detected after analysis with a Western blot.

Whole cell extracts were prepared by removing 1 ml of induced culture of each sample and centrifuging it at 13,000 rpm in a table top centrifuge, followed by the addition of sample buffer to adjust the O.D. of the samples. Protein samples were loaded on 15% SDS gels as both Pen-2 and Aph-1 are very small, and Western blots were performed using a monoclonal α-penta histidine

antibody (Sigma) for the detection of Aph-1, and a polyclonal α-Pen-2 antibody (Haass laboratory, Germany) for the detection of Pen-2.

Western blotting revealed that both Pen-2 and Aph-1 are produced in reasonably large amounts from pSA1 and pSA11 for Pen-2 and pSA2 and pSA12 for Aph-1 (Figure 3.3). In addition, co-expression of *ps-1* and *c99* with *pen-2* and *aph-1* did not facilitate increased production of neither Pen-2 nor Aph-1, and in some instances, it even resulted in reduced production levels of the recombinant proteins. Also in this instance, expression of *pen-2* without a his tag (pSA11) was higher than that of his tagged *pen-2* but this result was not replicated and therefore from then on, pSA1 was generally used for studies of Pen-2.

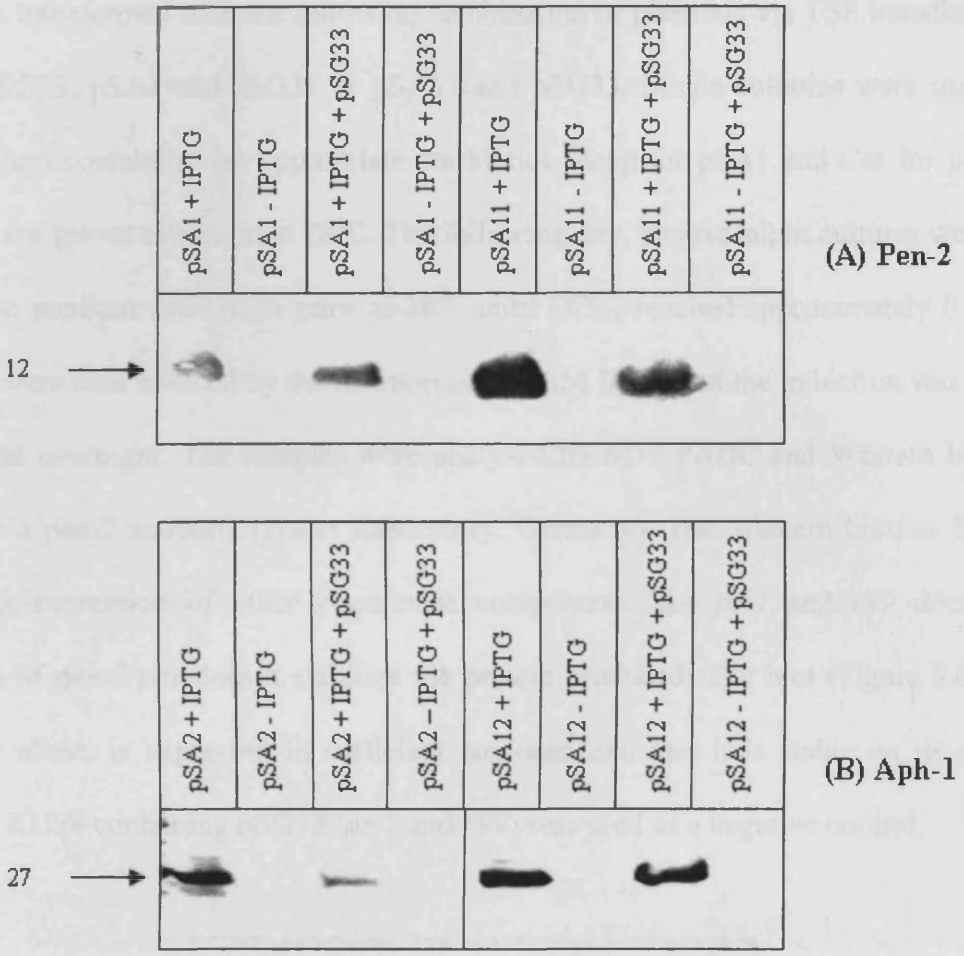


Figure (3.3) Western blot monitoring the expression of *pen-2* from pSA1 and pSA11, and the expression of *aph-1* from pSA2 and pSA12

The strain KU98 was transformed with each of the following combinations of plasmids by TSS transformation: (A) pSA1, pSA1 and pSG33, pSA11, pSA11 and pSG33, and (B) pSA2, pSA2 and pSG33, pSA12, pSA12 and pSG33. KU98 containing the plasmids was grown overnight at 28°C and then diluted 1/50 and grown again until $O.D_{600}$ reached about 0.5. Subsequently, expression was induced by the addition of 100 μM IPTG to the cultures at log phase. Un-induced samples were used as negative controls. The samples were then analysed by SDS gel electrophoresis and Western blotting using a monoclonal α penta histidine antibody from Sigma. Molecular weights are given in kDa.

3.2.1.4 Pen-2 expression is stable after overnight induction

KU98 was transformed with the following combination of plasmids via TSS transformation: pSA1, pSA11, pSG33, pSA1 and pSG33 or pSA11 and pSG33. Single colonies were used to inoculate NZA medium containing the appropriate antibiotics (Amp for pSA1 and Cm for pSG33) and the cultures were grown overnight at 28°C. The following day, the overnight cultures were diluted 1/50 in the same medium and left to grow at 28°C until OD_{600} reached approximately 0.5. The cells at log phase were then induced by the addition of 100 μM IPTG and the induction was carried out for 4 hours and overnight. The samples were analysed by SDS PAGE and Western blotting using a polyclonal α pen-2 antibody (Haass Laboratory, Germany). The Western blot, as before revealed that the co-expression of other γ-secretase components like *ps-1* and *c99* does not increase expression of *pen-2* nor does it stabilize the protein produced. The blot (Figure 3.4) also showed that *pen-2* alone, is expressed in sufficient amounts, and that it is stable on its own after O/N induction. KU98 containing pSG33 (*ps-1* and *c99*) was used as a negative control.

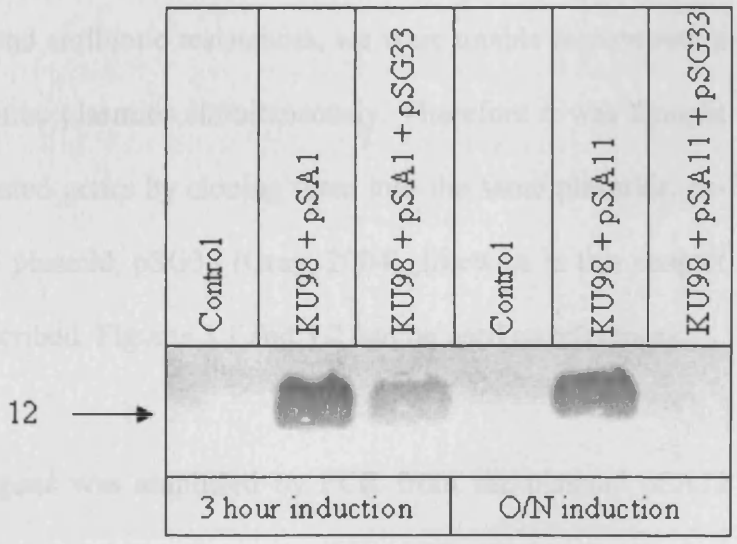


Figure (3.4) Western blot monitoring overnight expression of *pen-2* in KU98

KU98 cells transformed with either pSA1, pSA11, pSA1 with pSG33, pSA11 with pSG33 or just pSG33 as a control were grown to log phase and then induced with 100 μM IPTG. Samples were analysed by SDS PAGE and Western blotting using a polyclonal antibody directed against the C-terminus of Pen-2. Molecular weights are given in kDa.

3.2.1.5 Construction of pSA13 and pSA14 containing pen-2 and aph-1 both with N-terminal 6xhis tags

It was recently demonstrated that Pen-2, Aph-1, PS-1 and NicA represent the minimal set of components required for γ-secretase activity. *E. coli* represents an excellent system for reconstituting this activity because in contrast to eukaryotic cells and higher organisms, it contains no endogenous proteins that may interfere with the activity in question. One technique for reconstituting γ-secretase activity is to combine all four components in the one *E. coli* strain. The four proteins comprising the γ-secretase complex can then be produced and an enzyme assay can be carried out by the addition of the γ-secretase substrate C99, followed by the determination of the amount of Aβ produced by, for example, ELISA. Alternatively, the presence of PS-1 N- or C-terminal fragments, or the reduction in the amount of substrate, C99, can be detected by Western blotting.

Given the plasmids available in the laboratory collection, there are a number of origins of replication and antibiotic resistances, we were unable to construct a strain that could accommodate more than three plasmids simultaneously. Therefore it was thought necessary to combine some of our investigated genes by cloning them into the same plasmids. *ps-1* and *c99* were already present on the same plasmid, pSG33 (Grau, 2004), likewise in this chapter, the combination of *pen-2* and *aph-1* is described. Figures 3.1 and 3.2 can be used as references.

The *aph-1* gene was amplified by PCR from the plasmid pSA12 (Section 2.3.1, Materials and Methods) using the oligonucleotide "SDpGDR11/*SalI*" for the forward reaction and "AphI/*HindIII*/pGDR11 noncs" for the reverse reaction (Section 2.3.2, Materials and Methods). This resulted in a fragment containing *aph-1* with the flanked restriction sites *SalI* and *HindIII*. The vector pSA1, containing *pen-2* with an N-terminal His-tag was prepared via restriction digestion

with the same restriction enzymes. The *aph-1* PCR product was then ligated into the vector, the ligation reactions were transformed into electrocompetent MC4100 cells, and transformants were used to prepare plasmid DNA of the candidates for pSA14. Candidates were confirmed by restriction digestion and analysed by agarose gel electrophoresis. Sequencing of the insert excluded the presence of mutations in the *aph-1* sequence. The same procedure was repeated using pSA2 instead of pSA12 and this resulted in the plasmid pSA13, containing the splice variant of *aph-1* with *pen-2*, both with N-terminal his tags.

3.2.1.6 Pen-2 and Aph-1, are produced in E. coli from pSA13 and pSA14

To determine whether the cloned plasmids expressed *pen-2* and *aph-1*, pSA13 and pSA14 containing both *pen-2* and *aph-1* with N-terminal His tags, were transformed into KU98. Single colonies were used to inoculate NZA medium containing 200 μg/ml Amp and cultures were grown to saturation overnight at 28°C. Cultures were then diluted 1/50 in the same medium and grown until the $O.D_{600}$ reached approximately 0.5. The cells at log phase were then induced by the addition of 100 μM IPTG and the induction was carried out for 4 hours. The samples were analysed by SDS PAGE and Western blotting using a monoclonal α-penta histidine antibody from Sigma.

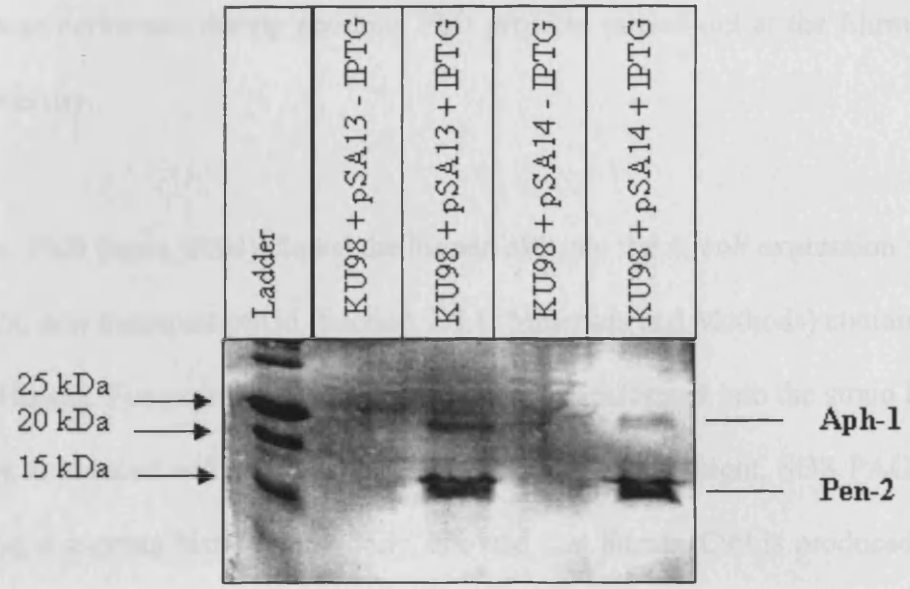


Figure (3.5) Western blot monitoring *pen-2* and *aph-1* expression from the plasmids pSA13 and pSA14

KU98 containing either pSA13 or pSA14 was grown at 28°C to an $O.D_{600}$ of approximately 0.5. Gene expression was then induced for 4 hours by the addition of 100 μM IPTG. The samples were analysed by SDS PAGE and Western blotting using a monoclonal α-penta histidine antibody from Sigma. Un-induced samples were used as controls, and molecular weight standards are given in kDa.

3.2.2 Cloning and Expression of *C99*, *PS-1* and *NicA*

This work was performed during previous PhD projects carried out at the Ehrmann laboratory, Cardiff University.

Sandra Grau (PhD thesis, 2004) cloned the human *c99* into the *E. coli* expression vector pCS19 producing the new construct pSG6 (Section 2.3.1, Materials and Methods) containing *c99* with a C-terminal His-tag. For expression studies, pSG6 was transformed into the strain KU98 and gene expression was induced with 10 μM IPTG for 4 hours and overnight. SDS PAGE and Western blotting using a α-penta histidine antibody, showed that human C99 is produced in *E. coli* and that it is stable after induction for 4 hours but it is degraded overnight at 28°C.

Mona Harnasch (PhD thesis, 2003) cloned and expressed *nicA*, *ps-1* and the gain of function mutant *ps-1Δexon9* which has been shown to have a role in early onset familial Alzheimer's disease (FAD). Human *ps-1* and *ps-1Δexon9* were cloned into the *E. coli* expression vector pGDR11, resulting in pMH12 and pMH13 containing *ps-1* and *ps1Δexon9* respectively, both with N-terminal His-tags. For expression studies, pMH12 and pMH13 were transformed into a number of strains including KU98. It was shown that in KU98, induction of gene expression with 100 μM IPTG resulted in the production of human PS-1 and PS-1Δexon9 in *E. coli*. It was found that at a high concentration the proteins migrated as a smear on SDS gels probably because of their hydrophobicity, but it was found that diluting the samples can aid the appearance of more distinct bands, which were verified by Western blotting using antibodies directed against the N-terminus of PS-1. The proteins were also stable after induction for 4 hours and overnight at 28°C in KU98. Human *nicA* was cloned into the *E. coli* vector pASK-IBA2 (Section 2.3.1, Materials and Methods) resulting in pMH42 containing *nicA* with a C-terminal *StrepII*-tag. For *nicA* expression, pMH42 was transformed into KU98 and gene expression was

induced with 100 nM A-tet for 3 hours. This revealed that human NicA can be produced in *E. coli* after induction for 3 hours at 28°C.

3.3 Topological studies of the human γ-secretase components in E. coli

Before any further studies can be performed with any of the γ-Secretase components, it is important to ensure that the human membrane proteins have reached their correct compartment in *E. coli* and that they are properly inserted into the bacterial cytoplasmic membrane.

3.3.1 Determining the topology of human Pen-2 and Aph-1 in the *E. coli* membrane

3.3.1.1 Construction of pen-2- and aph-1-alkaline phosphatase (phoA) fusions

To investigate the topology of Pen-2 and Aph-1 in *E. coli*, Pen-2-PhoA and Aph-1-PhoA hybrid proteins were constructed. PhoA is only active when it is translocated to the periplasm, where oxidising conditions allow its disulfide bonds, which are essential for its structure and therefore function, to be formed. The fusion of signal sequenceless *phoA* to appropriate *pen-2* or *aph-1* fragments aids determination of the localisation of the fusion joint of each Pen-2-PhoA or Aph-1-PhoA hybrid protein. High PhoA activity indicates a periplasmic localisation whereas low PhoA activity indicates a cytoplasmic localisation.

From their amino acid sequences, Pen-2 and Aph-1 were thought to have two and six TM segments respectively. Appropriate *pen-2* (1-2) and *aph-1* (1-6) fragments were amplified by PCR from the plasmids pSA1 and pSA2, using the "universal-fw-*XmaI* (Pen-2)" and "universal-fw-*XmaI* (Aph-1)" oligonucleotides for the forward PCR reaction for each of the *pen-2* and *aph-1* fragments respectively. The corresponding "N-*XbaI*-rev" oligonucleotide appropriate for each fragment was used for the reverse reaction. For a list of oligonucleotide sequences refer to section (2.3.2) in

Materials & Methods. These oligonucleotides were selected so that fragments ended directly in front of the predicted TM-segments (Figure 3.9).

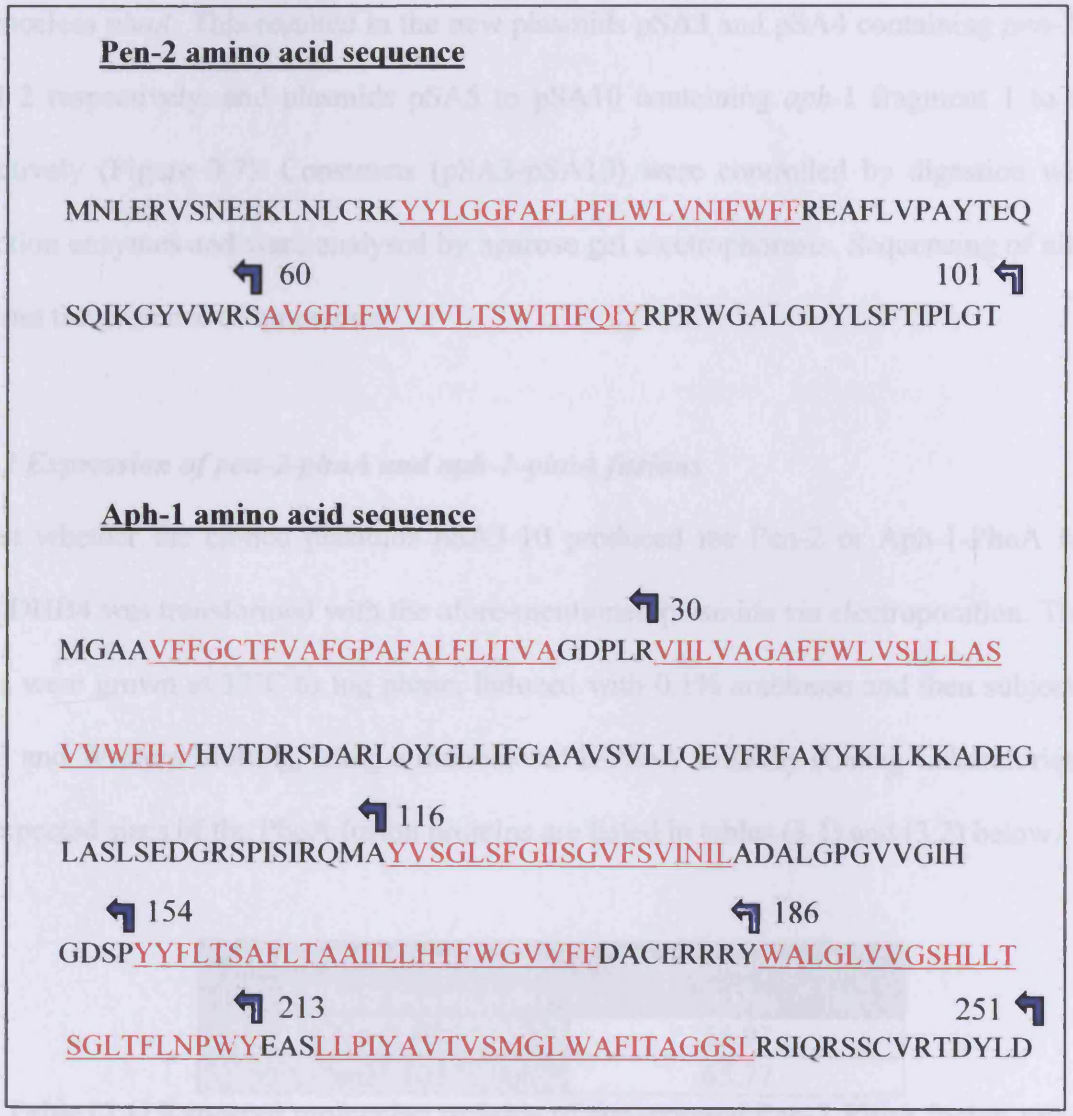


Figure (3.6) Amino acid sequence of Pen-2 and Aph-1.

Putative transmembrane regions are shown in red and are underlined, and the blue arrows indicate the positions of PhoA fusion joints to each of the transmembrane segments. The numbers of amino acids at the fusion joints are also shown.

The amplified fragments containing an *XbaI* site at the 5' and an *XmaI* site at the 3' end were cloned into the vector pEDIE3 upstream of *phoA* using these restriction sites. pEDIE3 has the arabinose promoter *pBAD*, an optimal translation initiation signal, a polylinker followed by signal sequenceless *phoA*. This resulted in the new plasmids pSA3 and pSA4 containing *pen*-2 fragments 1 and 2 respectively, and plasmids pSA5 to pSA10 containing *aph*-1 fragment 1 to fragment 6 respectively (Figure 3.7). Constructs (pSA3-pSA10) were controlled by digestion with suitable restriction enzymes and were analysed by agarose gel electrophoresis. Sequencing of all fragments ruled out the presence of mutations.

3.3.1.2 Expression of pen-2-phoA and aph-1-phoA fusions

To test whether the cloned plasmids pSA3-10 produced the Pen-2 or Aph-1-PhoA fusions, the strain DHB4 was transformed with the afore-mentioned plasmids via electroporation. The resulting strains were grown at 37°C to log phase, induced with 0.1% arabinose and then subjected to SDS PAGE and Western blotting, using a monoclonal α-PhoA antibody (Caltag Laboratories, Canada). The expected sizes of the PhoA fusion proteins are listed in tables (3.1) and (3.2) below.

Amino acid number at Pen-2-PhoA fusion joint	Molecular weight (kDa)
Pen-2 60	55.91
Pen-2 101	65.77

Table (3.1) Expected molecular weights of constructed Pen-2-PhoA fusion proteins

Amino acid number at Aph-1-PhoA fusion joint	Molecular weight (kDa)
Aph-1 30	51.58
Aph-1 116	61.21
Aph-1 154	64.92
Aph-1 186	68.73
Aph-1 213	71.70
Aph-1 251	75.85

Table (3.2) Expected molecular weights of constructed Aph-1-PhoA fusion proteins

Pen-2-PhoA fusions were shown to be produced and were stable after induction for 4 hours and overnight. However, Pen-2-PhoA fragment 1 which was encoded by pSA3 and postulated to have the fusion joint in the cytoplasm showed almost undetectable expression levels (Figure 3.8 C and D). Aph-1-PhoA fragments 1, 3 and 5 which were encoded by pSA5, pSA7 and pSA9 respectively were all expressed and were stable after induction for 4 hours and overnight. However, Aph-1-PhoA fragments 2, 4 and 6 which were encoded by pSA6, pSA8 and pSA9 respectively could not be detected by the α-PhoA antibody. This is common behaviour for membrane protein-PhoA hybrids that have PhoA translocated to the cytoplasm. This results in a misfolded proteolytic domain and protein removal by cellular proteases. Therefore, the protein hybrids could not be detected.

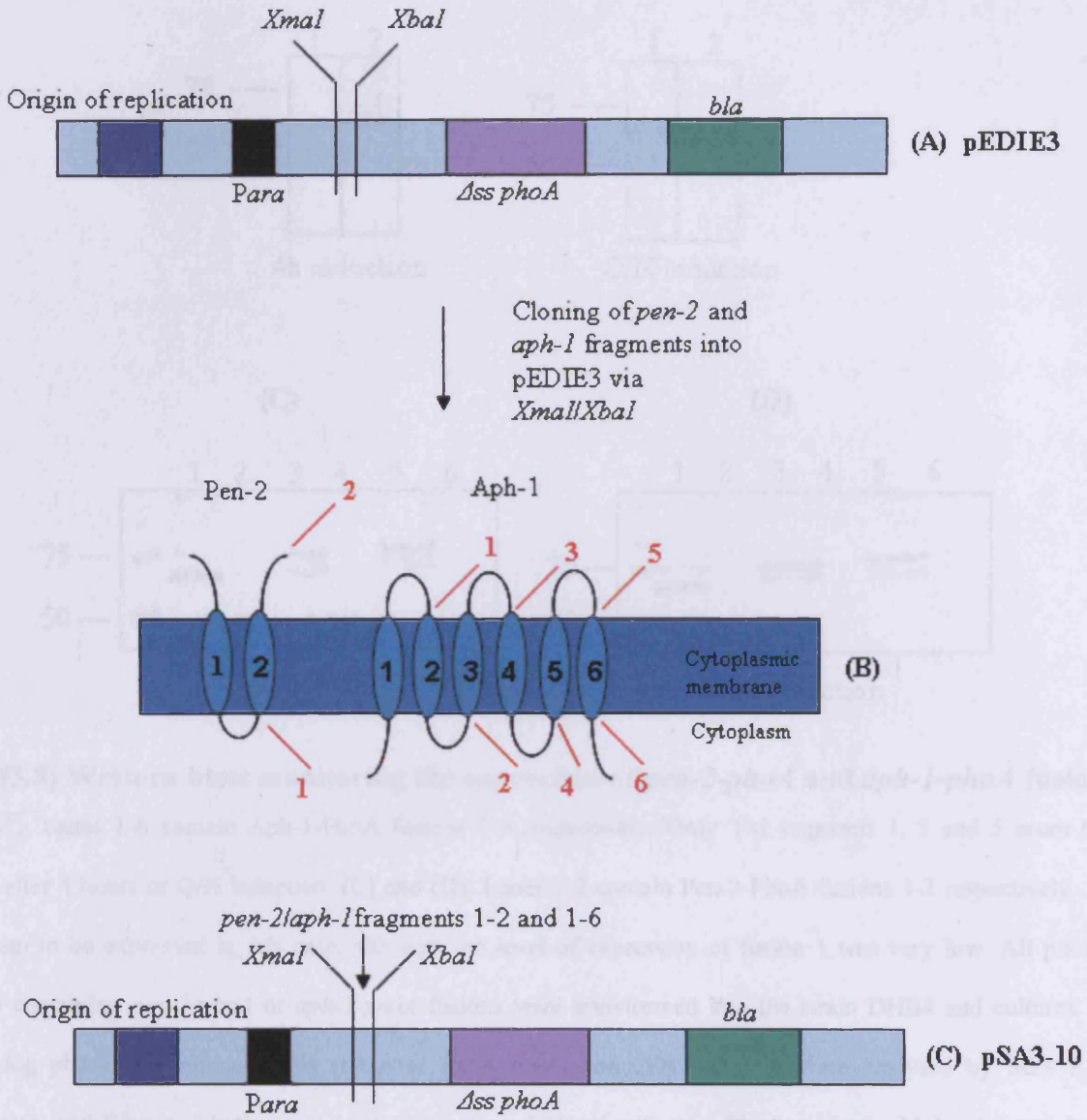


Figure (3.7) Schematic representation showing the cloning of *pen-2* and *aph-1* fragments into pEDIE3.

(A) Is the empty vector pEDIE3, which has an arabinose promoter (P*ara*) and signal sequenceless *phoA* (*Δss phoA)* preceded by the multiple cloning site. (B) Diagram of Pen-2 and Aph-1 showing their predicted transmembrane segments. The positions of the PhoA fusion joints are shown in red. (C) Cloning of *pen-2* and *aph-1* fragments into PEDIE3 via *XbaI/XmaI* to generate plasmids pSA3 and pSA4 for *pen-2-phoA* fragments 1 and 2 respectively, and plasmids pSA5 to pSA10 containing *aph-1-phoA* fragments 1 to 6 respectively.

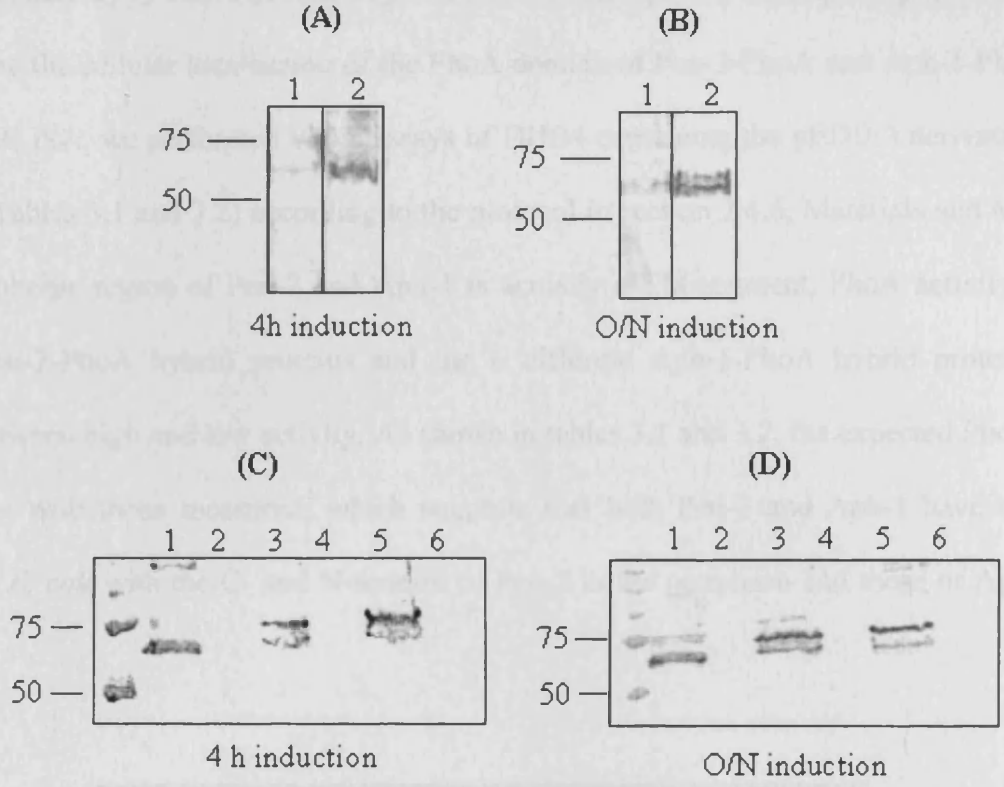


Figure (3.8) Western blots monitoring the expression of *pen-2-phoA* and *aph-1-phoA* fusions.

(A) and (B): Lanes 1-6 contain Aph-1-PhoA fusions 1-6 respectively. Only TM segments 1, 3 and 5 seem to be expressed after 4 hours or O/N induction. (C) and (D): Lanes 1-2 contain Pen-2-PhoA fusions 1-2 respectively. Both fusions seem to be expressed in this case, although the level of expression of fusion 1 was very low. All plasmids (pSA3-10) containing *pen-2-phoA* or *aph-1-phoA* fusions were transformed into the strain DHB4 and cultures were grown to log phase and induced with arabinose for 4 hours and O/N. Samples were analysed by SDS PAGE electrophoresis and Western blotting and the proteins were detected with an α-PhoA antibody. Molecular weights are given in kDa.

3.3.1.3 PhoA activity of PhoA domains of Pen2-PhoA and Aph-1-PhoA hybrid proteins

To determine the cellular localisation of the PhoA domain of Pen-2-PhoA and Aph-1-PhoA hybrid proteins in *E. coli*, we performed PhoA assays of DHB4 containing the pEDIE3 derivatives, pSA3 to pSA10 (Tables 3.1 and 3.2) according to the protocol in section 2.4.6, Materials and Methods. If each hydrophobic region of Pen-2 and Aph-1 is actually a TM-segment, PhoA activity for the 2 different Pen-2-PhoA hybrid proteins and the 6 different Aph-1-PhoA hybrid proteins should alternate between high and low activity. As shown in tables 3.1 and 3.2, the expected PhoA activity values agree with those measured, which suggests that both Pen-2 and Aph-1 have the correct topology in *E. coli* with the C- and N-termini of Pen-2 in the periplasm and those of Aph-1 in the cytoplasm.

Number of amino acid at the Pen-2-PhoA fusion joint	Expected PhoA activity	Measured PhoA activity (mU/mg protein)
60	Low	7
100	High	21

Table (3.3) PhoA Activities of Pen-2-PhoA fusions

Number of amino acid at the Aph-1-PhoA fusion joint	Expected PhoA activity	Measured PhoA activity (mU/mg protein)
30	High	11
116	Low	<1
154	High	16
186	Low	<1
213	High	16
251	Low	<1

Table (3.4) PhoA activities of Aph-1-PhoA fusions

3.3.2 Determining the topology of PS-1 in the *E. coli* membrane

Similarly, Mona Harnasch (PhD thesis, 2003) investigated the topology of human PS-1 in the *E. coli* membrane by constructing Pho-A fusions with different PS-1 fragments (Figure 3.9). PS-1 has always been described as an eight transmembrane domain protein (Li et al., 1997), however, Kyle and Doolittle hydropathy blots have revealed an extra hydrophobic region at the very C-terminus of the protein. Therefore, ten PS-1-PhoA hybrid proteins were constructed to verify the topology of the PS-1 expressed in *E. coli* and to investigate the possibility of a ninth transmembrane segment. This resulted in plasmids pMH2 to pMH11, containing *ps-1-phoA* fusions 1 to 10 respectively.

For expression studies pMH2 to pMH11 were transformed into the strain KU104 (Section 2.2.1, Materials and Methods), and gene expression was induced by the addition of 0.2% arabinose for 3 hours. Whole cell extracts were then analysed by SDS PAGE and Western blotting using a α-PhoA antibody (Caltag Laboratories, Canada). All *ps-1-phoA* hybrids were expressed in *E. coli*. PhoA assays were also performed with the PS-1-PhoA hybrid proteins to reveal the cellular location of the PS-1-PhoA fusion joints. The PhoA assays yielded results which were consistent with the eight transmembrane segment model of PS-1, but indicated the presence of a ninth transmembrane segment at the very C-terminus of the protein (Harnasch et al., 2004).

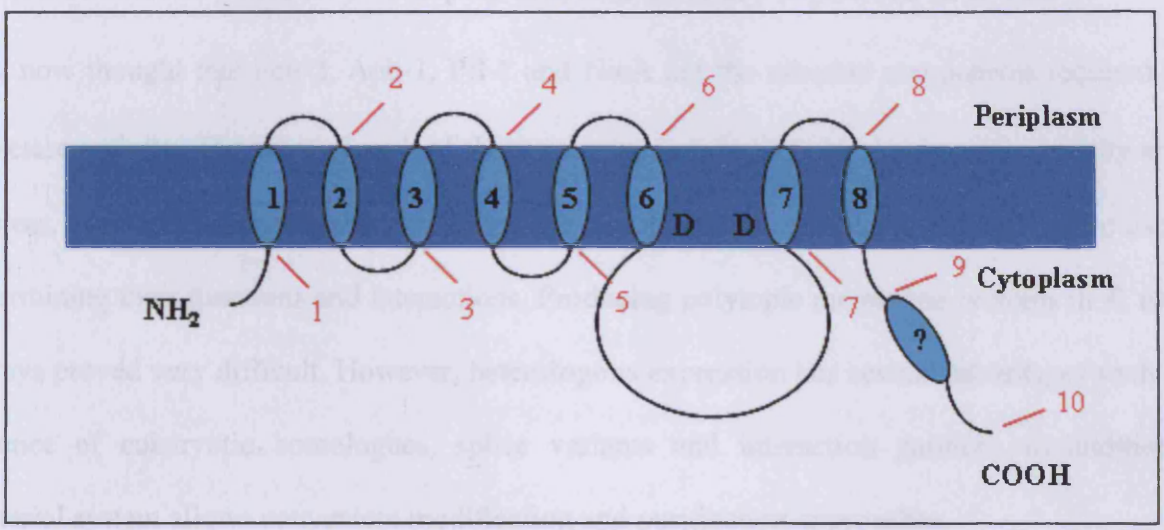


Figure (3.9) Schematic representation of PS-1.

The diagram shows the predicted transmembrane segments, and the PhoA fusion joints are indicated in red.

3.4 Chapter Discussion

3.4.1 The human γ-secretase components, and C99, are expressed in *E. coli*

It is now thought that Pen-2, Aph-1, PS-1 and NicA are the minimal components required for γ-secretase activity. The roles of each of these proteins in bringing about γ-secretase activity are still unclear, therefore producing these proteins in a simple system such as *E. coli* will prove useful in determining their functions and interactions. Producing polytopic membrane proteins in *E. coli* has always proved very difficult. However, heterologous expression has several advantages such as the absence of eukaryotic homologues, splice variants and interaction partners. In addition, the bacterial system allows convenient modification and purification approaches.

Here, we cloned the human genes *pen-2* and *aph-1* into an *E.* coli vector and produced the recombinant proteins in *E. coli* cells. Both proteins were produced in *E. coli* after 4 hours of induction with IPTG and Pen-2 was found to be stable overnight. Aph-1 was not detected after overnight induction which was probably due to degradation by *E. coli* proteases (Data not shown). It was thought that the presence of other γ-secretase components such as PS-1 and the γ-secretase substrate C99, may aid the stability of Aph-1 or increase the amount of Pen-2 and Aph-1 produced in the bacterial cells, but this was not found to be the case. The gain of function mutant PS1Δexon9 was found to be involved in FAD and therefore co-expressing this protein with Pen-2 or Aph-1 in the future may yield the expected result. Additionally, the level of Aph-1 expression from pSA13 and pSA14 at 28°C was found to be low and the growth of KU98 cells containing any plasmids containing *aph-1* was found to be very slow. Therefore, future expression of Aph-1 from these plasmids for the purposes of e.g. enzyme assays, were performed at 20°C because it appeared that producing Aph-1 at higher temperatures was stressful for the cells.

As mentioned before, it was important to combine some of the γ-secretase components in the same plasmids to allow their combined expression in one *E. coli* strain for the purpose of performing γ-secretase assays. As we were uncertain about what the best combinations of Pen-2 and Aph-1 would be and how the presence of N- or C-terminal His-tags will affect gene expression or protein topology, function and activity, more plasmids were constructed containing different combinations of *pen-2* and *aph1*. One of the plasmids constructed was pSA16 containing tag-less *pen-2* and *aph-1* with an N-terminal His-tag. This plasmid can also be used for co-purification studies as Aph-1 can be purified with a Ni^{2+} or Cu^{2+} column and the behaviour of Pen-2 could be observed. Also, as another alternative for the combination of *pen-2* and *aph-1*, pSA18 was constructed which contains *pen-2* and *aph-1* with no His-tags.

3.4.2 The human γ-secretase components are correctly inserted into the bacterial cytoplasmic membrane

There is a large body of evidence that suggests that the rules and signals that determine membrane insertion of complex integral cytoplasmic membrane proteins are conserved between species (van Geest and Lolkema, 2000). However, it is still important to ensure correct insertion of human membrane proteins into the *E. coli* membrane, as that represents an important step towards functional expression in bacteria. A genetic approach that involves using gene fusions to determine topology has been developed by Beckwith and co-workers and has been successfully used to determine the topology of over 100 integral cytoplasmic membrane proteins in *E. coli*. Originally constructed for topology studies of bacterial membrane proteins, the *phoA* fusion method was also successfully utilised for recombinant human membrane proteins in *E. coli*, such as the human beta 2-adrenergic receptor (7 TM-segments), the human vasopressin V2 receptor (7 TM-segments), cyclic nucleotide-gated ion channels (6 TM-segments) and human presenilin-1 (8/9 TM-segments) (Harnasch, 2003; Henn et al., 1995; Lacatena et al., 1994; Schulein et al., 1996). Here, we show

that the same technique can be used to determine the topology of human Pen-2, Aph-1 and PS-1 in *E. coli.*

To determine the topology of Pen-2 and Aph-1, eight plasmids were constructed (pSA3 to pSA10) that contain fusions of *pen-2* or *aph*-1 fragments to signal sequenceless *phoA*. The nucleotide sequence of Pen-2 has indicated that it probably has two TM-segments, and that both its N- and C-termini are in the periplasm. Whereas the nucleotide sequence of Aph-1 has indicated that it probably has 6 TM-segments and that both its N- and C-termini are in the cytoplasm. Expression of the gene fusions in pSA3 and pSA4 (corresponding to fusions 1 and 2 respectively) produced the Pen-2-PhoA hybrid proteins and they appeared to have approximately the molecular weight expected (Figure 3.11). However, the Pen-2-PhoA fusion 1 was not expressed as well. This could be due to the fact that this fusion joint resides within the cytoplasm and is therefore degraded by cellular proteases. Expression of the gene fusions in pSA5, pSA7 and pSA9 (corresponding to fusions 1, 3 and 5 respectively) produced the Aph-1-PhoA hybrid proteins and they appeared to have approximately the molecular weight expected. However, the gene fusions in pSA6, pSA8 and pSA10 (corresponding to fusions 2, 4 and 6 respectively) were not detected after induction of gene expression and Western blotting. This could, again, be due to degradation by cellular proteases. The PhoA assays of the PhoA domains of the hybrid proteins (Tables 3.3 and 3.4) have supported the expected topology for both Pen-2 and Aph-1. However, it can be argued that low PhoA activities were due to low or no expression of the corresponding *phoA* hybrids.

3.4.3 Conclusion

We describe previous cloning and expression of human *ps-1*, *ps1*Δexon9, *nicA* and *c99* and topological studies of PS-1, in *E. coli*. Additionally, in this chapter, successful cloning and expression of *pen-2* and *aph-1* and topological studies of the human proteins in *E. coli* were carried

out. This represented a significant first step towards studying the human γ-secretase complex in bacteria.

CHAPTER (4)

ANALYSIS OF THE γ-SECRETASE COMPONENTS

4 ANALYSIS OF THE HUMAN γ-SECRETASE COMPONENTS PRODUCED IN *E. COLI*

4.1 Introduction

As all four γ-secretase components (Pen-2, Aph-1, PS-1 and NicA) and the γ-secretase substrate (C99) were successfully expressed in *E. coli*, we decided to purify some of the proteins from *E. coli* cells. In this work, fast protein liquid chromatography (FPLC, Section 2.4.8, Materials and Methods) using Ni NTA was used to purify the recombinant human proteins from *E. coli* cells. Here we describe successfully purifying Pen-2 from the *E. coli* strain KU98. The availability of pure Pen-2 will be valuable for future assays aimed at further analysing the γ-secretase complex and its activity. Pure Pen-2 can be used for interaction studies and the production of antibodies directed against Pen-2. Additionally, the pure protein can be analysed by mass spectrometry to answer such questions as the oligomeric state of Pen-2 and post-translational modifications. Furthermore, the Pen-2 can be used in experiments that involve mixing of purified γ-secretase components to analyse the processing of C99 and Aβ production, and to answer stoichiometric questions in complex assembly.

In this chapter we also describe the successful purification of C99 from the strain KU98, and subsequently analysing the pure protein by mass spectrometry. Additionally, pure C99 was used as a substrate for γ-secretase assays performed in *E. coli* by either combining all four γ-secretase components in the same strain, or by combining membranes of individual components. γ-Secretase activity has been successfully reconstituted in eukaryotic cells but this did not rule out the presence of other endogenous proteins that could have been required for the activity. Therefore, bacteria provided a suitable system for the attempted reconstitution of γ-secretase activity, to assess whether these four components are in fact the minimum components required.

4.2 Purification studies of Pen-2

The *pen-2* gene, cloned into pSA1 contains an N-terminal His-tag. This allows the purification of Pen-2 by FPLC, using a Ni NTA chromatography column. Before the purification procedure could be performed, we needed to verify that Pen-2 was present in the bacterial membrane after expression. Hence, membranes containing the 6X histidine tagged Pen-2 were prepared.

4.2.1 Pen-2 is present in the membrane

A single colony of the strain KU98 containing the plasmid pSA1 (*pen-2* with an N-terminal *his* tag), was used to inoculate 200 ml of double NZA containing 200 μg/ml Amp and the culture was grown overnight at 28°C with shaking at 220 rpm. The overnight culture was then diluted 1/20 in the same medium (4 l) and grown until the $O.D_{600}$ reached approximately 0.5. Expression of *pen-2* was induced by the addition of 100 μM IPTG and the culture was grown further at 20°C overnight, given that Pen-2 was found to be stable after overnight induction (Section 3.2.1.4, Chapter 3). Membranes were prepared as described in section 2.4.8.4, Materials and Methods. To solubilise the membrane proteins, they were re-suspended in lysis buffer containing 1% SDS and the mixture was stirred at room temperature for 2 hours. Here, a strong detergent was used rather than a mild detergent such as CHAPSO because Pen-2 is predicted to have no tertiary structure. In addition, the SDS was removed at a later stage to allow oligomerisation to occur. Samples from the different stages of the preparation and solubilisation of membranes were collected and approximately 30 μg (total protein, measured using the BCA kit from Sigma, Section 2.4.8.5, Materials and Methods) of each sample were loaded on Schaegger gels (Table 2.8, Materials and Methods) and subjected to Coomassie staining and Western blotting to monitor the efficiency of the procedure. Figure (4.1) shows that Pen-2 was present in the membrane fraction, and that solubilisation of the membrane proteins with SDS was effective. Western blotting confirmed that the majority of the Pen-2 in the bacterial membrane was solubilised with 1% SDS. However, the purification of Pen-2, solubilised

from membranes was attempted a few times without success, as only a very small quantity of pure protein was collected after purification. Therefore it was suggested that Pen-2 was solubilised from whole cells to minimise loss of the protein during membrane preparation, solubilisation and FPLC.

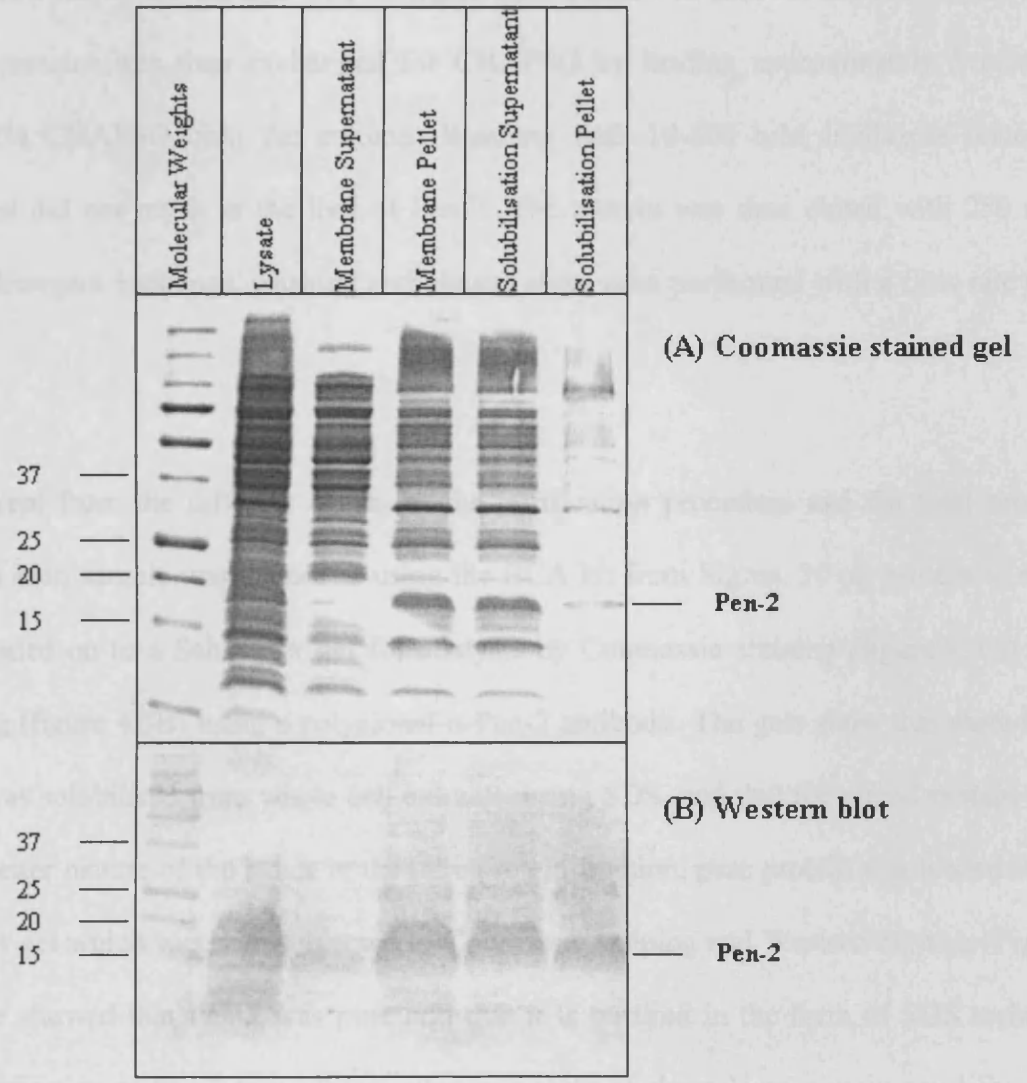


Figure (4.1) Coomassie stained gel (A) and Western blot (B) monitoring the preparation of KU98 membranes containing Pen-2.

A 4 l culture of KU98 containing pSA1 *(pen-2* with an N-terminal *his* tag) was grown to log phase, when gene expression was induced overnight by the addition of 100 μM IPTG. Membranes were prepared as described in Materials and Methods and proteins were solubilised with 1% SDS for 2 hours at room temperature. Samples were removed after every stage of the procedure and loaded on Schaegger gels for Coomassie staining and Western blotting using a polyclonal antibody directed against the C-terminus of Pen-2. Molecular weights are given in kDa.

4.2.2 Purification of Pen-2 from *E. coli* by FPLC

Pen-2 was purified from whole cells by FPLC using Ni NTA, as described in section 2.4.8 in Materials and Methods. Solubilised proteins from whole cell extracts were loaded onto the Ni NTA column with a flow rate of 0.5 ml/min. The detergent SDS which was used for the solubilisation of the membrane proteins was then exchanged for CHAPSO by loading approximately 5 column volumes of 0.2% CHAPSO onto the column. Washing with 10-100 mM imidazole removed contaminants but did not result in the loss of Pen-2. The protein was then eluted with 250 mM imidazole. All detergent exchange, washing and elution steps were performed with a flow rate of 2 ml/min.

Samples were kept from the different stages of the purification procedure and the total protein concentration in each sample was measured using the BCA kit from Sigma. 30 μg protein of each sample were loaded on to a Schaegger gel for analysis by Coomassie staining (figure 4.3A) and Western blotting (figure 4.3B) using a polyclonal α-Pen-2 antibody. The gels show that more than 50% of Pen-2 was solubilised from whole cell extracts, using SDS, and that the eluted protein was pure. To get a better picture of the bands in the pure protein fraction, pure protein was loaded on to a new Schaegger gel which was then subjected to Coomassie staining and Western blotting (Figure 4.4). This figure showed that Pen-2 was pure and that it is purified in the form of SDS resistant dimers. The purification of Pen-2 typically resulted in a yield of about 1 mg protein per 4 litres of culture.

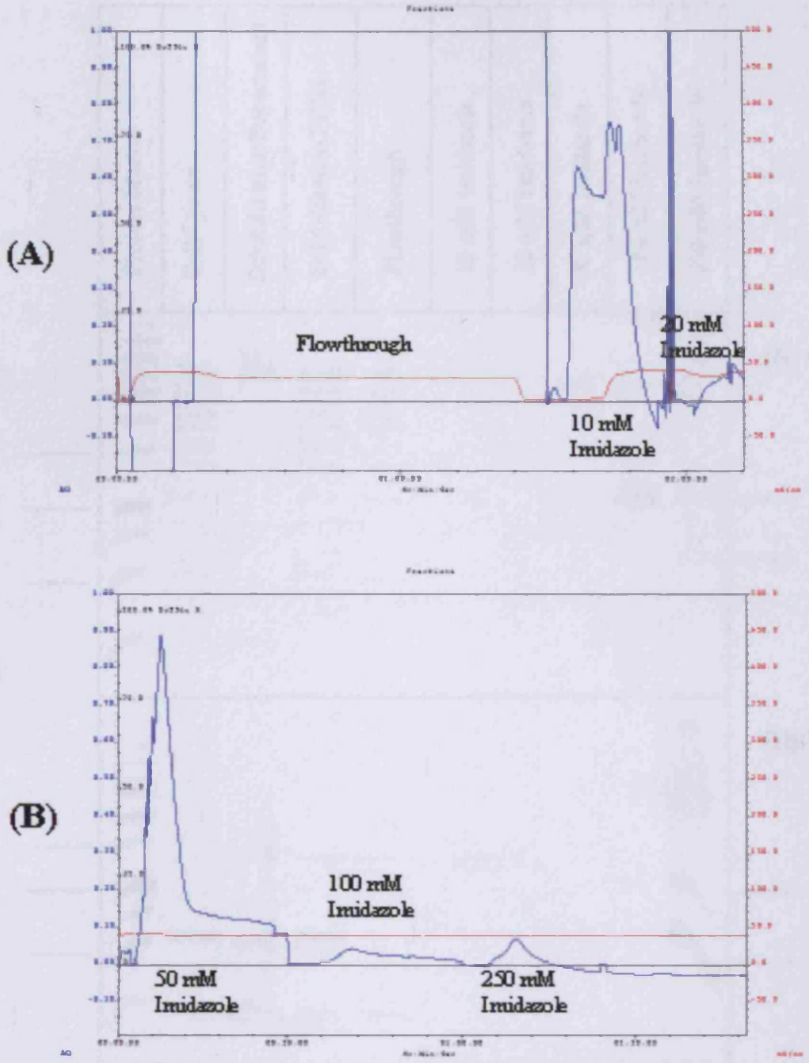


Figure (4.2) Purification profile of Pen-2

4 l of KU98 cells producing Pen-2 were stirred at room temperature with 1% SDS to solubilise all the membrane proteins. The mixture was centrifuged at 13,000 rpm for 10 minutes and the pellet was discarded. The supernatant containing the solubilised proteins was loaded onto a Ni NTA column. The column was washed with (A) 10 mM and 20 mM imidazole (B) 50 mM and 100 mM imidazole to remove contaminants, and then pure Pen-2 was eluted (B) with 250 mM imidazole.

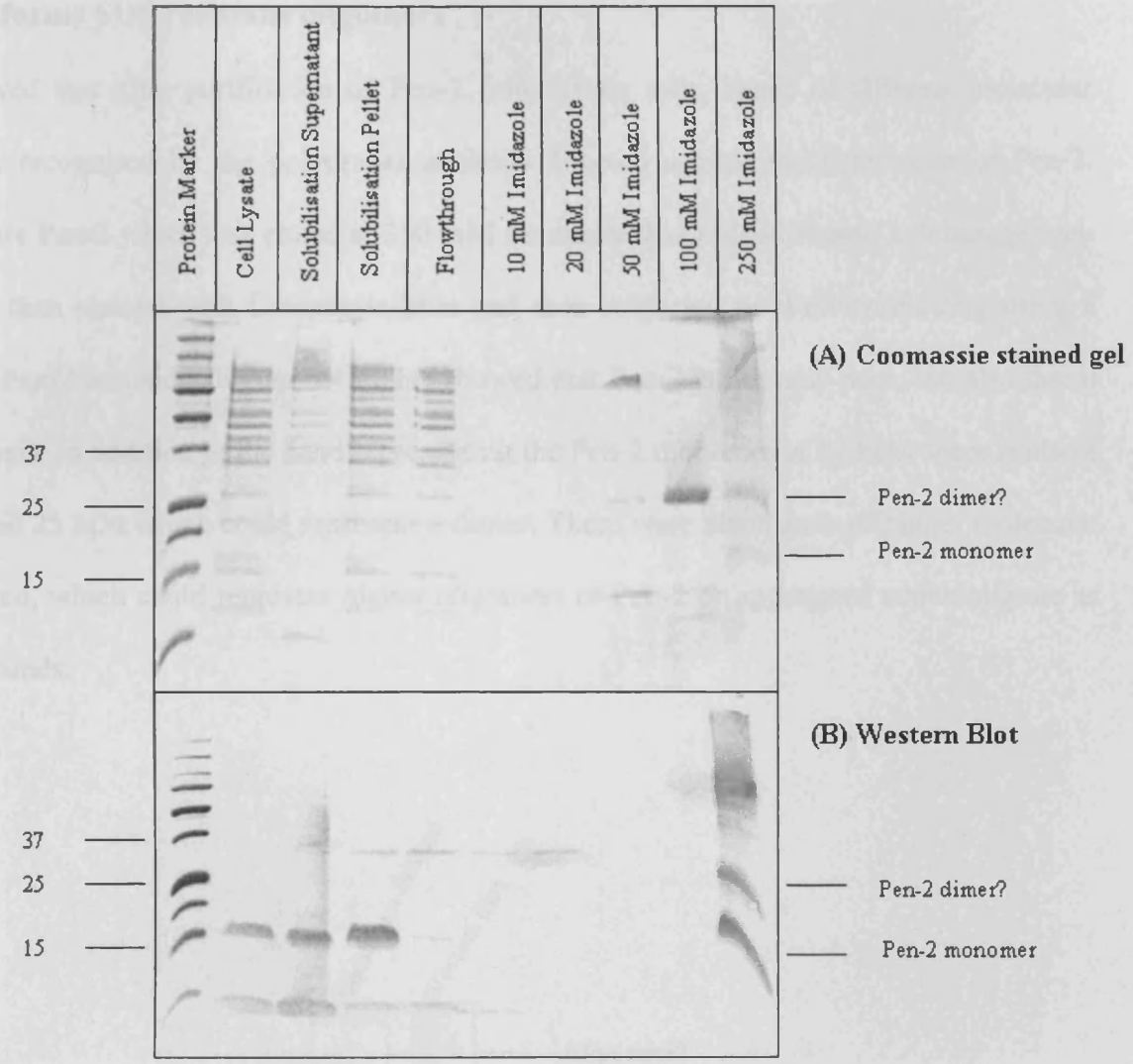


Figure (4.3) Coomassie stained gel and Western blot monitoring solubilisation and purification of Pen-2.

Cells from a 4 l culture were stirred at room temperature in 1% SDS for 2 hours. The supernatant was then loaded onto a Ni NTA column. The column was washed in increasing concentrations of imidazole and then pure pen-2 was eluted with 250 mM imidazole. The samples were analysed by Schaegger gels and Western blotting using a polyclonal α-Pen-2 antibody. Molecular weights are given in kDa.

4.2.3 Pen-2 forms SDS resistant oligomers

It was observed that after purification of Pen-2 from whole cells, bands of different molecular weights were recognised by the polyclonal antibody directed against the C-terminus of Pen-2. Therefore, pure Pen-2 which was eluted at 250 mM imidazole was re-loaded onto a Schaegger gel. The gel was then stained with Coomassie blue and then subjected to Western blotting using a polyclonal α-Pen-2 antibody (Figure 4.4). This showed that Pen-2 is not only pure, but also that it forms oligomers. In addition to the band representing the Pen-2 monomer at 12 kDa, there is also a band at around 25 kDa which could represent a dimer. There were also bands of higher molecular weight detected, which could represent higher oligomers of Pen-2 or aggregates which migrate as non-defined bands.

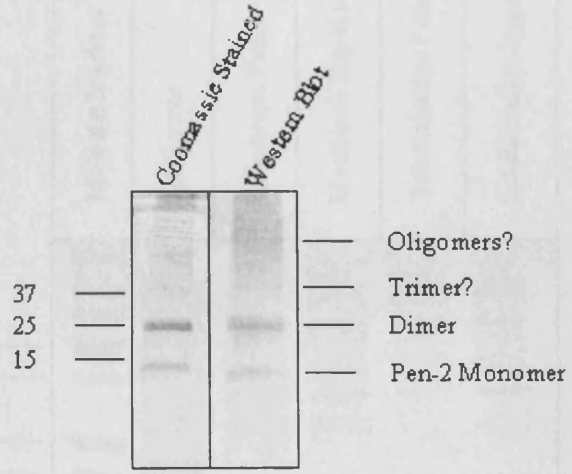


Figure (4.4) Gels monitoring the oligomerisation state of Pen-2.

Pure Pen-2 was loaded onto a Schaegger gel. In addition to the Pen-2 monomer, Coomassie staining and Western blotting with a polyclonal α-Pen-2 antibody revealed the presence of high molecular weight bands, which could represent oligomers of Pen-2. Molecular weights are given in kDa.

4.3 Purification of C99

This was performed as described in Sandra Grau, PhD Thesis, 2004. Membranes of the strain KU98 expressing C99 were prepared as described in Materials and Methods and solubilisation of the membrane proteins with 1% SDS at room temperature was successful. The cleared supernatants were then loaded onto a Ni NTA column with a flow rate of 0.5 ml/min. The detergent (SDS) was exchanged for 0.025% DDM which was loaded onto the column. Washing with 20 mM imidazole removed contaminants but did not result in the loss of C99. Pure C99 was then eluted with 500 mM imidazole. The purification of C99 typically yielded approximately 1.5 mg protein per 4 litres of culture.

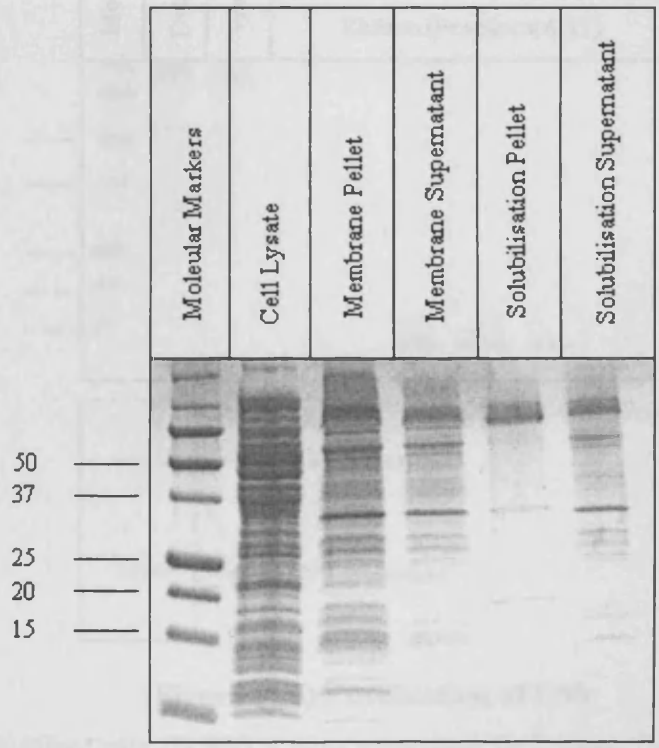


Figure (4.5) Coomassie stained schaegger gel monitoring the preparation of membranes and solubilisation of C99 from bacteria.

The Coomassie gel showed that membrane preparation from KU98 cells producing C99 was successful and that solubilisation worked well, as a larger amount of total protein was present in the supernatant fraction than that in the pellet fraction. Molecular weights are given in kDa.

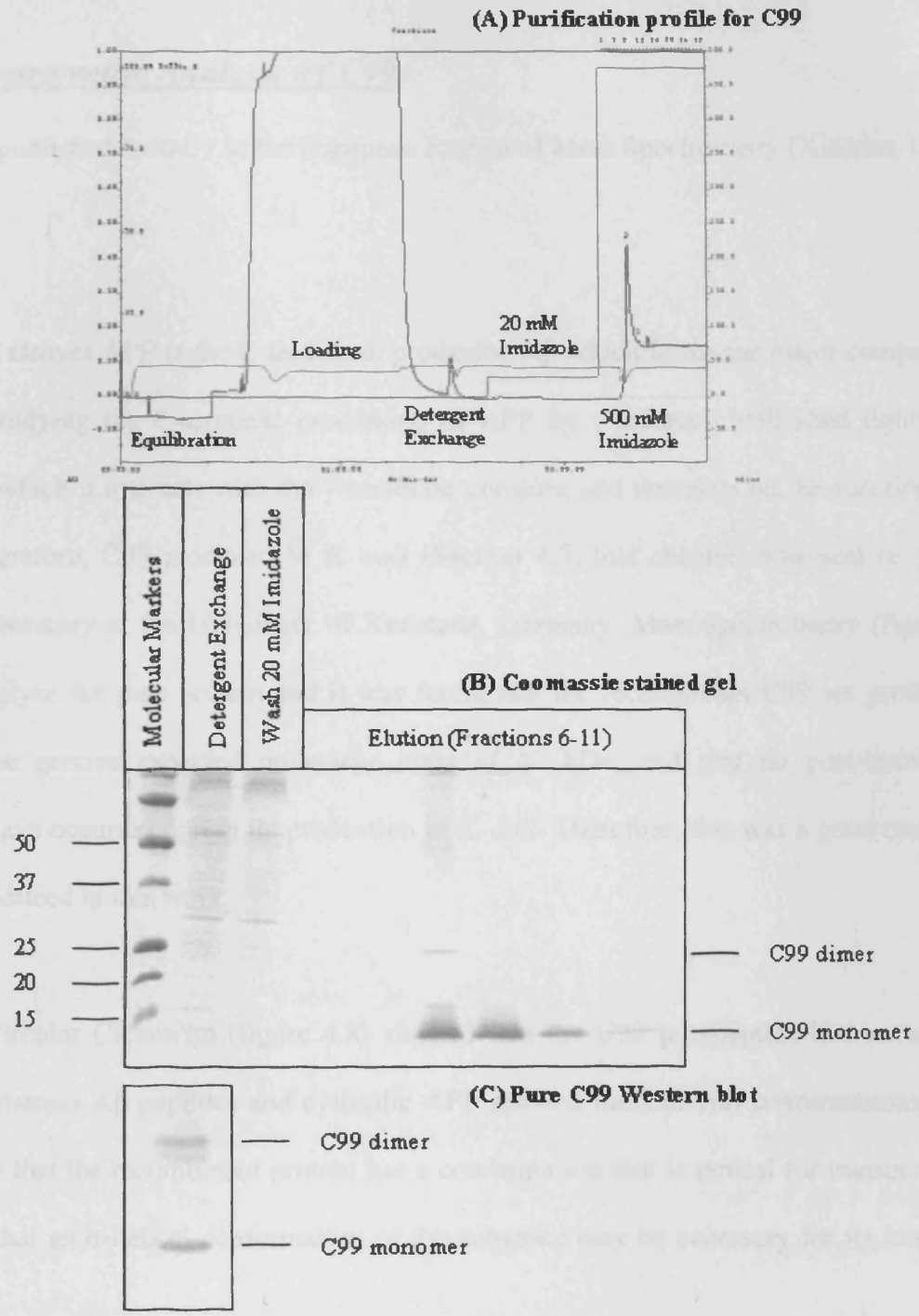


Figure (4.6) Purification of C99.

C99 membranes were solubilised with 1% SDS at room temperature for 2 hours. The cleared supernatant was loaded onto a Ni NTA column with a flow rate of 0.5 ml/min. The detergent (SDS) was exchanged for 0.025% DDM which was loaded onto the column. Washing with 20 mM imidazole removed contaminants but did not result in the loss of C99. Pure C99 was then eluted with 500 mM imidazole. Samples from different stages of the purification were loaded on Schaegger gels where they were Coomassie stained and subjected to Western blotting using a polyclonal α-C99 antibody. Molecular weights are given in kDa.

4.4 Mass Spectrometric Analysis of C99

This work was published recently in the European Journal of Mass Spectrometry (Xiaodan Tian, 2004).

The γ-secretase cleaves APP at the C-terminus, producing Aβ which forms the major component of AD plaques. Studying the C-terminal processing of APP by γ-secretase will shed light on the mechanism by which it interacts with the γ-secretase complex and therefore on the function of the γ-secretase. Therefore, C99 produced in *E. coli* (Section 4.3, this chapter) was sent to Michael Przybylski's laboratory at the University of Konstanz, Germany. Mass spectrometry (figure 4.7) was used to analyse the pure protein and it was found that the recombinant C99 we produced in bacteria had the precise expected molecular mass of 12 kDa, and that no post-translational modifications have occurred during its production in *E. coli*. Therefore, this was a good control for the material produced in this work.

Additionally, Circular Cichroism (figure 4.8) showed that the C99 polypeptide had an α-helical conformation whereas Aβ peptides and cytosolic APP showed random coil conformations. These results illustrate that the recombinant protein has a conformation that is typical for transmembrane segments, and that an α-helical conformation of the substrate may be necessary for its interaction with γ-secretase.

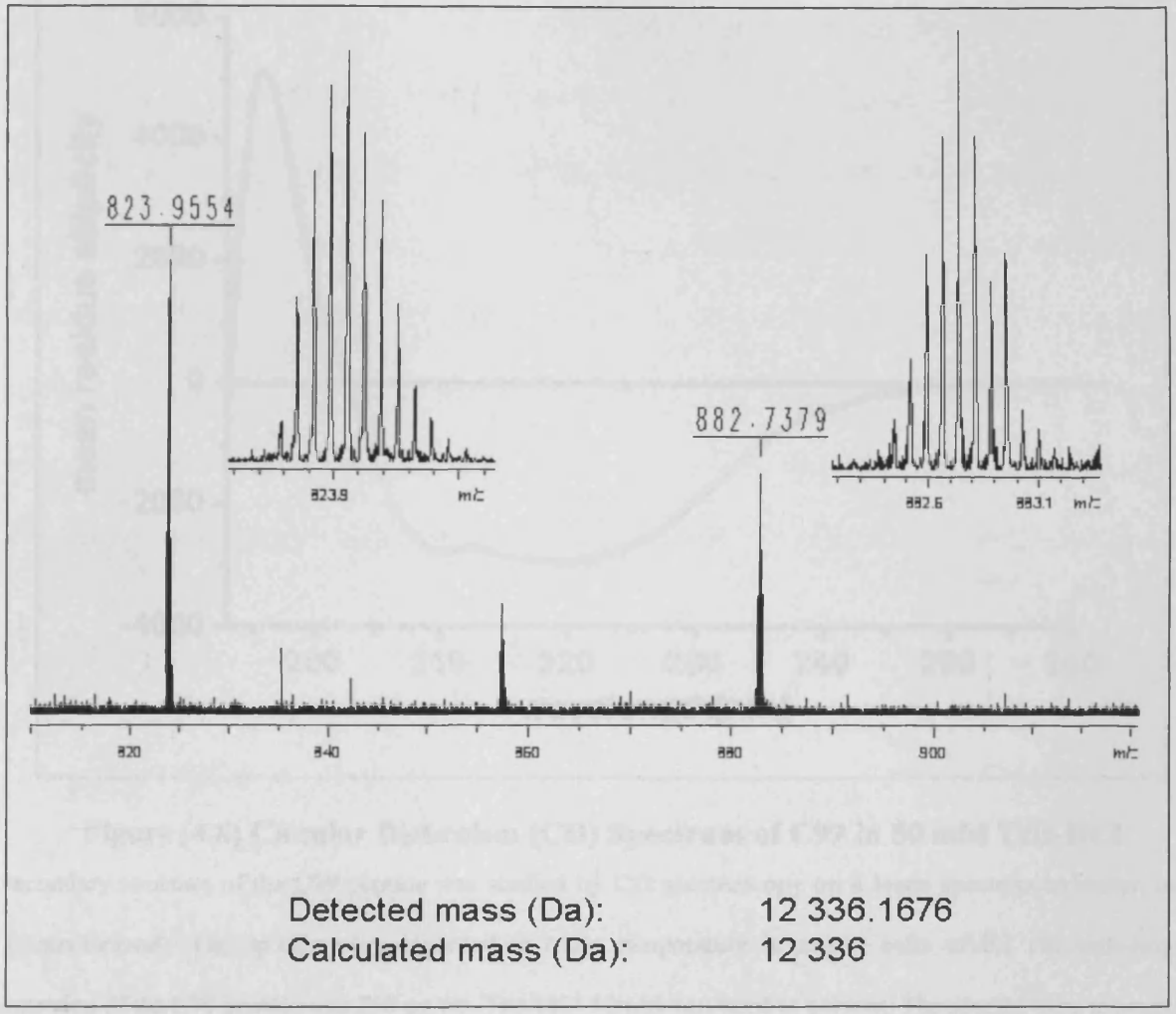


Figure (4.7) Mass spectrometric analysis of C99

The FTICR measurements were performed with a Bruker (Bruker Daltonik, Bremen, Germany) Apex II FT-ICR mass spectrometer equipped with a 7T superconducting magnet, a cylindrical infinity ICR analyser cell and an external Scout 100 fully automated X-Y target stage Maldi source with pulsed collision gas. A 100 mg/ml solution of 2,5-di-dihydroxybenzoic acid (DHB)in acetonitrile:0.1% trifluoroacetic acid in water (2:1) was used as matrix for sample preparation. Calibration was performed with a standard peptide mixture with an m/z range of approximately 2.500.

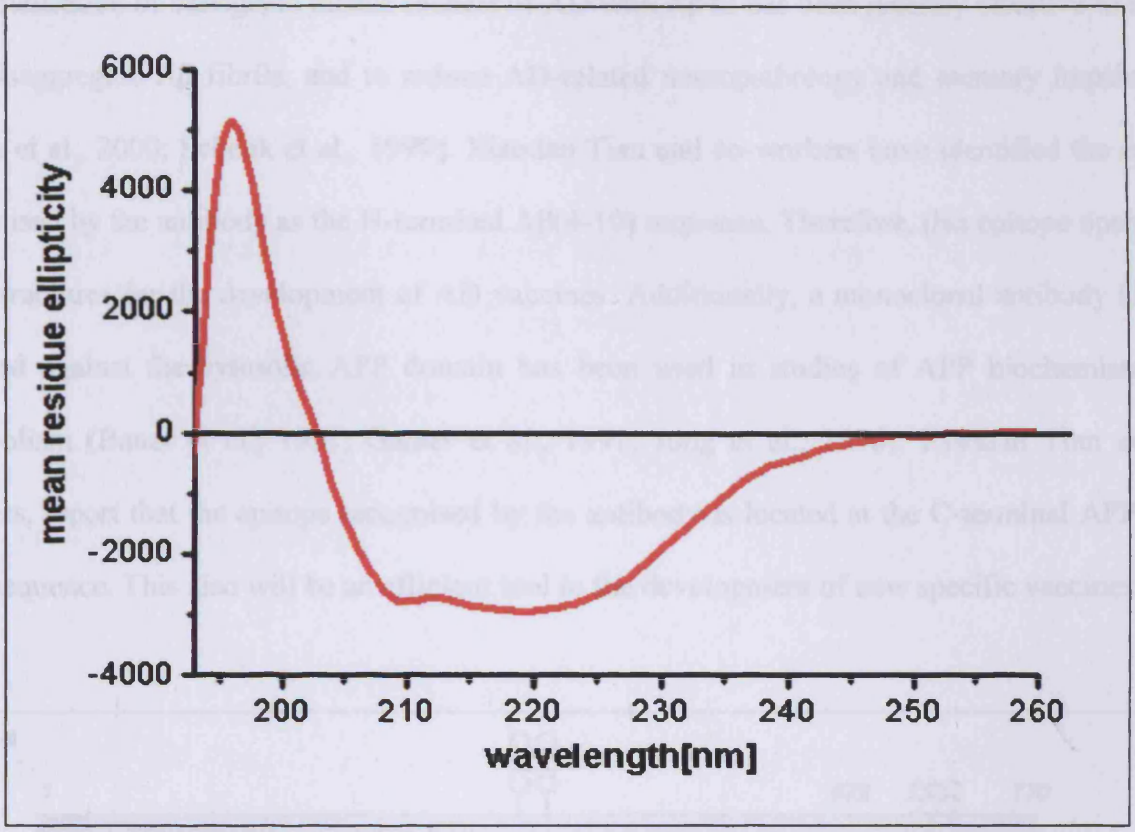


Figure (4.8) Circular Dichroism (CD) Spectrum of C99 in 50 mM Tris-HCl

The secondary structure of the C99 peptide was studied by CD spectroscopy on a Jasco spectropolarimeter, model J-720 (Jasco,Finland). The spectra were recorded at room temperature in quartz cells of 0.1 cm path-length The concentration of the C99 peptide was 250 μg/ml. Tris-HCl 50mM was used as solvent. The spectra were averages of six scans between λ=190 and 260 nm. Results are expressed in tems of mean residue ellipticity in units of deg cm2 dmol-1 after subtraction of buffer base-line

Immmunisation of transgenic mouse models of AD with Aβ42 has been recently effective to inhibit and disaggregate Aβ fibrils, and to reduce AD-related neuropathology and memory impairments (Janus et al., 2000; Schenk et al., 1999). Xiaodan Tian and co-workers have identified the epitope recognised by the antibody as the N-terminal Aβ(4-10) sequence. Therefore, this epitope opens new lead structures for the development of AD vaccines. Additionally, a monoclonal antibody (JmAb) directed against the cytosolic APP domain has been used in studies of APP biochemistry and metabolism (Bauer et al., 1991; Ganter et al., 1991; Jung et al., 1996). Xiaodan Tian and co-workers, report that the epitope recognised by the antibody is located at the C-terminal APP (740-747) sequence. This also will be an efficient tool in the development of new specific vaccines.

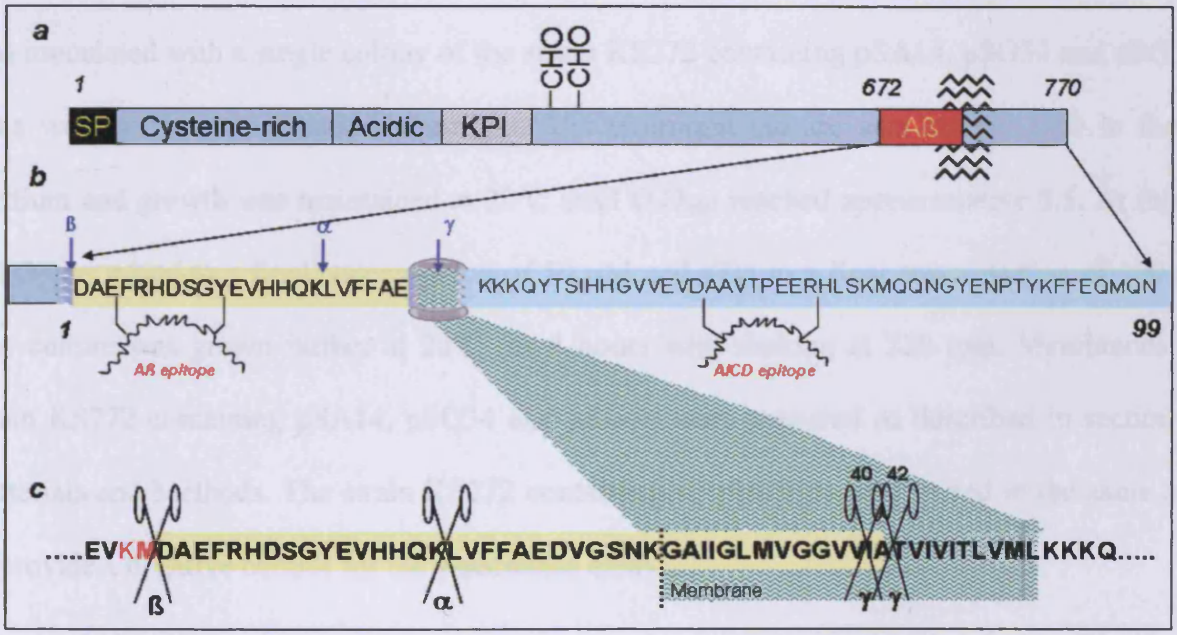


Figure (4.9) Schematic representation of APP showing important regions of the molecule.

(a) Structure of APP. The signal peptide is followed by the cysteine-rich region, an acidic region, the Kunitz-type protease inhibitor (*KPI*) and OX2 domains that occur in some APP isoforms, a glycosylation site, a transmembrane region, and a cytoplasmic tail; (b) Sequence and structure of C99 and the position of the Aβ and APP intercellular cytosolic domain (AICD) epitopes; (c) The Aβ region is shown which is a major component of Alzheimer's disease plaques, and the α-, β- and γ-secretase cleavage sites are indicated. Taken from Xiaodan Tian et al (2004).

4.5 γ-Secretase Assays

4.5.1 Measuring γ-secretase activity produced by components combined in one bacterial strain

One approach to investigate γ-secretase activity in *E. coli* involved combining the genes encoding γ-secretase components in up to 3 plasmids and subsequently transforming the generated plasmids into the same *E. coli* strain. Therefore, TSS transformation was used to transform pSA14 (*pen-2* and *aph-1*), pSG34 (*ps-1* and *c-99*) and pSG36 (*nicA*) into the strain KS272 (Sections 2.2.1 and 2.3.1, Materials and Methods).

For expression, double NZA containing Amp (200 μg/ml), Cm (15 μg/ml) and Kan (100 μg/ml) was inoculated with a single colony of the strain KS272 containing pSA14, pSG34 and pSG36 and cells were grown to saturation overnight. The overnight culture was diluted 1/20 in the same medium and growth was maintained at 28°C until $O.D_{600}$ reached approximately 0.5. At this point IPTG was added to a final concentration of 50 μM and aTet to a final concentration of 100 ng/ml. The culture was grown further at 20°C for 4 hours with shaking at 220 rpm. Membranes of the strain KS272 containing pSA14, pSG34 and pSG36 were prepared as described in section 2.4.8, Materials and Methods. The strain KS272 containing no plasmids was treated in the same manner to provide a negative control for the γ-secretase assay.

To perform the γ-secretase assay, KS272 membranes with or without γ-secretase components (200 μg/ml total protein) was added to 5 μg/ml pure C99 in lysis buffer (50 mM Tris-HCl pH 8.0 300 mM NaCl) containing 1% CHAPSO. Reactions were mixed thoroughly and incubated at 37°C and samples were removed after 7 and 24 hours. The reaction was then stopped by quickly freezing the samples at -80°C. As a second negative control, cells were incubated in lysis buffer without the substrate, C99.

4.5.1.1 Aβ ELISA (Enzyme-Linked-Immunosorbent-Assay)

To detect Aβ produced as a result of γ-secretase activity, an ELISA kit from the Genetics Company (Catalogue number: TK40HS) was used. The kit provides highly sensitive and selective analysis of human Aβ40 and it can be used to detect Aβ levels in the range of 25 to 500 pg/ml by comparison to a standard curve.

The antigen to be tested (Figure 4.10) is detected by monoclonal α-Aβ antibodies at two different binding sites, forming a sandwich complex. The surface of the micro titer plate is coated with an antibody which selectively recognises the C-terminal end of the antigen. During the test procedure, a α-Aβ antibody conjugate is incubated with the standard or sample and forms an antibody-Aβ-antibody complex. This complex is indirectly linked to an enzyme, which catalyses the conversion of a substrate into a coloured product and the colour intensity can be measured by means of photometry. The measured extinction correlates directly with the concentration of Aβ within the sample, and a quantification of the samples can be achieved by comparing the colour intensity to a standard curve using the concentrations of synthetic Aβ1-40 plotted against their colour intensity.

The assay samples from section 4.5.1 were analysed by the Aβ ELISA kit and the concentrations of Aβ produced as a result of the γ-secretase assay were calculated. Figure (4.11) shows that KS272 containing the γ-secretase components produced more Aβ than the control KS272. In addition, it is clear that the optimum length of time for γ-secretase activity to take place is between 7 and 24 hours, as Aβ levels begin to fall after 24 hours of incubation at 37°C.

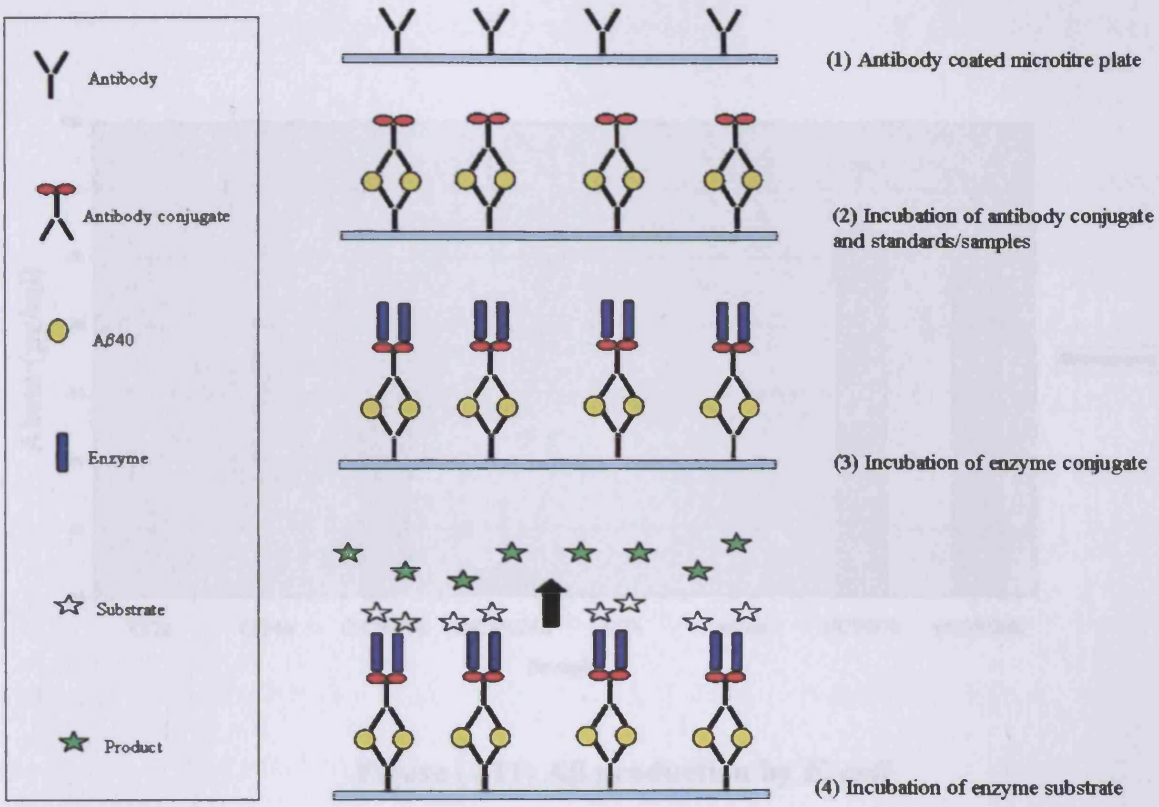


Figure (4.10) Schematic representation of the Aβ detection method produced as a result of γ-secretase activity, using an Aβ ELISA kit

The presence of Aβ is detected by two antibodies, forming a sandwich complex. The complex is linked with an enzyme which converts a substrate to a coloured product. The colour intensity of the product, which can be measured, directly corresponds to the concentration of Aβ present in the assay sample (Adapted from the manual for hAβ40 ELISA kit from the Genetics Company, catalogue number: TK40HS).

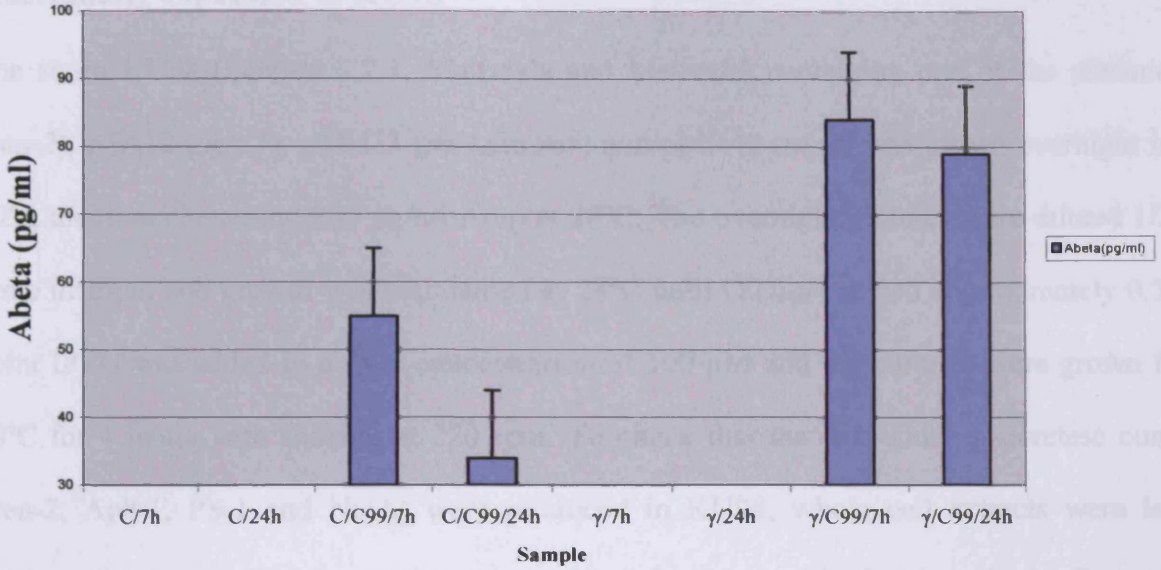


Figure (4.11) Aβ production by *E. coli*

The strain KS272, and KS272 containing the plasmids pSA14 (*pen-2* and *aph-1*), pSG34 (*ps-1* and *c-99*) and pSG36 (*nicA*) was grown at 28°C until $O.D_{600}$ reached 0.5. IPTG (50 μM) and aTet (100 ng/ml) were added to induce expression of the genes encoding the γ-secretase components. The culture was grown further at 20°C for 4 hours with shaking at 220 rpm. Membranes were prepared and γ-secretase assays were performed by mixing KS272 membranes with or without γ-secretase components (200 μg/ml) with 5 μg/ml pure C99 in lysis buffer (50 mM Tris-HCl pH 8.0 300 mM NaCl) containing 1% CHAPSO. Reactions were incubated at 37°C and samples were removed after 7 and 24 hours. To detect Aβ produced, the samples were analysed using an Aβ ELISA kit from the Genetics Company. C: Control KS272 membranes, γ: KS272 containing Pen-2, Aph-1, PS-1 and NicA.

4.5.2 Measuring γ-secretase activity produced by mixing of γ-secretase components, individually expressed in KU98

The strain KU98 (Section 2.2.1, Materials and Methods) containing one of the plasmids pSA1 (*pen-2*), pSA12 (*aph-1*), pMH13 (*ps-1Δexon9*) and pMH42 (*nicA*) was grown overnight in double NZA medium containing 200 μg/ml Amp at 28°C. The overnight cultures were diluted 1/20 in the same medium and growth was maintained at 28°C until $O.D_{600}$ reached approximately 0.5. At this point IPTG was added to a final concentration of 100 μM and the cultures were grown further at 20°C for 4 hours with shaking at 220 rpm. To check that the individual γ-secretase components (Pen-2, Aph-1, PS-1 and NicA) were produced in KU98, whole cell extracts were loaded on Schaegger gels and Western blots were performed using a polyclonal antibody directed against Pen-2, Aph-1, PS-1 or NicA. All components except Aph-1 were detected on Western blots (Figure 4.12). This was thought to be due to the fact that a newly obtained antibody was being used to detect Aph-1. Regardless, membranes of the strain KU98 containing one of pSA1, pSA12, pMH13 and pMH42 were prepared as described in section 2.4.8, Materials and Methods.

To perform the γ-secretase assay, KU98 membranes containing the different γ-secretase components were mixed in equal concentrations (100, 300, 1000 and 3000 μg total protein). One half of the samples was mixed with lysis buffer (50 mM Tris-HCl pH 8.0 300 mM NaCl) containing 1% CHAPSO, and incubated overnight at 37°C to allow the complex components to interact and to allow cellular proteases time to degrade, so that the level of background cleavage of C99 can be reduced. The mixture of components was then added to 40 μg pure C99. The other half of the samples was treated in the same manner but was not pre-incubated before the addition of C99. Reactions were mixed thoroughly and incubated at 37°C and samples were removed after 6, 24 and 30 hours. The reactions were then stopped by quickly freezing the samples at -80°C. As a negative control, samples were removed after incubation of the components with C99 for 0 hours.

The samples were then analysed using an Aβ40 ELISA kit from the Genetics Company, to test for the presence of Aβ40 and therefore γ-secretase activity (Figures 4.1).

The Aβ40 ELISA revealed that in general, pre-incubation of the γ-secretase components at 37°C before the addition of the substrate, C99, does not result in higher Aβ levels produced, or in lower levels of background. The results show that there is a general trend towards higher levels of Aβ produced as a result of the presence of a higher concentration of γ-secretase components as incubation of C99 with 3000 μg γ-secretase components produced more Aβ than samples containing lower concentrations of components. Also, it seems that incubation for 24 hours is sufficient to allow γ-secretase activity to take place.

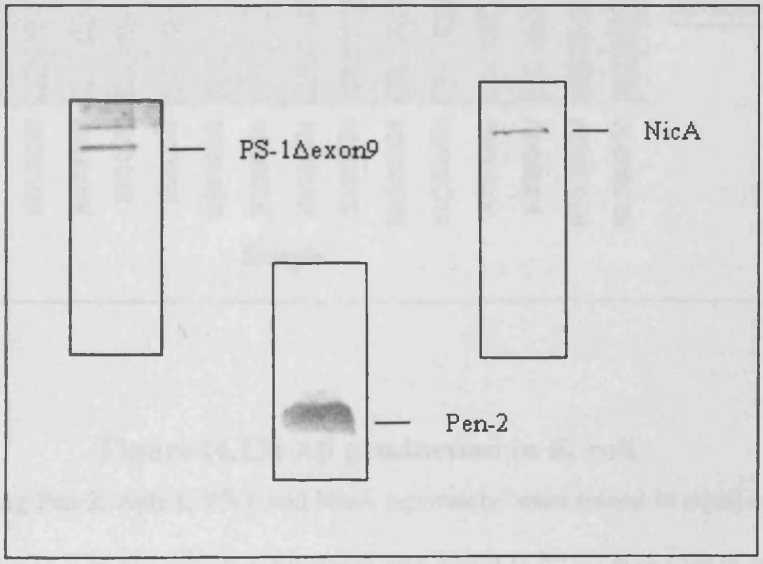
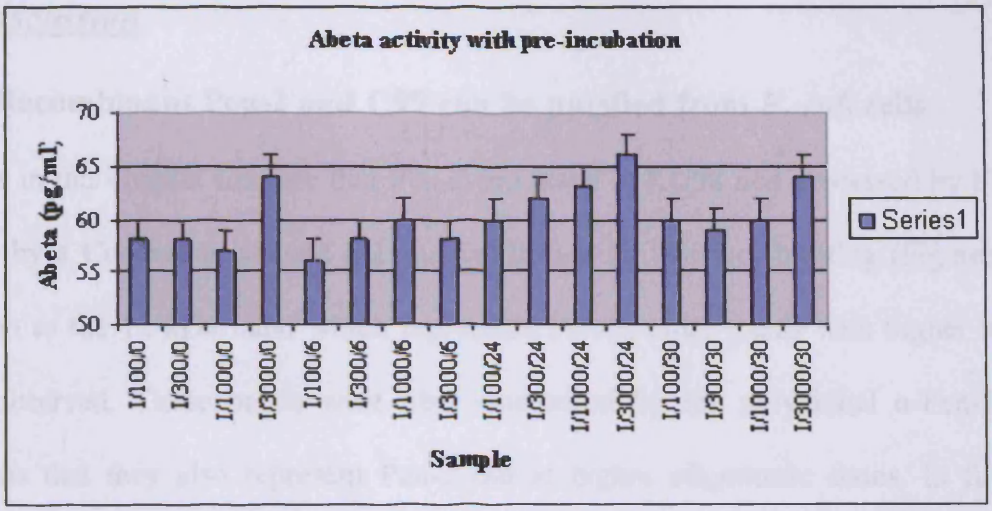


Figure (4.12) Western blots monitoring expression of γ-secretase components in KU98 for γ-secretase assays.

The strain KU98 containing either pSA1 (*pen-2*), pMH13 (*ps-1Δexon9*) or pMH42 (*nicA*) was grown at 37°C to log phase, when IPTG (100 μM) was added to induce gene expression. Cells were further grown at 20°C for 4 hours. Whole cell extracts were then loaded onto Schaegger gels and Western blots were performed using polyclonal antibodies directed against the individual components.

(A)



(B)

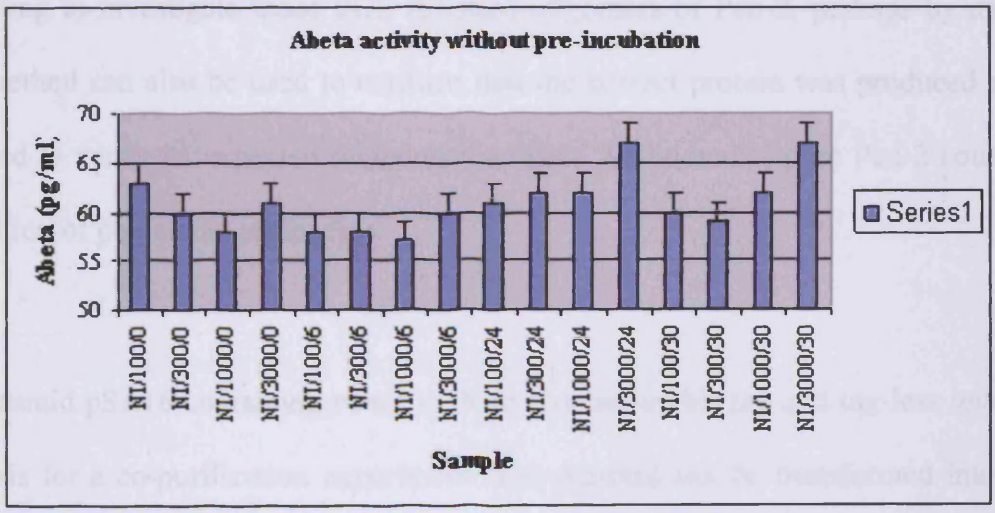


Figure (4.13) Aβ production in E. coli

KU98 membranes containing Pen-2, Aph-1, PS-1 and NicA separately, were mixed in equal concentrations (100, 300, 1000 and 3000 µg total protein). The mixture of membranes was added to 40 µg pure C99 in lysis buffer (50 mM Tris-HCl pH 8.0 300 mM NaCl) containing 1% CHAPSO, and incubated at 37°C to allow γ-secretase activity to take place. Samples were removed after 6, 24 and 30 hours and the reactions were stopped by quickly freezing the samples at -80°C. The same was repeated but membranes were incubated at 37°C for 24 hours before C99 was added. As a negative control, samples were also removed 0 hours after the addition of C99. In the figure: I= with pre-incubation, NI= no pre-incubation, 100/300/1000/3000= amount of membranes used in µg total protein, 0/6/24/30= number of hours assay at which sample was removed.

4.6 Discussion

4.6.1 Recombinant Pen-2 and C99 can be purified from *E. coli* cells

Results in this chapter indicate that Pen-2 produced in KU98 and processed by FPLC was pure, as shown by a Coomassie stained gel and confirmed by Western blotting (Figures 4.3 and 4.4). In addition to the 12 kDa band which represents Pen-2, other bands with higher molecular weights were observed. These bands were also recognised by the polyclonal α-Pen-2 antibody which confirms that they also represent Pen-2 but at higher oligomeric states. In future, it would be interesting to investigate those SDS resistant oligomers of Pen-2, perhaps by mass spectrometry. This method can also be used to confirm that the correct protein was produced and purified in *E. coli*, and to verify its expected molecular weight. Additionally, pure Pen-2 could be used for the production of polyclonal antibodies.

The plasmid pSA16 containing *pen-2* with an N-terminal his tag and tag-less *aph-1*, could provide the basis for a co-purification experiment. The plasmid can be transformed into the strain KU98 and expression of both *pen*-2 and *aph*-1 can be induced by the addition of IPTG and allowing the induction to take place for 4 hours, as Aph-1 was found to be unstable overnight. Following that, membranes can be prepared and the membrane proteins solubilised. However, Aph-1 has 6 transmembrane segments and probably considerable tertiary structure and therefore an alternative to solubilisation with SDS can be considered. Pen-2 can be produced with an N-terminal his tag and therefore purification with Ni NTA will be appropriate. The Elution fractions can then be tested for the presence of Aph1, which would indicate co-purification with Pen-2 and therefore a direct interaction between the two γ-secretase components.

Additionally, we describe the successful purification of the substrate C99 from *E.* coli cells. Moreover, Western blotting (figure 4.6) shows that C99 was purified as a monomer, but that higher

oligomers were also present, which seems to be common for this protein (Sandra Grau, PhD Thesis, 2004).

Pure C99 was then studied by mass spectrometry which confirmed that the genuine human recombinant protein was produced and purified in bacteria, and it verified that C99 had the expected molecular weight. Additionally, Tian and co-workers have identified crucial epitopes of the APP molecule that are recognised by specific antibodies. Knowledge of these epitopes could have great potential, as it may provide the basis for the design of artificial molecules for vaccination.

4.6.2 Aβ detected in the presence of γ-secretase components and C99, in initial assays

In the first γ-secretase assay (section 4.5.1), all γ-secretase components were combined by transforming the plasmids pSA14 (*pen-2* and *aph-1*), pSG34 (*ps-1* and *c-99*) and pSG36 (*nicA*) into the strain KS272. Expression of the recombinant human proteins was induced for 4 hours but with the exception of Pen-2, they were barely detectable on Western blots (data not shown). This could have been due to the fact that the presence of 3 plasmids in one strain was stressful for the bacterial cells and therefore expression was not properly induced. Additionally, one suggestion could be that expression of the four γ-secretase components could be optimised by testing varied concentrations of the inducers IPTG and aTet. However, it is possible that all γ-secretase components were in fact expressed in KS272, but that expression levels were so low that Western blotting was not sensitive enough to detect their presence. Therefore, the γ-secretase assay was carried out as described in section 4.5.1, and samples were analysed using the Aβ ELISA. The results showed that KS272 cells, producing γ-secretase components, produced more Aβ detected by ELISA, than control cells containing no components. They also showed that after 24 hours Aβ levels begin to fall therefore, the experiment needs to be repeated and samples removed between the time points of 7 and 24 hours to determine the optimum length of time for the assay.

Additionally, the Aβ ELISA revealed that lack of added purified C99 to cells containing the γ-secretase components resulted in no Aβ detected. This is surprising because KS272 contained the plasmid pSG34 which contains *c99*. However it is possible that Aβ is produced, but in small amounts that are then degraded by cellular proteases such as DegP (Clausen et al., 2002; Krojer et al., 2002; Spiess et al., 1999). Perhaps altering the incubation time for this assay could help with this problem.

In the second assay, each component was expressed individually in KU98 and then the membranes containing the individual components were combined. All components could be detected via Western blotting except Aph-1. This was thought to be due to the fact that a new Aph-1 antibody was obtained and its use was not yet optimised. However on some Western blots (data not shown), Aph-1 could be detected as a very faint band. Therefore, it was thought that perhaps the protein was in fact produced but in very low amounts that could not be detected using Western blotting. γ-secretase assays followed by Aβ ELISAs in this case showed that there was not much difference between test and control samples, although test samples still produced higher levels of Aβ. Incubation for 24 hours again was sufficient for Aβ production and higher concentrations of γ-secretase components in the assay samples resulted in higher levels of Aβ detected. However, pre-incubation of the membranes containing the γ-secretase components at 37°C for 24 hours did not result in higher levels of Aβ. Also, excess concentrations (40 μg) of the substrate C99 were used in the assays, which could have resulted in higher background levels of Aβ detected as C99 is also degraded by cellular proteases still remaining even after pre-incubation for 24 hours.

However, both assays produced result values in the region which can be detected by the ELISA kit but they were still in the lower range of the scale and therefore may not be very accurate. It was also observed the second assay carried out by mixing of γ-secretase components individually expressed in the strain KU98, produced higher levels of background. This was thought to be due to

the higher concentration of total membrane proteins used in this assay when compared to the first assay. And although there is no endogenous γ-secretase activity in *E. coli*, cellular proteases could be responsible for cleaving C99 and producing Aβ.

Akiyama and Ito (Akiyama and Ito, 2003) have created a new experimental technique for studying the action of proteases. They studied *E. coli* FtsH which is a membrane bound and ATP-dependent protease which brings about degradation of numerous membrane proteins. Among the ATP-dependent proteases (e.g. Lon) of *E. coli*, FtsH is unique in that it is a transmembrane protein and that its substrates include integral membrane proteins e.g. the SecY subunit of protein translocase (Akiyama et al., 1998). The membrane localisation of FtsH is essential for its role as a protease for membrane proteins(Akiyama and Ito, 2000). Furthermore, the proteolytic activity of FtsH is stimulated by the proton motive force (PMF) across the membrane (Akiyama, 2002).

In vitro reaction systems using detergent solubilised enzyme and substrates were unsuitable for characterising the activities of FtsH to degrade membrane integrated substrates, because such important features as the dislocation and the PMF stimulation could not be addressed. It was also difficult to prepare membrane vesicles carrying both FtsH and its degradation substrate. Therefore, Akiyama and co-workers prepared separate membrane vesicles, one carrying the enzyme and the other carrying a substrate. These membrane vesicles could then be fused to enable the enzyme-substrate interaction, by using PEG3350 to cause an ATP degradation of the substrate (Akiyama and Ito, 2003). Similarly, it was thought that this reaction system could adapted to demonstrate γ-secretase activity. Membranes of a suitable *E. coli* strain containing one of the plasmids containing *pen-2* and *aph-1*(pSA14, pSA16, pSA18) can be prepared, in addition to those containing the other γ-secretase components (e.g. pSG34 containing *ps1Δexon9* and *c99*, and pSG36 containing *nicA*). The membranes could then be fused following the protocol outlined by Akiyama and co-workers,

followed by the addition of the substrate C99 to perform the assay and test for occurrence of γ-secretase activity.

4.6.3 Conclusion

Pen-2 and the γ-secretase substrate, C99, have been successfully purified from bacteria which will allow them to be utilised in various assays investigating γ-secretase activity, and enable further analysis of their structure, function and interaction with other γ-secretase components. Additionally, initial assays involving the four γ-secretase components in *E. coli* demonstrate a lot of potential for the system; however additional work needs to be carried out to optimise the assay conditions and to investigate the reaction further.

CHAPTER (5)

THE *E. COLI* TWO HYBRID SYSTEM

5 THE *E. COLI* TWO-HYBRID SYSTEM FOR THE DETECTION OF HUMAN PROTEIN INTERACTIONS

5.1 Introduction

To aid the study of the function of each of the components of the γ-secretase complex (Pen-2, Aph-1, PS-1 and NicA) and their roles in bringing about γ-secretase activity, their interactions with each other and with the substrate APP needed to be analysed. As already mentioned in the introduction (Chapter 1), *E. coli* two-hybrid systems seem more suitable than yeast two-hybrid systems for such a study. The smaller genome complexity of *E. coli* and greater evolutionary distance from higher eukaryotes mean that *E. coli* two-hybrid systems should not generate as many false positives and negatives due to interactions between endogenous proteins and eukaryotic bait and prey. Additonally, *E. coli* grows much faster than yeast and can be transformed with higher efficiency, allowing better coverage in library-based screens. (Hu et al., 2000).

It has been shown that interaction between any two proteins can activate transcription in *E. coli* provided one of the interacting proteins is bound to the DNA via a DNA binding domain (DBD) for example the lambda repressor *λcI*, and the other interacting protein is bound to an RNA polymerase (RNAP) subunit for example the ω-subunit (Dove et al., 1997). Here, we construct plasmids containing some of the γ-secretase components (*aph-1*and *nicA*), fused to either *λcI* or the ω-subunit of RNAP according to the protocol outlined by Dove and co-workers, Harvard Medical School, Boston (Dove and Hochschild, 1998). Subsequently, the plasmids containing the investigated proteins, along with the reporter plasmid pRW50/417 containing the *lacZ* gene (Section 2.3.1, Materials and Methods) were transformed into an *E. coli* strain. When the two investigated proteins interact, transcription of the *lacZ* gene is activated producing a phenotype which can be measured by performing β-galactosidase assays.

5.1.1 The interaction between PS-1 and the γ-secretase substrate, C99

There is strong evidence to show that presenilins are able to interact directly with APP. Complex formation between APP and PS-1 and -2 has been demonstrated by co-immunoprecipitation of both proteins (Waragai et al., 1997; Weidemann et al., 1997; Xia et al., 1997). Consequently, work recently done in this lab has shown that PS-1 and C99 interact in an *E. coli* two-hybrid system (Harnasch et al., 2004). The plasmid pCB6 containing PS-1 fused to the λ repressor and C99 fused to the ω-subunit of RNAP was constructed (Section 2.3.1, Materials and Methods). A β-galactosidase assay in the presence of pCB6, in addition to the reporter plasmid pRW50/417 (Section 2.3.1, Materials and Methods) produced a high signal. In addition, Western blots with antibodies against PS-1 and C99 showed that the two proteins are stably co-expressed (Figure 5.1).

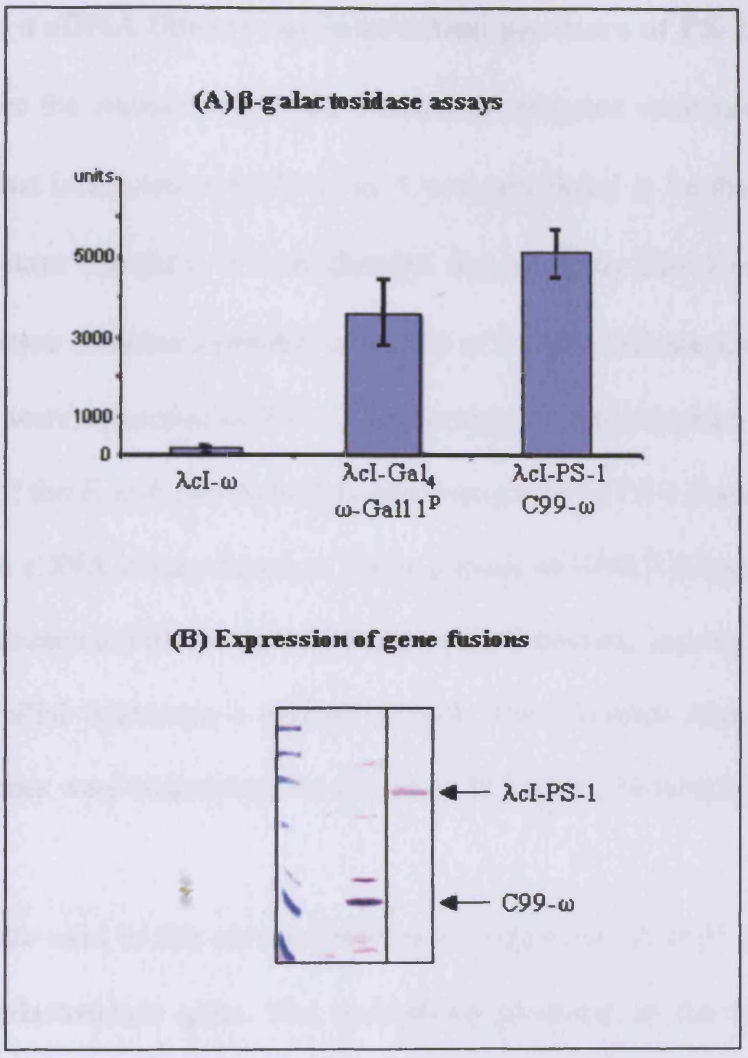


Figure (5.1) The interaction between PS-1 and C99 in an *E. coli* two-hybrid system

(A)The β-galactosidase assays indicate a low level response in the presence of the empty vector controls. The yeast Gal_4 and $Gal11^P$ proteins, serving as a positive control, lead to a positive signal in the β-galactosidase assays. The signal from the λcI-PS-1/C99-ω construct is even higher. β-galactosidase assays were performed in whole *E. coli* cells under standard conditions. (B)Western blot with antibodies against C99 and PS-1 showing stable expression of full length constructs (Pictures from Christian Behrend, Diplom thesis, 2002)

5.1.2 Screening a cDNA library for interaction partners of PS-1 and PS-1Δexon9

Additionally, before the components of the γ-secretase complex were known, it was interesting to identify proteins that interacted with PS-1, as it was postulated to be the γ-secretase or the active site of the γ-secretase complex. It was thought that proteins that interact with PS-1, may be involved in γ-secretase complex assembly or export of PS-1 to the relevant cellular compartment or alternatively, they were substrates of PS-1. Our strategy for identification of interaction partners of PS-1 was the use of the *E. coli* two-hybrid system composed of PS-1 fused to the λ repressor, and a human foetal brain cDNA library fused to the ω-subunit of RNAP (Figure 5.2). We also screened for proteins that interacted with the gain of function PS-1 mutant, lacking exon 9 (PS-1Δexon9), as it is linked to familial Alzheimer's disease (FAD). The plasmids containing the PS-1 and PS-1Δexon9 fusion genes were constructed by Christian Behrends (Behrends, 2002).

The reporter cassette used in this screen has been incorporated on an F' episome and contains the Amp and the β-galactosidase gene. The activatable promoter in the reporter gene casette is a modified *lac* promoter that contains a single λ operator (O_R2) centred at position –62, replacing the CRP-binding site originally associated with the *lac* promoter. To enhance the reporter gene expression, the Shine-Dalgarno translational signal is also included upstream of the reporter genes.

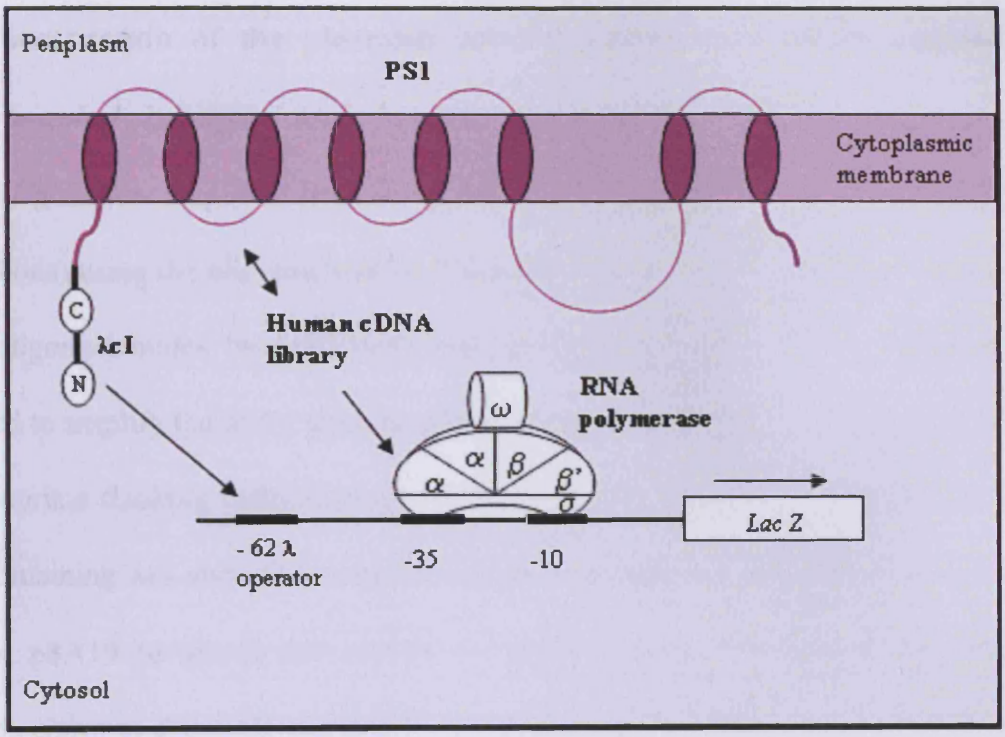


Figure (5.2) Schematic representation of the interaction between proteins encoded by the cDNA library and PS-1 or PS-1Δexon9.

5.2 Detection of two-hybrid interactions between γ-secretase components

5.2.1 Construction of the plasmids pSA19, pSA20 and pSA21 containing the gene fusions *ω-aph-1*, *λcI-aph-1* and *ω-nicA*, respectively

The *aph-1* gene was amplified from the plasmid pCDNA (zeo) Aph1a251 (Section 2.3.1, Materials and Methods) using the oligonucleotides "cI-AphI/*NotI*" and "cI-AphI/*BglII*" to allow fusion to *λcI* and the oligonucleotides "w-AphI/*NotI*" and "w-AphI/*SalI*" (Section 2.3.2, Materials and Methods) were used to amplify the *aph-1* gene to allow fusion to the ω-subunit of RNAP. PCR products with the appropriate flanking restriction sites were produced, which were then ligated into the vectors pCB1 containing λcI and pBRω containing the ω-subunit of RNAP (Figure 5.3). The resulting plasmids, pSA19 *(ω-aph-1)* and pSA20 (*λcI-aph-1*) were controlled by digestion with suitable restriction enzymes and were analysed by agarose gel electrophoresis.

Similarly, the *nicA* gene was amplified from the plasmid pMH42 (Section 2.3.1, Materials and Methods) using the Oligonucleotide "NicA-w/*NdeI*" as a forward oligonucleotide and "NicA-w/*NotI*" as a reverse oligonucleotide (Section 2.3.2, Materials and Methods). PCR products with the appropriate flanking restriction sites were produced, which were then ligated into the vector pBRω containing the ω-subunit of RNAP. The resulting plasmid, pSA21, was controlled by digestion with suitable restriction enzymes and analysed by agarose gel electrophoresis. Figure (5.3) can be used for reference.

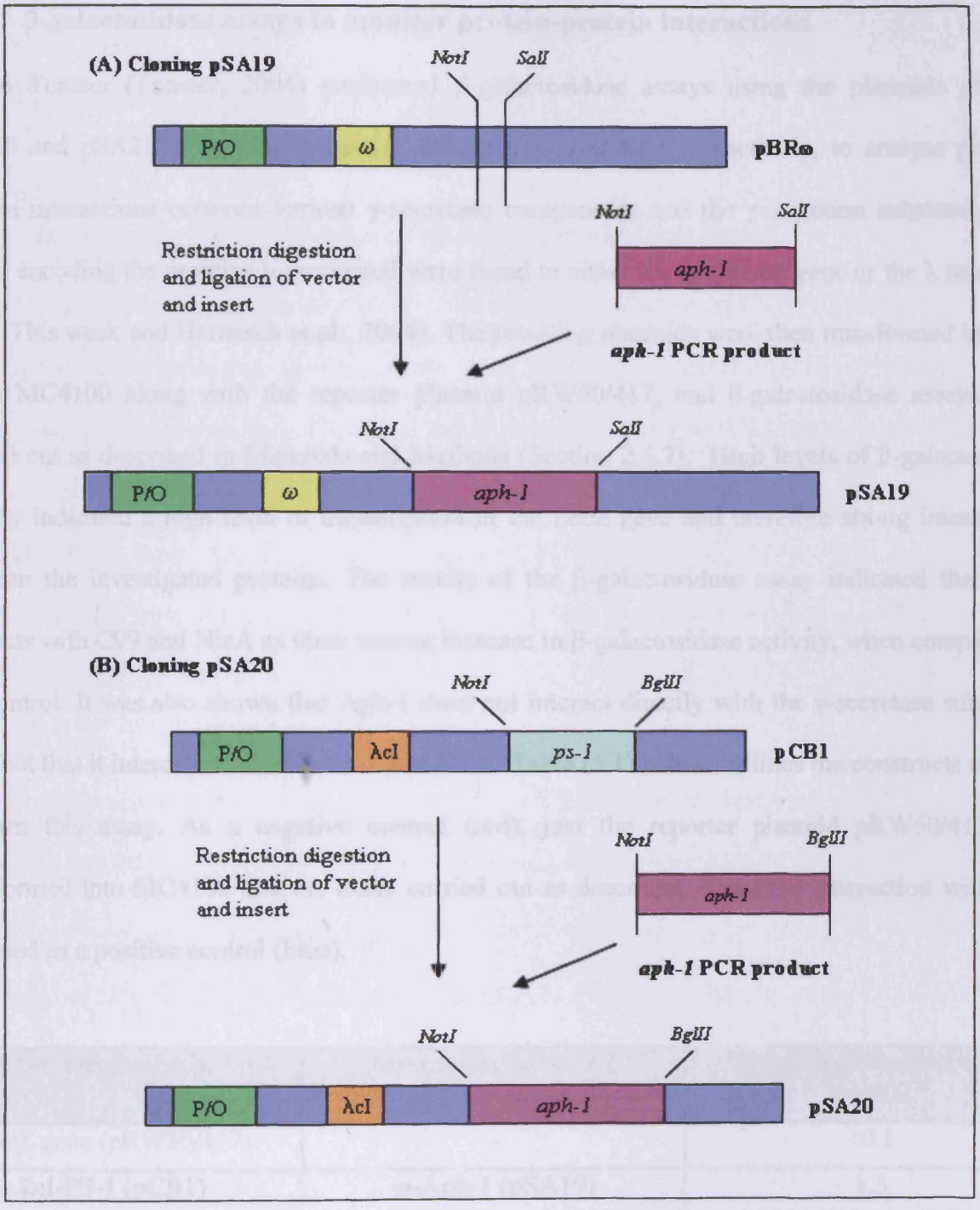


Figure (5.3) Schematic representation of the construction of pSA19 and pSA20 containing *ω-aph-1* and *λcI-aph-1* respectively

The *aph-1* gene was amplified from the plasmid pCDNA (zeo) Aph1a251. For the construction of pSA19, the *aph-1* PCR product with flanking restriction sites was ligated into pBRω via *SalI* and *NotI*, and for the construction of pSA20, the *aph-1*PCR product was ligated into pCB1 via *BglII* and *NotI*, replacing the *ps-1* sequence.

5.2.2 β-galactosidase assays to monitor protein-protein interactions

Simon Tunster (Tunster, 2004) performed β-galactosidase assays using the plasmids pSA19, pSA20 and pSA21 containing *ω-aph-1*, *λcI-aph-1* and *ω-nicA* respectively, to analyse protein-protein interactions between various γ-secretase components and the γ-secretase substrate, C99. Genes encoding the proteins investigated were fused to either the *ω* subunit gene or the λ repressor gene (This work and Harnasch et al., 2004). The resulting plasmids were then transformed into the strain MC4100 along with the reporter plasmid pRW50/417, and β-galactosidase assays were carried out as described in Materials and Methods (Section 2.4.7). High levels of β-galactosidase activity indicated a high level of transcription of the *LacZ* gene and therefore strong interactions between the investigated proteins. The results of the β-galactosidase assay indicated that PS-1 interacts with C99 and NicA as there was an increase in β-galactosidase activity, when compared to the control. It was also shown that Aph-1 does not interact directly with the γ-secretase substrate, C99, but that it interacts with both PS-1 and NicA. Table (5.1) below outlines the constructs used to perform this assay. As a negative control (red), just the reporter plasmid pRW50/417 was transformed into MC4100 and the assay carried out as described. The PS-1 interaction with C99 was used as a positive control (blue).

Interacting protein 1	Interacting protein 2	β-galactosidase activity (units)
LacZ gene (pRW50/417)	-	<0.1
λcI-PS-1 (pCB1)	ω-Aph-1 (pSA19)	1.3
λcI-PS-1 (pCB1)	C99-ω (pCB2)	0.9
λcI-Aph-1 (pSA20)	C99-ω (pCB2)	<0.1
λcI-PS-1 (pCB1)	ω-NicA (pSA21)	1.15
λcI-Aph-1 (pSA20)	ω-NicA (pSA21)	1.0

Table (5.1) Interaction between γ-secretase components measured by β-galactosidase activity

5.3 Screening a human foetal brain cDNA library for interaction partners of PS-1 and PS-1Δexon9 using the E. coli two-hybrid system

5.3.1 Isolation and amplification of the cDNA library supplied

The cDNA library was cloned so that it is fused to the α-subunit of RNAP in the plasmid pTRG XR (Tet resistant) from Stratagene. In order to be able to use library DNA repetitively, the cDNA library needed to be amplified. XL1-blue MRF' Kan cells (Section 2.2.1, Materials and Methods) containing the cDNA library inserts were grown at 28°C, since from previous experience we established that membrane proteins demonstrated improved stability when cells producing them were grown at lower temperatures. Additionally, in order to screen for non-membrane proteins, cells were grown at 37°C as well.

The isolation and amplification procedure of the cDNA library is described in figure (5.4). 1 ml XL1-blue MRF' Kan cells were thawed, and 150 µl were mixed with 20 ml SOB liquid medium and 100 µl were plated on each of 200 NZA agar plates containing 15 µg/ml Tet. 190 plates were incubated at 28°C whereas 10 plates were incubated at 37°C overnight. A lawn of cells grew on the plates and cells were harvested by scratching them off the plates. Subsequently, DNA was isolated using the QIAquick midipreparation kit from Qiagen. This yielded 2 ml of cDNA library inserts from 28 °C and 1 ml from 37°C.

Additionally, 1 l of LB liquid medium was inoculated with 100 µl library cells and incubated O/N at 28°C and the same was repeated and the culture was incubation at 37°C. 300 ml of the overnight culture grown at 28°C, and 300 ml of the culture grown at 37°C were used to isolate DNA via midipreparation. This yielded 1 ml cDNA library from each temperature. The rest of the 1l LB

culture from each temperature (700 ml) was frozen at -20°C. Two minipreparations were performed with the remaining 650 µl of thawed XL1 MRF' Kan cDNA library cells. This yielded 0.2 ml of unamplified cDNA library.

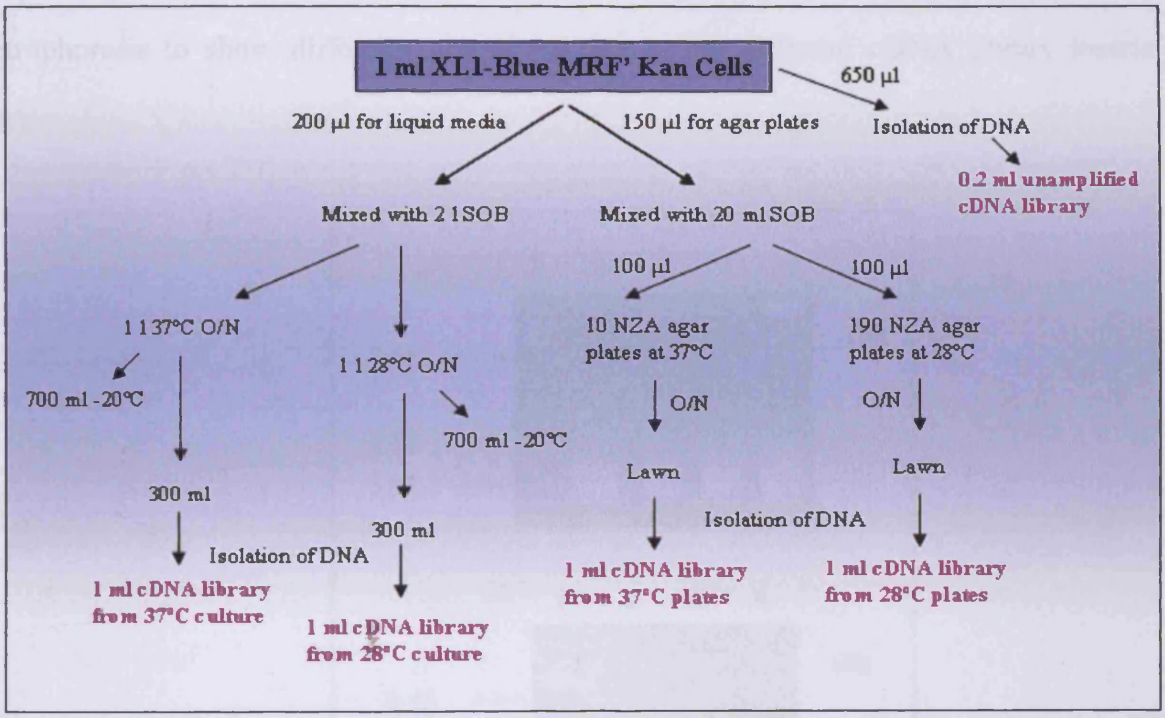


Figure (5.4) Schematic representation of the procedure performed to isolate the cDNA library.

5.3.2 Analysis of the cDNA library isolated

To determine the concentration of cDNA library DNA isolated, 2 µl of each sample of amplified DNA and 5 µl of the unamplified library, were loaded on 1% agarose gel (Figure 5.5A). The gel shows the quantities of cDNA library DNA isolated. Abundant DNA was isolated as compared with that of the unamplified cDNA library, and it was sufficient for use in the cDNA library screening experiments.

Additionally, as we had no experience in using this library from Stratagene, it was necessary to rule out the possibility that all the cDNA library inserts were identical. Hence, the cDNA library DNA isolated was transformed into MC4100. Four of the transformants obtained were used for the minipreparation of plasmid DNA. 10 µl aliquots of each preparation were analysed by agarose gel electrophoresis to show different migrations due to the different cDNA library inserts (Figure 5.5B).

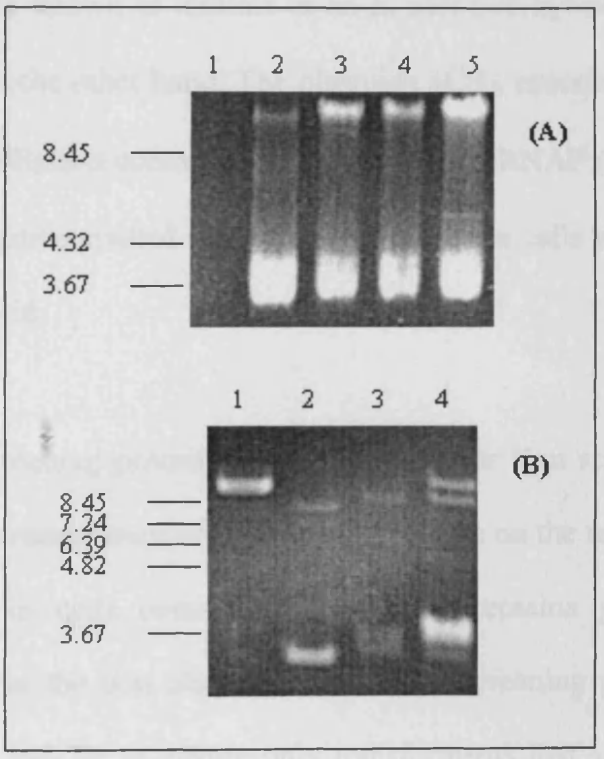


Figure (5.5) Agarose gels analysing the cDNA library isolated

The cDNA library was amplified from XL1-blue MRF' Kan cells (Stratagene) by growing the cells on agar plates and in liquid cultures of SOB medium, at 28°C and 37°C. The DNA was then isolated using mini- or midi-preparation kits from Qiagen, and loaded onto 1% agarose gels. (A) The lambda DNA-BstEII digest from NEB was used as a ladder. Lane 1: Unamplified cDNA library (10 µl). Lanes 2 & 3: DNA from 1 l cultures at 37°C and 28°C respectively. Lanes 4 & 5: DNA from agar plates at 37°C and 28°C respectively. (B) DNA isolated from XL1 MRF' kan cells was transformed into the strain MC4100 and isolated using a midipreparation kit (Qiagen). Lambda DNA-BstEII from NEB was used as a DNA ladder and Lanes 1-4 are the different cDNA library inserts showing different migrations on agarose. Sizes of DNA fragments are given in kilo basepair (kb).

5.3.3 Determining the type of agar plates to be used in the screening procedure

Before screening the cDNA library for interaction partners of PS-1 and PS-1Δexon9, it was necessary to decide what type of agar plates were to be used for the screening procedure. The plasmids pACLGF$_2$ containing *λcI* fused to *Gal4* (Cm resistant) and pBRαGal11^P containing the the α subunit of RNAP fused to *Gal11^P* (Tet resistant), were transformed into the strain CB4 already containing the reporter *lacZ* gene(Kan resistant). This was used as a positive control as the proteins Gal4 and Gal11^P are known to interact in an *E. coli* two-hybrid system, as mentioned in Chapter (1) of this thesis. On the other hand, The plasmids pCB1 containing *λcI* fused to *ps-1* (Cm resistant) and the plasmid pBRstarα containing the α subunit of RNAP (Tet resistant), in the strain MC4100 were used as a negative control. This was because these cells will contain the bait hybrid and an in-complete prey hybrid.

All plasmids used in the screening procedure were Cm, Tet or Kan resistant. However, protein-protein interactions cause increased transcription of the *bla* gene on the reporter plasmid, conferring Amp resistance, resulting in cells containing interacting proteins growing on Amp plates. Therefore, it was decided that the best plates for use in the screening procedure were NZA agar plates containing Amp, Cm and Tet to ensure only transformants that grow on Amp are analysed further, MacConkey lactose plates containing Cm, Tet and Kan for visualising protein-protein interactions as a colour change, and NZA agar plates containing Cm, Tet and Kan to ensure the MC4100 cells contained all the desired plasmids. It was found that the positive control grew on Amp and caused the appearance of red colonies on MacConkey agar plates, and the negative control did not grow on Amp and caused the appearance of white colonies on McConkey agar plates.

5.3.4 The screening procedure

5.3.4.1 Isolation of candidate interaction partners of PS-1wt and PS-1Δexon9

This procedure is outlined in figure (5.6).The plasmid pTRG XR (Section 2.3.1, Materials and Methods) containing the cDNA library fused to the α-subunit of RNAP was transformed using the high yield method of electrotransformation (Section 2.3.8.2, Materials and Methods) into the electrocompetent cells CB4 containing pCB1 (*λcI-ps-1*) and CB4 containing pCB13 (*λcI-ps-1Δexon9*). Following a 1 hour period of phenotypic expression in 1 ml NZA at 28°C and 37°C, the *E. coli* cells were plated out (100 µl and 900 µl) on NZA Amp plates (300, 500, 750 and 1000 µg/ml final concentration). Different concentrations of Amp were used in order to enable us to determine what Amp concentration is best for minimising background growth.

After the overnight incubation, the transformation plates were inspected and a lawn was observed on all the plates. Therefore, all the plates were replicated on NZA agar plates containing the appropriate Amp concentration and incubated overnight at the appropriate temperature. Subsequently, single colonies were observed on some of the plates. A few colonies were picked and patched on NZA plates containing Amp, NZA plates containing Amp, Cm and Tet, and MacConkey plates containing Cm, Tet and Kan. The plates were then incubated overnight at the appropriate temperatures. No positive candidates were obtained from these plates.

A lawn was observed on some of the replica plates therefore, those plates were replicated again and some cells were patched on the same plates as before. Also, single colonies obtained from the replica plates were patched as before. Cells that grew on Amp and had a red phenotype on MacConkey lactose plates were considered to be positive candidates. The purified single colonies were then purified and re-tested 4 times on NZA and MacConkey plates containing Cm, Tet and

Kan to verify that they were pure and homogenous. If the patches were heterogenous, then the single colonies were streaked out again and patched, until it could be verified that they were pure, otherwise they were not investigated further. NZA medium with the appropriate antibiotics was inoculated with a single colony of *E. coli* containing a positive candidate for use in the preparation of DMSO stocks for storage of the strains and mini preparations of plasmid DNA.

5.3.4.2 Validation of detected protein-protein interactions

91 positive candidates were isolated, but in order to exclude, for example, promoter-up mutations in the reporter gene or any other mutation that could cause a false positive phenotype, plasmid DNA of the candidates was re-tranformed into cells that were not subjected to selection and screening media. Therefore, candidates were transformed into the strain CB4 containing pCB1 (*λcI-ps-1*) and CB4 containing pCB13 (*λcI-ps-1Δexon9*). The single colonies from the transformantion plates were picked and patched as before, and cells that contain a bait hybrid and an incomplete prey hybrid (CB4 with pCB1 and pBRα or CB4 with pCB13 and pBRα) were used as negative controls. 30 candidates were found to be false positives, and those that could reproducibly produce a positive phenotype on plates were analysed further.

Subsequently, the target plasmids containing the remaining 61 positive candidates were transformed into CB4 cells containing pACλcI3.2. The plasmid pACλcI3.2 is an incomplete bait hybrid because it does not encode the bait protein (PS-1 or PS-1Δexon9). 11 transformants produced a positive phenotype on plates and so they were regarded as false positives. This could occur when the positive candidate itself contains a DNA binding domain. In this case, the bait hybrid fused to λcI is not required to bind to the DNA. Instead, transcription of the *LacZ* is activated as a result of the prey hybrid itself binding to DNA, resulting in a false positive phenotype.

Single colonies of the cells containing the remaining positive target plasmids were transformed into MC4100 and incoculated overnight for DMSO stocks and minipreparations of plasmid DNA. Plasmid DNA was then sequenced according to the protocol in section 2.3.10, Materials and Methods. Simultaneously, they were re-transformed into the strain CB4 containing pCB1 and CB4 containing pCB13 and patched to verify that they were positive and homogenous.

5.3.4.3 Further analysis of verified positives

50 verified positives were obtained which grew on Amp and produced a light to intensive red colour on McConkey lactose plates. To identify the protein encoded by the target DNA, the nucleotide sequence of the target DNA was determined and the sequence was then compared to the human genome sequence in the Blast database, to identify related proteins and the chromosomal locations of the candidates were obtained (Table 5.2). One of the positive candidates (not shown) had no sequence similarities in the databases. This was surprising because at the time of the screen, the whole human genome sequence was known. An explanation for this could be that when the human cDNA library was constructed, it was contaminated with sequences from another organism whose genome had not been sequenced at the time.

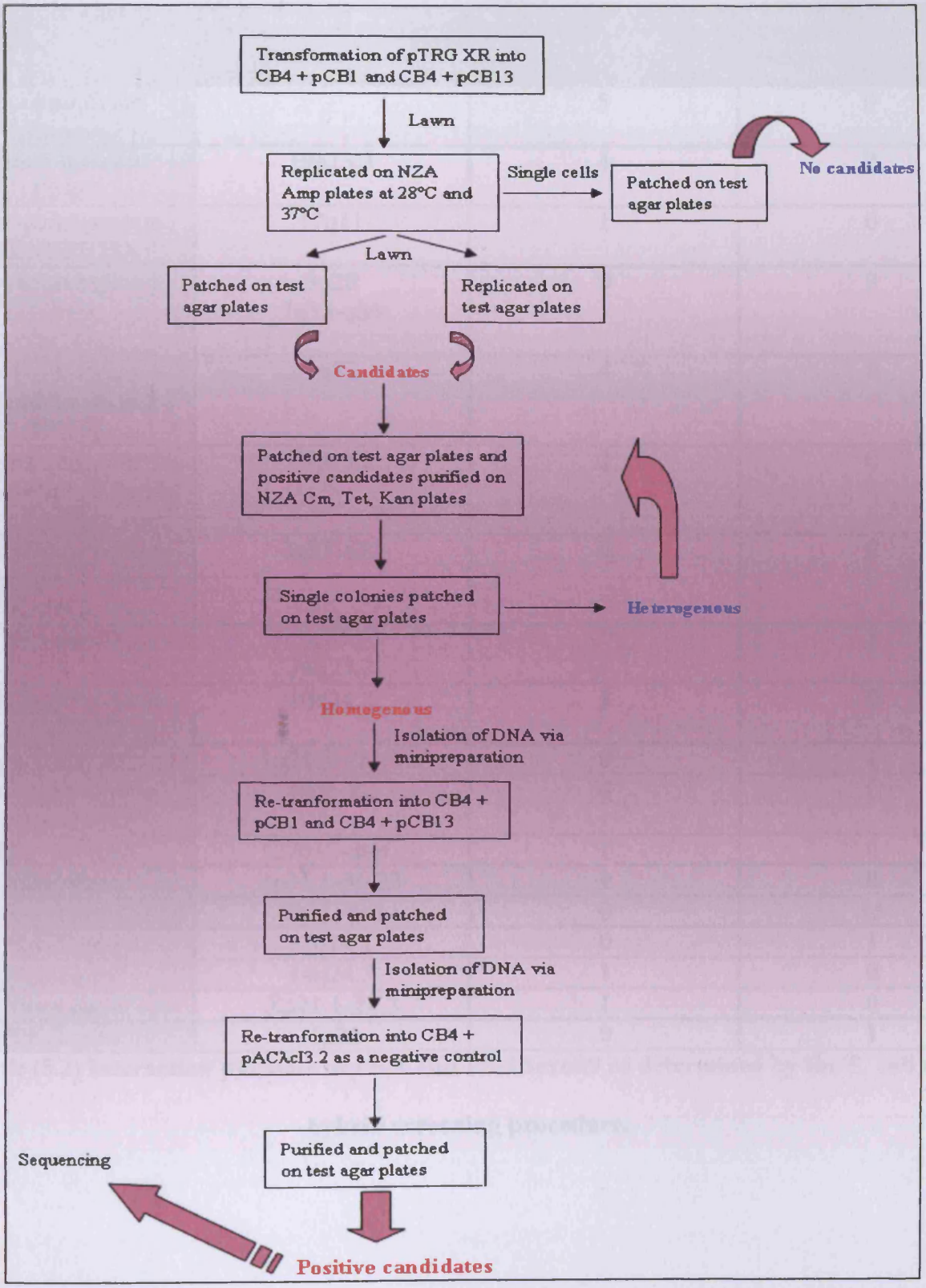


Figure (5.6) Schematic representation of the procedure used for screening a human cDNA library for interaction partners of PS-1 and PS-1Δexon9 using the *E. coli* two-hybrid system

Gene	Chromosomal location	Number of times interacts with PS-1	Number of times interacts with PS-1Δexon9
Mitochondrion genome	-	5	0
Beta 5 tubulin (TBB5)	19p13.3	4	2
Ribosomal protein L23a (RPL23A)	17q11	1	0
Ets-variant gene 5 (ETV5)	3q28 7q33-q36	0	2
Interferon gamma-inducible protein 16 (IFI16)	1q22	4	0
GTP-binding protein, RAS oncogene family (RAB3A)	19p13.2	1	0
Lantibiotic synthase component C-like 1 (LANCL1)	2q33-q35	0	2
KIAA0614	12q24.21 14q24.3		1
Ring finger protein 27 (RNF27)	10q24.3	1	0
Lamin A/C (LMNA)	1q21.2-q21.3	0	1
New gene following KIAA1337	1p36.22	4	0
HS1 protein	22q12-qter	1	0
Novel gene	1p35.1-36.23	1	0
Novel gene	7q33-q36	0	15
Novel gene	12q24.1	0	1
Novel gene	14q24.3	1	0
Novel gene	Xp21.1-21.3	1	0
Novel gene	Xp22	0	1

Table (5.2) Interaction partners of PS-1 and PS-1Δexon9 as determined by the E. coli two-hybrid screening procedure.

5.4 Discussion

5.4.1 Two-hybrid interactions are detected between γ-secretase components, and the substrate C99

The *E. coli* two-hybrid system was successfully utilised to identify a number of interactions that occur between components of the γ-secretase complex. It is now widely accepted that PS-1 comprises the active site of the γ-secretase complex, harbouring the two active site aspartates in transmembrane domains 6 and 7, in the N- and C-terminal fragments of PS-1, possibly generated by autoproteolysis (Prokop et al., 2005). Therefore, an interaction between PS-1 and C99 was expected and it was shown using the *E. coli* two-hybrid system. However, it was not clear whether other γ-secretase components interacted with each other or with C99 prior to the γ-secretase complex assembly. It has been suggested that other components are required for assembly, stabilisation and maturation of the complex and that NicA may be involved in the recognition of substrates (Steiner, 2004).

Results in this chapter indicated that Aph-1 does not directly interact with C99, as was shown by the β-galactosidase assays performed. However, interactions were observed between Aph-1 and PS-1. This was thought to be due to the fact that this interaction was necessary for forming the "scaffold" for the γ-secretase complex (De Strooper and Woodgett, 2003). The *E.* coli two hybrid system in this work suggested that PS-1 and NicA interact, and therefore it is probably also necessary for Aph-1 to interact with NicA, so that the PS-1/NicA intermediate can be stabilised. Additionally, Shirotani and co-workers (Shirotani et al., 2004) suggested that there is a stable interaction between NicA and Aph-1, independent of PS-1 and Pen-2. Furthermore, it was suggested that Pen-2 is necessary for the stabilisation of the PS NTF and CTF (Prokop et al. 2005), therefore it would be interesting to use the *E. coli* two hybrid system to analyse that possible interaction.

Additionally, regardless of the lack of evidence for successful expression of *ω-aph-1*, *λcI-aph-1* and *ω-nicA* from the plasmids pSA19, pSA20 and pSA21, respectively, β-galactosidase assays were still carried out. The results of the assay not only indicate that the γ-secretase components interact but also that they must be stably expressed. Perhaps they were expressed in very small amounts that could not be detected well using Western blotting but that these amounts were sufficient for a two-hybrid interaction.

5.4.2 Screening the cDNA library using the *E. coli* two-hybrid system, identified candidate interaction partners of PS-1 and PS-1Δexon9

The cDNA two-hybrid screen yielded 50 "hits" from the screening procedure. After sequencing and identification (where possible) of the cDNA library inserts, 18 different potential interaction partners of PS-1 and PS-1Δexon9 were obtained.

Looking at table (5.2) in the Results section, it is shown that some of the candidates isolated by the screening procedure interact with either PS-1 or PS-1Δexon9. It would be interesting in the future to re-transform the isolated cDNA library insert into a reporter strain with the other presenilin to determine whether the interaction observed in the screening is specific to PS-1 or PS-1Δexon9. For example, Ribosomal protein (L23a) was isolated as an interaction partner of PS1, but not PS1Δexon9, so if it was re-transformed into a strain containing the reporter plasmid and pCB13 (λcI-PS1Δexon9) then the importance of exon 9 for the interaction can be determined.

A few interesting genes were isolated as interaction partners of at least one of the presenilins. For example, RAB3A, a GTP-binding protein belonging to the RAS oncogene family was isolated as an interaction partner of PS1. This is interesting because previously Dumanchin et al. (Dumanchin

et al., 1999) found that RAB11 interacts with PS1, which suggested that presenilins might be involved in vesicular routing, as mentioned in the Introduction. Additionally, the N-terminus of PS-1 was shown to bind to RabGDI which is a regulatory factor also involved in vesicular transport (Scheper et al., 2000). Furthermore, Scheper and co-workers have found that in fibroblasts Rab6 membrane association is PKC dependent and that this regulation of Rab6 membrane association is dependent on PS-1 (Scheper et al., 2004).

Other isolated interaction partners include Ets variant gene 5 (ETV5), which encodes a Pea3 subfamily ETS domain transcription factor. Recent results show that the Pea3 subfamily members play a role in epithelial-mesenchymal interactions during lung organogenesis (Liu et al., 2003).

Another interesting interaction partner is Ring Finger Protein 27 (RNF27) which was found to interact with PS-1. This is a protein motif related to the zinc finger motif, found on the sequence of the RING1 gene. This motif was later found in 27 proteins, all of which have putative DNA binding functions (Lovering et al., 1993). Protein DNA interactions are involved in many of the fundamental processes that occur inside cells for example, transcription and replication. To meet such a demand, a number of protein sequence or structural motifs have evolved to allow DNA interaction (Freemont et al., 1991; Harrison, 1991). This is interesting because it brings about the question of whether this protein actually interacted with PS-1, or if the presence of a DNA binding protein resulted in transcriprional activation without a protein-protein interaction. This was of course accounted for in the screening procedure, but RNF27 was only isolated once and found to interact with just PS-1, which makes it possible that was a false positive. However, presenilin is known to interact with a number of different proteins and to be involved in signaling processes therefore its interaction with RNF27 should not be ruled out.

Lamin A/C which was isolated as a candidate interaction partner of PS-1Δexon9. Nuclear lamins are filament proteins that were originally identified as components of the nuclear lamina (Aebi et al., 1986; McKeon et al., 1986). It was originally thought that the nuclear lamina functions to provide structural support and organisation to the nuclear envelope (Csoka et al., 2004; Lammerding et al., 2004; Lenz-Bohme et al., 1997; Newport et al., 1990; Smythe et al., 2000; Sullivan et al., 1999; Vigouroux et al., 2001), but recent findings have shown that lamins exist throughout the nucleoplasm suggesting a structural contribution to a nuclear matrix (Bridger et al., 1993; Goldman et al., 1992; Hozak et al., 1995), and roles in DNA replication (Moir et al., 2000), transcription (Spann et al., 2002) and RNA processing (Jagatheesan et al., 1999; Kumaran et al., 2002; Muralikrishna et al., 2001). Additionally, recent work in mouse and human ES cells has shown that lamin A/C is predominantly expressed in differentiated cells which suggests a role in the maintenance of a differentiated state (Constantinescu et al., 2005).

HS1 protein (hematopoetic lineage cell-specific protein 1) was isolated via its interaction with PS-1. It is a substrate for protein tyrosine kinases in lymphocytes, which binds to F-actin and promotes actin polymerization (Hao et al., 2005). Actin is associated with the plasma membrane where it triggers new behaviour in the cell when its environment changes, by membrane re-modelling to create tubules or vesicles (Revenu et al., 2004). As presenilin is also found in the plasma membrane, this supports a role for presenilin in vesicle formation.

Also, interestingly, a few of the hits obtained by the screening, were found to have chromosomal locations similar to factors thought to be involved in AD, or interaction partners of APP or the presenilins. This could have important implications. Such hits should be investigated further, to determine whether there is a link between chromosomal location, and a role in AD. Figure (5.7)

shows "hits" on chromosome 1 (same as PS-1, NicA and Aph-1) and chromosome 19 (same as ApoE)

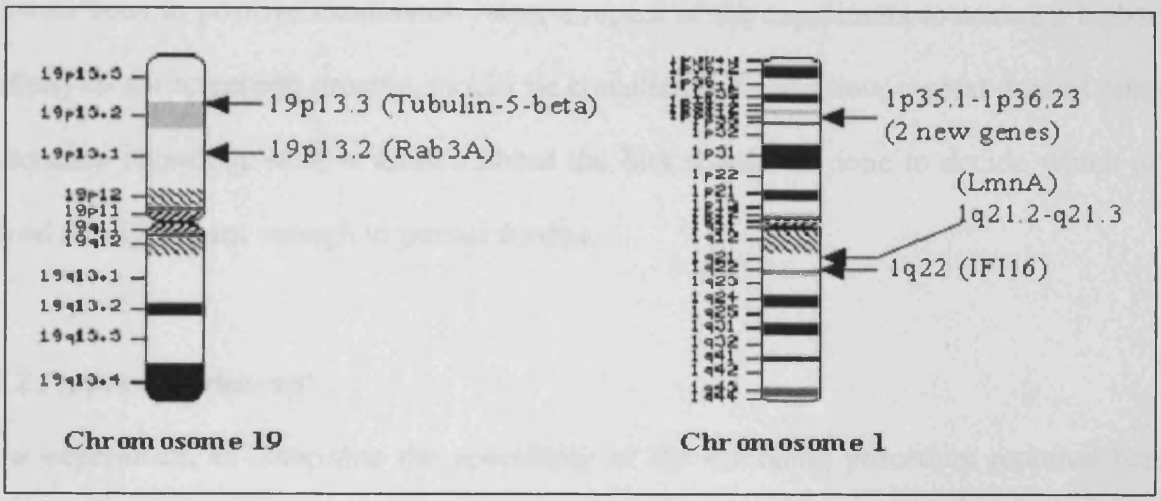


Figure (5.7) chromosomal map showing the chromosomal locations of some of the cDNA two-hybrid hits.

According to the screening results, the PS-2, Aph-1 and NicA genes are mapped to chromosome 1 in addition to LmnA, IFI16 and two new genes. ApoE is mapped to chromosome 19 in addition toTBB5 and Rab3A.

5.4.2.1 Evaluation of the screen results

Although the results of the screening of the cDNA library are promising, caution should be taken when considering the results. False positives are a known problem with two-hybrid systems, and although using an *E. coli* two-hybrid system for detecting human protein interactions should result in fewer false positives observed, the system still does not completely rule them out, especially when such a large number of hits is obtained. Additionally, it is cause for concern that none of the proteins found in the literature to interact with PS-1 (e.g. Notch) and the now known γ-secretase components, were isolated by this screening experiment. Furthermore, the cDNA library used from Stratagene is a commercial library, therefore we are not certain about its quality.

Consequently, the degree of reliability of the results obtained needs to be evaluated. A decision needs to be made about whether enough measures were taken while performing this experiment, to ensure only candidates that interact specifically with the bait proteins (PS-1 or PS-1Δexon9 in this case) were seen as positive candidates. Also, a repeat of the experiment to ensure a higher level of specificity in the screening process should be considered. In addition, a great deal of research into the literature regarding what is known about the hits should be done to decide which of the hits obtained are significant enough to pursue further.

5.4.2.2 Future experiments

A new experiment, to determine the specificity of the screening procedure reported here can be performed. The cDNA library already isolated can be transformed into a reporter strain containing the bait plasmids as described. However, instead of Ampicillin plates, the transformants can be plated out on NZA plates containing Cm, Tet and Kan to ensure that all 3 plasmids are present in the strain, without exerting too much pressure on the cells to express the LacZ gene. The transformants should then be replicated onto indicator plates, where a positive protein-protein interaction produces a colour change.

Spiess et al., (1999) showed that DegP, the *E. coli* homologue of the human L56 had dual chaperone and protease activity at different temperatures. It was suspected that L56 may have a role in AD, and Christian Behrends (Diplom thesis, 2002) detected a positive protein-protein interaction between L56 and PS-1. Therefore, once the screening procedure is optimised, the *E.* coli two-hybrid system can be used to screen for interaction partners of L56 and an L56 mutant (L56SA), as well as other proteins known to interact with PS-1 or thought to have a role in AD.

A large number of novel genes were isolated by the screening procedure, therefore these genes as well as others can be used for sequence homology to try and identify protein families (if any) that they might belong to, and then a speculation may be made about how and why these proteins interact with PS-1 and PS-1Δexon9. Additionally, the cDNA library contains portions of genes therefore it may be informative to clone the full length genes of the candidate interaction partners of PS-1 and PS-1Δexon 9 and repeat the entire procedure.

5.4.3 Conclusion

This chapter describes a convenient and simple system in *E. coli* for studying the complex interactions of human membrane proteins involved in AD, and those proteins thought to interact with members of the γ-secretase complex. The system still needs to be optimised for each particular experiment, but the results so far are promising.

CHAPTER (6)

GENERAL DISCUSSION

6 GENERAL DISCUSSION

6.1 Alzheimer's Disease (AD) to date

AD is a disease affecting the brain, and it is the most common neurodegenerative disease in the world. Nowadays, and especially in industrialised countries it is a major cause of death after heart diseases, stroke and cancer. Improved health care and a better standard of living in the west, resulted in an older average population age. Subsequently, as the risk of AD increases with age, this meant that AD has become a major problem in such countries. The disease causes a lot of emotional and physical strain to the patients and others around them, especially in the later stages. And in addition to the social implications of the disease, the economic cost has started to cause a major problem in our societies. However, so far there is no definite diagnosis for AD and no current cure.

A few laboratories are investigating the chain of events which leads to AD pathology, and in particular the γ-secretase pathway which causes proteolytic cleavage of C99, resulting in the production of Aβ peptides which aggregate and form the amyloid plaques found in the brains of sufferers. However, to date all experiments in other laboratories, which were focused on the γ-secretase complex have so far been performed in eukaryotic systems. We postulate that even though organisms like yeast may have no endogenous γ-secretase activity, it is possible that other endogenous proteins could interact with PS-1, NicA, Pen-2 or Aph-1 and could aid the reconstitution of γ-secretase activity in eukaryotes. However, to completely rule out the involvement of other factors in bringing about γ-secretase activity, a simpler experimental system would be of use when analysing the complex. Here we describe the use of the gram negative bacteria *E. coli* to investigate the γ-secretase pathway and to verify that PS-1, NicA, Pen-2 and Aph-1 are the minimal components required for an active γ-secretase complex.

6.2 E. coli as a model system for the investigation of γ-secretase activity

The production of human membrane proteins in bacteria is still considered as very difficult as various problems are generally incurred during the process. These problems are not only relevant for high level production but also for basic structure/function analyses or for protein-protein interaction studies.

Difficulties in gene expression include degradation by *E. coli* proteases and mutations in the transformed plasmids. Therefore in this project we describe the use of suitable expression vectors such as pGDR11 or pCS19, and a suitable *E. coli* strain such as KU98 (Chapter 3). An additional problem may be inclusion bodies that are the result of a high number of un- or not completely folded proteins in the cytoplasm. Folding of human proteins often requires a longer amount of time in *E. coli*. Consequently, aggregation of hydrophobic regions (such as TM-segments in polytopic membrane proteins e.g. PS-1 and Aph-1) leads to the formation of inclusion bodies that are often described as a result of attempts to over-express human genes in bacteria.

However we show that human membrane protein production is possible in *E. coli*. We describe how the simple system of *E. coli* can be used to produce human membrane proteins, determine their topology in the *E. coli* membrane and to analyse their protein-protein interactions. Heterologous expression has numerous advantages such as the absence of homologues and splice variants. The bacterial system also allows convenient modification and purification approaches.

6.3 Components of the γ-secretase complex are successfully produced in E. coli

6.3.1 Expression studies of the γ-secretase complex components, and the substrate C99

Chapter (3) of this thesis describes successful cloning of the genes encoding Pen-2 and Aph-1 from a cDNA library, and their subsequent expression in *E. coli*. Expression of both *pen-2* and *aph-1* from the plasmids encoding them, pSA1 and pSA11 for Pen-2; and pSA2 and pSA12 for Aph-1, resulted in the production of a high amount of each protein as verified by Western blotting (Figure 3.3). Pen-2 was found to be stable after 4 hours and overnight induction while Aph-1 was stable after 4 hours of induction but was degraded overnight, presumably by *E. coli* proteases. Additionally despite initial suspicions, it seems that the presence of a His-tag fused to the N-terminus of Pen-2 does not affect the levels of expression of the protein, nor does it affect its topology. Therefore the plasmid pSA1, encoding Pen-2 with an N-terminal His-tag was used in all subsequent experiments as the presence of a His-tag was useful for the detection of protein expression and subsequent protein purification (Chapter 4).

It was thought that co-expression of *ps-1* which encodes one of the γ-secretase complex components, and *c99* which encodes the γ-secretase substrate may aid the stabilisation of Aph-1 after overnight induction, but this was not found to be the case. Co-expression of *ps-1* and *c99* (pSG33) with *aph-1* (pSA2 and pSA12) did not stabilise Aph-1 overnight, nor did it increase the levels of Pen-2 or Aph-1 produced in the strain KU98. However, as we are uncertain about the sequence of events governing the assembly of the γ-secretase complex or how the individual components interact with each other, it was thought that stabilisation of Aph-1 could possibly be achieved by co-expression of the of the genes encoding the other γ-secretase components NicA, and Pen-2. Additionally, the PS-1 gain of function mutant PS1Δexon9 was found to be involved in FAD so the co-expression of *ps1Δexon9* and *c99* (pSG34) with either *pen-2* or *aph-1* may result in higher levels of expression of the genes and/or the stabilisation of Aph-1 overnight. A suggestion

for a new experiment could be to combine the four γ-secretase components, in the presence and absence of C99 in the same protease minus strain such as KU98 and to compare that to co-expression of the four components in a number of strains containing different combinations of the γ-secretase components. This was not possible to accomplish in this project as a result of the different antibiotic resistances conferred by the plasmids containing the γ-secretase components' genes, and the different origins of replication. However, additional strains should be investigated and it is also a possibility to clone the genes encoding the γ-secretase components into alternative plasmids. Such an experiment could result in an achievement of an even higher level of over-expression for the γ-secretase components. The individual or combination of proteins can then be used for purification studies and enzyme assays.This will also give us an insight into which of the γ-secretase components directly interact with each other and the sequence of events leading to the γ-secretase cleavage of C99. This will provide a very good and detailed sequence of events that can be targeted for drug design and future treatment of AD.

Chapter (3) described the construction of a plasmid containing *pen-2* and *aph-1* to aid the combination of all the γ-secretase components in the same bacterial strain. We successfully cloned and expressed *pen-2* and *aph-1* in the plasmid pGRD11 (pSA13 and pSA14) (Figure 3.5). The production of Pen-2 and Aph-1 in the strain KU98 was detected by incubating the PVDF membrane containing both proteins with a monoclonal α-penta histidine antibody (Qiagen). The purification of Pen-2 in chapter (4) has revealed that pure Pen-2 can form dimers. The pen-2 dimer migrates at roughly the same molecular weight as an Aph-1 monomer (25 kDa), therefore in hindsight we are aware that using the α-penta histidine antibody for the detection of both proteins present on the same membrane is not advisable, as it could be very difficult to differentiate between an Aph-1 monomer and a Pen-2 dimer. However, at the time of the experiment there was no alternative antibody that could have been used for the detection of Aph-1. Additionally, Pen-2 has

only been shown to form dimers when purified, or very rarely when expressed in very high amounts and the SDS gels are overloaded. Such behaviour was also observed during over-expression and purification of the γ-secretase complex substrate C99. Furthermore, even if the band at 25 kDa (Figure 3.5) represented Pen-2, this does not rule out the possibility that Aph-1 is also expressed but at very low levels and that it is migrating at the same molecular weight.

6.3.2 Topological Studies

Chapter (3) described how the well established method of *phoA* fusions was used to ensure that the human γ-secretase components expressed in *E. coli* (Manoil and Beckwith, 1986) have the correct topology because that is essential for their function. Previous work (Fortna et al., 2004) has shown that Aph-1 spans the membrane seven times. However, according to the amino acid sequence of Aph-1, it was predicted that the protein will have six transmembrane segements (Kyle and Doolittle, 1982). This work has supported this prediction by expression studies and the performance of PhoA assays. Additionally, Pen-2 was found to span the bacterial membrane twice, which is what was predicted from its amino acid sequence.

6.3.3 Purification studies and mass spectrometry

Chapter (4) of this thesis describes successful purification of Pen-2 via FPLC by using its N-terminal His-tag. The purity of the Pen-2 produced was ascertained by loading the pure protein onto a Shaegger gel and subsequently Coomassie staining to check for the lack of other proteins, and Western blotting with a polyclonal α-Pen-2 antibody to check that all bands seen represented Pen-2. Mass spectrometric studies on pure Pen-2 should be performed to check the protein's exact molecular mass, and to determine if it has undergone any post-translational modifications in bacteria. Other uses for pure Pen-2 could be the production of polyclonal antibodies directed

specifically against Pen-2 which would be useful for future work involving this protein investigating, for example, the γ-secretase complex or the interaction of Pen-2 with other proteins.

This chapter also describes the purification of Pen-2 from whole cell extract. This could be a disadvantage because it means that any Pen-2 that was not properly translocated to the bacterial membrane was also purified. However, figure (4.1) shows that most of the Pen-2 was located in the membrane. However, the purification of pen-2 from membranes was not possible when attempted in this case. Thus, some thought should be invested into how membranes of Pen-2 could be prepared and the protein purification performed with the least amount of protein loss possible. On the other hand, the purification scheme employed here is probably unproblematic as the small Pen-2 protein lacks a complex tertiary structure. After the dilution of detergent, Pen-2 should be well able to fold into its proper conformation.

Figure (4.4) shows Pen-2 obtained as a result of purification by FPLC. In addition to the protein migrating at the correct molecular weight of approximately 12 kDa, other bands were observed that could be oligomers of Pen-2. This behaviour was also seen with C99. However, as the bands are undefined and migrate as a smear there is also a possibility that they could be aggregates of Pen-2 and that pen-2 shows similar behaviour to PS-1 when a large amount of protein is loaded onto a gel (Mona Harnasch, PhD Thesis, 2003).

Co-purification studies of C99 and PS-1 have indicated that the two proteins interact (Harnasch et al., 2004). To investigate the interaction of the other γ-secretase components, or interactions between the γ-secretase components and the substrate C99, additional co-purification experiments could be performed. An example is the use of the plasmid pSA16 which encodes Pen-2 without a tag and Aph-1 with an N-terminal His-tag. The His-tag could be used to purify Aph-1 with a Ni

NTA column, and it would be interesting to see if Pen-2 will co-purify. This experiment would give us an insight into a possible interaction between the γ-secretase components Pen-2 and Aph-1.

6.4 An investigation into the activity of the γ-secretase complex in E. coli

6.4.1 γ-Secretase Assays and Aβ ELISA

An attempt at reconstituting γ-secretase activity in *E. coli* in initial assays was described in chapter (4). Two experiments were carried out. The first involved co-expression of *ps-1, c99, pen-2, aph-1 and nicA* in one *E. coli* strain, KS272 whereas the second assay involved the expression of the genes encoding the individual components in the strain KU98 separately and subsequently mixing the membranes containing the γ-secretase components.

It seems that the co-expression experiment was more successful at reconstituting γ-secretase activity, compared to the experiment involving the mixing of bacterial membranes containing the γ-secretase components. The first experiment resulted in higher γ-secretase activity and lower background levels. However, the second assay should not be completely excluded as perhaps a combination of the two could be achieved. For example, the γ-secretase components could be purified individually or in combinations, and subsequently the pure proteins could be mixed together. Additionally, because the Aβ ELISA is carried out on a 96-well plate, various conditions could be conveniently tested for their effect on the γ-secretase activity measured. Different detergents could be used to solubilise the γ-secretase components from membranes, the assay could be carried out in various concentrations of salts and different pH values. Despite the fact that the ELISA detected relatively low levels of Aβ, the amounts still correlated with what is so far published in the literature (Fraering et al., 2004).

6.4.2 The E. coli two-hybrid system unveils interactions between γ-secretase components and isolates candidate interaction partners of PS-1 and PS-1Δexon9

Recent studies have identified the gene products that comprise the γ-secretase and established their physical interaction is required for γ-secretase activity. It was previously shown that under non-denaturing conditions, the γ-secretase is associated with a molecular mass which corresponds to the combined apparent molecular mass of PS, NicA, Pen-2 and Aph-1 (Kimberly et al., 2003). However, little is known about their specific roles in intramembrane proteolysis or the nature of the individual protein-protein interactions within the active complex. Additionally, any information regarding the interaction between different γ-secretase components will give us insight into the structural organisation of the active γ-secretase complex. This is essential for determining the biochemistry of the γ-secretase complex involved in the processing of Notch and several other type I transmembrane proteins, most importantly for us, APP.

It was established by Dove and co-workers (1997) that any protein-protein interaction can activate transcription in *E. coli* provided one protein of interest is bound to the DNA via a DNA binding domain (e.g. λcI), and the other protein of interest is bound to a subunit of RNA polymerase. Previous work in this laboratory where PS-1 was fused to the λcI and C99 was fused to the ω subunit of RNAP, showed that the two proteins interact in an *E. coli* two-hybrid system (Harnasch et al., 2004). It was then interesting to see if other γ-secretase components interacted which would help us build a picture of how the γ-secretase cleavage step takes place and the sequence of events involved.

Firstly, to analyse interactions between the known γ-secretase components, a number of gene fusions were generated (Chapter 5). *aph-1* was fused to the ω subunit of RNAP and the λcI ; and *nicA* was fused to the ω subunit of RNAP. Successful cloning of the genes into appropriate

expression vectors is described. However, although expression of the gene fusions was not optimised, β-galactosidase assays indicated not only that the protein fusions were produced, but also that members of the γ-secretase complex interact with each other. The results indicated that Aph-1 interacts with PS-1 and NicA but not with C99 and that NicA interacts with PS-1, which correlates with the literature (see next paragraph).

The PS heterodimer is thought to be the active form of the protein (Capell et al., 1998; Podlisny et al., 1997; Thinakaran et al., 1996; Yu et al., 1998). All presenilins contain two conserved intramembraneous active site residues, the mutation of which reduces Aβ secretion and PS endoproteolysis. This together with the fact that γ-secretase inhibitors can be covalently linked to PS1 NTF and CTF (Esler et al., 2000; Li et al., 2000a), suggests that PS NTF/CTF heterodimer comprise the active site of γ-secretase. Furthermore, co-immunoprecipitation experiments in multiple cell lines using antibodies against Aph-1 reveal a selective association between Aph-1 and NicA. This shows that Aph-1 and NicA directly interact early in the γ-secretase assembly, forming a stable intermediate prior to the incorporation of PS and Pen-2, PS endoproteolysis and the final glycosylation of NicA all of which are known to correlate with mature, active γ-secretase (LaVoie et al., 2003). Fraering and co-workers have shown that the detergent DDM can dissociate active γ-secretase complexes in a concentration dependent manner into two major complexes (mature NicA and Aph-1, Pen-2 and PS-1-NTF; and two minor complexes NicA, Aph-1 and PS-1-CTF; and PS-1CTF and PS-1 NTF. This is likely to reflect the physical interactions that exist in the active γ-secretase complex (figure 6.1).

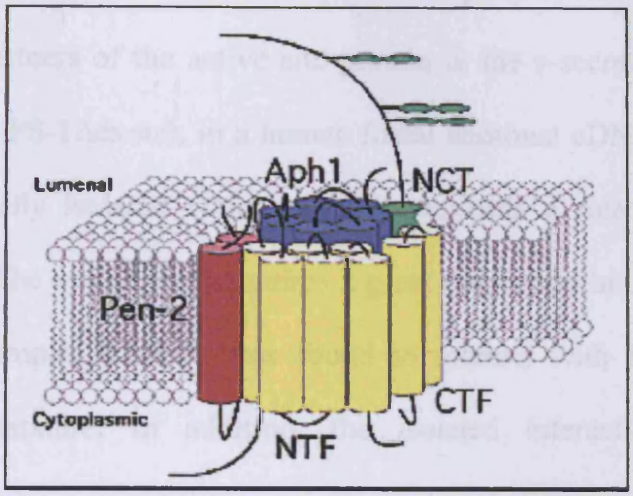


Figure (6.1) Schematic representation of the subunit organization within the active γ-secretase complex.

This model is based on the protein-protein interactions identified in the complexes generated by the DDM-mediated dissociation of γ-secretase: Pen-2 interacts directly with PS1-NTF, mNicA (Here NCT) interacts directly with Aph1, PS1-CTF interacts directly with PS1-NTF, and PS1-CTF interacts with NicA and Aph1. Taken from Fraering et al (2003).

Evidence suggests that Aph-1 stabilises the complex while Pen-2 is required for endoproteolytic processing of PS-1 in mammalian cells (Luo et al., 2003; Takasugi et al., 2003) and in yeast (Edbauer et al., 2003). Because proper trafficking and compartmentalization of all four these proteins as well as full glycosylation of NicA are essential for formation of functional γ-secretase complex, Baulac and co-workers analysed the interactions of these newly identified members using immunofluorescence microscopy in chinese hamster ovary (CHO) cells stably expressing the human proteins. They report that all four proteins interact in the Golgi/*trans*-Golgi network (TGN)-enriched compartments where they observe γ-secretase cleavage of APP C-terminal fragments (C99, C89, C83) and generation of Aβ and AICD. Immunofluorescence studies provide evidence for the localisation of the components in the Golgi/TGN compartments rather than in the ER (Baulac et al., 2003).

Work on this project has also shown that the *E. coli* two-hybrid system can be utilised to screen for candidate interaction partners of the active site protein in the γ-secretase complex, PS-1; and the gain of function mutant PS-1Δexon9, in a human foetal neuronal cDNA library. A few interaction partners were successfully isolated which all point towards a role for PS-1 in many various processes (Chapter 5). The system still requires a great deal of optimisation, but the results so far are promising. For example, RAB3A was found to interact with PS-1 which correlates with information in the literature. In addition, the isolated interaction partners await further investigation.

6.5 Conclusion

This thesis shows that *E. coli* represent a simple and efficient system that can be used to analyse human membrane proteins. Components of the γ-secretase complex were successfully cloned and expressed in *E. coli* and their topologies in the bacterial membrane were studied using *phoA* fusions. Furthermore, interactions of the γ-secretase complex components were investigated using the *E. coli* two-hybrid system, and intitial studies to reconstitute γ-secretase activity using the components produced in *E. coli* were carried out. This was an interesting project, which opened up many possibilities for the study of the γ-secretase complex in *E. coli* and presented potential targets and strategies for the treatment of Alzheimer's disease. For example, convenient production of the active γ-secretase complex could improve high throughput screens for γ-secretase inhibitors or small molecules interfering with γ-secretase complex assembly.

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