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A

THE CHARACTERIZATION OF THE SECRETASES INVOLVED IN BETA-APP PROCESSING IN YEAST AND MAMMALS

By

DANIEL OMAR ESPINOZA

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2001

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Abstract

The Characterization of The Secretases Involved in Beta-APP Processing in Yeast and Mammals.

By

Daniel Omar Espinoza

Adviser: Dr. David L. Miller

Accumulation of β-amyloid peptide (Aβ) is thought to be one of the principal causes of Alzheimer's disease (AD) pathology. Aβ is a metabolic product of the β-amyloid precursor protein (APP), a type I transmembrane glycoprotein. The enzymes that cleave APP within the Aβ domain are designated α-secretases. The enzymes that generate the amino and carboxyl termini of Aβ are respectively designated β- and γ-secretase(s). None of these secretases had been identified when this study was started. Much progress has been made in the last 2-3 years. Two β-secretases were recently identified, and there are now strong candidates for α- and γ-secretases. The identification of these secretases is a major goal of AD research because they regulate accumulation of Aβ. The yeast *Saccharomyces cerevisiae* was proposed as a model for the identification of human α-, β- and γ-secretase(s) candidates from a human brain cDNA library. We and others have reported the existence of α-secretase activity in yeast that has the same specificity as the human α-secretase(s), but no β- nor γ-secretase activities could be detected. Two homologous aspartyl proteases, Yps1p and Yps2p seem to be responsible for most of the yeast α-secretase. The deletion of two other *YPS1*-homologous genes did not reduce yeast α-secretase activity. A yeast system was developed to screen for human APP secretases

from a human cDNA library. The system consists of a yeast acid phosphatase fused at its carboxyl terminus to the last 106 amino acids of human APP, and a yeast strain with almost no APP-secretase activity. This yeast system was tested with reported human secretase candidates. It was found that full length β-secretases are not expressed at detectable levels in yeast. On the other hand, expression of mature forms of these enzymes can be detected when they are fused to the yeast α-mating factor signal sequence. Presenilin 1 was also expressed in yeast and tested as a γ-secretase candidate, but no γ-secretase activity could be detected. Other cofactor(s) that are not present in yeast may be needed to activate γ-secretase.

To my parents Jorge and Josefina.
To my sisters Marlene, Jessie, Marilu, and Josefina and my brother Jorge.
For all their love and support throughout my life.

Acknowledgments

I would like to thank many people that directly or indirectly helped me during my studies:

Dr. David Miller, my advisor, for all his guidance, support and friendship since I joined his laboratory at the NYS Institute for Basic Research (Staten Island, NY).

Dr. Anna Potempska and Ms. Kathryn Mack for their friendship and all the help with Western blots and transfection experiments.

Dr. Julia Currie for her friendship, help in searching and analyzing DNA and protein sequences from databases, and for many suggestions.

Dr. Carlos Monge and Dr. Fabiola Leon-Velarde from the Universidad Peruana Cayetano Heredia in Peru, for all their guidance, friendship and support since I started my Masters studies in Peru many years ago.

Dr. James Larrick, Dr. Josefina Coloma and Dr. Sherrie Morrison for introducing me to the exciting world of Molecular Biology.

Dr. David Soifer, Dr. Ekkhart Trenkner and Ms. Diane Cocozza, for their support to all the students that join the CSI/IBR Center for Developmental Neuroscience, not only in the form of fellowships, but also for their friendship and for welcoming many foreign students like myself.

Dr. Wei Zhang, my former lab partner and fellow student, for his friendship and for introducing me to the yeast system.

Dr. Yu-Wen Hwang for his advice when I had problems cloning some of the genes.

Ms. Patricia Casiano, former secretary of our department, for her friendship all these years.

Dr. Sandrine Funes for her friendship, for listening my practice talks and for pushing me to finish the writing of this thesis.

To my thesis committee members, for all their guidance, friendship and support during my studies.

To all the people that kindly provided me with plasmids, cDNA, antibodies, etc.

To the CSI/IBR Center for Developmental Neuroscience and NIH grant # AG04220 for the financial support during my studies.

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5-FOA: 5-fluoro-orotic acid
αCTF: α-secretase cleaved C-terminal fragment
α-sAPP: soluble N-terminal fragment of APP after α-secretase cleavage.
βCTF: β-secretase cleaved C-terminal fragment
β-sAPP: soluble N-terminal fragment of APP after β-secretase cleavage.
Aβ: β-amyloid peptide
AD: Alzheimer's disease
ADAM: A Disintegrin And Metalloprotease
AEBSF: 4-(2-aminoethyl) - benzenesulfonyl fluoride
ApoE: apolipoprotein E - in human chromosome 19
APP: β-amyloid precursor protein
APPsw: APP carrying the Swedish mutation
BACE: β-secretase site APP Cleaving Enzyme
C99: the last 99 amino acids of APP (starting at the β-secretase cleavage site)
C106sw: the 106 C-terminal amino acids of human APPsw
C-ter: Carboxyl terminal end of a protein
CSM: Complete synthetic medium
CTF: Carboxyl terminal fragment
DS: Down's syndrome
ELISA: Enzyme-linked immunosorbent assay
ER: Endoplasmic reticulum
FAD: familial Alzheimer's disease
N-ter: Amino terminal end of a protein
NTF: Amino terminal fragment
PCR: Polymerase Chain Reaction
PMA: Phorbol 12-myristate 13-acetate
PDBu: phorbol 12,13-dibutyrate
PS1: presenilin 1 (or S182) - located in human chromosome 14
PS2: presenilin 2 - located in chromosome 1
TACE: Tumor necrosis factor Alpha Converting Enzyme
TGN: Trans Golgi network
TM: transmembrane domain
TS: temperature-sensitive
Tricine: N-tris(hydroxymethyl)methylglycine

Introduction

Alzheimer's Disease (AD):

As the life expectancy of the human population increases due to better diet, advances in biomedical research, and more healthful life styles, a new challenge emerges for the aging population: to maintain a reasonably functional mind and body. It is thought that the increase in the number of elderly people will be accompanied by a significant rise of the number of people with dementia (Maj and Sartorious, 2000). Memory impairment is common in older people. The causes of this impairment are several: poor diet, poor blood circulation, strokes, accumulative effects of medicines or drugs, etc. (Ashford and Zec, 1993). Neurodegenerative diseases, particularly Alzheimer's disease, become highly prevalent beyond the seventh decade of life (Selkoe, 1998).

Approximately 50 disorders are known to cause dementia, of which AD is recognized as the most common. AD is responsible for about 50% of all dementias, with an additional 15-20% of dementias having a combined AD and vascular pathology (Albert et al. 1999). Vascular dementia alone, due to strokes that destroy areas of the brain involved in memory, is probably the second leading cause. Less common causes of dementia include Parkinson's disease, severe alcohol abuse, Creutzfeldt-Jakob disease, Huntington's disease, Pick's disease, and fronto-temporal dementias (Henderson and Jorm, 2000). Genetic and environmental factors affect the development of many of these diseases.

With the aging of the population, AD is becoming an increasing health problem. It is estimated that 1.5 to 3 million people in the US currently have the disorder, and AD accounts for 40% to 50% of nursing home admissions and between 120,000 to 200,000 deaths per year (Zec, 1993). The annual cost of this disease in the US in 1990 exceeded $80 billion (Zec, 1993).

The beginning stages of AD are characterized by the failure to recall recent events or to process new information, but with no apparent motor impairments. As the disease advances, old memories are affected, the person becomes disoriented, and motor functions start to fail. These impairments have been correlated with lower than normal metabolism in brain areas involved in these functions, as seen by PET scan studies (Parks et al., 1993). The presence of amyloid plaques and tangles of intracellular fibrils are also characteristic in brains of AD patients examined post-mortem (these structures will be detailed later in the text), especially in brain areas such as the limbic system, entorhinal cortex, and association cortices (Hyman et al., 1993; Selkoe, 1994).

As previously stated, although memory impairment may be due to several causes, a progressive loss of memory is a characteristic of AD; however, the definitive diagnosis is only done post-mortem. Several risk factors have been identified for the onset of AD, including: old age, family history of AD, Down's syndrome, point mutations in the APP and the presenilin genes, and the presence of the Apolipoprotein E4 (ApoE4) allele (Zec, 1993; Selkoe, 1997; and others). Other risk factors for which there is no clear evidence include: national and ethnic background, head trauma, aluminum in the water supply, and occupational exposure to magnetic fields (reviewed in Henderson and Jorm, 2000). Use of anti-inflammatory drugs, estrogen replacement therapy and a higher education level are considered possible protective factors against the risk of developing the disease (reviewed in Nourhashemi et al. 2000).

Brains of AD patients show two typical structures initially described by Alois Alzheimer (Bick et al., 1987): senile plaques and neurofibrillary tangles (NFT). Senile plaques are extracellular structures whose main proteinaceous component is the β-amyloid peptide (Aβ), often accompanied by glial cells and dystrophic neurites (Selkoe, 1998; Miller et al., 1993; Masters et al. 1985). NFT are intraneuronal structures that consist, at least in part, of insoluble cytoskeletal proteins, τ (tau) being one of the main components (Hyman, et al., 1993). These structures develop over many years or decades as a person ages, but AD pathology develops just in certain people (Selkoe, 1997). However, some risk factors, as the ones previously described, favor the early, or late, onset of AD. In familial AD cases, which represent about 10-15% of all AD cases (Saftig et al., 1999), mutations in a few genes have been correlated with the onset of AD. Risk factors that favor the early onset of AD include mutations in the βAPP gene (on human chromosome 21), presenilin 1 (on chromosome 14), and presenilin 2 (on chromosome 1). The ApoE4 polymorphism (chromosome 19) seems to predispose a late onset of AD (Selkoe, 1997). APP mutations include the Swedish (APPsw) double mutation, K595N and M596L (APP695 numbering) and the London mutation (V642I), among others. The Swedish mutation increases the production of Aβ, and preferentially, the more amyloidogenic form terminating at Ala638 in tissue culture cells and in patients carrying the mutation (Citron et al., 1992; Citron et al., 1994; Selkoe, 1997). The London mutation does not result in an overall increase of Aβ levels, but favors instead the production of the more amyloidogenic form (Selkoe, 1994). A similar increase in Aβ production is seen in hereditary hemorrhage of the Dutch type, where there is a mutation in the APP gene (E693Q) (Selkoe et al., 1996a). The clinical, neuropathological and biochemical abnormalities of familial AD cases are very similar, if not identical, to sporadic AD cases (Saftig et al., 1999; St. George-Hyslop, 2000).

Even though the etiology of AD is not known yet, it seems that the accumulation of Aβ in the brain is an early and invariant event in the development of AD (Selkoe, 1997). In this aspect, AD resembles other diseases where proteins are excessively accumulated in the extracellular spaces of tissues and which are collectively called amyloidoses (Selkoe, 1996a).

The β-amyloid Precursor Protein (APP)

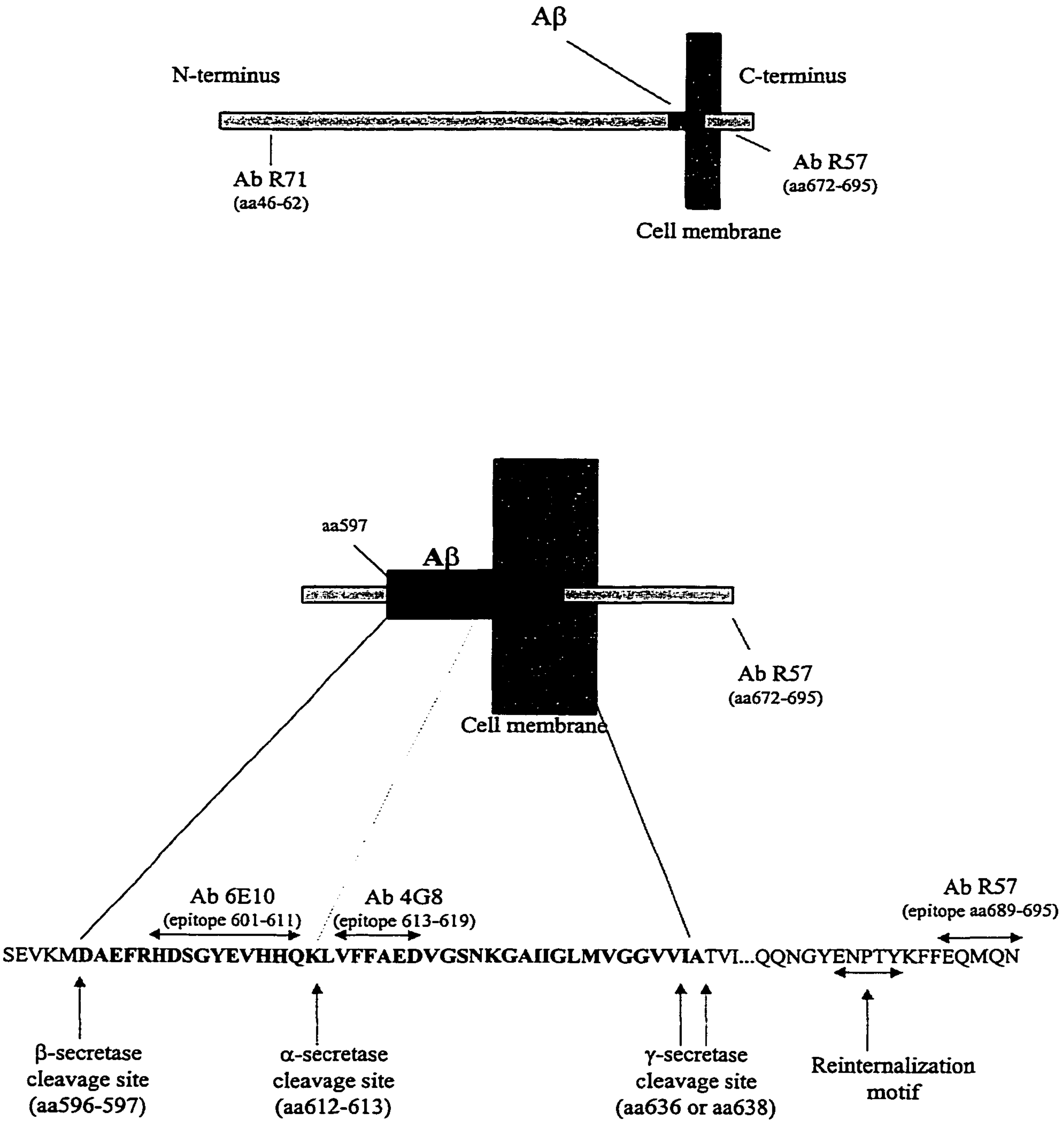
Aβ is a metabolic product of a much larger transmembrane glycoprotein called β-amyloid precursor protein (APP). APP is a member of a conserved gene family found in most taxonomic classes, including the fruit fly (*Drosophila melanogaster*) (Rosen et al., 1989), mammals (Yamada et al., 1987; Sandbrink et al, 1996), amphibians (*Xenopus*) (Okado and Okamoto, 1992), *Caenorhabditis elegans* (APP-like) (Daigle and Li., 1993), and chickens (Sarasa, Rodolosse and Sorribas – unpublished - Genbank acc# AAG00594).

The APP gene maps to the long arm of human chromosome 21 and consists of 19 exons which are alternatively spliced into at least 8 variants (Konig et al., 1992; Sandbrink et al, 1996). These different isoforms originate from alternative splicing of APP mRNA, and it involves exons 7, 8, and 15 (reviewed in Sandbrink et al, 1996). The single transmembrane domain is encoded by exon 17. Aβ, a 39 to 42 residue peptide, is encoded by portions of exons 16 and 17 (Beyreuther et al., 1993). APP is expressed in a variety of tissues. High levels of expression are found in brain, lung, kidney, muscle, and spleen (Tanaka, 1989; Neve, 1988). The expression of APP isoforms is cell or tissue specific and also varies with the state of differentiation or activation of the cell (Currie et al., 1997). The APP695 isoform is predominantly expressed in mammalian brains (Neve, 1988) and it is regulated during brain development (Loffler and Huber, 1992).

Full length APP's are type I membrane bound glycoproteins, each with a large N-terminal lumenal domain, a transmembrane domain, and a short C-terminal cytoplasmic region (Figure 1). During its synthesis, APP is translocated to the endoplasmic reticulum by its signal peptide and undergoes N- and O-glycosylation and sulfation in the ER and Golgi to become a mature protein (reviewed in Selkoe, 1994). APP then follows the constitutive secretory pathway, as was initially shown in primary cultured neurons (Overly et al., 1991), microglia and astrocytes (Haass et al., 1991). After it reaches the plasma membrane, APP may be secreted or else reinternalized to be further processed or recycled to the plasma membrane. APP is phosphorylated in its ectodomain during its transport through the secretory pathway (Selkoe 1998).

Figure 1: APP is a type I transmembrane protein.

Diagram represents APP695 isoform and shows the postulated cutting sites of the different secretases involved in APP processing. R71, R57, 6E10, and 4G8 are different antibodies generated against different regions of human APP. The lower diagram is a more detailed representation of the C-terminal portion of APP695 and shows the reinternalization motif (NPTY).

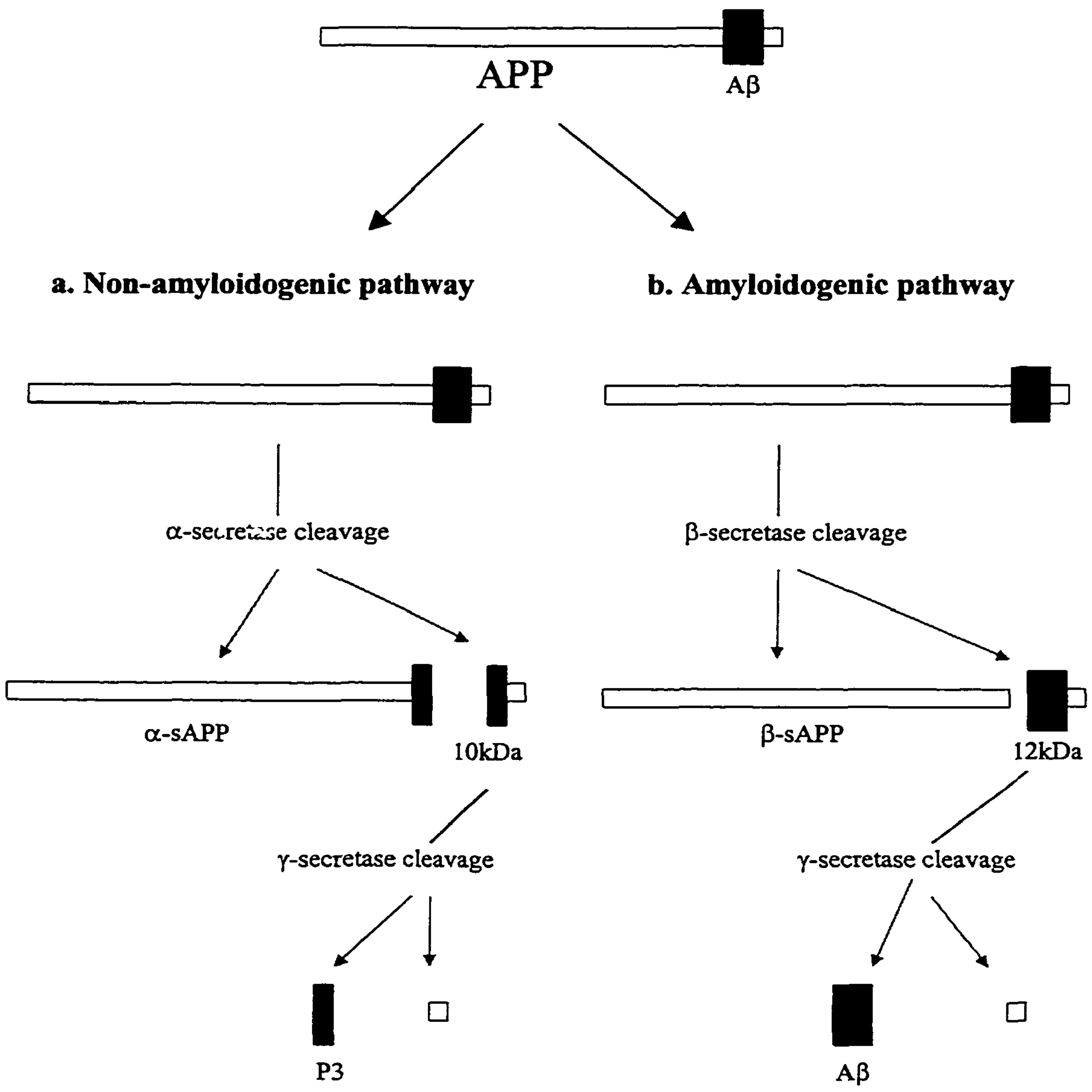


APP reinternalization and targeting to the endosomal/lysosomal compartment was suggested by the fact that intact APP can be recovered from clathrin-coated vesicles (Nordstedt et al., 1993) and by the presence of the NPXY clathrin adapter binding motif in its cytoplasmic portion (Figure 1). APP reinternalization was shown by labeling surface APP with biotin or iodinated antibodies, and by deletion of the NPXY motif. The labeled intact molecules can be detected in endosomes/lysosomes, but when the NPXY motif is deleted, surface APP is retained longer on the plasma membrane (Selkoe, 1996a). Late endosomes/lysosomes isolated from these cells contain full length APP and smaller fragments containing Aβ (potentially amyloidogenic fragments). The half-life of surface APP molecules was found to be 5 to 10 minutes (Koo et al. 1996). Internalized APP can recycle back to the cell membrane within 10 min (Yamazaki et al., 1996).

APP processing can follow either an amyloidogenic pathway, which leads to Aβ formation, or a non-amyloidogenic pathway, which prevents Aβ formation (Figure 1, Figure 2). It appears that only about 30% of all biosynthesized APP molecules are processed following either of these pathways, while the reminder of the full length APP molecules are found inserted into internal membranes, particularly in the Golgi (Weidemann et al., 1989; Selkoe, 1996a) and nuclear membranes (Currie et al., 1997). These APP molecules are turned over by non-secretory processes (Weidemann et al., 1989).

Figure 2: Processing of APP by α-, β-, and γ-secretases.

APP can follow an amyloidogenic pathway (leading to the formation of Aβ by the action of β- and γ-secretases) or a non-amyloidogenic pathway (Aβ formation is precluded by the action of α-secretase). Accumulation of Aβ is thought to be one of the leading causes of AD pathology.



Secretases and Amyloid β (Aβ) Formation

Aβ is generated by normal metabolic processing of APP by enzymes called secretases (Haass et al. 1992; Haass and Selkoe, 1993). Secretases have been described as a group of enzymes that release (solubilize) membrane associated proteins (Hooper et al., 1997). A review by Nigel Hooper et al. (1997) lists several membrane associated proteins that have soluble isoforms generated by proteolysis that are probably products of one or more secretases. These membrane associated proteins include type I and II, as well as glycosyl-phosphatidyl-inositol (GPI) membrane-anchored proteins. Among these proteins one can find cell-adhesion molecules (e.g. CD14, CD18, VCAM-1, etc), leukocyte antigens (e.g. class I MHC, CD43, CD44, etc.), growth factors (e.g. TGF-α, TNF-α, CSF-1, etc.), receptors (e.g. NGFr, CFS-1r, IL1r, IL6r, etc.), ectoenzymes (e.g. angiotensin converting enzyme), and APP (for a complete review and appropriate references see Hooper at al., 1997).

Aβ is generated by at least 2 processing enzymes that cleave at the N-terminus (β-secretase) and at the C-terminus (γ-secretase) of the peptide. α-secretase(s) cleaves within Aβ, precluding Aβ formation (Figure 1, Figure 2). Until recently none of these secretases had been identified. Two β-secretases have now been reported (Vassar et al., 1999; Hussain et al., 1999; Hussain et al., 2000; Yan et al. 1999; Sinha et al. 1999; Acquati et al. 2000, Lin et al 2000), and there are now strong candidates for α- and γ-secretases.

The cellular compartment (especially membranous), lack of specificity, and lack of an appropriate system to follow secretase activity when attempting their purification are probably some of the reasons their identities had not been revealed. The fact that they could become masked by other proteases released in cell lysates or that more than one enzyme could cleave at the same site, or very close to it, make their characterization difficult.

α-Secretase Activity Precludes Aβ Formation

Cleavage of APP by α-secretase renders a large amino-terminal (N-terminal) soluble fragment (α-sAPP) and an approximately 10kDa carboxyl-terminal fragment (CTF), which precludes Aβ formation (Figure 2). The 10kDa CTF is further processed by γ-secretase to render a 3kD fragment (P3). The α-secretase usually cleaves APP before Leu17 or at Val18 (Selkoe, 1994) of the Aβ sequence. In fact this has been difficult to demonstrate unequivocally, owing to the potential for amino- or carboxy-peptidase removal of the Lys16 residue after the primary cleavage event (Hooper et al., 1997). Neither substitutions of amino acid in the neighborhood of the α-secretase cleavage site nor addition or deletion of amino acid residues between the α-secretase cleavage site and the transmembrane domain greatly affect the rate of cleavage by α-secretase; mutant sequences are cleaved at the same distance from the membrane as wild type. This may indicate that the enzyme is not sequence specific and what seems to be important is the tertiary structure around this site (Zhong et al., 1994; Hooper et al., 1997). In fact, APP is predicted to have a helical structure around the α-secretase cleavage site and some, but not all, amino acid substitutions that are thought to disrupt α-helix structures are also effective in preventing α-secretase cleavage (Checler, 1995; Hooper et al., 1997). Hooper and Turner (1995) also suggest that α-secretase might recognize bulky residues at the P_1' position (nomenclature of Schecter and Burger, in which the enzyme cutting site is between P_1 and P_1', P_1 being at the N-terminus, and P_1' at the C-terminus).

α-secretases in CV-1 monkey fibroblasts expressing recombinant APP cleave the precursor at Lys16 (Aβ numbering) 60% of the time and at Phe19 40% of the time (Zhong et al, 1994). Parallel studies using fibroblasts from a Down's syndrome patient found that the α-secretase activity in these fibroblasts generate C-terminal products cleaved at Phe19, Glu22, and Gly25, but not at Lys16. APP secretion may involve multiple secretases varying according to cell background (Zhong et al., 1994).

Several studies have shown that α-secretase can act either at the plasma membrane or intracellularly. The use of inhibitors to block the four major classes of proteases in N2a neuroblastoma cells did not affect secretion of α-sAPP, indicating that α-secretase was not accessible to the inhibitors, and thus is located most likely in an intracellular compartment (De Strooper et al., 1992). This was confirmed by Sambamurti et al. (1992) using rat adrenal pheochromocytoma PC12 cells. Several studies with agents that interfere with acidic compartments (e.g. NH_4Cl or chloroquine) showed that secretion of α-sAPP is prevented, but that the organelles where α-secretase is located do not include lysosomes since inhibitors that affect lysosomal proteolysis did not effect sAPP secretion.

This suggests that α-secretase activity is located in an acidic compartment, but one distinct from lysosomes. De Strooper et al. (1993) established that α-secretase activity likely occurred in a late compartment of the secretory pathway. Also, α-sAPP was found in intracellular vesicles in PC12 and human neuroblastoma H4 cells, suggesting that α-secretase likely occurs in the TGN or in a late trans-Golgi compartment (Sambamurti et al., 1992a; Kuentzel et al., 1993).

Evidence for plasma membrane-associated α-secretase activity comes from experiments labeling cell surface APP by biotinylation or the use of iodinated antibodies specific for APP. The labeling is done at 4°C and then the cells are washed and incubated at 37°C in fresh medium. After some time labeled α-sAPP is detected in the medium. This is supported by experiments in which the NPTY motif is deleted from the APP cytoplasmic domain. In this case APP reinternalization decreases but an increase of α-sAPP is detected in the medium, suggesting that α-secretase is at the plasma membrane (Checler, 1995; Selkoe, 1996a; Parvathy et al. 1999).

Some candidates for intracellular or plasma membrane-associated α-secretase have been proposed based on the use of model peptides (short peptides) that include the α-secretase cleavage site. The intracellular candidates are expected to have a maximum activity at an acidic pH. Cathepsin B (Tagawa et al., 1991), the multicatalytic proteinase complex (Kojima and Omori, 1992) and a 105-120 KDa metalloproteinase isolated from human brain (McDermott and Gibson, 1991) are some of the candidates for the intracellular α-secretase activity. Similarly, other candidates have been proposed for the membrane associated form, and these include endopeptidase 3.4.24.11 (Marks et al., 1994) and a metalloproteinase present in rat brain cortex (Allsop et al., 1991), among others. These candidates have not been shown to cleave full length membrane-associated APP, thus, unambiguous identification still awaits further studies (Checler, 1995).

α-secretase is inhibited by hydroxamate-based zinc metalloproteinase inhibitors such as batimastat, BB2116 and marimastat, but neither the level of full length APP nor its cleavage by β-secretase are affected by these inhibitors (Parvathy et al., 1998a; Parvathy et al., 1998b). α-secretase differs from matrix metalloproteinases in its enzymatic properties. For example, batimastat has to be used at a 1000 fold dose compared to that used to inhibit collagenase, and several potent matrix metalloproteinase inhibitors have no effect on the activity of α-secretase (Hooper et al., 2000).

The release of surface proteins seems to involve a common mechanism or a few secretases (Arribas et al., 1996; Mullberg et al., 1997). Arribas et al. (1996) isolated a mutant cell line that was defective in the shedding of at least two unrelated proteins, APP and proTGF-α. The metalloproteinase inhibitor TAPI is able to block the release of Steel factor, type II IL-1R and IL-2Rα from transfected COS-7 cells, and PMA (Phorbol 12-myristate 13-acetate) can accelerate the release of these molecules (Mullberg et al., 1997). α-secretase is also postulated to be a metalloproteinase and it is also activated by PMA through PKC (Hooper at al., 1997). This suggest that a secretase, such as the tumor-necrosis-factor-α (TNF-α) converting enzyme (TACE), could have α-secretase activity (Arribas et al., 1996; Mullberg et al., 1997).

There is some evidence that TACE is involved in the regulated α-secretase cleavage of APP (Buxbaum et al., 1998). TACE cleaves pro-TNF-α (26kDa) to release TNF-α (17kDa) from the membrane, but it also seems to be involved in the release of a number of cell-surface proteins (Peschon et al. 1998). TACE is a type I transmembrane protein that belongs to the ADAM (A Disintegrin And Metalloprotease) family of proteases. TACE is also known as ADAM 17. ADAM 10 has also been involved in the basal and protein kinase C-stimulated α-secretase activity in HEK 293. The proteolytically activated form of ADAM 10 was localized by cell surface biotinylation in the plasma membrane, but the majority of the proenzyme was found in the Golgi (Lammich et al., 1999).

There are some similarities between α-secretase and the secretase that releases angiotensin converting enzyme (ACE) from the cell surface (Oppong and Hooper, 1993; Parvathy et al., 1997), but this activity seems to be distinct from TACE (Parvathy et al., 1998a; Parvathy et al., 1998b). ACE plays a key role in the control of blood pressure homeostasis and inhibitors of the enzyme are used clinically in the treatment of hypertension and congestive heart failure. Like APP, ACE is a type I transmembrane glycoprotein which is subjected to post-translational proteolytic processing event that releases a soluble form of the protein from the cell-surface.

α-secretase and ACE secretase display similar inhibition profiles with a variety of hydroxamate-based zinc metalloproteinase inhibitors (Parvathy et al., 1998a; Parvathy et al., 1998b). Both cleave their respective substrates between a basic and a hydrophobic residue (Lys-Leu and Arg-Leu) and are stimulated by phorbol esters, and both secretases appear to be integral membrane proteins (reviewed in Checler, 1995). TACE does not seem to be involved in ACE cleavage, since ACE cleavage is normal in TACE knockout mice (Sadhukhan et al., 1999). TACE involvement in α-secretase cleavage may be cell type dependent (Hooper and Turner, 2000).

β- and γ-Secretase Activities Produce Aβ

β-secretase cleaves APP to produce a ca. 16 residue shorter N-terminal fragment than α-sAPP (β-sAPP) and an approximately 12kDa C-terminal fragment that can be cleaved by γ-secretase to produce the 4kDa amyloidogenic Aβ (Figure 2). Sequencing of the 4kDa peptide shows some heterogeneity at both ends of Aβ purified from AD brain. Asp1 (Aβ numbering) is usually the first N-terminal residue, but minor species begin at Val3, Phe4, Ile6, and Glu11 (Haass et al., 1992; Miller et al., 1993). This may be an indication that β-secretase activity is due to more than one enzyme.

β- and γ-secretase(s) have been shown to be normally active in cells. Aβ is found in the conditioned media of several cell types and it is continuously produced during normal cellular metabolism (Haass et al., 1992a). Pulse-chase experiments suggest that Aβ is made after full maturation of APP and involves acidic compartments other than lysosomes, probably early endosomes or late Golgi (Haass et al., 1993a). This is supported by experiments in which various agents that affect the pH gradients in the vesicular compartments reduced Aβ formation drastically (Shoji et al., 1992; Haass et al., 1993b). Koo and Squazzo (1994) have shown that at least a portion of the secreted Aβ is made during the early endocytic trafficking of APP.

Aβ formation seems to be initiated by β-secretase cleavage and it seems to occur in a different compartment than the γ-secretase cleavage (Dyrks et al., 1993). Aβ has not generally been detected inside cells, and it has been suggested that Aβ is made very close to the plasma membrane, where the γ-secretase activity would be located (Shoji et al., 1992; Haass et al., 1993a). Higaki et al. (1995) reported β-secretase activity in endocytic vesicles and found that a specific calpain inhibitor also inhibited β-secretase but calpain has not been shown to have β-secretase activity. The cytoplasmic domain of APP does not seem to be necessary for Aβ formation, as long as APP remains attached to the membrane. If membrane attachment is compromised, then Aβ production is abolished (Citron et al., 1995).

Many β-secretase candidates have been proposed: gelatinase (Miyasaki et al., 1993), cathepsin G (Sahasrabudhe et al., 1993), calcium-activated serine protease (Abraham et al., 1991), multicatalytic protease (Ishiura et al., 1989), chymase or mast cell protease (Nelson et al., 1993), clipsin (Nelson and Siman, 1990), calpain I (Siman et al., 1990), the metalloprotease EC 3.4.24.15 (McDermott et al., 1992; Papastoitsis et al., 1994), and cathepsin D (Ladror et al., 1994). These candidates are able to cleave small peptides containing the β-secretase site, but failed to cleave full length APP at the expected site. Cathepsin D was considered by some investigators to be a strong candidate because it is found associated with senile plaques, it is the major endosomal/lysosomal aspartyl endopeptidase, it cuts the APP containing the Swedish mutation at a higher degree than wild type APP, it is sensitive to pepstatin, and cuts synthetic peptides containing β-secretase cleavage sites at the expected places (Chevallier et al., 1997). Others suggest that cathepsin D may be the β-secretase in the processing of the APP containing the Swedish mutation, and perhaps there is a different enzyme for the wild type APP (Brown et al., 1996). However, P. Saftig et al. (1996) demonstrated that hippocampal neurons devoid of cathepsin D (derived from cathepsin D knock out mice) can still process APP, produce CTF, and secrete Aβ. This was the case for wild type APP or for APP containing the Swedish or the London mutations, and normal levels of α- and β-cleaved APP were detected (Saftig et al., 1996).

Recently several groups have reported that a membrane associated aspartyl protease (variously named BACE, BACE1, Asp 2, and Memapsin 2) is a β-secretase (Vassar et al. 1999; Sinha et al. 1999; Yan et al. 1999; Hussain et al. 1999; Lin et al. 2000). BACE is located in human chromosome 11. Also reported was a BACE homologue (variously named BACE2, Asp1, memapsin1) whose gene is located in the Down's syndrome critical region of chromosome 21 (Acquati et al., 2000; Hussain et al., 2000).

β-secretases BACE1 and BACE2

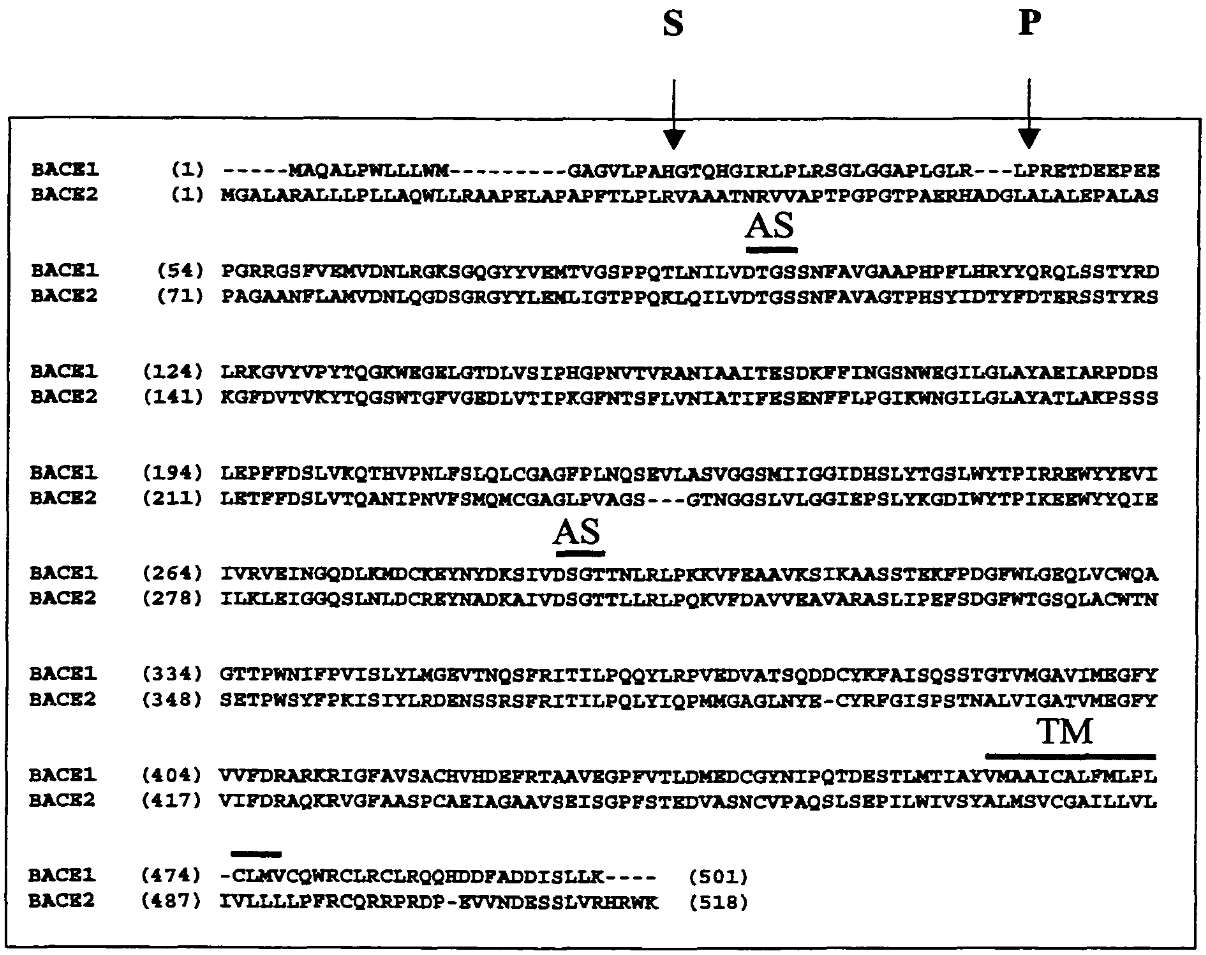
For the purpose of this thesis, BACE (for β-site APP Cleaving Enzyme) will be referred as BACE1 and its homologue in human chromosome 21 as BACE2. BACE1 and BACE2 share a high degree of homology (49% identity; 66% similarity) (Table 1, Figure 3). They belong to the pepsin family of peptidases, but they differ from the rest of the family because of the presence of a putative transmembrane domain. Both are type I transmembrane glycoproteins, with a large lumenal (N-terminus) domain, a transmembrane domain, and a short cytosolic (C-terminus) domain. The BACE1 amino acid sequence is 501 residues long, while BACE2 is 17 residues longer.

BACE1 has been studied more extensively because it is thought to be the main β-secretase in brain (Vassar et al. 1999). Both BACE1 and BACE2 are apparently synthesized as pro-enzymes. Mature BACE1 starts at residue Glu46 (Vassar et al. 1999, Shi et al., in press J Biol Chem). Furin or a furin-like activity cleaves BACE1 pro-domain (Bennet et al., 2000; Benjannet et al., in press J Biol Chem). The N-terminus of mature BACE2 has not been characterized that well.

BACE1 has all the expected properties of β-secretase (Vassar et al. 1999; Lin et al 2000). Its mRNA is highly expressed in the brain and pancreas (Vassar et al. 1999; Lin et al 2000), it cleaves APP at the β-secretase site, and it cleaves the APP Swedish mutant more readily than wild-type APP (Vassar et al. 1999; Lin et al 2000; Shi et al., in press J Biol

Figure 3: Amino acid sequence of BACE1 and BACE2.

Sequence alignment performed using Vector NTI software (Informax Inc.). Amino acid sequences were translated from Genbank accession numbers AF190725 (BACE1) and AF050171 (BACE2). Arrows indicate putative cleavage sites between signal peptide and pro-domain (S) and pro-domain and mature peptide (P), respectively. Conserved aspartyl proteases active sites (AS) and putative transmembrane domains (TM) are indicate with a bar on top of the corresponding sequence



```
BACE1    (1) -----MAQALPWLLLWM---------GAGVLPAHGTQHGIRLPLRSGLGGAPLGLR---LPRETDEEPEE
BACE2    (1) MGALARALLLPLLAQWLLRAAPELAPAPFTLPLRVAAATNRVVAPTPGPGTPAERHADGLALALEPALAS

BACE1   (54) PGRRGSFVEMVDNLRGKSGQGYYVEMTVGSPPQTLNILVDTGSSNFAVGAAPHPFLHRYYQRQLSSTYRD
BACE2   (71) PAGAANFLAMVDNLQGDSGRGYYLEMLIGTPPQKLQILVDTGSSNFAVAGTPHSYIDTYFDTERSSTYRS

BACE1  (124) LRKGVYVPYTQGKWEGELGTDLVSIPHGPNVTVRANIAAITESDKFFINGSNWEGILGLAYAEIARPDDS
BACE2  (141) KGFDVTVKYTQGSWTGFVGEDLVTIPKGFNTSFLVNIATIFESENFFLPGIKWNGILGLAYATLAKPSSS

BACE1  (194) LEPFFDSLVKQTHVPNLFSLQLCGAGFPLNQSEVLASVGGSMIIGGIDHSLYTGSLWYTPIRREWYYEVI
BACE2  (211) LETFFDSLVTQANIPNVFSMQMCGAGLPVAGS---GTNGGSLVLGGIEPSLYKGDIWYTPIKEEWYYQIE

BACE1  (264) IVRVEINGQDLKMDCKEYNYDKSIVDSGTTNLRLPKKVFEAAVKSIKAASSTEKFPDGFWLGEQLVCWQA
BACE2  (278) ILKLEIGGQSLNLDCREYNADKAIVDSGTTLLRLPQKVFDAVVEAVARASLIPEFSDGFWTGSQLACWTN

BACE1  (334) GTTPWNIFPVISLYLMGEVTNQSFRITILPQQYLRPVEDVATSQDDCYKFAISQSSTGTVMGAVIMEGFY
BACE2  (348) SETPWSYFPKISIYLRDENSSRSFRITILPQLYIQPMMGAGLNYE-CYRFGISPSTNALVIGATVMEGFY

BACE1  (404) VVFDRARKRIGFAVSACHVHDEFRTAAVEGPFVTLDMEDCGYNIPQTDESTLMTIAYVMAAICALFMLPL
BACE2  (417) VIFDRAQKRVGFAASPCAEIAGAAVSEISGPFSTEDVASNCVPAQSLSEPILWIVSYALMSVCGAILLVL

BACE1  (474) -CLMVCQWRCLRCLRQQHDDFADDISLLK----  (501)
BACE2  (487) IVLLLLPFRCQRRPRDP-EVVNDESSLVRHRWK  (518)
```

Table 1: BACE1 homologues

BACE1 amino acid sequence was compared to human and yeast protein sequences using BLAST (NCBI). A list of known BACE1 closest homologues in human and yeast is given below. Human homologues: BACE2, Cathepsin D (lysosomal aspartyl protease), renin precursor and angiotensin-forming enzyme precursor. *Saccharomyces cerevisiae* homologues: Pep4p (vacuolar proteinase A), Bar1p (extracellular protease synthesized in a-cells that cleaves and inactivates alpha factor) and Ylr121cp (putative aspartic proteinase).

Protein	Identities	Similarities
BACE2	222/448 (49%)	302/448 (66%)
Cathepsin D	106/368 (28%)	160/368 (42%)
Progastricsin (pepsinogen C)	88/355 (24%)	159/355 (44%)
Renin precursor	83/356 (23%)	156/356 (43%)
Pep4p [S. cerevisiae]	88/347 (25%)	139/347 (39%)
Bar1p [S. cerevisiae]	93/385 (24%)	158/385 (40%)
Ylr121cp [S. cerevisiae]	65/264 (24%)	114/264 (42%)

Chem). BACE1 knockout mice have no detectable levels of Aβ in the brain (Citron et al., 2000 - Abstract #14.1 SFN 2000). Antisense oligos complementary to BACE1 mRNA inhibit both BACE1 mRNA and β-secretase activity in parallel almost completely in HEK 293 cells (Vassar et al., 1999) while antisense inhibition of BACE2 did not significantly reduce Aβ levels (Yan et al., 1999). BACE2 does not seem to compensate for the loss of BACE1 function in these cells. On the other hand BACE2 mRNA levels are higher in peripheral tissues than in brain (highest levels in spleen and prostate - Lin et al., 2000). Developmental analysis of the comparative tissue expression of mouse BACE1 and BACE2 suggest that BACE1 but not BACE2 is a good candidate for β-secretase in the brain (Benjannet et al., JBC in press). However, it has been reported that BACE1 and BACE2 protein levels and distribution are similar in monkey and rodent brain, and both seem to be associated with neuronal elements with little glial immunoreactivity (Lah et al., 2000 – abstract # 14.7 SFN 2000).

γ-secretase / Presenilin 1 ?

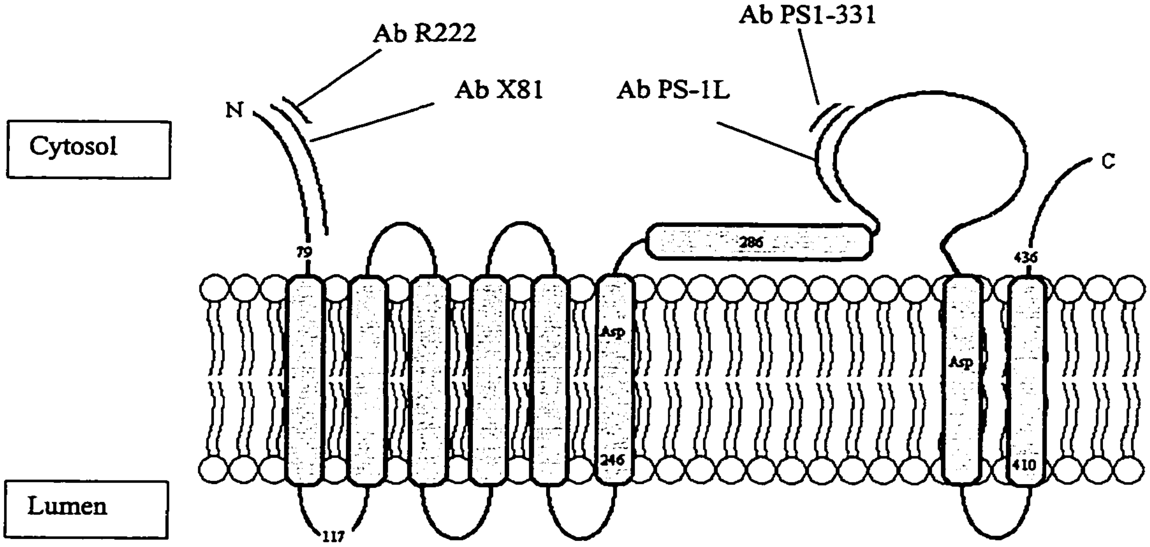
APP endoproteolysis by γ-secretase within its transmembrane domain (Figure 1, Figure 2) is not completely understood, but there seems to be at least two other proteins that have similar processing: SREB (Sterol Regulatory Element Binding Protein – Tomita et al. 1998; reviewed by Brown and Goldstein, 1997) and Notch (De Strooper et al., 1999). γ-secretase has been postulated to act very close to the plasma membrane (Shoji et al., 1992; Haass et al., 1993b).

Some recent evidence suggests that presenilins are involved in the γ-secretase cleavage of APP, and perhaps they themselves are γ-secretases (Wolfe et al., 1999). To date, 43 different AD-related PS1 mutations have been identified, while only 3 mutations have been identified in PS2. Most FAD-linked PS1 mutations are missense mutations close to the membrane region (Figure 4). One splice site mutation in PS1 resulting in the in-frame skipping of exon 9 (Δ9) has been identified (Saftig et al., 1999). All these mutations increase Aβ levels, specially Aβ1-42, the more amyloidogenic form of the peptide. Expression levels of APP and α- and β-secretase cleavages do not seem to be affected (reviewed in Saftig et al. 1999). Two aspartates located in two transmembrane domains of PS1 have been shown to be required for PS1 endoproteolysis and γ-secretase activity (Wolfe et al., 1999). Also, in PS1-/- mice γ-secretase cleavage is not present, and several other proteins are not cut properly, including Notch (reviewed in St. George-Hyslop, 2000).

The presenilins are conserved through evolution. The presenilins are highly homologous sequences (67% identity between PS1 and PS2), and also highly homologous to *SEL-12*, a *Caenorhabditis elegans* protein involved in the Notch signaling pathway (Levitan et al. 1996, Li and Greenwald 1996). Wild type, but not mutant, PS1 is able to rescue the egg-laying deficiency in *C. elegans* caused by null mutation of *SEL-12* (Baumeister et al., 1997; Levitan et al. 1996). Lack of PS1 or the use of γ-secretase inhibitors in mammalian cells reduces the proteolytic release of the Notch intracellular domain (NICD), suggesting a specific step altered by PS1 deficiency (De Strooper et al., 1999). A second PS1

Figure 4: Presenilin 1 (PS1) - γ-secretase candidate?

Presenilin 1 (PS1) is a multipass transmembrane protein. The most accepted model is shown in the diagram below (adapted from Hardy and Hutton, 1997). N- and C-termini face the cytosol. Recognition sequences of the antibodies used in this study are indicated in figure.



homologue in *C. elegans*, *HOP1*, seems to have similar functions to *SEL-12* (Li and Greenwald, 1997) (Table 2).

The presenilins seem to be associated with a large detergent soluble fraction that has γ-secretase activity (Li et al., 2000). In this study, γ-secretase activity could also be immunoprecipitated with anti-PS1 antibodies. Just one other protein, named nicastrin, has been reported to form part of this large γ-secretase complex (Yu et al., 2000). Transition state analogues designed to target the γ-secretase active site labeled PS1 (Li et al., 2000a; Esler et al., 2000), which suggested a direct involvement of PS1 in γ-secretase cleavage.

Wild type APP or a mutant APP lacking the cytoplasmic domain can be co-immunoprecipitated with PS1, suggesting that these proteins may be interacting through their membrane domains, and perhaps PS1 is directly involved in γ-secretase cleavage (Xia et al. 1997).

The techniques thus far employed could not unambiguously determine whether these secretases are directly or indirectly involved in APP processing. Since it was found that APP is required to be membrane bound to be processed by the α- and β-secretases (Sisodia, 1992; Selkoe, 1994; Hooper et al., 1997a), this has to be considered when screening for secretase candidates. The yeast system proposed here may reveal some other candidates that will have to be carefully analyzed in mammalian cells.

Table 2: Presenilin homologues in *C. elegans*

PS1 homologues in C. elegans: sel-12/sum-1/F35H12.3 Presenilin affecting LIN-12 and GLP-1 signal transduction, it plays a role in cell fate decisions (Length = 444); hop-1/C18E3.8, required for the appropriate neurite connectivity of a small subset of interneurons, including AIY (Length = 358); spe-4/ZK524.1, required for spermatogenesis (Length = 465). Source: Yeast Proteome Database (Costanzo et al., 2000).

Protein	Identities	Similarities
Sel12	214/412 (52%)	290/412 (70%)
Hop1	119/383 (31%)	197/383 (51%)
Spe-4	103/415 (25%)	180/415 (43%)

Specific aims

The goal of this research is to identify and characterize the human secretases (α, β, and γ) involved in the processing of human β-amyloid precursor protein (APP) and to contribute in this way to a better understanding of the proteolytic processing pathways of the secretory systems of yeast and mammals. *Saccharomyces cerevisiae* was used as a working model because it is a relatively easy model in which to do genetic manipulations. It is used routinely for the expression of homologous and heterologous proteins either in the cytoplasm or directed to the secretory pathway (Moir and Davidow, 1991), and also because yeast α-secretase activity has been found previously by our group (Hines, 1994) and others (Zhang, 1994). No β- or γ-secretase activity has been detected in our yeast strain (Figure 5). Two aspartyl proteases, Yap3p and Mkc7p, have been proposed as α-secretase candidates in yeast. A yeast strain carrying a double deletion of these two genes has only about 14 % α-secretase activity of the wild type strain, and *YAP3* deletion was found to be responsible for most of this decrease (Figure 6, Zhang et al. 1997). Based on these findings, other yeast and human aspartyl proteases are postulated to have α-secretase activity. By protein sequence homology searches in the Yeast Proteome Database, two sequences highly homologous to Yap3 were identified: ORF YIR039c and ORF YLR121c (Table 3), although it is not known if these coding sequences are translated into protein.

Figure 5: Expression of human APP in yeast

Yeast strain DS7 was transfected with APP695-containing yeast expression vector (pDE195) or vector alone (p416Gal1). APP expression was induced by growing cells in galactose-containing medium for 8hr. Cells were harvested and sonicated for 20 sec in lysis buffer (1% TritonX-100, 20mM Tris-HCL pH 8.0) (see Materials and Methods). 7.5 μg of protein were loaded onto 7/15% acrylamide gel, electrophoresed and transferred to nitrocellulose membrane. Figure shows a representative Western blot immunodetected with Ab R57 (whose epitope is at the very end of the C-terminal of human APP, Figure 1). Untransfected mammalian cells (COS-7) were used as control. Yeast is able to cleave human APP at the α-secretase site, but no β- nor γ-secretase activities can be detected (Hines et al., 1994; Zhang et al., 1994).

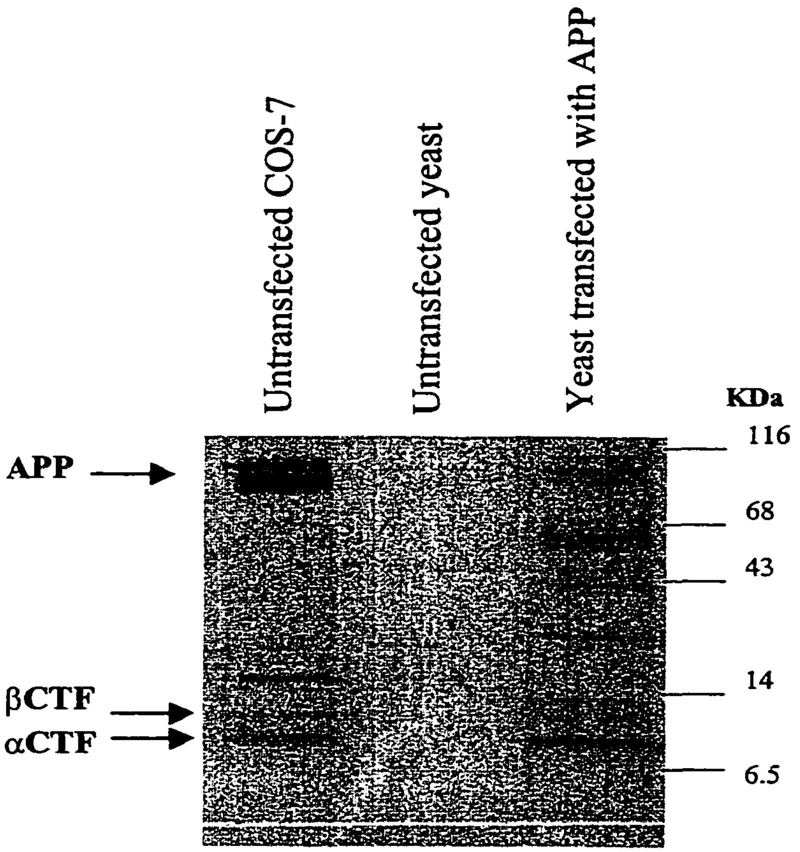


Figure 6: Aspartyl proteases Yap3 and Mkc7 are responsible for most of the yeast α-secretase activity

Processing of wild type APP695 and APP(k612Q) in yeast strains bearing deletions in *YAP3* and *MKC7*. Cells were labeled for 10min with ^{35}S-TRANS (ICN) and chased for 30min at 30oC. APP and C-terminal fragments were immunoprecipitated with R57 and subjected to SDS-PAGE. The radioactivity was quantified by phosphorimaging, and the fractions of radioactivity in APP (all forms) at t=0 that appeared in CTF at 30min was calculated. Error bars indicate the standard deviations of two or three independent experiments. Strains: DS7 (*YAP3 MKC7*), YBAD1 (*yap3 MKC7*), DE12 (*YAP3 mkc7*), DE9 (*yap3 mkc7*). Data reported in Zhang et al. (1997).

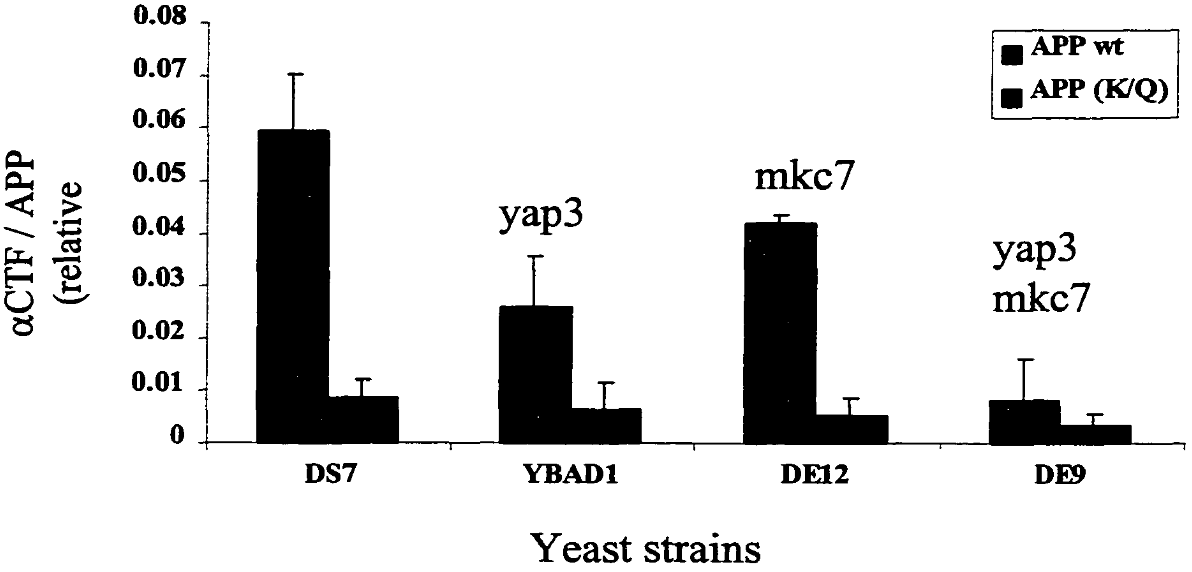


Table 3: Yeast yap3p homologues

Proteins with the highest degree of homology to Yap3p are included. Yap3, Mkc7, Ylr121c, and Yir039c are included in the yapsin family of yeast aspartyl proteases. All are GPI anchored. BAR1 is a secreted pepsin-like protease that degrades α-factor. Yap3 has been renamed Yapsin1, and the name "YAP3" has been assigned to a different gene (source: Yeast Proteome Database – Costanzo et al., 2000).

Protein	Identities	Similarities
Mkc7p (Yps2p)	285/521 (55%)	359/521 (69%)
Ylr121cp (Yps3p)	259/552 (47%)	338/552 (61%)
Yir039cp (Yps6p)	207/587 (35%)	307/587 (52%)
Yil015wp (BAR1p)	167/506 (33%)	261/506 (52%)

S. cerevisiae was also used to develop a system for the identification of human α-, β-, and γ-secretase(s) candidates from a human brain cDNA library. Because of recent findings in the Alzheimer's disease area, the actual screening of the cDNA library was not carried out. Instead, reported secretase candidates were cloned and tested in the yeast and mammalian systems to confirm their proposed role as secretases and to test the yeast screening system.

The proposed specific aims were:

1. Test the secretase activity of other yeast Yap3p homologues (including ORF YLR121c and ORF YIR039c) identified in the Yeast Proteome Database.
2. Develop a yeast system for the screening of a human brain cDNA library for APP secretase candidates.
3. Study the effect of reported human secretase candidates on APP processing in yeast and mammalian cells.

Materials and Methods

Reagents

All chemicals used were of reagent grade available from commercial providers.

Yeast strains

Many strains were developed from DS7 (*MATα MFα1/α2::LEU2 ade2 his3 leu2 trp1 ura3*) (Bourbonnais et al., 1993). Additionally JSC310 (MATa leu2 ura3-52 prb1-1122 pep4-3 prc1-407 GAP-ADR1::G418 (Hines at al. 1994) and YBAD1 (*MATα YAP3::HIS3 MFα1/α2::LEU2 ade2 his3 leu2 trp1 ura3*) (Bourbonnais et al., 1993) were used for the expression of some human cDNAs.

Bacteria strains

E. coli strains DH5α (*supE*44 *Δlac U169* (Φ80 *lacZΔM*15) *hsdR*17 *recA*1 *endA*1 *gyrA96 thi*-1 *relA*1) and HB101 (*supE*44 *hsdS*20($r^-_B m^-_B$) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rspL*20 *xyl*-5 *mtl*-1) were routinely used for cloning and subcloning purposes. Both bacterial strains are commercially available from GIBCO-BRL, Life Technologies.

Growth and maintenance of yeast and bacteria strains

Bacterial strains were grown and maintained according to Sambrook et al. (1989). Yeast strains were grown and maintained according to Ausubel et al. (1987).

Plasmid DNA preparations

Bacterial plasmid DNA was prepared using routine methods (boiling or alkaline lysis preparations) according to Sambrook et al. (1989). Genomic yeast DNA was prepared using a modification of the method reported in Mín et al. (1995). Briefly, to the pellet from a 1.5 ml overnight culture approximately 0.1 g of glass beads and 600 µl of lysis buffer (50mM Tris pH 7.5, 62.5 mM EDTA, 2% SDS) were added, the sample was agitated in a vortex mixer for 1 minute, followed by incubation for 1 min at room temperature. The supernatant was then transferred to a clean 1.5 ml microcentrifuge tube and spun down for 1 min. This supernatant was then mixed with 1 ml of a DNA-binding resin (Wizard DNA kit from Promega), the resin was then rinsed with a wash buffer and dried. The DNA was eluted from the resin by adding 50 - 100 µl of warm dH_2O or TE buffer. When high purity DNA was needed (e.g. for DNA sequencing or transfection of mammalian cells) commercial kits were used (Qiagen)

DNA sequencing

DNA sequencing of the different plasmids was done almost exclusively by Genemed Synthesis (South San Francisco, CA)

Bacteria transformations

For most cloning and subcloning procedures heat shock was the method of choice for introducing DNA in bacteria (Sambrook et al. 1989). Briefly, the pellet of a fresh bacterial culture was treated with 50 mM $CaCl_2$ (or a combination of salts, depending on the method of choice) to make them competent (ideally the culture should have an OD_{600nm} of about 0.5-1.0). The cells were then incubated on ice with the appropriate DNA for 30 min, after which the mixture was incubated at 42°C for 1-2 min (depending of volume of competent cells) and put back on ice for 2 min. The cells were allowed to grow for 1 hr at 37°C before spreading them on selective medium plates. The plates were then incubated overnight at 37°C to allow colony formation.

Yeast transformations

Electroporation was the method of choice to transform yeast. Competent cells were made according to the suggested protocol from the electroporator manufacturer (BTX Inc., San Diego, CA). Briefly, 100 ml of a fresh yeast culture (OD_{600nm} of about 0.5 – 1.0) was spun down and the cell pellet rinsed twice with 100 ml of chilled water and resuspended with 5ml of 1M sorbitol. Cell suspension was incubated on ice for 30 min. Cells were pelleted by centrifugation and resuspended with 100 μL of 1M sorbitol. The procedure was carried out at 4°C and under sterile conditions. Cells were concentrated 1000-fold at the end of procedure. Then, an aliquot of cells (typically 50μl) was transferred to an electroporation cuvette and incubated with the DNA of interest on ice for 30 min (approximately 500 ng of DNA was used). The electroporator settings were adjusted according to the manufacturer's specifications. After electroporation, cells were spread on appropriate selective medium plates.

Mammalian Transfections

A-204 (human rhabdomyosarcoma) and COS-7 (monkey kidney fibroblast-like) cells were transfected with expression vectors using Lipofectamine Plus (Gibco BRL, Life Technologies) according to manufacturer's protocol. Transfection efficiency for A-204 cells was about 5-10%, and that for COS-7 cells about 30-40%.

^{35}S-Met Labeling

Cells (approximately $1\mathrm{x}10^6$) are usually incubated for 10min with 100uCi of Tran^{35}S-label (*E. coli* Hydrolysate labeling reagent, containing $\geq$ 70% L-methionine, [^{35}S]) from ICN Pharmaceuticals, Inc. - Irvine, CA) at the appropriate temperature (usually 37°C for mammalian cells and 30°C for yeast cells) in a Met-, Cys-free medium. The cells are then rinsed and chased for the desired time.

Mutagenesis of yeast genes

1. Disruption by homologous recombination: The *MKC7* gene was disrupted by insertion of the *TRP1* gene via homologous recombination (Rothstein, 1991). PCR primers were designed flanking the *MKC7* gene based on the reported sequence in Genbank. *MKC7* was PCR-amplified from yeast chromosomal DNA and cloned into pGEM-T (Promega). The *TRP1* gene from YDp-W (kindly donated by Rolf Sternglanz, Stony Brook, NY) was introduced as a SalI/BamHI fragment to replace the 680bp SalI/BamHI fragment in the *MKC7* gene. This plasmid was digested with PvuII and transfected into yeast strains

YBAD1 and DS7 by electroporation. Colonies that grew on Trp$^-$ medium were analyzed by PCR to confirm the presence of the *TRP1* insertion into *MKC7* (Figure 7).

2. Deletion by CRE/loxP recombination

YAP3, YLR121c, YIR039c, PHO5, PHO3, PHO11, and *PHO12* were deleted from the yeast genome by the CRE/loxP method reported by B. Sauer (1994). The system consist of a inducible CRE recombinase-containing plasmid and a deletion cassette that contains a selectable marker flanked by *lox*P sequences, that are flanked at the same time by 5' and 3' homologous sequences to the target gene. CRE recombinase and loxP sequences are derived from *Escherichia coli* phage P1. CRE will recombine loxP sequences and excise any other sequence in between. Both the CRE recombinase-containing plasmid and the deletion cassette have to be transfected into the yeast strain of interest. The deletion cassette would replace the wild-type gene by homologous recombination, and expression of CRE will recombine loxP sites excising the selectable marker (Sauer, 1994). If desired, a different deletion cassette can be introduced and the process can be repeated many times (Figure 8). DNA fragment containing selectable marker is lost from the cell by unkown mechanisms. Loss of marker can be confirmed by growing the cells in appropriate selective medium. CRE containing-plasmid can also be excluded from cells by growing them in non-selective or in 5-fluorootic acid (5-FOA) medium. URA3+ strains are unable to grow in media containing the pyrimidine analog 5-FOA (Boeke et al., 1987). Loss of CRE containing plasmid can be confirmed by growing the cells in appropriate selective medium. This system can also be applied to insert genes at desired loci, as it is shown in this dissertation.

Figure 7: *YAP3* and *MKC7* deletions

MKC7 was disrupted by insertion of the *TRP1* gene (Zhang et al. 1997). *YAP3* was disrupted using the CRE/loxP system. Disruption of the genes was verified by PCR using genomic DNA from yeast strain carrying the double deletion of these genes (yDE77) and sets of specific PCR primers for *YAP3* and *MKC7* flanking sequences, as shown in diagram. Small arrows indicate position of primers used: A= MKC7 2019, B=MKC7 149, C=5'YAP3 set1, D=3' YAP3 set2. Primer sequences are given in the Materials and Methods section. PCR products were run on 1% agarose gel (containing 200 ng of ethidium bromide / mL). In picture below: 1-4, yDE77 (carries the double yap3/mkc7 deletion); 5, DS7 parent strain; *, 1Kb DNA ladder (Gibco BRL, Life Technologies).

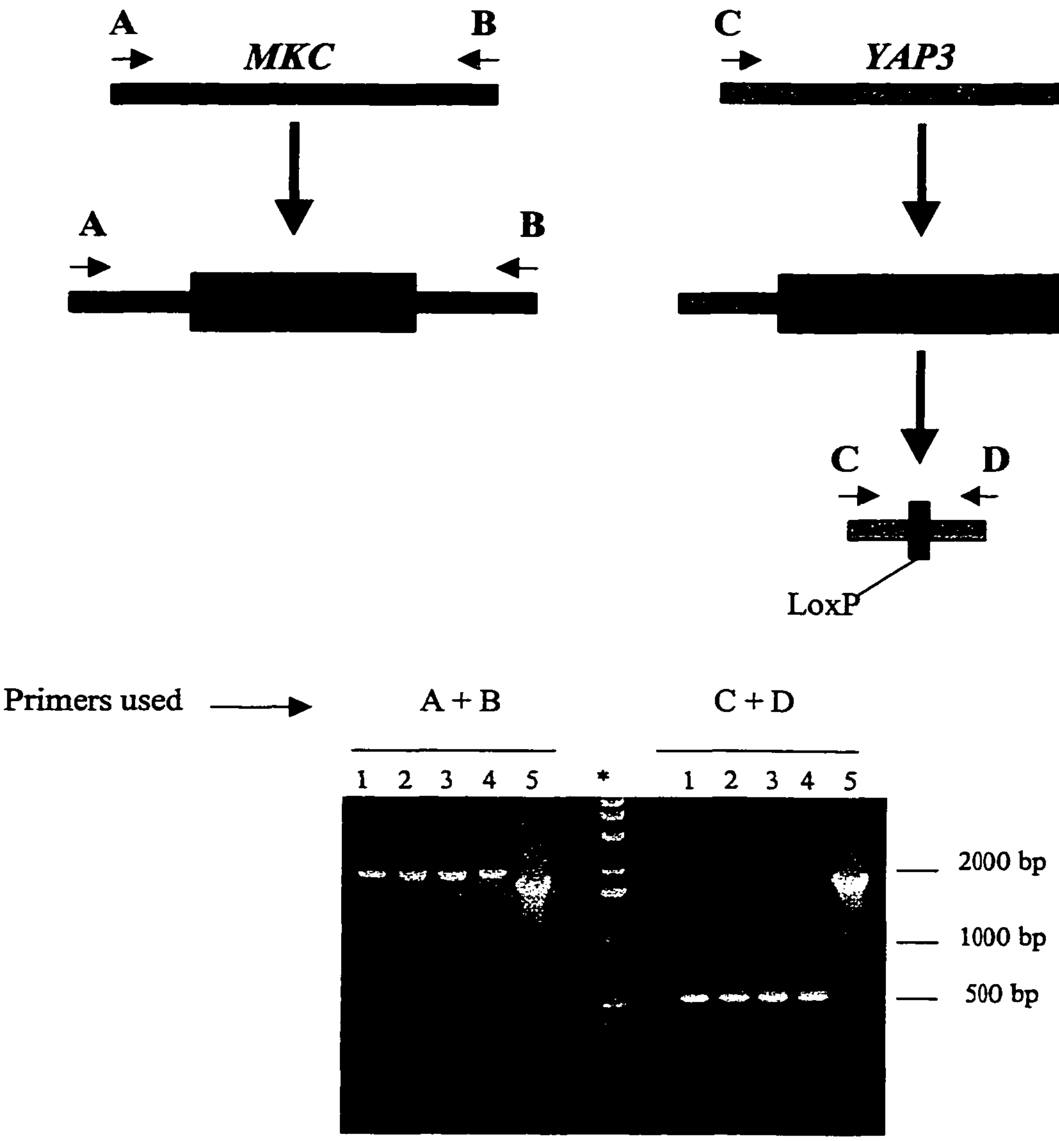


Figure 8: Deletions of genes using the CRE/loxP system

The CRE/loxP system consists of a plasmid containing CRE recombinase and a deletion cassette that contains a selectable marker flanked by loxP sequences, that at the same time are flanked by homologous sequences to the target gene. The basic strategy is depicted in the diagram below. 1, desired yeast is transfected with plasmid containing the CRE recombinase (pBS39). 2, introduce deletion cassette. Cassette will replace the wild-type gene by homologous recombination. 3, CRE expression is induced (by growing cells in galactose-containing medium) and it recombines loxP sequences, excising intervining sequence. 4, pBS39 is lost from yeast strain by growing yeast under non-selective conditions or on 5-FOA plates (see Materials and Methods).

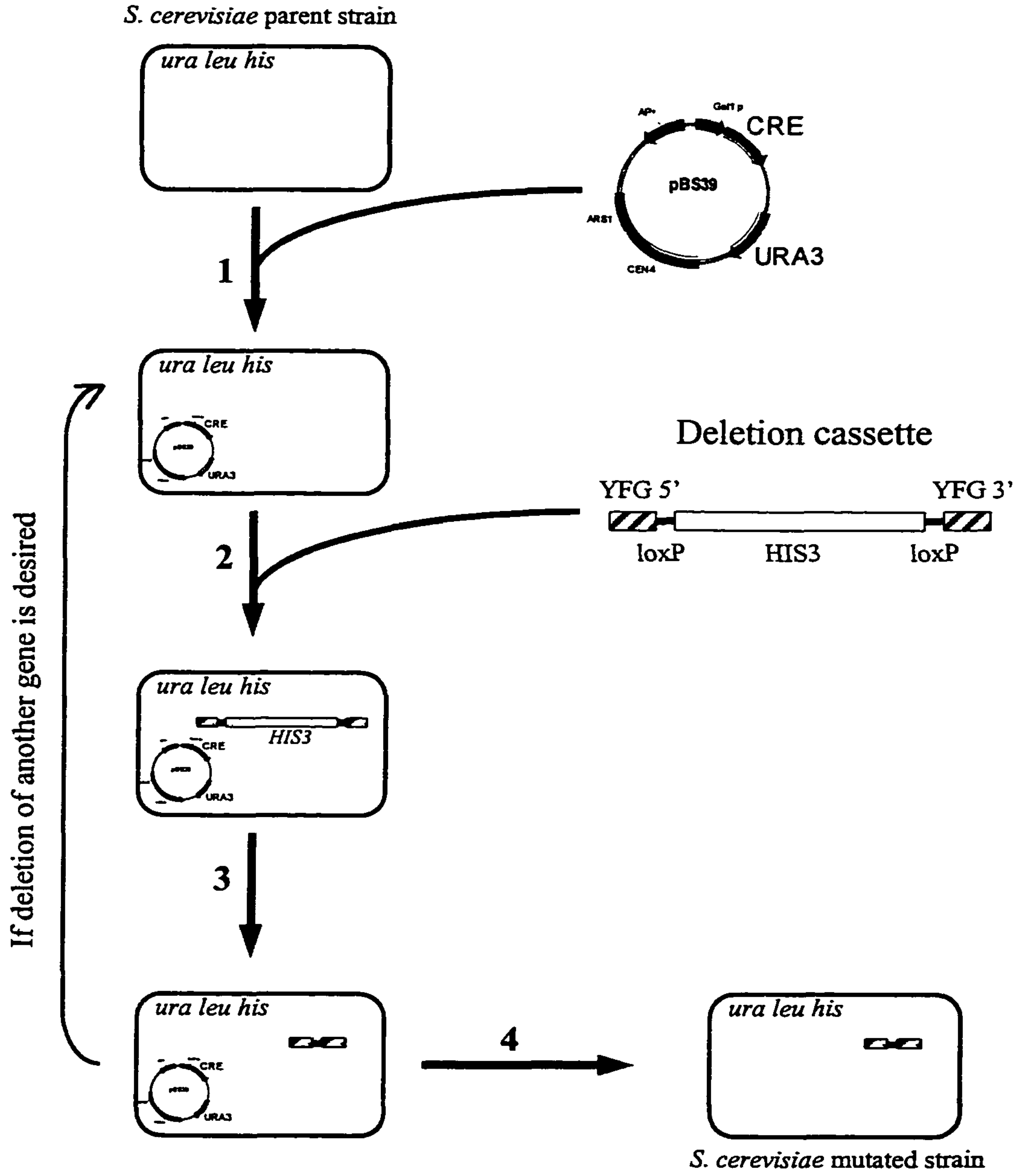
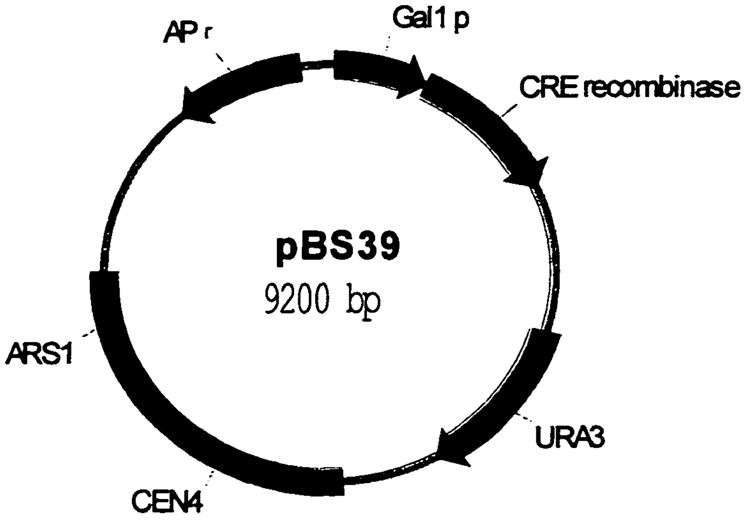


Figure 9 shows the CR/loxP system plasmids used in this study. pBS39 is a plasmid that expresses the CRE recombinase in the presence of galactose (but not glucose) in the growth medium. It carries URA3 as a selectable marker. Cells containing pBS39 should be able to grow in medium without uracil. Plasmids to make deletion, or insertion, cassettes are available carrying different selectable markers (Sauer, 1994) and can be obtained from Life Technologies. Plasmid pBS346, that carries *HIS3* as selectable marker, was used in this study because more information was available to us about its sequence, and this allowed a better manipulation of the plasmid.

To make the YAP3 deletion cassette, a 5' sequence flanking YAP3 coding region was PCR-amplified from yeast strain DS7 genomic DNA, using oligos 5' YAP3 set1 and 3' YAP3 set1 (Table 4), and inserted into plasmid pBS346, as a NotI / EcoRI fragment, to make plasmid pDE030 (Figure 10). Next, a 3' sequence flanking YAP3 coding region was PCR-amplified from the same yeast genomic DNA, using oligos 5' YAP3 set2 and 3' YAP3 set2 (Table 4), and inserted into plasmid pDE030, as a NotI / SpeI fragment, to make plasmid pDE032 (Figure 10). To insert YAP3 deletion cassette into yeast, 2 μg of NotI digested pDE032 were used to transform yeast strain DS7 (previously transformed with plasmid pBS39) by electroporation. Cells were then grown on complete synthetic medium lacking histidine and uracil (CSM-His-Uracil) at 30°C. Insertion of deletion cassette at the right locus was confirmed by PCR using an oligo that primes to the inserted HIS3 marker and another oligo that primes outside of the deletion cassette (Table 5), as it is explained below. Colonies were then grown in galactose containing medium

Figure 9: CRE / loxP system plasmids used

pBS39 expression vector carries CRE recombinase behind inducible Gal1 promoter, that will be activated in the presence of galactose (but not glucose) in the growth medium. Plasmid pBS346 was used to make deletion, or insertion, cassettes in this study, although other plasmids are available with different selectable markers (Sauer, 1994).



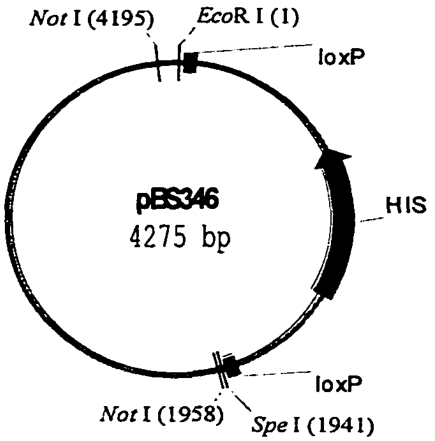


Table 4: PCR oligos used to make deletion cassettes for CRE / loxP system.

Oligo name	Restriction sites	Oligo sequence
5’YAP3 set 1	NotI-XbaI	5' GG GCGGCCGC TCTAGA TATTAAGAAGAACGCAATTGG 3’
3' YAP3 set 1	EcoRI	5' GG GAATTC GTCGTCGTCGCGCTTGTTTGC 3’
5' YAP3 set 2	SpeI	5' GG ACTAGT AGCGCCGTAAAGGCACCAGGC 3’
3' YAP3 set 2	NotI-XhoI	5' GG GCGGCCGC CTCGAG AAGAAGAGAAATTAATGTGAG 3’
5' YIR set1	XbaI	5' GG TCTAGA TGGAACTGCTTTCAAAGTTGA 3’
3' YIR set1	EcoRI	5' GG GAATTC GGTAGAAACATCCTGTGTGCA 3’
5' YIR set2	SpeI	5' GG ACTAGT TCAAGTAGTGGCGGCTCTAGC 3’
3' YIR set2	XhoI	5' GG CTCGAG CCTGGGGAAAAAGTGTGCACT 3’
5' YLR set1	XbaI	5' GG TCTAGA TTGTAGTAGAAATTATTGGCG 3’
3' YLR set1	EcoRI	5' GG GAATTC TAAGACTGCTAATGTAGCCAC 3’
5' YLR set2	SpeI	5' GG ACTAGT CATAAAAGCAAGAGGGCACTC 3’
3' YLR set2	XhoI	5' GG CTCGAG AGGAGGCAGTGCTATGTGAGG 3’
5’ matAP-APP	XbaI	5' CC TCTAGA TAAAAGAGGTACCATTCCCTTAGGC 3'
3’ PHO5 set1	EcoRI	5' CC GAATTC TCTACCAACCATTTGCAGTTG 3'
5’ PHO3	SpeI	5' GG ACTAGT CAAGGTGCTCGTGTCTACACC 3'
3’ PHO3	XhoI	5' GG CTCGAG GGTGACGTTACTGACACTGCT 3'
5' PHO11/12 set1	XbaI	5’ GG TCTAGA GAAATAGGAAGGAAAGAG 3’
3' PHO11/12 set1	EcoRI	5’ GG GAATTC ACGGCTGACTTCAACATT 3’
5' PHO11/12 set2	SpeI	5’ GG ACTAGT ATAATCGATGACAAAAAT 3’
3' PHO11/12 set2	XhoI	5’ CC CTCGAG TTACTGTTTTAATAAAGT 3’

Table 5: Oligos used to screen for insertion of deletion cassettes at the right locus

Oligo name	Oligo sequence
nHIS3-SCREEN	5' GGAAATACGCTCACGTAC 3'
nYAP3-SCREEN	5' GTGAAGTAAAAATGAAGACCG 3'
nYLR121C-SCREEN	5' GCGAAAAGCCACTCAGTTGAG 3'
nYIR039C-SCREEN	5' TCACTGCTTTTTAGCACTGTG 3'
PHO5/3 - SCREEN	5' TGCCGCTAATCTACAAACTCG 3'
5 CAPP	5’AAGATGCAGTACACATCCATTCATCATGGT 3’

for 8hr and then spread on CSM-Uracil plates. Deletion of the gene was confirmed by PCR (Figure 7).

Because the making of deletion cassettes using pBS346 involves doing partial digestions of the plasmid with NotI, plasmid pDE032 (containing the YAP3 deletion cassette) was used instead to make the rest of the deletion cassettes since unique cloning sites (XbaI and XhoI) were introduced at the time of the PCR-amplification of the 5' and 3' YAP3 flanking sequences (Figure 10).

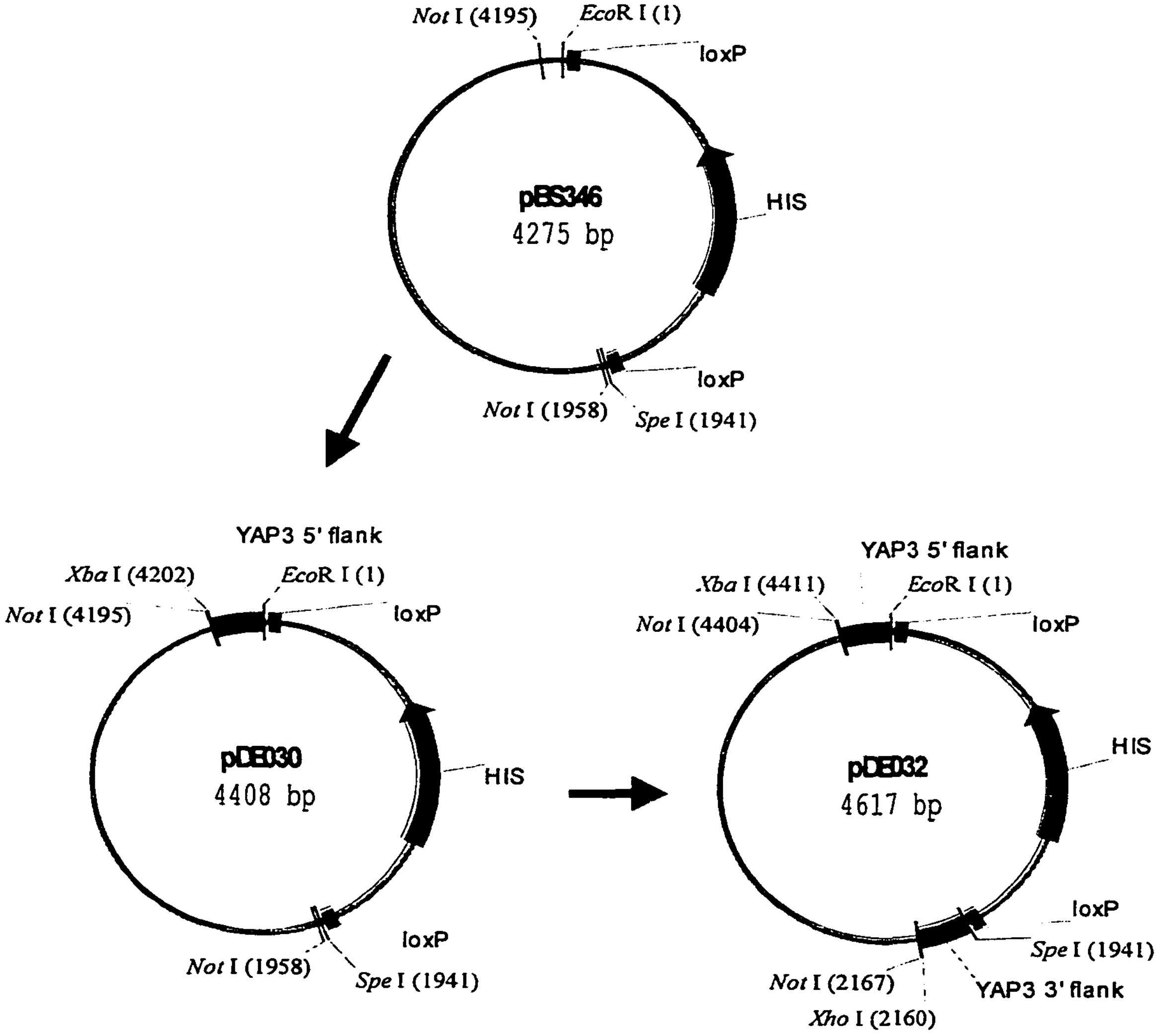
Loss of pBS39 was achieved by growing cells in YPD medium overnight at 30°C and then spreading cells on 5-FOA plates. Lost of pBS39 was confirmed by the inability of the cells to grow in uracil deficient medium.

PCR conditions

Taq polymerase (Promega Corp.) was used when proof reading activity was not necessary (e.g. to screen bacteria or yeast colonies for the presence of a given plasmid or insert, to screen for mutations in the DNA, or to screen for deletion or insertion of genes). To minimize PCR errors, Vent, DeepVent (New England Biolabs) or Pfu Turbo (Stratagene) DNA polymerases was used. Pfu Turbo was actually the polymerase of choice. PCR conditions were set according to the manufacturer of the polymerase. For screening colonies from solid medium plates, the starting conditions were: 94°C for 5 min, 30 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds to 3 minutes, depending on the length of the target sequence), and a last step of 72°C for 10 min. The

Figure 10: Making of YAP3 deletion cassette using CRE/loxP system

The deletion of yeast aspartyl protease YAP3 was achieved by inserting a deletion cassette using the CRE/loxP system (see Materials and Methods). 5' and 3' flanking sequences were PCR-amplified from yeast genomic DNA using oligos 5' YAP3 set1 and 3' YAP3 set1 for the 5' flanking region, and 5' YAP3 set2 and 3' YAP3 set2 for the 3' flanking region (Table 4). A 5' YAP3 flanking region was inserted into plasmid pBS346 between NotI (4195) and EcoRI sites to generate plasmid pDE030. To complete the deletion cassette, a 3' YAP3 flanking region was inserted into plasmid pDE030 between NotI (1598) and SpeI sites to generate plasmid pDE032. This plasmid was later used to make other deletion cassettes because it offers unique cloning sites to insert sequences flanking loxP sites (partial NotI digestions are necessary when using pBS346).



first step is enough to lyse the cells. When Pfu Turbo was used, the denaturing temperature was 98°C. Annealing temperature was varied according to the melting point (Tm) of the primer (an approximate value for Tm can be calculated from the formula: Tm= (wA+xT) * 2 + (yG+zC) * 4, where w,x,y,z are the number of the bases A,T,G,C in the sequence, respectively). The extension time was varied according to the length of the target sequence, giving 1 minute per 1000 bp of target sequence.

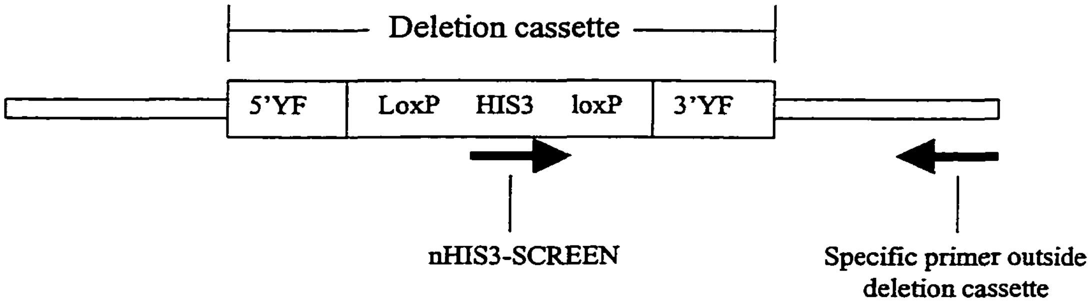
PCR primers

Most primers had at least 15 to 18 bases homologous to the target sequence (or its complement). When a restriction site was added to the very 5' end of the primer, two extra bases, usually GG or CC, were added to the primer to facilitate cleavage by the restriction enzyme. The majority of primers were synthesized by Biosource International (Camarillo, CA).

Primers used to make deletion cassettes of genes using the CRE/loxP system

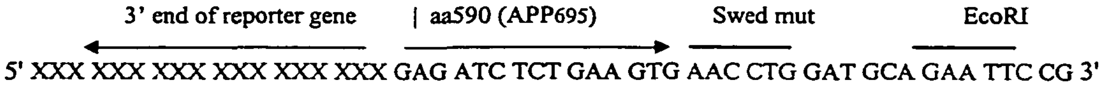
As it was previously stated the *YAP3* deletion cassette was made using plasmid pBS346 (Figure 10). All the rest of deletion cassettes were done using plasmid pDE032 (Figure 10), because it offers unique cloning sites for the insertion of sequences to flank loxP sites. For this reason, flanking sequences to make YLR121c and YIR039c deletion cassettes include XbaI / EcoRI and SpeI / XhoI sites for the 5' and 3' flanking sequences, respectively (Table 4). Table 4 also includes oligos to make deletion cassettes for the yeast endogenous phosphatases, as will be explained later.

Insertion of deletion cassettes at the correct locus was verified by PCR. A sense oligo that primes in the HIS3 sequences (nHIS3-SCREEN) and an antisense oligo that primes outside the deletion cassette (Table 5), as illustrated in the following diagram (arrows indicate primer positions):



Fusion of APP to reporter proteins

The basic strategy to make the fusion proteins was to place the reporter gene upstream of the codons for amino acids 590 to 600 of the APP695 Swedish mutant (C106sw), in such a way that both sequences are in frame. This was accomplished by PCR primers used to amplify the reporter genes. The 3' PCR primer included the DNA coding sequence for the last six amino acids of the reporter protein and amino acids 590 to 600 of APPsw. This primer contains a convenient EcoRI restriction site, which was ligated to the naturally occurring EcoRI site present in C106, and at the same time it introduces the codons for the Swedish mutation.



The 5' PCR primer for the reporter gene contains the first six codons for the marker protein and a XbaI restriction site upstream from it to fuse to the pro-α-mating factor sequence.

XbaI | 5' of reporter gene
5' GG TCT AGA XXX XXX XXX XXX XXX 3'

A complete sequence for each of the primers used is given in Table 6.

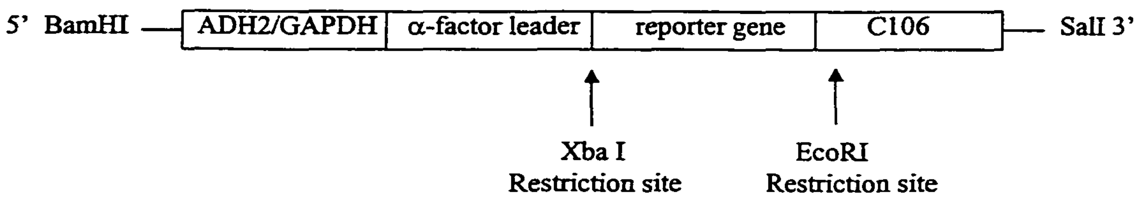
Table 6: Oligos used to make APP reporter molecules

Oligo name	Oligo sequence
5' EGFP-APP	5' CCTCTAGATAAAAGAATGGTGAGCAAGGGCGAG 3'
3' EGFP-APP	5' CGGAATTCTGCATCCAGGTTCACTTCAGAGATCTCCTTGTACAGCTCGTCCAT 3'
5' GFPs65t-APP	5' CCTCTAGATAAAAGAATGGGTAAAGGAGAAGAA3'
3' GFPs65t-APP	5' CCCTGCAGCTTGTATAGTTCATCCAT 3'
5' PHO5-APP	5' CCTCTAGATAAAAGAGGTACCATTCCCTTAGGC 3'
3' PHO5-APP	5' CGGAATTCTGCATCCAGGTTCACTTCAGAGATCTCCTGCAGTTGTCTCAATAGACTGGC 3'

The PCR-amplified reporter gene then had a XbaI site at the 5' end and an EcoRI site at the 3' end of the product.

5' XbaI — reporter gene — EcoRI 3'

This product was inserted into plasmid pBluescript SK + II (Stratagene, CA) and transformed and amplified in E. coli DH5-α (GIBCO BRL, Life Technologies Inc). Clones were selected and their structures confirmed by restriction analysis and DNA sequencing. The insert was excised using XbaI and EcoRI and ligated to yeast expression vector pBS6α, which contains the mature form of wild type APP695 (Figure 11), digested with the same enzymes. The gene fusion is then under the control of the hybrid ADH2/GAPDH promoter, and the α-factor leader would direct the protein for secretion, as shown in the following scheme:



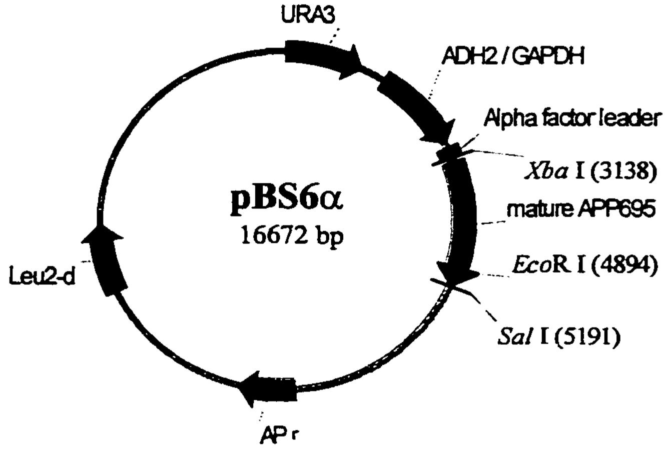
The green fluorescent protein from the jellyfish *Aequorea victoria* and the major secreted *S. cerevisiae* acid phosphatase (Pho5p) were chosen as reporter molecules to fuse to C106sw. This APP fragment seems to be an adequate substrate for proper APP processing by the α-, β-, and γ-secretases (Pappolla et al. 1994; Citron et al. 1995) (Figure 12).

Construction of mature PHO5-C106sw fusion

The mature form of yeast secreted phosphatase *PHO5*, was amplified from yeast genomic DNA using the following primers: 5' PHO5-APP (to fuse mature yeast acid phosphatase pho5 to alpha leader) includes XbaI site to fuse to alpha leader, codons for KEX2 cutting sequence and codons for amino acids 18 to 23rd of PHO5 coding sequence (mature *PHO5*); and 3' PHO5-APP (to fuse acid phosphatase to APP) includes codons for the last

Figure 11: Yeast shuttle vector pBS6α

This 2μm based yeast shutle vector has been described previously in Hines et al. (1994). It contains the mature human APP695 isoform behind the pre-pro-α-mating factor. Expression of APP in this vector is under the control of the ADH2/GAPDH inducible promoter. PBS6α backbone was initially used in this study to express PHO5-C106sw fusion (pDE041), but later on, the use of centromeric vectors with Gal1 promoters were preferred because of their high stability, uniform copy number inside cells, and better control of induction provided by the Gal1 promoter.



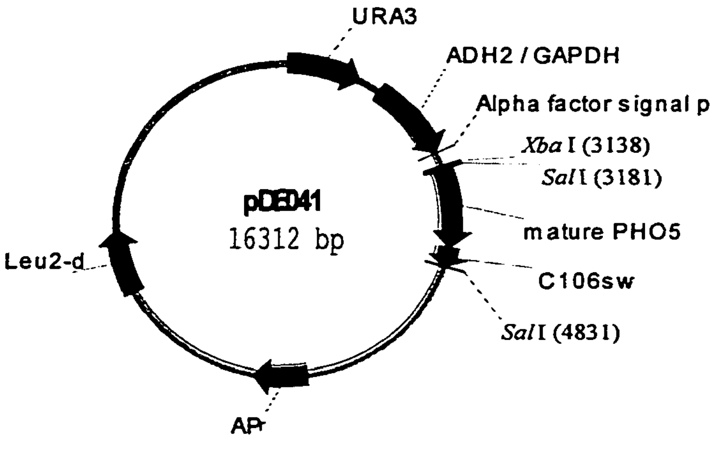
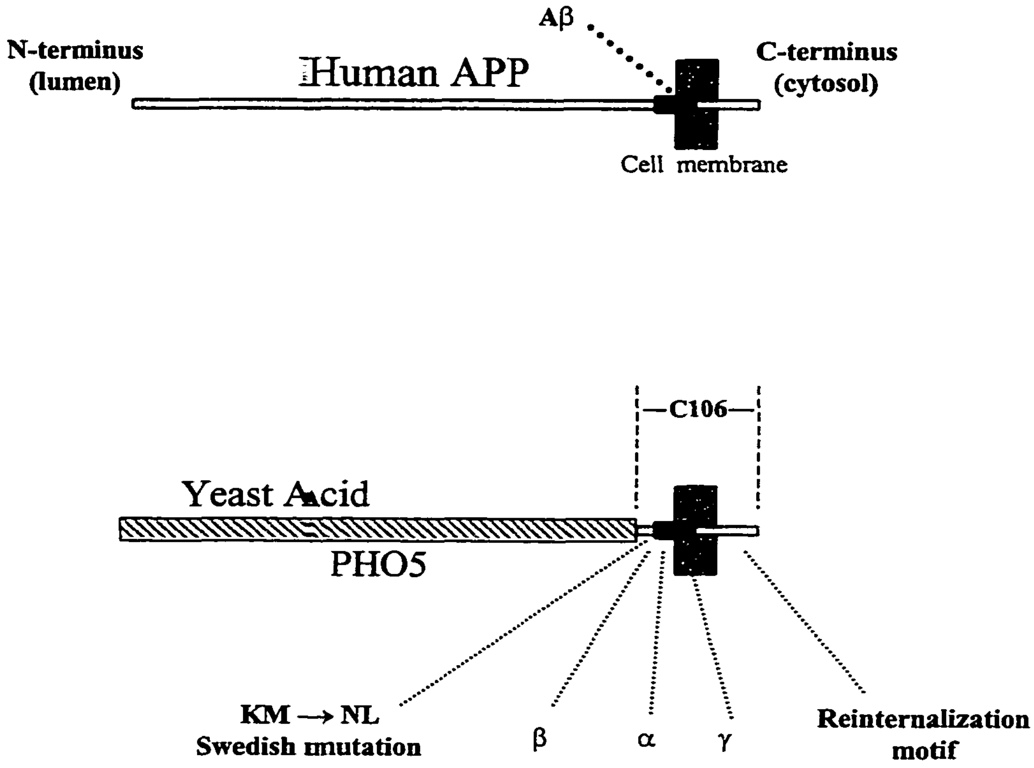


Figure 12: Yeast phosphatase-human APPsw reporter (PHO5-C106sw)

PHO5-C106sw reporter molecule was made by replacing most of the human APP N-terminus with mature Pho5p. Fusion was done at the DNA level. *PHO5* cDNA was PCR-amplified from yeast strain DS7 and fused to DNA sequences coding for the last 106 amino acids of the human APP. Codons for the APP Swedish mutation were introduced with the PCR primers used to amplify *PHO5* (see Materials and Methods).



6 amino acids of PHO5, a PstI site (to facilitate exchange of gene marker), and codons for amino acids 590 (APP695) to 2 bases upstream the EcoRI site of APP. It also introduces the Swedish mutation (Table 6). *PHO5*-C106sw sequences were introduced in plasmid pBS6α (Figure 11) as a XbaI/SalI fragment (replacing APP695 sequence in pBS6α) to make pDE041 (Figure 11). *PHO5*-C106sw fusion in this plasmid is under the ADH2/GAPDH inducible promoter.

Construction of EGFP-C106sw fusion

An enhanced form of GFP, EGFP, was PCR-amplified from plasmid pEGFP-N3 (Clontech) using the following oligos: 5' EGFP-APP (to fuse EGFP to alpha leader), which contains first 6 codons from GFP coding sequence, a XbaI site and codons for Kex2 cutting site added upstream, plus two extra G's at the 5' of the primer; and 3' EGFP-APP (to fuse EGFP to aa590 of APP695) which includes the 6 last codons of GFP and APP codons for aa590 to 2 bases upstream EcoRI site. It also includes codons for the APP Swedish mutation (Table 6).

Construction of GFPS65T-C106sw fusion

GFPs65t was PCR-amplified from plasmid pGFPs65t (Clontech) using the following oligos: 5' GFPs65t-APP (contains first 6 codons for GFPs65t, an XbaI site and the codons for Kex2 cutting site added upstream, plus two extra G's at the 5' of the primer); and 3' GFPs65t-APP (includes last 6 codons of GFP and a BglI site to fuse to APP) (Table 6).

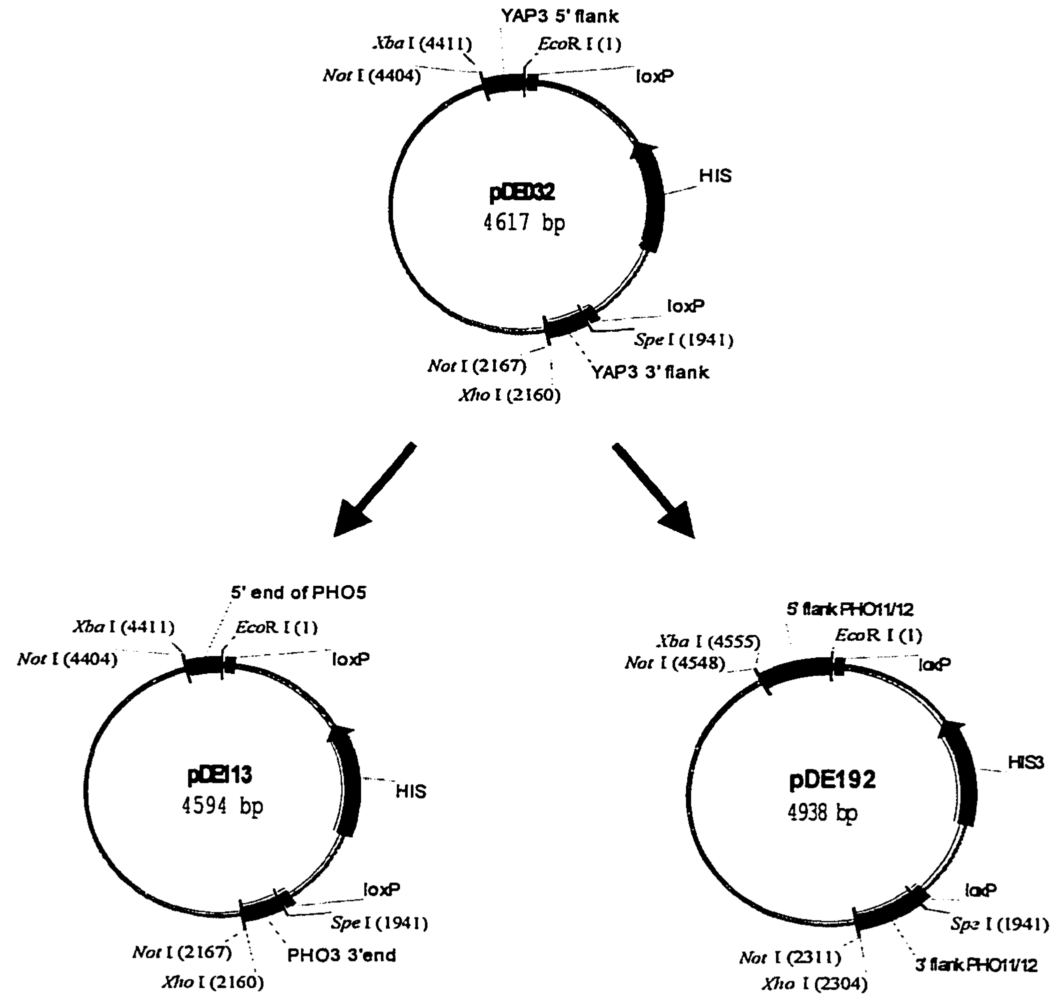
Deletion of endogenous secreted yeast phosphatases

Deletion of secreted yeast phosphatases was necessary in strains carrying the *PHO5*-C106sw reporter. Deletions of the genes coding for the 4 secreted acid phosphatases (*PHO3, PHO5, PHO11, and PHO12*) was achieved using the CRE/loxP system by replacing flanking regions in plasmid pDE032 (Figure 10). Both *PHO5* and *PHO3* were deleted at the same time, since these genes are in tandem in yeast chromosome II (The Saccharomyces Genome Database (SGD) - at http://genome-www.stanford.edu/Saccharomyces). The 5' flanking region to make the deletion cassette corresponds to a fragment of the 5' PHO5 coding region that was PCR-amplified using oligos 5' matAP-APP and 3' PHO5 set1 (Table 4). The 3' flanking region of the deletion cassette corresponds to a region from the 3' end of *PHO3* obtained by PCR-amplifying DS7 genomic DNA using primers 5' PHO3 and 3' PHO3 (Table 4). The resulting plasmid carrying the PHO5/PHO3 deletion cassette was named pDE113 (Figure 13).

PHO11 and *PHO12* are located in different chromosomes but their sequences are basically identical, for this reason the same deletion cassette was used to target these two phosphatases. *PHO11/12* sequences were PCR-amplified from DS7 genomic DNA using primers 5' PHO11/12 set1 and 3' PHO11/12 set1, for the 5' flanking sequence, and 5' PHO11/12 set2 and 3' PHO11/12 set2 for the 3' flanking sequences. These sequences were replaced in plasmid pDE032 to generate plasmid pDE192 (Figure 13).

Figure 13: Making of phosphatase deletion cassettes

Since APP reporter molecule contains a secreted phosphatase, endogenous secreted phosphatases had to be deleted. This was achieved by the CRE/loxP system. *PHO5* and *PHO3* were deleted t the same time, since these genes are located in tandem in yeast chromosome II. To this end, *YAP3* flanking sequences in plasmid pDE032 (Figure 10) were replaced by *PHO5* and *PHO3* flanking sequences to make plasmid pDE192 (see Materials and Methods for details). *PHO11* and *PHO12* are located in different chromosomes, but their sequences are basically identical, for this reason *PHO11* and *PHO12* were deleted using the same deletion cassette. To this end, *YAP3* sequences in pDE032 were replaced by *PHO11/12* flanking sequences to make plasmid pDE192 (see Materials and Methods for details).



Phosphatase assay

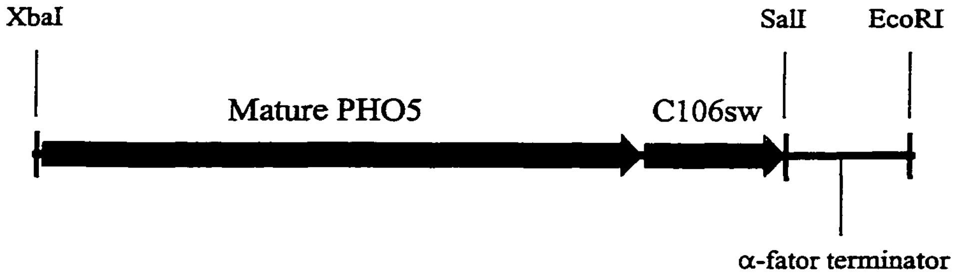
Phosphatase assays were done in 96 well flat bottom microtiter plates. 50 μl of conditioned medium, or yeast culture, were incubated with 50 μl of 1mg/ml p-nitrophenyl phosphate (Sigma 104 – phosphatase substrate tablets, Sigma) in 0.1 M acetate buffer pH 4.0. The reaction was stopped after 1hr of incubation at 37°C by adding 100μl saturated Na_2CO_3. The plate was read at 405nm in an ELISA plate reader (Biomek 1000 – Beckman). If a cell suspension was used, the samples were spun down after adding Na_2CO_3 and OD_{405nm} was determined in the cleared supernatants.

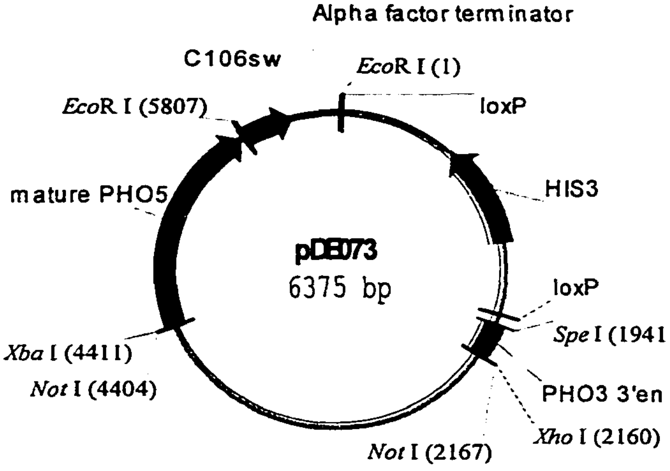
Insertion of PHO5-C106sw fusion into the yeast chromosome

PHO5-C106sw reporter was inserted into the yeast chromosome II at the PHO5/PHO3 locus using the CRE/loxP system. A PHO5-C106sw insertion cassette was made by fusing PHO5-C106sw sequences from plasmid pDE041 (Figure 11), XbaI/SalI digested, to α-factor terminator sequences that were PCR-amplified from the same plasmid using primers 5' ALPHA TERM (5' GGGTCGACTTTGTTCCCACTGTAC 3') and 3' ALPHA TERM (5' GGCAGCTGGAATTCTCTTAGGATTCGA 3') and digested with SalI and EcoRI. This fusion was introduced into pDE032 (Figure 10) digested with XbaI and EcoRI. The 3' flanking region of the cassette consists of the same PHO3 3' flanking region used to make PHO5/PHO3 deletion cassette (Figure 13), and it was inserted as a XhoI/SpeI fragment to make the final cassette. Plasmid containing cassette was named pDE073 (Figure 14).

Figure 14: Making of *PHO5*-C106sw insertion cassette

A *PHO5*-C106sw insertion cassette was made by taking XbaI/SalI fragment of pDE041 and fusing it to α-factor terminator sequence PCR-amplified from the same plasmid and digested with SalI/EcoRI. Cassette was inserted into pDE032 (Figure 10) digested with XbaI/EcoRI. A *PHO3* 3' flanking region (same sequence used to make *PHO5/PHO3* deletion cassette) replaced the XhoI/SpeI fragment in the same vector. Resulting vector was named pDE073.





Expression vectors

Yeast expression vectors p413Gal1, p423Gal1, p416Gal1, and p426Gal1 (Figure 15) were obtained from the American Tissue Culture Collection (ATCC). Table 7 summarizes the characteristics of each vector.

Table 7: Yeast expression vectors

Name	Promoter	Yeast marker	Yeast replic. seq	# of copies in cell
p413Gal1	Gal1	HIS3	ARS4/CEN6	1
p423Gal1	Gal1	HIS3	2 μ	multiple
p416Gal1	Gal1	URA3	ARS4/CEN6	1
p426Gal1	Gal1	URA3	2 μ	multiple

Mammalian expression vectors are derived from pCR3.1-UNI (Invitrogen) (Figure 16).

APP yeast expression vectors

APP695 sequences from pBS6α (XbaI/SalI) were fused to the pre-pro α-mating factor sequences (PCR-amplified from pBS6α and digested with BamHI/XbaI) and inserted into p416GalI (Figure 15) digested with BamHI/SalI to make expression vector pDE195. This puts APP695 under the inducible Gal1 promoter.

Insertion of APP695 into the yeast chromosome

Mature APP695, behind the Gal1 promoter and α-factor pre-pro sequence, was inserted into the yeast chromosome II at the PHO5/PHO3 locus using the CRE/loxP system. (Figure 17). Cassette was built by putting together three pieces: pDE073 backbone

Figure 15: Yeast expression vectors

Yeast expression vectors were obtained from ATCC. Expression of cDNAs in the vectors used are under the control of the Gal1 promoter (that is induced by galactose but inhibited by glucose).

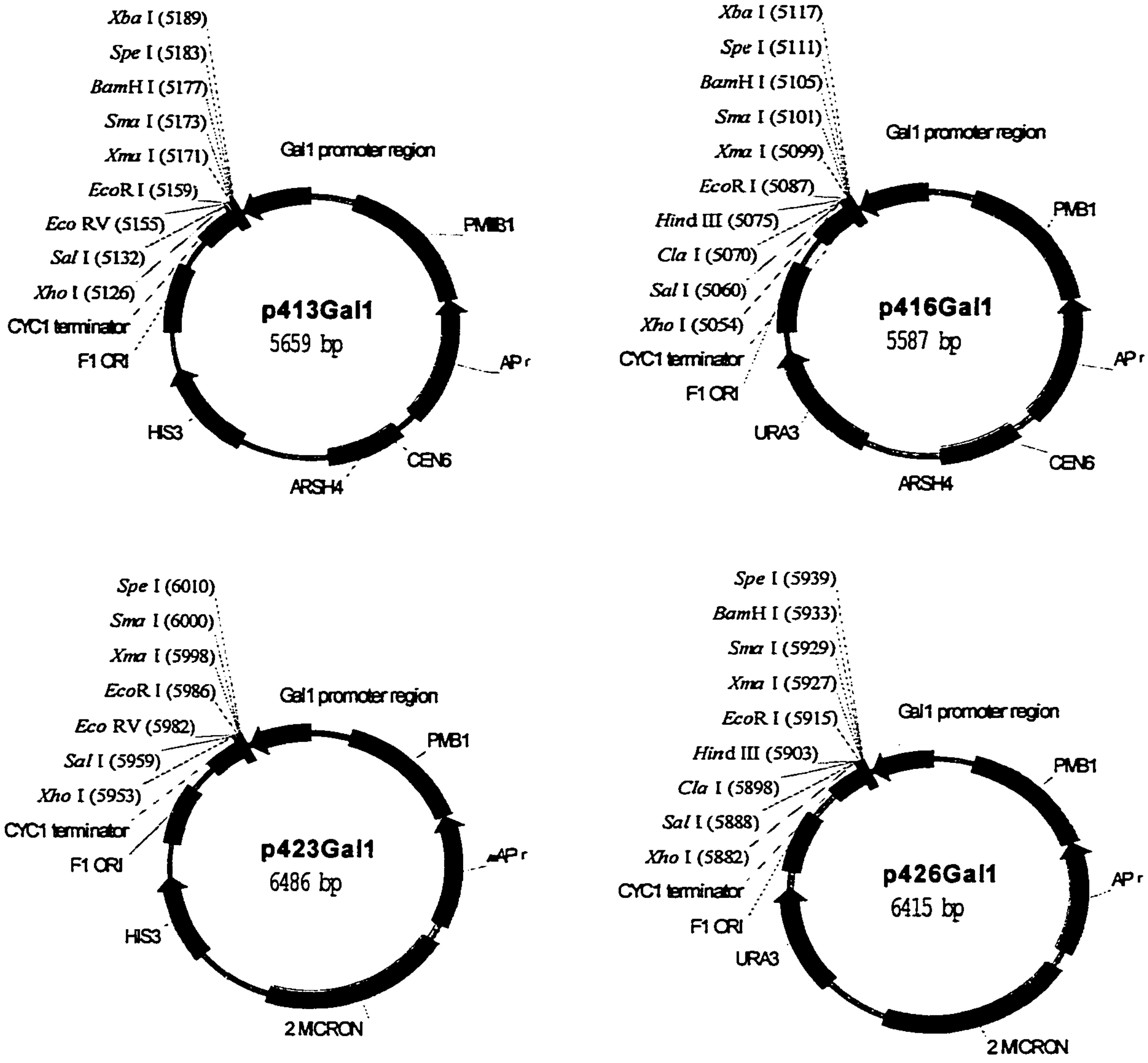


Figure 16: Mammalian expression vector

PCR3.1 uni (Invitrogen) was the vector of choice for mammalian expression. Proteins are constitutively expressed from this vector under the strong CMV promoter.

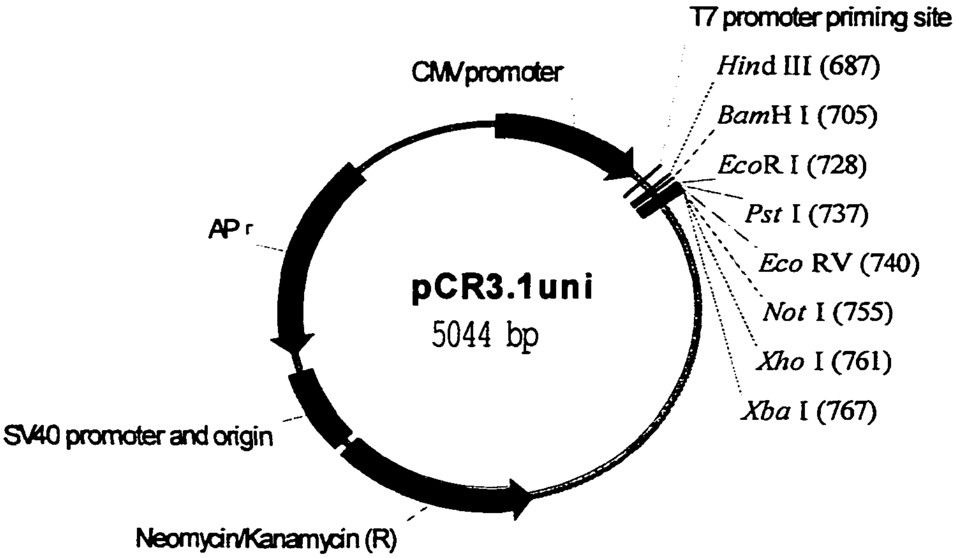
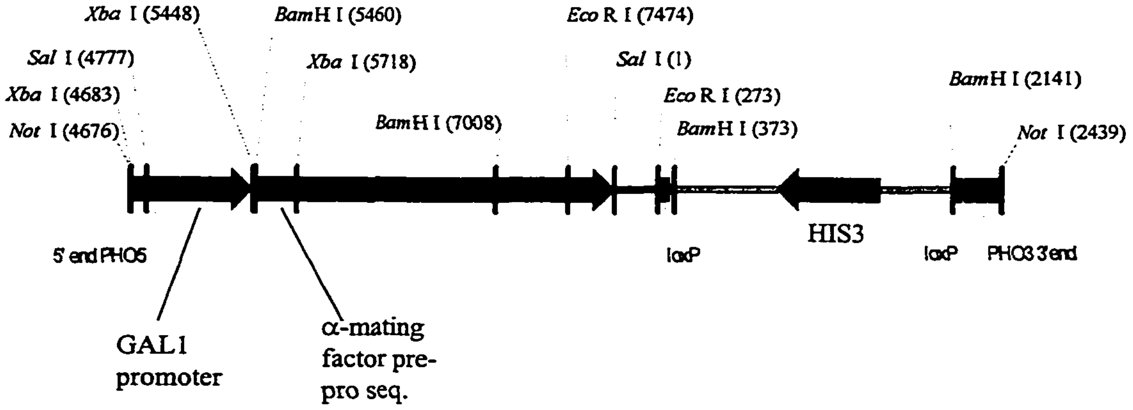


Figure 17: APP695 cassette for insertion into yeast chromosome

A cassette was engineered to introduce mature APP695 into the yeast PHO5/PHO3 locus. Mature APP695 was put behind the α-mating factor signal and pro-domain to target protein to the secretory pathway. Fusion protein is under the control of the inducible Gal1 promoter. Insertion cassette was based on the CRE/loxP system to target insertion to the desired place. Cassette was built by putting together three pieces pDE073 backbone (XbaI/SalI) + Gal1p-APP695 from pDE195 (SalI/SacIp) + a 5' region of PHO5 PCR amplified from DS7 genomic DNA using primers 5'matAP-APP and 3' PHO5 set1, digested with XbaI / SacI (see methods for details on the making of this construct). Diagram shows part of plasmid pDE228 that contains APP695 insetion cassette.



(XbaI/SalI) + Gal1p-APP695 sequences from pDE195 (digested with SalI/SacI partial) + a 5' region of PHO5 PCR amplified from DS7 genomic DNA using primers 5'matAP-APP (5' CCTCTAGATAAAAGAGGTACCATTCCCTTAGGC 3') and 3' PHO5 set1 (5' CCGAATTCTCTACCAACCATTTGCAGTTG 3'), digested with XbaI / SacI. Resulting vector was named pDE228.

Making of APP C99 – a γ-secretase substrate – yeast expression vector

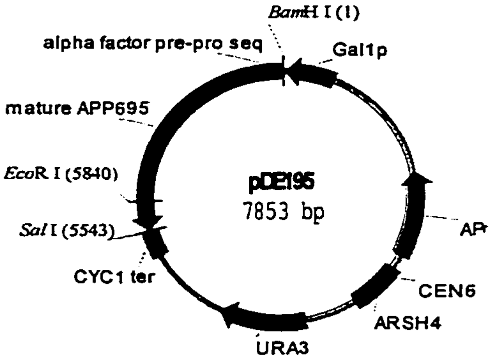
APP C99 (C99, a γ-secretase substrate) was cloned into yeast expression vector p416Gal1. C99 was engineered by replacing the alpha factor pre-pro sequence and most of the mature APP695 in vector pDE195 (EcoRI/BamHI fragment) with that of the α-factor signal sequence PCR-amplified from pDE195 using oligos C99 3'Alpha-AS (an antisense oligo that contains codons for the last 6 amino acids of α-factor signal sequence and codons from the beginning of beta amyloid to the EcoRI site - 5' CGGAATTCTGCATCAGCTAATGCGGAGGATGC 3') and pYES2 start (sense oligo that contains sequences in the Gal1 promoter region, about 30bp before polylinker region - 5' CTCTATACTTTAACGTCAAG 3') (Figure 18). The resulting vector was named pDE264.

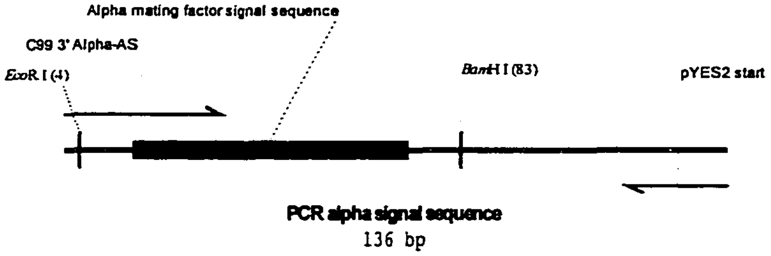
Induction of yeast expression vectors

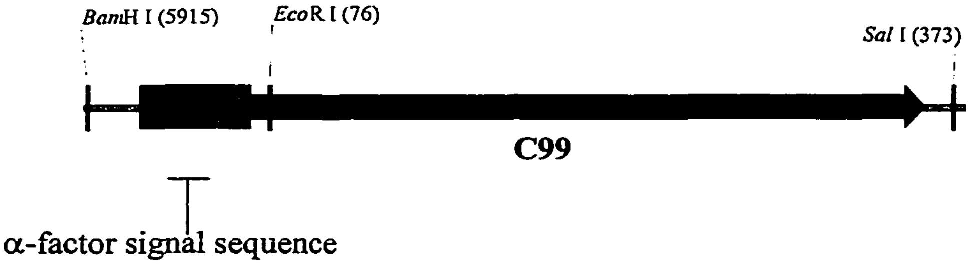
Cells were grown in 2% raffinose-plus the appropriate amino acid mixture-until the culture reached an OD_{600nm} between 1 and 1.5, after which the cells were sedimented and

Figure 18: Making of APP C99, a γ-secretase substrate

In order to test γ-secretase candidates in the yeast system, APP C99 (C99, a γ-secretase substrate) was cloned in a yeast expression vector. C99 was engineered by replacing the alpha factor pre-pro sequence and most of the mature APP695 in vector pDE195 (EcoRI/BamHI fragment) with the α-factor signal sequence PCR-amplified from pDE195 using oligos C99 3'Alpha-AS and pYES2 start (see Materials and Methods section for oligo sequences). The resulting vector was named pDE264.







supernatants discarded. Cell pellets were resuspended in 5 times the original volume in a medium containing 4% galactose. Cells, and media when appropriate, were collected after 8 hr of induction at 30°C.

Addition of MYC-6xHis tags to expression vectors:

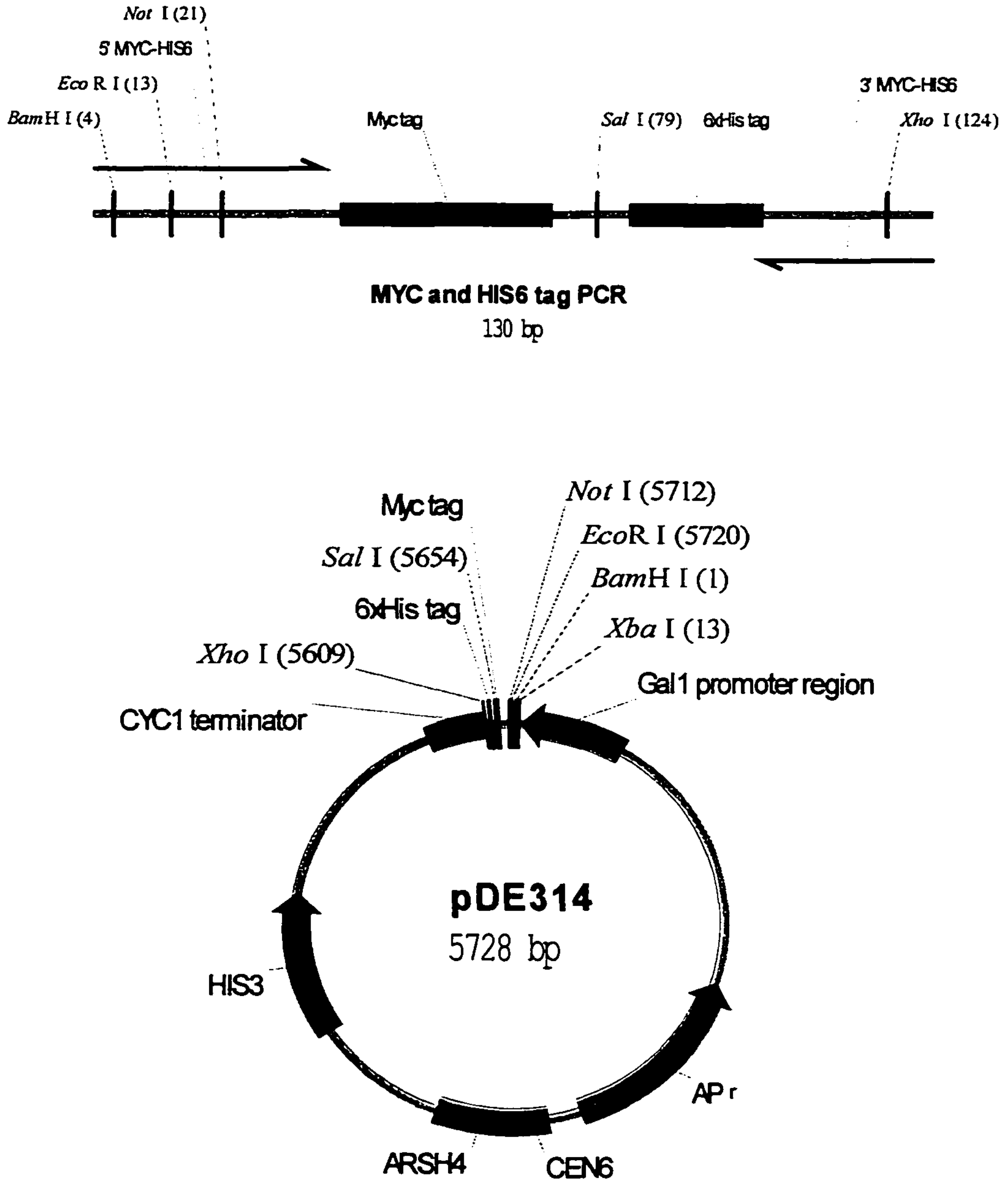
Myc-6xHis tags were PCR-amplified from a commercially available vector (pPICZ-A, Invitrogen Corp.) using the following primers: 5' MYC-HIS6 (5' CCGGATCCGAGGAATTCGGCGGCCGCCAGCTTGGG 3') and 3' MYC-HIS6 (5' CCCTCGAGATGTCTAAGGCTACAAAC 3'). PCR product was inserted into p413Gal1 yeast expression vector as a BamHI/XhoI fragment to generate plasmid pDE314 (Figure 19). This plasmid is intended to have proteins expressed with a Myc-6xHis tag at their C-terminus. But it is also possible to have the proteins fused just to the 6xHis tag by taking advantage of the SalI site included between the tags, or delete the 6xHis tag by digesting plasmid with SalI and XhoI and re-ligating the backbone (Figure 19).

Cloning of BACE1 and BACE2 with myc-6xHis tags

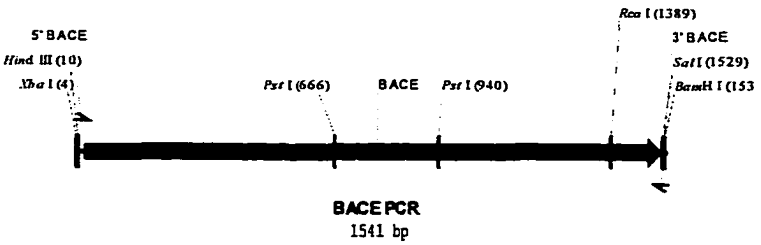
BACE1 and BACE2 cDNA were PCR-amplified from human spleen cDNA (Marathon ready, Clontech Inc.) using primers designed from reported sequences (Genbank accession # AF190725 and AF050171, respectively). Primers were designed to fuse BACE1 and BACE2 PCR products to a myc-6xHis tag in yeast expression vector pDE314 (Figure 19) to generate plasmids pDE316 and pDE326, respectively. Correct clones were confirmed by DNA sequencing. The PCR primers used to amplify BACE1 were: 5' BACE (starts 10 bases before coding region and includes GG-XbaI-HindIII

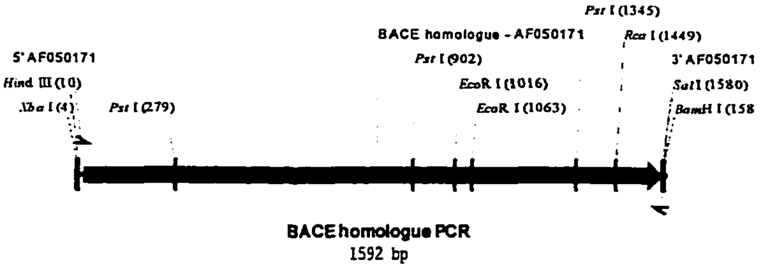
Figure 19: Adding myc-6xHis tags to expression vectors

Myc and 6xHis tags were PCR-amplified from plasmid pPICZ-A (Invitrogen Corp.) and inserted into yeast expression vector p413Gal, as a BamHI / XhoI fragment, to generate expression vector pDE314 (bottom diagram). Correct clone was confirmed by DNA sequencing. This vector allow expression of proteins with a myc-6xHis tag at their C-terminus



sequence at the 5' end) and 3' BACE (that includes codons for the last 6 amino acids, without STOP codon, and SalI-BamHI-CC sequence at the 5' end). The PCR primers used to amplify BACE2 were: 5' AF050171 (starts 10 bases before coding region and includes GG-XbaI-HindIII sequence at the 5' end) and 3' AF050171 (that includes codons for the last 6 amino acids, without STOP codon, and SalI-BamHI-CC sequence at the 5' end) (Table 8).





Wild-type BACE1 and BACE2

Myc-6xHis tag was replaced by a stop codon in plasmids pDE326 and pDE316 to obtain wild-type forms of BACE1 and BACE2, respectively. For this purpose the BACE1 sequence was PCR-amplified using primers 5'BACE (described above) and 3' BACE STOP (that includes last 6 codons of BACE1, a STOP codon and XhoI-GG sequence at the 5' end). BACE2 was PCR-amplified using primers: 5' AF050171 (described above)

Table 8: PCR primers to amplify BACE1 and BACE2

Primers used to make the different BACE1 and BACE2 constructs. All primers include two extra bases at the 5' end to facilitate cleavage by the restriction enzymes (restriction sites are in bold).

Oligo name	Restriction sites	Oligo sequence
5' BACE	Xba-HindIII	5' GGTCTAGAAAGCTTGGGGCCCACCATGGCCCA 3',
3' BACE	BamHI-SalI	5' GGGGATCCGTCGACCTTCAGCAGGGAGATGTC 3'
5' AF050171	XbaI-HindIII	5' GGTCTAGAAAGCTTCGCCGTGGGCATGGGCGC 3'
3' AF050171	BamHI-SalI	5' GGGGATCCGTCGACTTTCCAGCGATGTCTGAC 3'
3' BACE STOP	XhoI	5' GGCTCGAGTCACTTCAGCAGGGAGATGTC 3'
3' AF050171 STOP	XhoI	5' GGCTCGAGTCATTTCCAGCGATGTCTGAC 3'
3' sol BACE-1	BamHI-SalI	5' GGGGATCCGTCGACTGACTCATCTGTCTG 3'
3' sol AF050171	BamHI-SalI	5' GGGGATCCGTCGACGGGCTCGCTCAAAGA 3'
New 5' BACE	XbaI	5' GGTCTAGAAACAAACAAAATGGCCCAAGCCCTG 3'
New 5' AF050171	XbaI	5' GGTCTAGAAACAAACAAAATGGGCGCACTGGCC 3'
5' mat BACE-1	XbaI	5' CCTCTAGATAAAAGAGAGACCGACGAAGAG 3'
3' mat BACE-1	NotI	5' GGGCGGCCGCTCTTCAGCAGGGAGAT 3'
5' mat BACE-2	XbaI	5' CCTCTAGATAAAAGAGCCCTGGAGCCTGCC 3'
3' mat BACE-2	NotI	5' GGGCGGCCGCTTTTCCAGCGATGTCT 3'

and 3' AF050171 STOP (that includes last 6 codons of BACE2, a STOP codon and XhoI-GG sequence at the 5' end) (Table 8).

Secreted forms of BACE1 and BACE2

Secreted forms of BACE1 and BACE2 with a C-terminus myc-6xHis tag were designed for purification and activity measurement purposes. Ectodomains of BACE1 and BACE2 were PCR-amplified from plasmids pDE326 and pDE316, respectively. BACE1 ectodomain was amplified using primers 5' BACE (described above) and 3' sol BACE1 (contains 6 codons for amino acids before putative transmembrane sequence and SalI-BamHI-GG sequence at the 5' end). BACE2 ectodomain was amplified using primers 5' AF050171 (described above) and 3' sol AF050171 (contains 6 codons for amino acids before putative transmembrane sequence and SalI-BamHI-GG sequence at the 5' end) (Table 8).

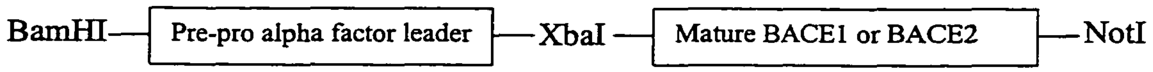
BACE1 and BACE2 with yeast ribosomal binding site

To boost BACE1 and BACE2 expression in yeast, DNA sequences corresponding to bases –1 to –10 of the yeast TDH3 gene (AACAAACAAA) were introduced just before BACE1 and BACE2 coding regions in plasmids pDE326 and pDE316, respectively. BACE1 was PCR amplified from pDE326 using primers New 5' BACE1 (that includes codons for the first 5 amino and AACAAACAAA-XbaI-GG sequence at the 5' end) and 3' BACE (described above). BACE2 was PCR amplified from pDE316 using the following primers: New 5' AF050171 (that includes codons for the first 5 amino and AACAAACAAA-XbaI-GG sequence at the 5' end) and 3' AF050171 (described above) (Table 8).

Mature forms of BACE1 and BACE2 for yeast expression

Mature forms of BACE1 and BACE2 (Figure 3) were obtained by replacing the putative signal and pro-domain wild-type sequences of BACE1 and BACE2 in plasmids pDE326 and pDE316, respectively, with that of the yeast pre-pro α-mating factor sequence. α-factor sequence was taken from plasmid pDE195 as a BamHI/XbaI fragment. Codons for Kex2 cleavage site were introduced just before BACE1 and BACE2 mature sequences by PCR. This would allow cleavage of the fusion proteins by Kex2 protease to give the mature forms of both BACE1 and BACE2.

Mature BACE1 was PCR-amplified from plasmid pDE326 using the following primers: 5' mat BACE-1 (that contains a XbaI site and codons for KEX2 cleavage site just before mature BACE1 sequence) and 3' mat BACE-1 (contains a NotI restriction site and an extra base before it to put it in frame with myc-his tag. Mature BACE2 was PCR-amplified from plasmid pDE316 using the following primers: 5' mat BACE-2 (that contains a XbaI site and codons for KEX2 cleavage site just before mature BACE2 sequence) and 3' mat BACE-2 (contains a NotI site and an extra base before it to put it in frame with myc-his tag) (Table 8).



Mature forms fused to the α-factor sequence were then ligated to corresponding BamHI/NotI sites in yeast expression vector pDE314 (Figure 19) that provides the myc-6xHis tags to these proteins.

PS1 expression in yeast

Human PS1 wild type cDNA was obtained from plasmid pGEX-NT-BL21 (kindly provided by Dr. Nikolaos Tezapsidis, New York University, NY) as a 1,857 bp BamHI/EcoRI fragment and inserted into p413Gal1 and p416Gal1 to make pDE167 and pDE284 expression vectors, respectively. The PS1 Polish mutant P117L was obtained by digesting plasmid P117L (kindly provided by Dr. Karol Dowjat, NYS Institute for Basic Research, Staten Island, NY) with EcoRI and inserting fragment into p413Gal1 EcoRI digested to make plasmid pDE277.

Cloning of human nicastrin

Nicastrin (Genbank accession # AF240468) cDNA was PCR-amplified from HeLa cells cDNA (Marathon ready, Clontech, Inc.) using primers 5' NICAS (that includes bases –10 to +8 of reported mRNA sequence and GG-XbaI-BglII at the 5' end, 5'GGTCTAGAAGATCTGAGAGGCAAGATGGCTAC3') and 3' NICAS (that includes codons for the last 6 amino acids, and a NotI site for cloning and linking to myc-6xHis tag. Also includes an extra base between coding sequence and NotI site to put C-ter tags in frame with nicastrin, 5'GTCCGCGGCCGCTGTATGACACAGCTCCTGG3'). The PCR product was digested with XbaI/NotI and inserted into yeast expression vector pDE314 (Figure 19) to generate plasmid pDE379. The correct clone was confirmed by DNA sequencing. To have a nicastrin mammalian expression vector, its sequence was cleaved from plasmid pDE379 as a BglII/NotI fragment and inserted into a mammalian vector derived from pcDNA3.1 Uni (Invitrogen Corp.) that carries the myc-6xHis tags.

Cloning of the human tumor necrosis factor-α converting enzyme (TACE)

A human TACE cDNA was previously cloned in our lab and inserted into pCR3.1. TACE cDNA from this vector was transferred to yeast expression vector p413Gal to make pDE157. The DNA sequence determination of TACE in pDE157 revealed the presence of a STOP codon before putative transmembrane sequence. Attempts to repair mutation were unsuccessful. TACE was PCR-amplified again from human spleen Marathon-Ready cDNA (Clontech) using primers 5' TACE 2000 (starts at position –10 of coding region and includes a BamHI and XbaI sites for subcloning purposes, 5' GGGGATCCTCTAGAGGCCGGGAACATGAGGCA 3') and 3' TACE 2000 (includes codons for last 6 amino acids, but no STOP codon, an EcoRI site, and four extra bases to be able to clone it into GFP containing vector, 5' TTCCGAATTCGCACTCTGTTTCTTTGCT 3'). The PCR product was inserted into mammalian expression vector pcDNA3.1CT-GFP-TOPO (Invitrogen), that allows expression of a GFP tagged protein. DNA sequencing of one of these TACE clones (pDE365) revealed the presence of the same STOP codon mutation as previous TACE clone. For this reason TACE cloning was not further pursued.

Protein extraction from mammalian and yeast cells

Cell pellets from 1.5-3.0 ml bacteria or yeast cultures were resuspended with 100 to 200μl of Triton (1% Triton-X100, 20mM Tris pH8.0) or SDS (1% SDS, 50mM Tris pH8.0, 50mM NaCl, 5mM EDTA) extraction buffers, containing a cocktail of protease inhibitors (Boehringer Mannheim cat # 1697498). Samples were then sonicated for 20 seconds. Cell extracts were cleared by centrifuging at 100k x g for 10 min in a refrigerated bench top ultracentrifuge (Beckman TL100). Protein concentration was determined using BCA assay (BioRad).

Western Immunoblots

Routinely, 7.5ug of protein were loaded per lane on 7/15% or 9% acrylamide-bisacrylamide, depending on the size of the protein of interest, with 4% stacking gel. Tricine-Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was the method of choice (Schagger and von Jagow, 1987). After electrophoresis the proteins were transferred onto nitrocellulose membrane at 100V constant for 1hr according to Schagger and von Jagow (1987). After transfer the blots were incubated with blocking buffer (1xPBS, 0.2% (w/v) BSA, 0.1% (v/v) Tween 20) for at least 1hr at room temperature. Blots were then incubated with appropriate primary antibodies, diluted in blocking buffer, for 1hr at room temperature or overnight at 4°C. Blots were then washed 2 times with blocking buffer (incubating 10 minutes each time) and then incubated with appropriate alkaline phosphatase conjugated secondary antibodies, also diluted in blocking buffer, for 1hr at room temperature. Blots were then washed twice with blocking buffer, as before, and twice with dH_2O. Finally, blots were developed by incubating membranes in buffer containing 5-bromo-4chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

Antibodies

APP antibodies: R57, a rabbit polyclonal antibody (epitope residues 672-695 of human APP695) was used to detect APP and APP C-terminal fragments (i.e. C99 and C83). R162 is a rabbit polyclonal antibody specific for Aβ1-40 (raised against residues 32 to 40 of Aβ sequence) and does not cross-react with Aβ1-42. R71 is a rabbit polyclonal antibody raised against residues 46-60 of human APP. 6E10 mouse monoclonal raised to residues 8 to 16 of Aβ sequence. 4G8 mouse monoclonal specific for residues 17 to 24 of Aβ sequence. The rabbit antibodies we provided by Dr. Pankaj Mehta and colleagues (NYS Institute for Basic Research). The monoclonal antibodies were provided by Dr. K.S. Kim (NYS Institute for Basic Research) (Table 9).

BACE antibodies: R264 and R265 are rabbit polyclonal antibodies generated at the NYS Institute for Basic Research (Staten Island, NY) by Dr. Pankaj Mehta and colleagues. R264 was raised to residues 486 to 501 of human BACE1 sequence. R265 was raised against residues 509 to 518 of human BACE2 sequence (Table 9).

PS1 antibodies: Two antibodies targeted to the N-terminus (R222 and X81) and two to the loop region (PS-1L and PS1-331) of human PS1were used. R222 is a rabbit polyclonal antibody generated at the NYS Institute for Basic Research (Staten Island, NY) (immunogen residues 2-12 of human PS1) and it was epitope-affinity-purified. X81, a rabbit antibody raised against residues 1 to 81 of human PS1, was obtained from Dr. Weiming Xia (Harvard Medical School, Boston). PS-1L is a rabbit polyclonal antibody purchased from Zymed Laboratories, and it is also epitope-affinity-purified (immunogen residues 319-357). PS1-331, a rabbit polyclonal antibody (epitope narrowed to residues 331 to 355), was obtained from Dr. W.K. Dowjat (NYS Institute for Basic Research, Staten Island, NY).

Anti-myc antibody (Invitrogen) was used to detect myc-tagged proteins. Anti-TACE SC-6417 (Santa Cruz Biotechnologies). Goat polyclonal anti-TNFα SC-1350 (Santa Cruz Biotechnologies). Antibodies against organelle markers: rabbit polyclonal anti-Calreticulin (Affinity Bioreagents, Inc), mouse monoclonal anti-Golgi 58K protein (Sigma), mouse monoclonal anti-β-COP (Sigma).

Fluorescent labeled antibodies: Alexa488 or Alexa568 (Molecular Probes), anti mouse or anti rabbit, accordingly, were used for confocal microscopy.

Immunocytochemistry

$5x10^4$ cells were seeded on microscope slides and processed after 24 hours of seeding, or after 24hr post-transfection (if they were transfected). Cells were fixed and permeabilized as follows: cells were briefly rinsed two times with PBS and fixed with 2% paraformaldehyde in RPMI for 10 min, followed by two rinses with PBS for 10 min each time. 2% goat serum in RPMI was added, as a blocking agent, and incubated for 20-30 minutes. Cells were then incubated with appropriate primary antibody diluted in 1% goat serum RPMI (0.05% Saponin, 0.01% NaN3, 1% goat serum in RPMI) for 1 hr. Cells were then rinsed twice with PBS, 10 min each time. Cells were then incubated with appropriate fluorescent secondary antibody (such as Alexa 488 or Alexa 568, Molecular Probes) for 1hr at room temperature and protected from light. Cells were then rinsed once more with PBS and coverslips mounted on glass slides. Cells were then examined under confocal fluorescent microscope.

Table 9: Antibodies used

The following is a list of the antibodies used in this study:

Ab Name	Species in which Ab was raised	Antigen (residues)
R57	Rabbit	APP695 (672-695)
R3	Rabbit	APP695 (649-671)
R71	Rabbit	APP695 (46-62)
6E10	Mouse	Aβ (Epitope 8-16)
4G8	Mouse	Aβ (Epitope 17-24)
R162	Rabbit	Aβ (32-40)
R264	Rabbit	Human BACE1 (486-501)
R265	Rabbit	Human BACE2 (509-518)
R222	Rabbit	Human PS1 (2-12)
X81	Rabbit	Human PS1 (1-81)
PS-1L	Rabbit	Human PS1 (319-357)
PS1-331	Rabbit	Human PS1 (331-355)
Anti-myc	Mouse	Myc epitope

Results

Yeast α-secretase activity of putative aspartyl proteases *YLR121c* and *YIR039c*

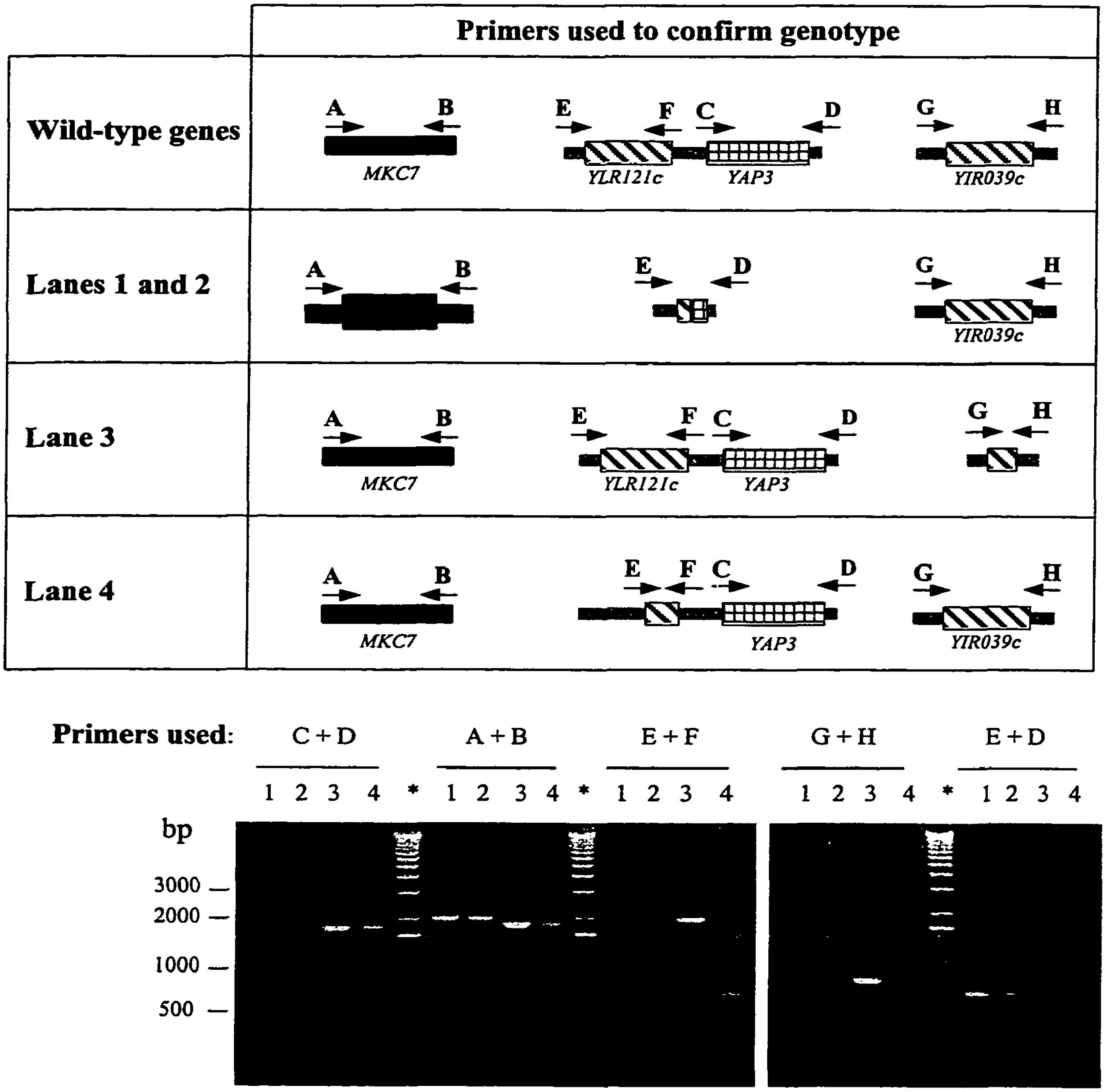
To test whether *YLR121c* or *YIR039c*, two other *YAP3* homologues, had any α-secretase activity, their genes were deleted from the yeast chromosome. A series of yeast mutants were generated from DS7. These mutants carry single, double, triple or quadruple deletions of the four yeast aspartyl proteases (Table 10). Deletion of these genes was confirmed by PCR, and by loss of α-secretase activity in the case of *YAP3* and *MKC7* double deletion (Figure 6, Figure 7).

The *MKC7* gene was disrupted by insertion of *TRP1* via homologous recombination (Rothstein, 1991) (see methods section). *YAP3*, *YLR121c*, and *YIR039c* were disrupted by the CRE/loxP system because it allows the disruption of multiple genes without the exhaustion of selectable markers commonly used to disrupt genes in S. cerevisiae (see Materials and Methods). Deletion of these genes was confirmed by PCR (Figure 20). Radiolabeling experiments suggested that disruption of *YLR121c* or *YIR039c* does not significantly affect α-secretase cleavage (Figure 21). It is not known whether these proteases are potential APP secretases. Their levels of expression in DS7 were not determined.

For the purpose of developing a yeast system to screen for APP secretase candidates, the strain that carries the double yap3/mkc7 deletion was chosen as the host cell (to minimally disturb the host cell, since roles of *YLR121c* and *YIR039c* in yeast are unknown). This strain has a residual α-secretase activity of approximately 14% of the parent strain DS7 (Figure 6, Figure 21).

Figure 20: Deletion of yeast *YAP3* homologues *YLR121c* and *YIR039c*

YLR121c and *YIR039c* were deleted in *S. cerevisae* strains dereived from DS7 by the CRE/loxP system. Disruption of the genes was verified by PCR using genomic DNA and sets of specific PCR primers for *YAP3*, *MKC7*, *YLR121c*, and *YIR039c*, as shown in diagram below. Primers used (see Materials and Methods): A= MKC7 2019, B=MKC7 149, C=5'YAP3 set1, D= 3' YAP3 set2, E = YLR121c set1, F= 3'YLR121c set2, G= 5'YIR039c set1, H= 3' YIR039c set2. PCR products were run on 1% agarose gel (containing 200 ng of ethidium bromide / mL). **Lanes 1 & 2**: 2 clones from yDE78 (*yap3 mkc7 ylr121c YIR039c*); **Lane 3**: yDE94 (*YAP3 MKC7 YLR121c yir039c*); **Lane 4**: yDE95 (*YAP3 MKC7 ylr121c YIR039c*).



(*) =1Kb DNA ladder (Gibco BRL, Life Technologies).

Figure 21: *YAP3* homologues *YLR121c* and *YIR039c* do not have significant α-secretase activity

α-secretase activity was measured in yeast strains carrying mutations in the α-secretase genes (*yap3* and *mkc7*) and in either *YLR121c* or *YIR039c*. The graph shows the results of three pulse-chase experiments where cells were labeled with ^{35}S-TRANS (ICN) and chased for 30min. APP and C-terminal fragments were immunoprecipitated with R57 and subjected to SDS-PAGE. The radioactivity was quantified by phosphorimaging. α-secretase activity is expressed as the signal obtained from α-secretase cleaved C-terminal fragment (αCTF) over signal obtained from total APP (all forms), calculated for t=0 and t=30. Neither deletion of ylr121c nor yir039c significantly reduced α-secretase activity any lower than that of the double yap3/mkc7 double knockout.

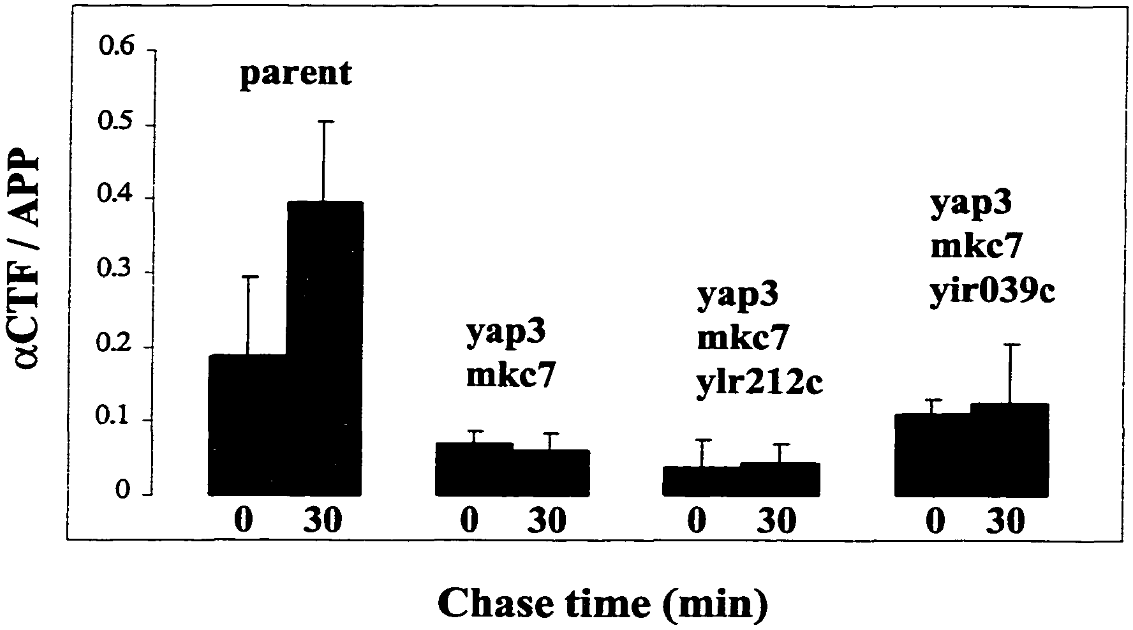


Table 10: Yeast strains with aspartyl proteases deleted

Plus sign indicates that the wild-type gene is present, the minus sign indicates that the gene has been deleted. *MKC7* was deleted by insertion of *TRP1* gene via homologous recombination. The rest of genes were deleted using the CRE/loxP system. Deletion of genes was confirmed by PCR (Figure 7 and Figure 20 show the PCR results from some of these strains).

Strain name	YAP3	MKC7	YLR121c	YIR039c
DS7	+	+	+	+
yDE94	+	+	+	-
yDE95	+	+	-	+
yDE77	-	-	+	+
yDE78	-	-	-	+
yDE79	-	-	+	-
yDE76	-	-	-	-

APP-reporter molecules

To facilitate the detection of secreted APP products, sequences for the last 106 amino acids of APP Swedish mutant (C106sw) were fused to reporter molecules. The last 106 amino acids of APP contain all the cleavage sites for α-, β-, and γ-secretases, and has been shown to be an adequate substrate for these enzymes (Pappolla et al. 1994; Citron et al. 1995). The APP Swedish mutant is cleaved by β-secretase more efficiently than wild-type APP, for this reason this mutant is preferred to make APP-reporter molecules. Two different forms of GFP (EGFP and GFPs65t – Clontech Inc.) and the mature form of the major secreted yeast acid phosphatase (Pho5p) were fused at their C-termini to C106sw. To ensure that the fusion protein is brought to the secretory pathway, the yeast α-factor signal and pro sequences were added to the N-terminus of the fusion proteins. Fusions were done at the DNA level (see Materials and Methods). PHO5-C106sw was expressed from 2μ-based plasmid pDE041 (derived from pBS6α, Hines et al. 1994). GFP fusion proteins were expressed from plasmid p416Gal (Figure 15). Each fusion protein and its corresponding αCTF (after α-secretase cleavage) could be detected with antibody R57 on Western blots (Figure 22). Therefore both PHO5-C106sw and GFPs65t-C106sw seem to be processed by yeast α-secretases similarly to wild-type APP695. Some additional cleavage, or staining, occurs with the GFPs65t, probably due to the much higher expression levels than the other APP constructs. Immunoprecipitation experiments on extracts of cells expressing pDE041 suggest that the cleavage occurs at the α-secretase site (Figure 1 and Figure 23). The sizes of the CTFs were similar to that of αCTF that had previously been confirmed by sequencing (Hines et al., 1994; Zhang et al., 1994). Furthermore, the CTFs react with mAb 4G8 (epitope residues 17 to 24 of Aβ sequence), but not with mAb 6E10 (specific for residues 8-16 of Aβ).

No fluorescence could be detected in the conditioned medium of GFP-C106sw transfected cells. When GFP-C106sw transfected cells were examined under a fluorescent microscope, it was observed that not all the cells were fluorescent, as would be expected from a cloned population (Figure 24). On the other hand, phosphatase activity could be easily detected in the conditioned medium of PHO5-C106sw transfected cells (Figure 25).

Figure 22: Expression of APP and APP reporter proteins in yeast

APP and APP reporter proteins were transfected and expressed in yeast strains DS7 (except for lane 4, in which JSC310 was used). Cell pellets from 1.5 ml cultures were sonicated in 1% Triton X-100 20mM Tris pH 8.0. Extracts were cleared by centrifugating at 100k x g for 10min. 7.5 μg of cleared extracts were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Western blot was immunodetected with antibody R57 (Figure 1).

1. PHO5-C106sw expressed from multicopy plasmid pDE041
2. APP695 expressed from multicopy plasmid pBS6α (Hines et al. 1994) in DS7
3. APP695 expressed from multicopy plasmid pBS6α (Hines et al. 1994) in JSC310
4. APP695 expressed from centromeric plasmid p416Gal1
5. APP695 expressed from multicopy plasmid p426Gal1
6. GFPs65t-C106sw expressed from centromeric plasmid p416Gal1 (this strain was examined under confocal microscope to check for fluorescence, see Figure 24)
7. GFPs65t-C106sw expressed from multicopy plasmid p426Gal1

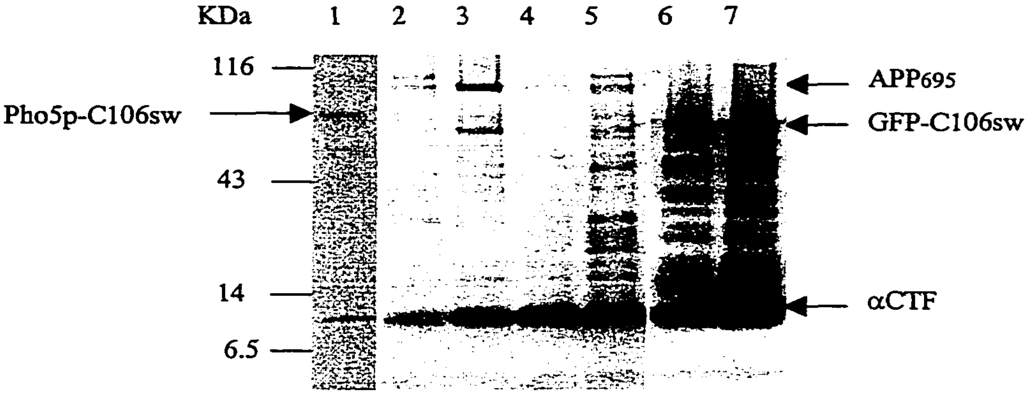


Figure 23: Cleavage of Pho5p-C106sw is similar to that of full length APP.

A Triton X-100 cell extract of yDE059, yeast strain DS7 transfected with pDE041 (PHO5-C106sw containing vector), was immunoprecipitated with antibody 4G8, subjected to SDS-PAGE, blotted on nitrocellulose membrane, and detected with antibody 6E10 (lane 2) or R57 (lane 3). Lane 1 is the material used for immunoprecipitation and detected with antibody R57.

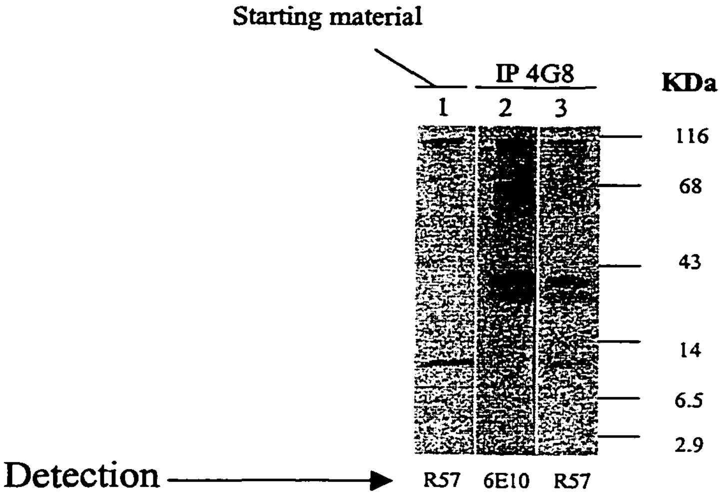


Figure 24: Fluorescence of GFPs65t-C106sw yeast

Yeast strain yDE233, expressing GFPs65t-C106sw, was observed under confocal fluorescent microscope. Note that although the protein is expressed (Figure 22) not all the cells are fluorescent, as would be expected from a cloned population. GFP fluorescent cells are very bright (green), while some other cells give a dimmer background fluorescence (yellow/red).

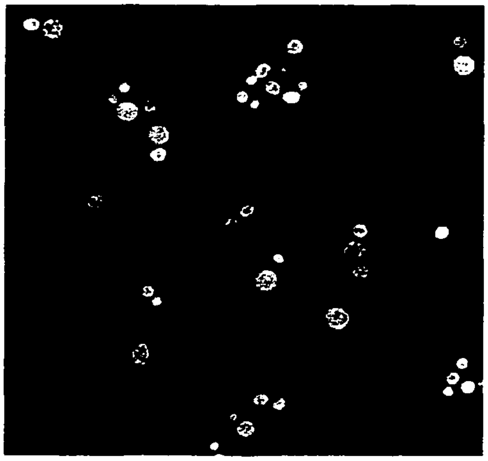
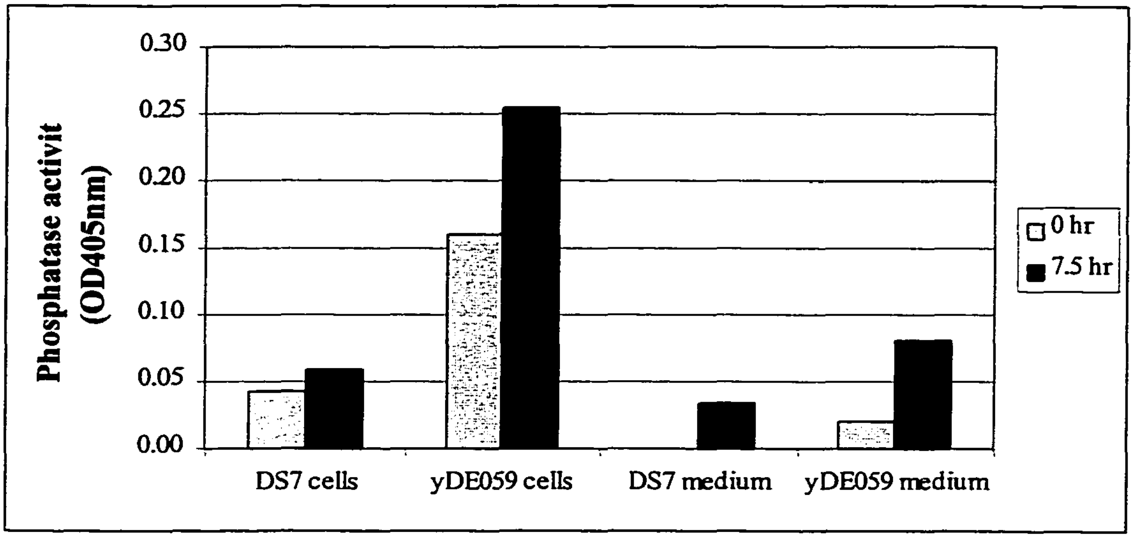


Figure 25: Phosphatase activity from PHO5-C106sw expressed from pDE041

Equivalent number of cells of yeast strains DS7 and yDE059 (contains plasmid pDE041, PHO5-C106sw plasmid) were grown in 0.05% glucose-containing medium to induce Pho5p-C106sw expression. Phosphatase activity was determined from 50μl of cells suspension or supernatants harvested at t=0hr and t=7.5hr. Samples were incubated with 50 μl of a 1mg/ml solution of pNPP for 1hr at 37°C (see Materials and Methods).



Insertion of PHO5-C106sw into the yeast chromosome

To reduce the variability in expression levels of the *PHO5*-C106sw reporter, this was inserted into the yeast chromosome using the CRE/loxP system (Figure 26). The insertion has also the advantage of not using up selectable markers, important if co-expression of more than one plasmid is desired in the same yeast strain. The *PHO5*-C106sw fusion was inserted into the yeast chromosome II at the PHO5/PHO3 locus by engineering an insertion cassette that contains PHO5-C106sw sequences fused at their 3' end to the α-mating factor terminator sequences (Figure 14; see Materials and Methods for details). This cassette replaces the *PHO5* and *PHO3* sequences, eliminating these two major endogenous secreted yeast acid phosphatases. Fusion is then under the control of the *PHO5* promoter. Insertion in the right place was verified by PCR (Figure 27) and by the induction of expression in the presence of low Pi levels in the growth medium (Figure 28). Protein expression levels of PHO5-C106sw under the PHO5 promoter are slightly lower than expression of APP695 from plasmid pBS6α (multicopy plasmid that carries APP695 and is described in Hines et al. 1994) (Figure 29).

Yeast system to screen for human APP secretase candidates

To screen a human cDNA library for human APP secretase candidates, a yeast system was developed using a *S. cerevisiae* strain. The system consists of a host cell with almost no secretase activity (yeast strain yDE77, carrying yap3/mkc7 double deletion and about 14% residual secretase activity) and a reporter protein consisting of the yeast acid phosphatase pho5p fused to the last 106 amino acids of human APP (Pho5p-C106sw). To enhance the sensitivity of the reporter molecule, secreted yeast acid phosphatase, all the

Figure 26: Deletion of yeast phosphatases and insertion of PHO5-C106sw into the yeast chromosome

A: Major yeast acid phosphatase genes *PHO5* and *PHO3* are in tandem in S. cerevisiae chromosome II.

B: Disrupted *PHO5/PHO3* genes by insertion of deletion cassette using CRE/loxP system.

C: Insertion of PHO5-C106sw into yeast PHO5/PHO3 locus using CRE/loxP system.

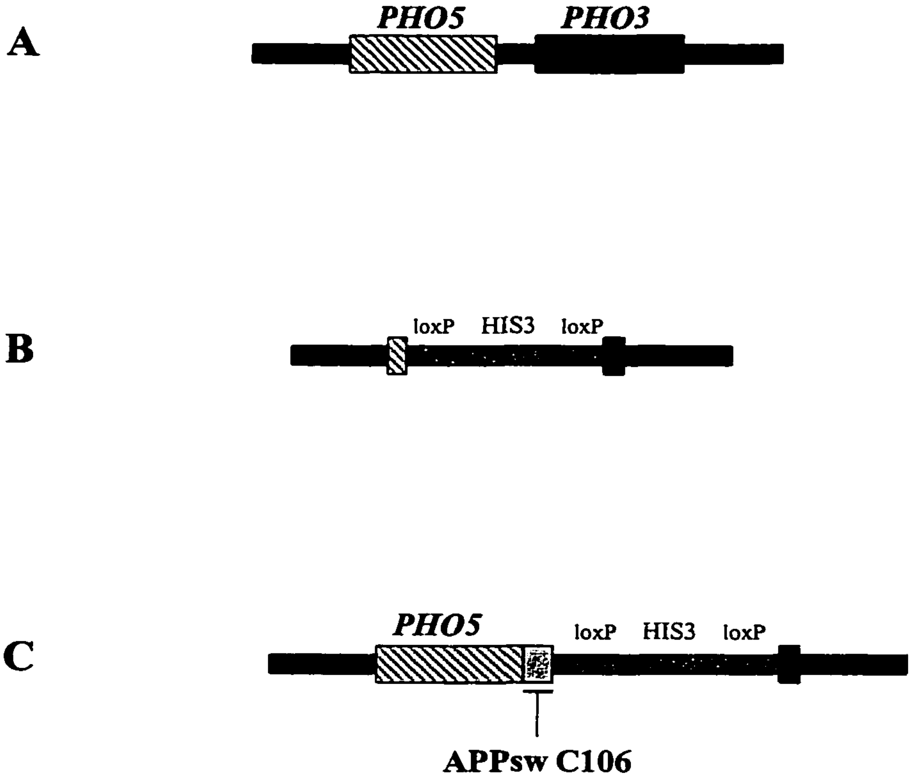
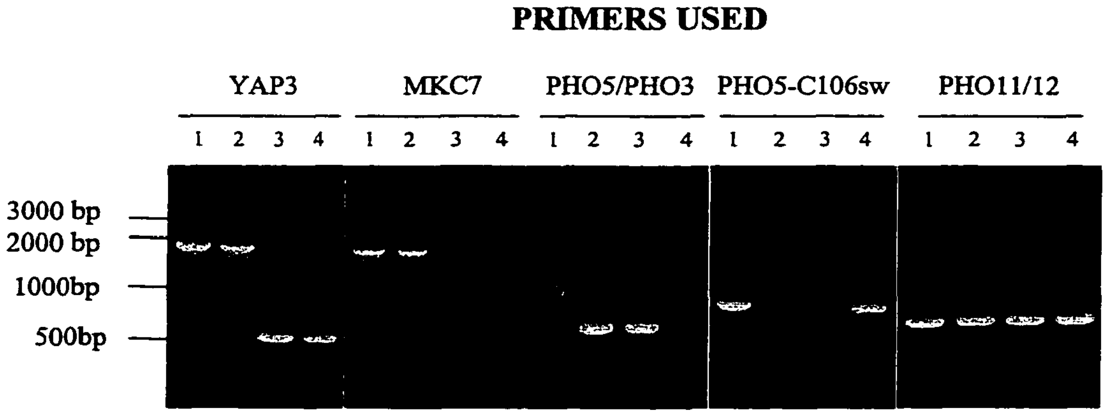


Figure 27: PCR to confirm deletions of phosphatases and insertion of PHO5-C106sw into PHO5/PHO3 locus

Genomic DNA was extracted from mutant strains and PCR-amplified with primers flanking *YAP3*, *MKC7*, *PHO5/PHO3*, and *PHO11/PHO12*. Primers used for PHO5/PHO3 flanking regions were used also to confirm insertion of PHO5-C106sw into PHO5/PHO3 locus. This was corroborated with a second set of primers, one specific for the C106sw sequence and PHO5/PHO3 screen (see Table 4 and Table 5 for primer sequences).

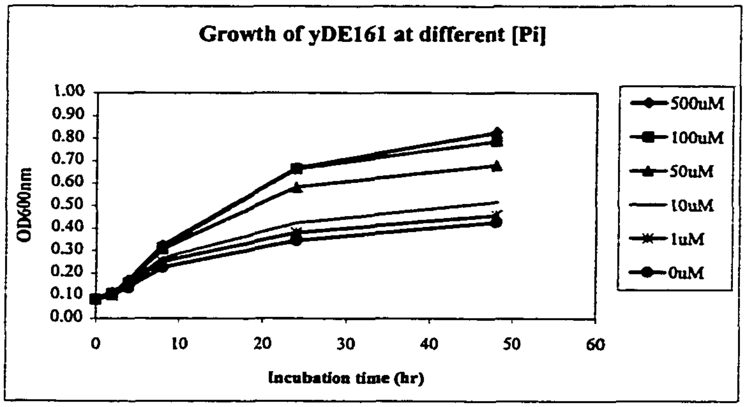


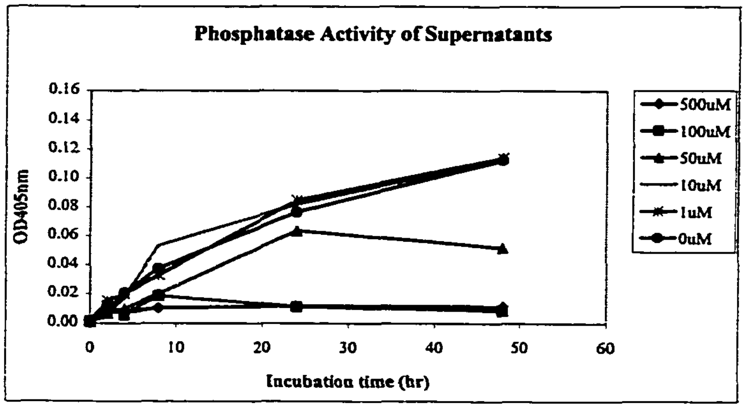
Lane	Relevant Genotype
1	*YAP3 MKC7* pho5/pho3::PHO5-C106sw/loxP pho11::loxP pho12::loxP
2	*YAP3 MKC7* pho5/pho3::loxP pho11::loxP pho12::loxP
3	yap3 mkc7 pho5/pho3::loxP pho11::loxP pho12::loxP
4	yap3 mkc7 pho5/pho3::PHO5-C106sw/loxP pho11::loxP pho12::loxP

EXPECTED SIZES OF PCR PRODUCTS			
Genotype	**5' Primer**	**3' Primer**	**Expected size (bp)**
YAP3	5' YAP3 set 1	3' YAP3 set 2	1796
yap3::loxP	5' YAP3 set 1	3' YAP3 set 2	503
MKC7	MKC7 2019	MKC7 149	1868
mkc7::TRP1	MKC7 2019	MKC7 149	2020
PHO5 PHO3	5' matAP-APP	PHO5/PHO3 screen	3301
pho5/pho3::loxP	5' matAP-APP	PHO5/PHO3 screen	635
pho5/pho3::PHO5-C106sw/loxP	5' matAP-APP	PHO5/PHO3 screen	2416
pho5/pho3::PHO5-C106sw/loxP	5 CAPP	PHO5/PHO3 screen	881
pho11::loxP	5' PHO11/12 set1	3' PHO11/12 set2	About 650
pho12::loxP	5' PHO11/12 set1	3' PHO11/12 set2	About 650

Figure 28: Induction of PHO5-C105sw expression by low [Pi] in growth medium

To determine the optimal [Pi] concentration for the growth and induction of the *PHO5*-C106sw reporter, yeast strain yDE161 (*YAP3 MKC7* pho5/pho3::*PHO5*-C105sw/loxP) was grown in CSM containing different concentrations of inorganic phosphate (Pi).





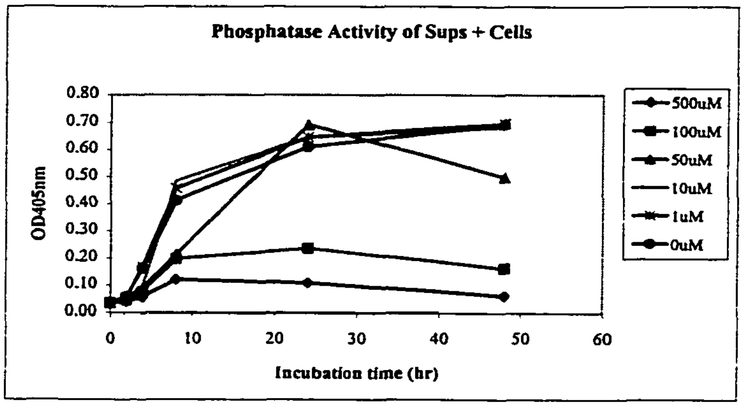
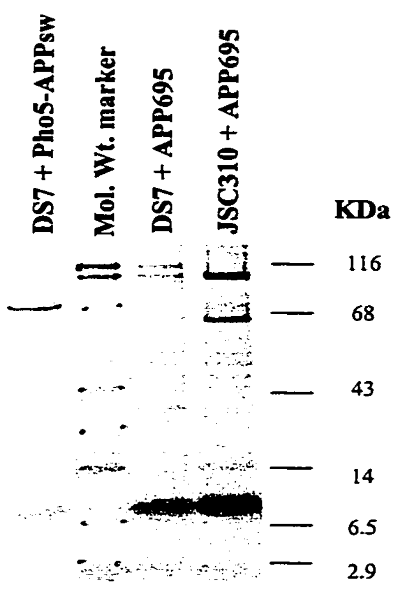


Figure 29: Expression of *PHO5*-C106sw from yeast chromosome

PHO5-C106sw reporter was introduced into yeast chromosome II by the CRE/loxP system (see Materials and Methods). Figure shows a Western blot of Triton-X100 extracts of different yeast strains expressing human APP695. 7.5 μg of protein were loaded per lane in a 7/15% tris-tricine gel, and immunoblotted with antibody R57 that recognizes the very end of human APP (Figure 1).



endogenous secreted yeast acid phosphatases (*PHO3, PHO5, PHO11* and *PHO12*; see Table 11) were deleted from the yeast genome by the CRE/loxP system (see Materials and Methods). The deletion of these genes was performed in yeast strain yDE77, and was confirmed by PCR (Figure 27) and by lack of phosphatase activity in the conditioned medium of cells grown under low extracellular Pi, which induces the phosphatase system (Figure 30). The host cell has then almost no secretase activity as well as no endogenous secreted phosphatase activity. The only secreted phosphatase activity comes from the PHO5-C106sw reporter molecule when it is cleaved by the yeast α-secretases (Figure 30). The reporter protein, Pho5p-C106sw, seems to be processed in yeast similarly to full length APP in the sense that characteristic αCTF is formed (Figure 22, Figure 23).

To test the yeast screening system, a secretase was introduced in the form of a expression vector (Figure 31). The major yeast α-secretase, *YAP3*, was reintroduced inserted in plasmid p413Gal1 (resulting plasmid named pDE156) into yeast strain yDE152 (*yap3 mkc7 pho5/pho3::PHO5*-C106sw/loxP). After reintroduction of *YAP3*, secretase activity was regained, and secreted phosphatase activity was detected (Figure 32). yDE152 transfected with vector alone does not show the same effect. Strains containing pDE156 (*YAP3* plasmid) and p413Gal (vector alone) were named yDE165 and yDE166, respectively.

When yDE165 and yDE166 were grown on solid medium plates and overlaid with 5 μM fluorogenic phosphatase substrate DiFMUP (6,8-difluoro-4-methylumbelliferyl

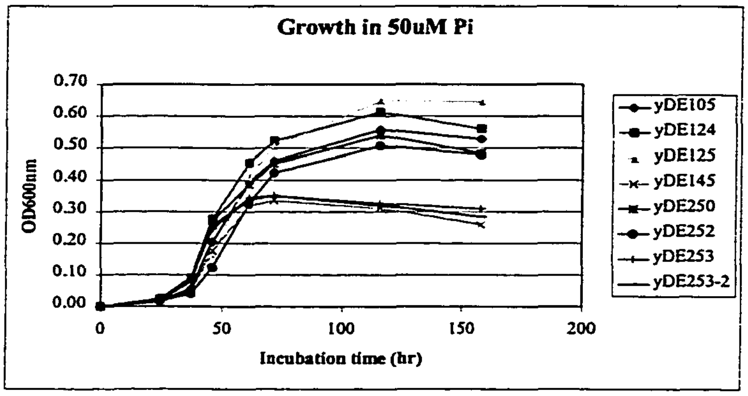
Table 11: Secreted yeast acid phosphatases

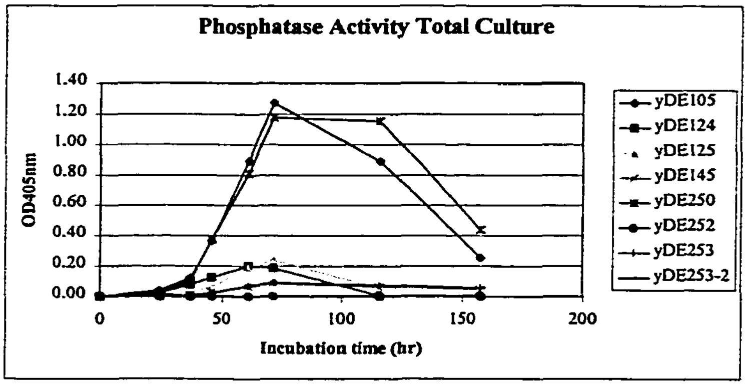
Four genes that code for secreted acid phosphatases were deleted from the yeast genome by the CRE/LoxP system. *PHO5* is responsible for about 90% of the secreted phosphatase activity. *PHO3* contributes with about 5%. *PHO11* and *PHO12* are responsible for the other 5% of secreted phosphatase activity. Even though the genes for these phosphatase are highly homologous sequences (more than 80% identity at the DNA level), the differences in the non-coding regions seem to be responsible for their different protein expression levels. There is also a highly homologous sequence in chromosome IV, but that does not seem to be expressed.

Gene	**Chromosome**
PHO5	**II**
PHO3	**II**
PHO11	**I**
PHO12	**VIII**
PHO5 **vs**	
PHO3	Identities = 1217/1403 (86%)
PHO11	Identities = 1174/1403 (83%)
PHO12	Identities = 1172/1403 (83%)
CHR4: Chromosome IV Sequence Identities = 969/1406 (68%)	

Figure 30: Phosphatase activity of yeast strains lacking endogenous phosphatases

Strain	Relevant genotype
yDE105	YAP3 MKC7 pho5/pho3::PHO5.APP.loxP
yDE124	YAP3 MKC7 pho5/pho3::loxP
yDE125	yap3 mkc7 pho5/pho3::loxP
yDE145	yap3 mkc7 pho5/pho3::PHO5.APP.loxP
yDE250	YAP3 MKC7 pho5/pho3::PHO5-APP/loxP pho11::loxP pho12::loxP
yDE252	yap3 mkc7 pho5/pho3::loxP pho11:loxP pho12::loxP
yDE253	yap3 mkc7 pho5/pho3::PHO5-APP/loxP pho11:loxP pho12::loxP





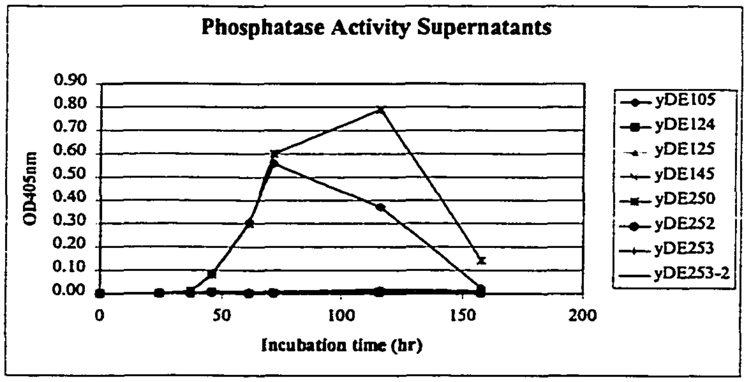


Figure 31: Test of yeast screening system with a secretase

Yeast system consists of a host cell with ideally no secretase activity present (our host cell has about 14% of residual secretase activity) and a reporter molecule, *PHO5*-C106sw, integrated in the yeast PHO5/PHO3 locus. Additionally the host cell does not have any endogenous secreted phosphatases, all phosphatase activity should come from the reporter Pho5p-C106sw molecule. The system was put to test by introducing a expression vector that carries a yeast secretase (see Figure 32).

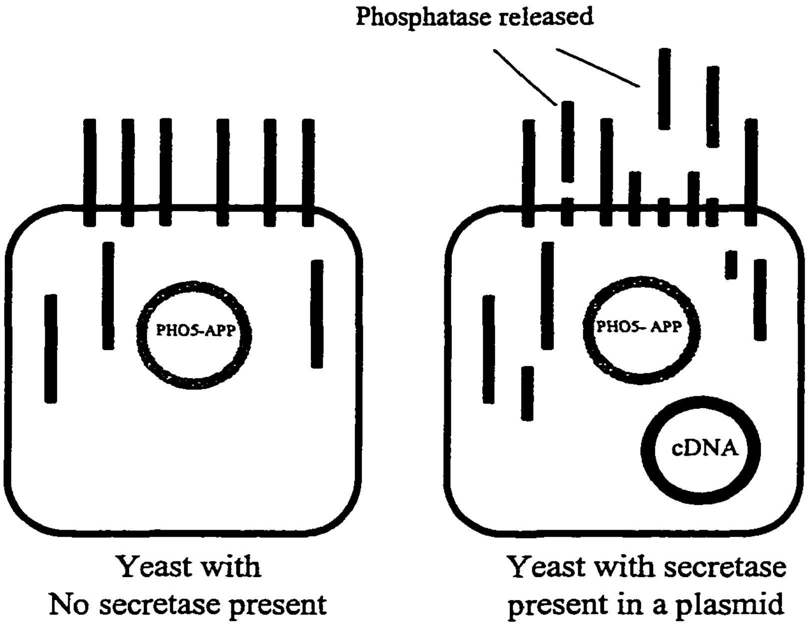
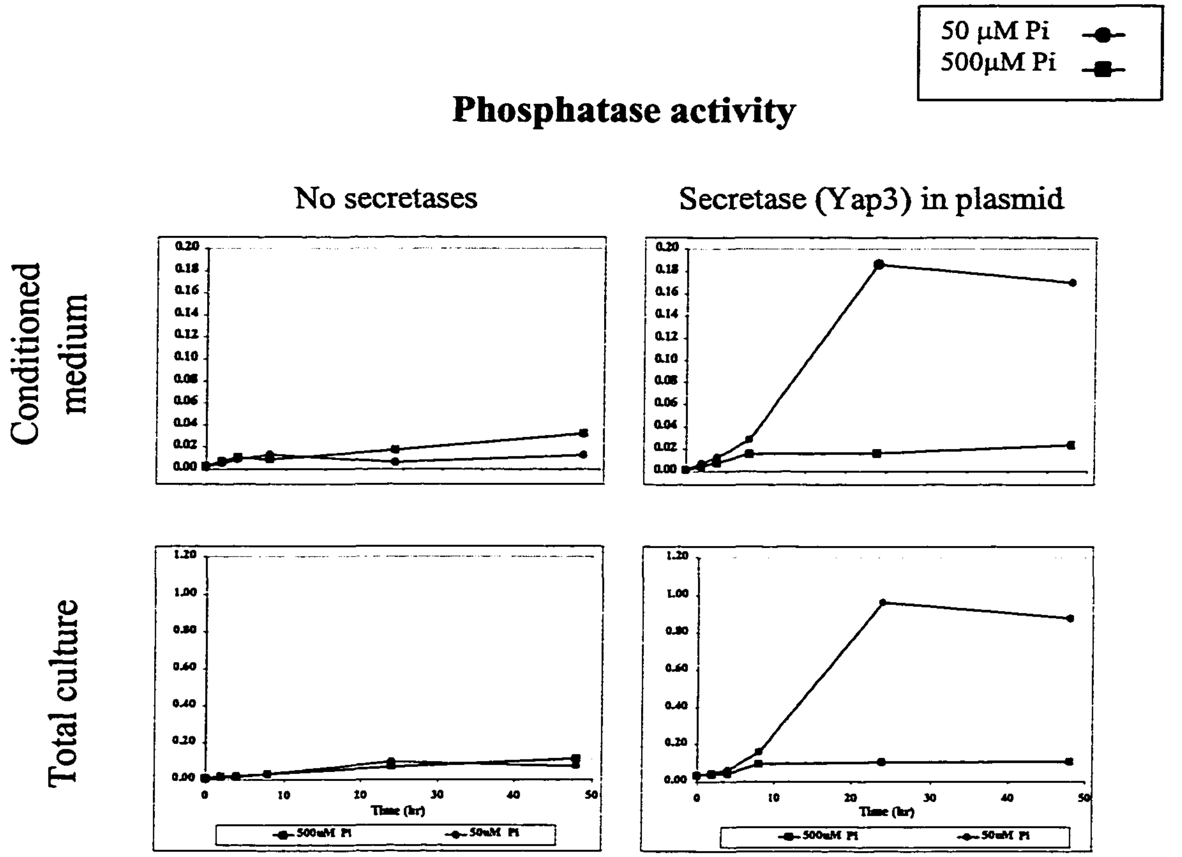


Figure 32: Secretase activity regained in secretase-deficient strain after introducing yeast secretase.

Yeast strain carrying the *PHO5*-C106sw reporter inserted in the chromosome was transfected with a plasmid control, p413Gal1 (left panels), or a plasmid containing *YAP3*, pDE156 (right panels). Resulting strains named yDE165 and yDE166, respectively. Phosphatase activity was measured after induction of PHO5-C106sw reporter molecule (growing in 50μM Pi containing medium) and of the expression vectors in conditioned media (upper panels) or total cultures (lower panels). Phosphatase activity is not induced when cultures are grown at high levels of extra cellular Pi (500μM), which agrees with the fact the *PHO5*-C106sw reporter is under the control of the inducible PHO5 promoter.



phosphate, Molecular Probes, Inc.), a clear fluorescent halo is formed around colonies that express the secretase (yDE166), but not in the ones that do not express secretases (yDE165)(Figure 33). This would allow the selection of colonies that contain plasmids coding for secretase candidates right on the plate. An interesting observation was to see that the growth of strains expressing the Pho5p-C106sw reporter and lacking the two major yeast α-secretases is arrested (Figure 34). A strain carrying the same gene deletions, but not the PHO5-C106sw insertion, does not show this phenomenon (Figure 34). This is perhaps due to blockage of the yeast secretory pathway by the accumulation of the reporter molecule inside the cell. All this evidence suggests that the yeast screening system works in the presence of a secretase, at least of a yeast secretase. Testing of the yeast system with some of the reported human secretase candidates will be described in the next section.

Characterization of APP secretase candidates in yeast and mammalian cells

To determine whether the yeast screening system would work with human APP secretases, some of the recently reported secretase candidates were cloned and their expression attempted in yeast and mammalian cells. As it was mentioned before, two β-secretases have been recently reported (BACE1 and BACE2). TACE (ADAM17) and ADAM10 seem to be involved in α-secretase cleavage, and PS1 seems to form part of the γ-secretase complex, or perhaps it itself is γ-secretase (Figure 35).

Figure 33: Colonies expressing secretase activity can be selected on plates

Strains yDE165 (contains vector alone) and yDE166 (contains YAP3 expression vector pDE156) were grown on solid medium containing a phosphate ester (as Pi source) and galactose (2%) to allow expression of inserted plasmid, and if a phosphatase fluorogenic substrate (DiFMUP – Molecular Probes) is overlaid on top of the colonies, a clear fluorescent halo can be seen around the colonies expressing the secretase, but not in the ones containing a plasmid control. This would facilitate the selection of colonies that have plasmids expressing secretase candidates right on the plate.

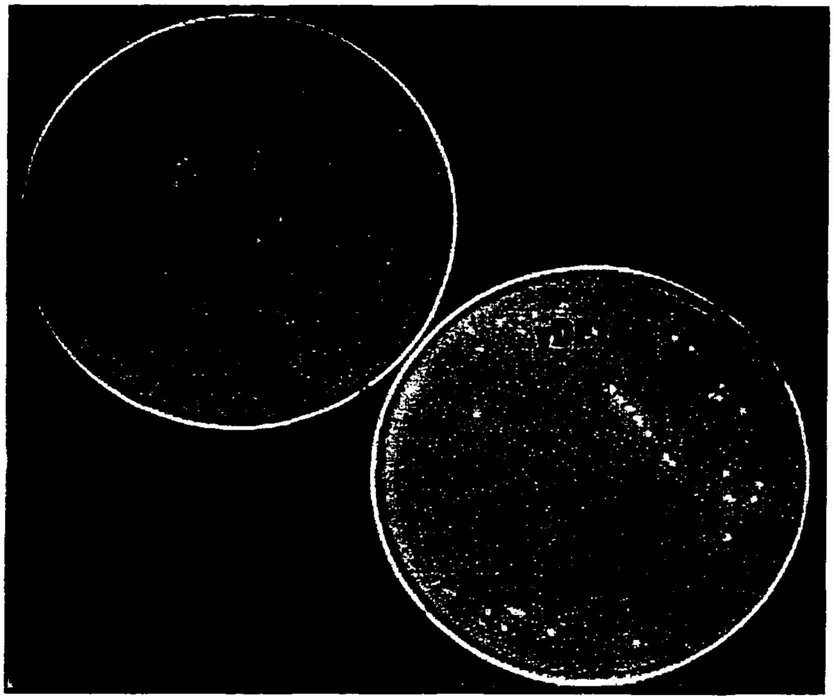


Figure 34: Secreted phosphatase activity and growth of yeast strains expressing Pho5p-C106sw

YDE250 (*YAP3 MKC7* pho5/pho3::PHO5-C106sw/loxP pho11::loxP pho12::loxP), YDE252 (*yap3*::loxP *mkc7::TRP1 pho5/pho3*::loxP *pho11*::loxP *pho12*::loxP), and YDE253 (*yap3*::loxP *mkc7::TRP1 pho5/pho3*::PHO5-C106sw/loxP pho11:loxP pho12::loxP) were grown in CSM containing 50μM Pi. Optical density and phosphatase activity of cultures was followed for more than 7 days. Growth was arrested in yeast strains carrying the PHO5-C106sw reporter and lacking the major yeast α-secretases, while a strain carrying the same deletions but not the PHO5-C106sw reporter grew as well as strains carrying the PHO5-C106sw reporter and had the secretases present (for clarity only three strains are included in the graph. Same experiment with other strains is shown in Figure 30).

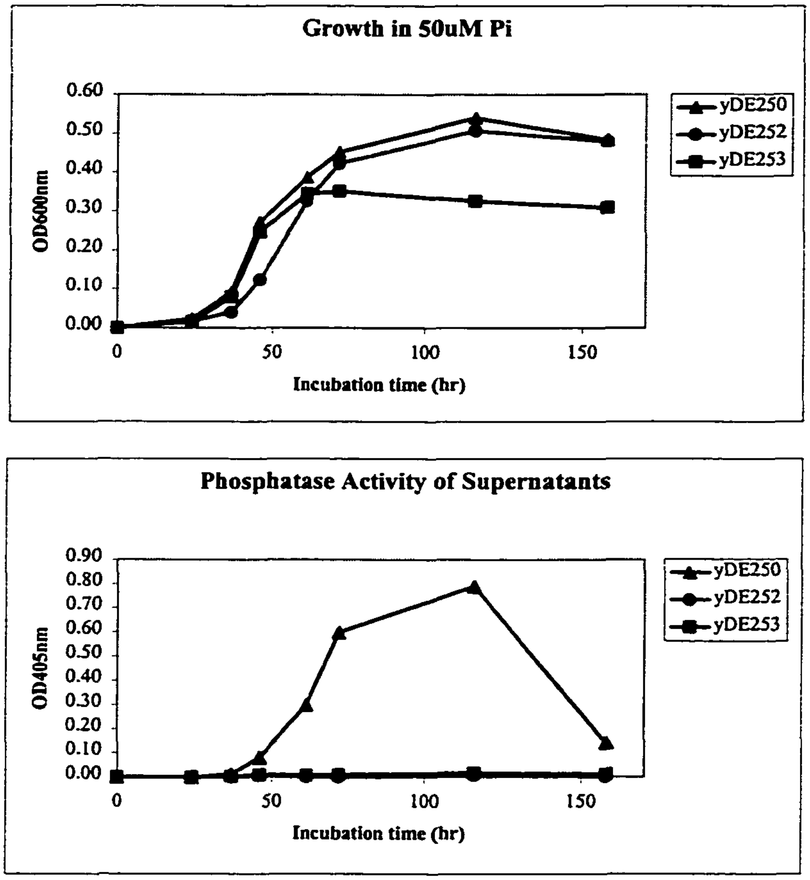
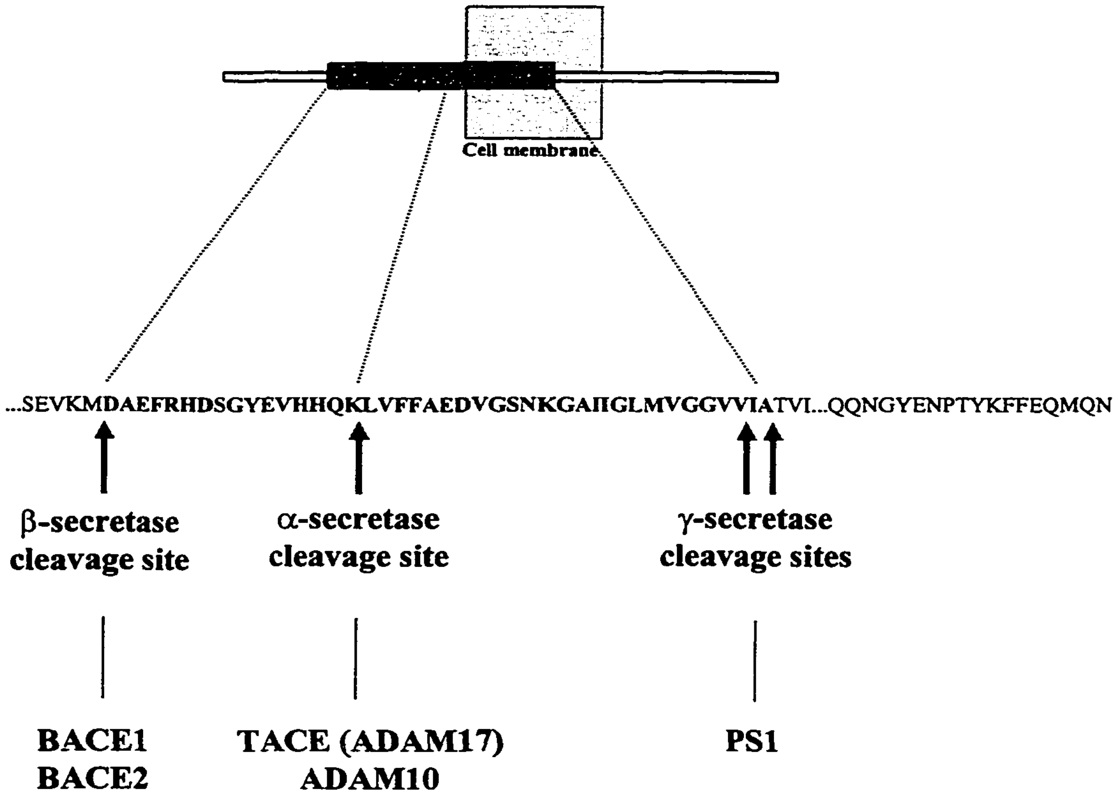


Figure 35: Human APP secretase candidates

Much progress has been made in the AD field the recent years. Two β-secretases have been identified (BACE1 and BACE2), TACE (ADAM17) and ADAM10 seem to be involved in α-secretase cleavage, and PS1 seems to be part of the γ-secretase complex or it itself is γ-secretase.



With the purpose of co-expressing these secretase candidates together with full length APP, and any natural substrate or co-factor known for such secretase candidate (such as TNFα in the case of TACE), mature APP695 with the α-mating factor signal and pro domain (α-factor-APP) was introduced into the yeast genome. α-factor-APP was engineered to be expressed under the Gal1 promoter (see Materials and Methods), and this cassette was introduced into the PHO5/PHO3 locus in chromosome II (Figure 17).

Cloning of BACE1 and BACE2

To test β-secretase activity of BACE1 and its homologue, BACE2, in the yeast and mammalian systems, BACE1 and BACE2 cDNA were PCR-amplified from human spleen cDNA (Marathon Ready - Clontech Laboratories, Inc) and inserted into pDE314 (Figure 19), a yeast expression vector that contains a myc-6xHis tag (see Materials and Methods for details). The resulting vectors were named pDE326 and pDE316, respectively. To make the mammalian expression vectors, each insert from the yeast expression vectors, containing the appropriate cDNA fused to the myc-6xHis tag, was inserted into a vector derived from pCR3.1-UNI (Invitrogen Corp.), resulting in the expression vectors pDE327 and pDE328, respectively. Cloned cDNAs include 10 bases before the starting ATG of the wild-type sequence. Sequences were designed to include myc-6xHis tags to facilitate detection of the proteins. BACE1 and BACE2 wild-type sequences were later obtained by replacing the myc-6xHis tags with a stop codon (see Materials and Methods).

Expression of BACE1 and BACE2 in yeast

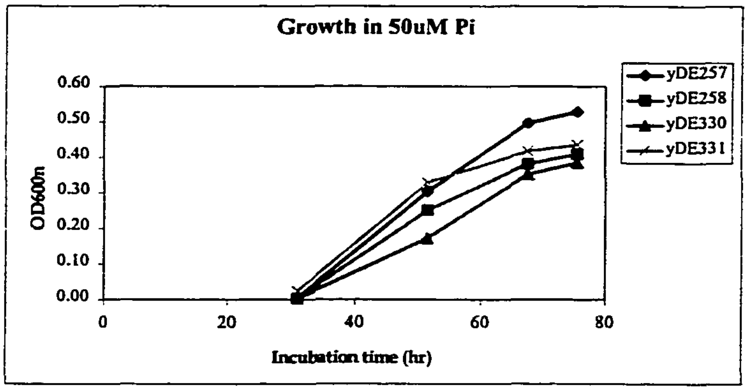
To test whether BACE1 and BACE2 could act as β-secretases in our yeast reporter system, plasmids pDE326 (BACE1-myc-6xHis) and pDE316 (BACE2-myc-6xHis) were introduced into yeast strain yDE256 (relevant genotype: *yap3 mkc7 pho5/pho3::PHO5*-C106sw *pho11* and *pho12* – see Materials and Methods). After induction of Pho5p-C106sw and the β-secretases, no secreted phosphatase activity was detected above control vector-transfected cells (Figure 36). Simultaneously, expression of BACE1 and BACE2 was analyzed by Western blotting, but neither BACE1 nor BACE2 could be detected using anti-myc antibody (Invitrogen) or antibodies against wild-type C-terminus R264 and R265. Similar results were obtained when wild type (without any tag) BACE1 or BACE2 were expressed in yDE256.

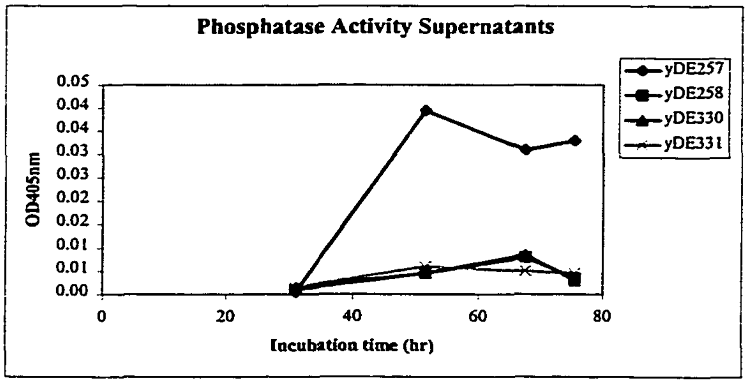
To determine whether BACE1 or BACE2 would be able to cleave full length APP695, even if protein levels were undetectable, pDE326 (BACE1-myc-6xHis) and pDE316 (BACE2-myc-6xHis) were transfected into yeast strain yDE276, which carries full length APP695 inserted into the PHO5/PHO3 locus and has the yap3/mkc7 deletion (yDE276 relevant genotype: *yap3 mkc7 pho5/pho3*::Gal1p-APP695/loxP *pho11*::loxP *pho12*::loxP). Resulting yeast strains were named yDE323 and yDE234, respectively. APP CTFs were analyzed by Western blotting with antibody R57 (Figure 37). If BACE1 or BACE2 act as β-secretases in yeast, a βCTF band of approximately 2Kda above the αCTF should be seen. As expected, mature APP695, α-pre-pro-APP695 (fusion unprocessed by Kex2 protease) and αCTF are clearly seen as reported before (Zhang et al., 1997). A fragment of about 12KDa can also be seen in all the lanes. This fragment may be the same as the one seen before, but not characterized, by Hines et al. (1994). This fragment reacted with antibodies 6E10 and 4G8 (Hines et al. 1994) which suggested that it might contain Aβ. If this is in fact a β-secretase product, it should be increased in the presence of active BACE1. This does not seem the case in Figure 37. On the other hand, this fragment seems to increase in the presence of PS1, which it is thought to be involved in the γ-secretase cleavage. This will be discussed with more detail in the PS1 section of this dissertation.

In an attempt to solve the expression problem of BACE1 and BACE2 in yeast, the 5' untranslated regions of BACE1 and BACE2 sequences were modified by replacing the human ribosomal binding sites from these cDNAs with the one from the yeast gene TDH3. This modification did not seem to produce a protein either. On the other hand, when the entire BACE1 and BACE2 pro-domains were replaced with the yeast α-mating factor signal and pro-domain sequences in plasmids pDE326 and pDE316 (making pDE358 and pDE359, respectively) low levels of expression of BACE1 and BACE2 could be detected (Figure 38, Figure 39). Since β-secretase cleaves APP Swedish mutant almost 100 times better than it cleaves wild-type APP (Vassar et al. 1999), β-secretase activity of these constructs is being tested by co-expressing them with PHO5-C106sw, full length APP695, and full length APP695sw in yeast.

Figure 36: Secretase activity of BACE1and BACE2 in yeast with PHO5-C106sw

Growth and phosphatase activity were determined in yeast strain yDE256 (relevant genotype: *yap3 mkc7 pho5/pho3*::PHO5-C106sw/loxP *pho11*:loxP *pho12*::loxP) transfected with pDE156 (YAP3), p413Gal1 (vector alone), pDE326 (BACE1-myc-6xHis), or pDE316 (BACE2-myc-6xHis). The strains were named yDE257, yDE258, yDE330, and yDE331, respectively.





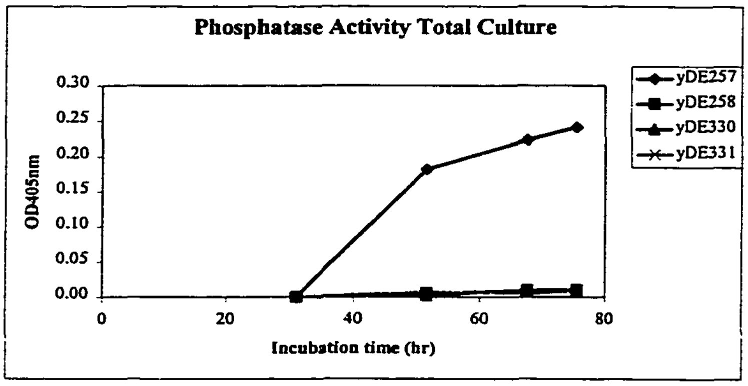


Figure 37: BACE1 and BACE2 cleavage of APP695 in yeast

Yeast strain yDE276 (carrying the double *yap3/mkc7* deletion and APP695 inserted in PHO5/PHO3 locus) was transfected with the following plasmids: pDE326 (BACE1-myc-6xHis), pDE316 (BACE2-myc-6xHis), or a plasmid control (pDE284, a PS1 containing plasmid). After 8hr induction with galactose, cell pellets were sonicated in 1% Triton X-100 buffer and subjected SDS-PAGE and Western blotting with antibody R57 to visualize APP and CTFs.

Lane 1: Untransfected yDE276
Lane 2: yDE276 transfected with pDE326 (BACE1-myc-6xHis)
Lane 3: yDE276 transfected with pDE316 (BACE2-myc-6xHis)
Lane 4: yDE276 transfected with pDE284 (PS1)

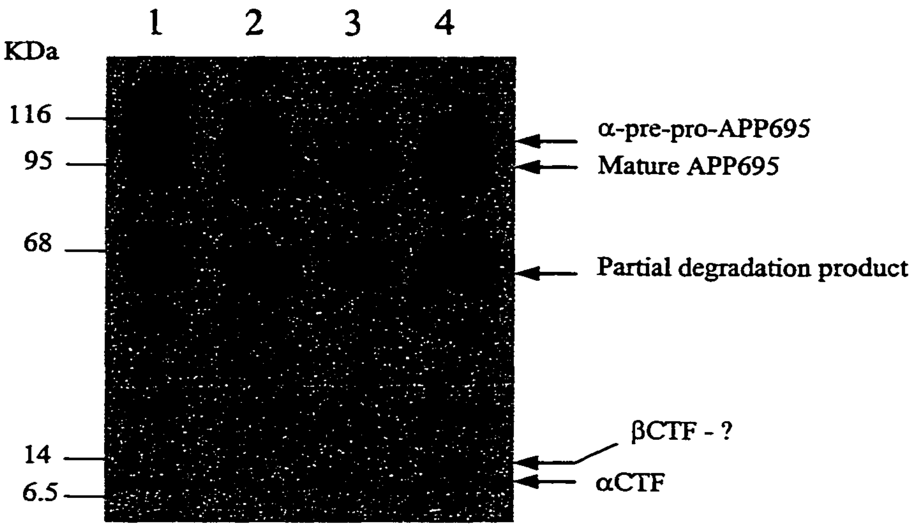


Figure 38: Expression of BACE1 and BACE2 in yeast

BACE1 and BACE2 cDNAs were PCR amplified from human spleen cDNA (Clontech). Constructs designed with (A) or without (B) a myc-6xHis tag at their C-terminus, but neither of these clones seems to express a protein. 5' end of BACE1 and BACE2 were modified to attempt solve expression problem. Initially the human ribosomal binding site was replaced with the corresponding yeast sequence from gene TDH3 (C), but this modification did not produce a protein either. Replacement of the entire BACE1 and BACE2 pro-domains with the signal and pro-domain sequence of the yeast α-mating factor (D) increased expression to detectable levels (Figure 39).

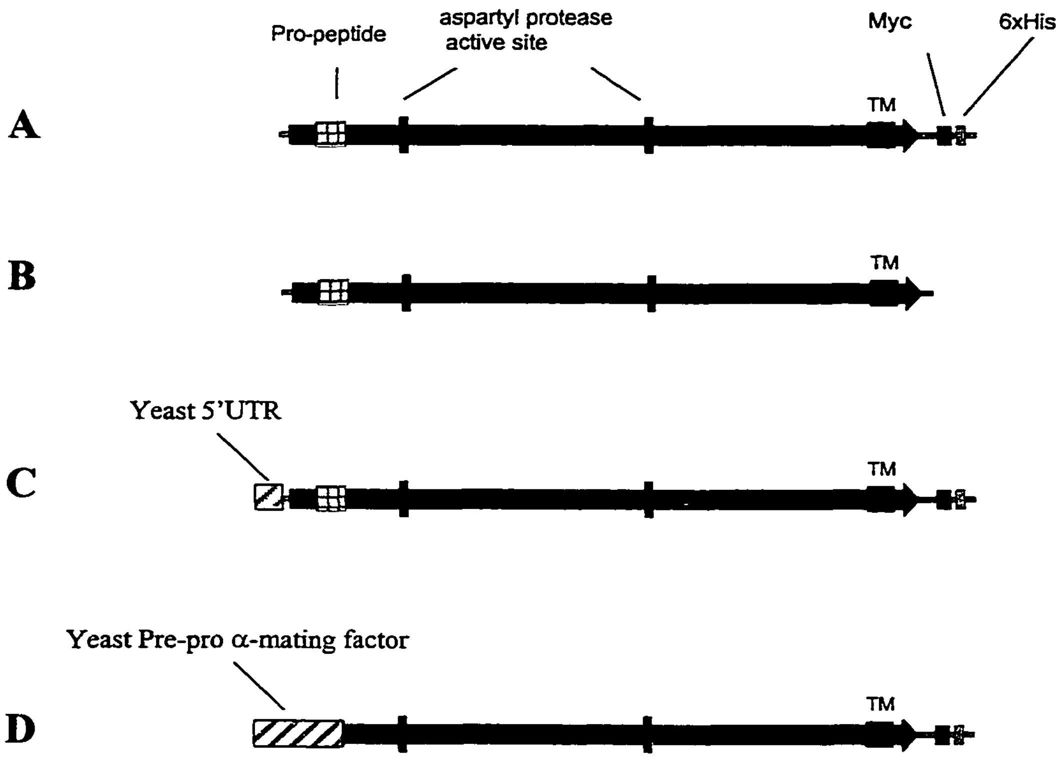
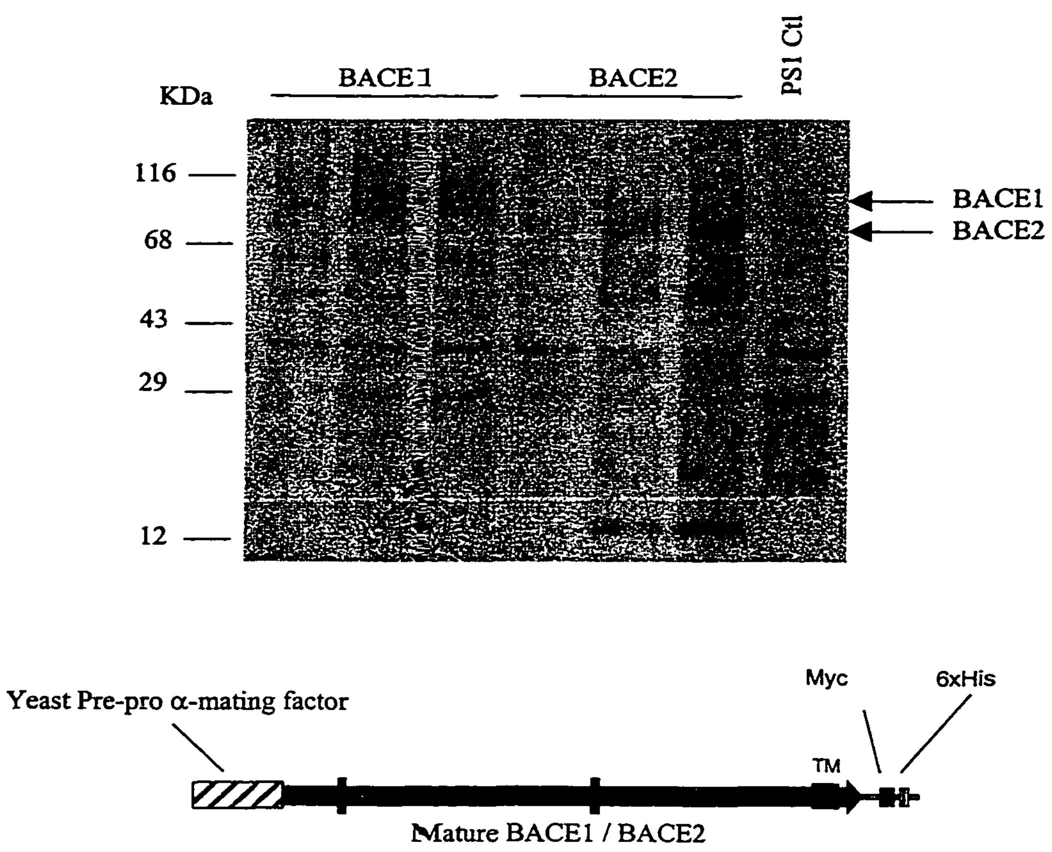


Figure 39: Mature BACE1 and BACE2 expression in yeast

Expression vectors pDE358 (α-factor pre-pro-BACE1) and pDE359 (α-factor pre-pro-BACE2) were transfected into yeast DS7. Cell pellets were harvested 8hr after induction of expression and sonicated in the presence of 1% Triton X-100 20mM Tris pH 8.0. Cell extracts were cleared and subjected to SDS-PAGE using 9% gels. Proteins were then transferred to nitrocellulose membrane. Blots were detected with anti-myc antibody. Replacement of BACE1 and BACE2 pro sequences with that of the signal and pro-α-mating factor sequences allows low level of expression of both secretases. Figure shows expression from 3 BACE1 and 3 BACE2 clones. A control lane included (DS7 expressing wild-type PS1).



Expression of BACE1 and BACE2 in mammalian cells

To test whether the expression problem in yeast was intrinsic to the BACE1 and BACE2 constructs that we made, both BACE1 and BACE2 cDNAs with and without the myc-6xHis tags (Figure 40) were transferred to a mammalian expression vector derived from pcDNA3.1 Uni (Figure 16 - Invitrogen Corp. Inc) to generate expression vectors pDE327 and pDE328 (BACE1 and BACE2 carrying myc-6xHis tags, respectively), and pDE340 and pDE341 (BACE1 and BACE2 without tags, respectively). When these mammalian expression vectors were transiently transfected into COS-7 and A204 cells, they were easily detectable either by western blots (Figure 41) or immunocytochemistry (Figure 42). Functional assays confirmed that we had active proteins in mammalian cells, as will be detailed later.

BACE1 and BACE2-myc-6xHis were transiently co-transfected in COS-7. When visualized by immunocytochemistry and confocal microscopy they seem to co-localize in Golgi-like structures (Figure 43). COS-7 cells were also transfected with either BACE1 or BACE2, both myc-6xHis tagged, co-immunostained with antibodies against myc tag and different organelle markers and visualized by confocal microscopy. Results suggest that BACE1 localizes preferentially to the Golgi complex and early endosomes, while BACE2 localizes preferentially to Golgi and the endoplasmic reticulum (Figure 44).

To determine the effects of BACE1 and BACE2 on APP processing, A204 and COS-7 cells were transiently transfected with BACE1 expression vectors pDE327 and pDE340, and BACE2 expression vectors pDE328 and pDE41 (constructs with and without myc-

Figure 40: Cloning of BACE1 and BACE2 in mammalian vectors

Human BACE1 and BACE2 cDNAs were cloned from spleen cDNA (Marathon ready – Clontech) with *(A)* or without *(B)* a myc-6xHis tag. BACE1 and BACE truncated before their putative transmembrane domains *(C)* with myc-6xHis tag were also cloned (see Materials and Methods). TM= putative transmembrane domain.

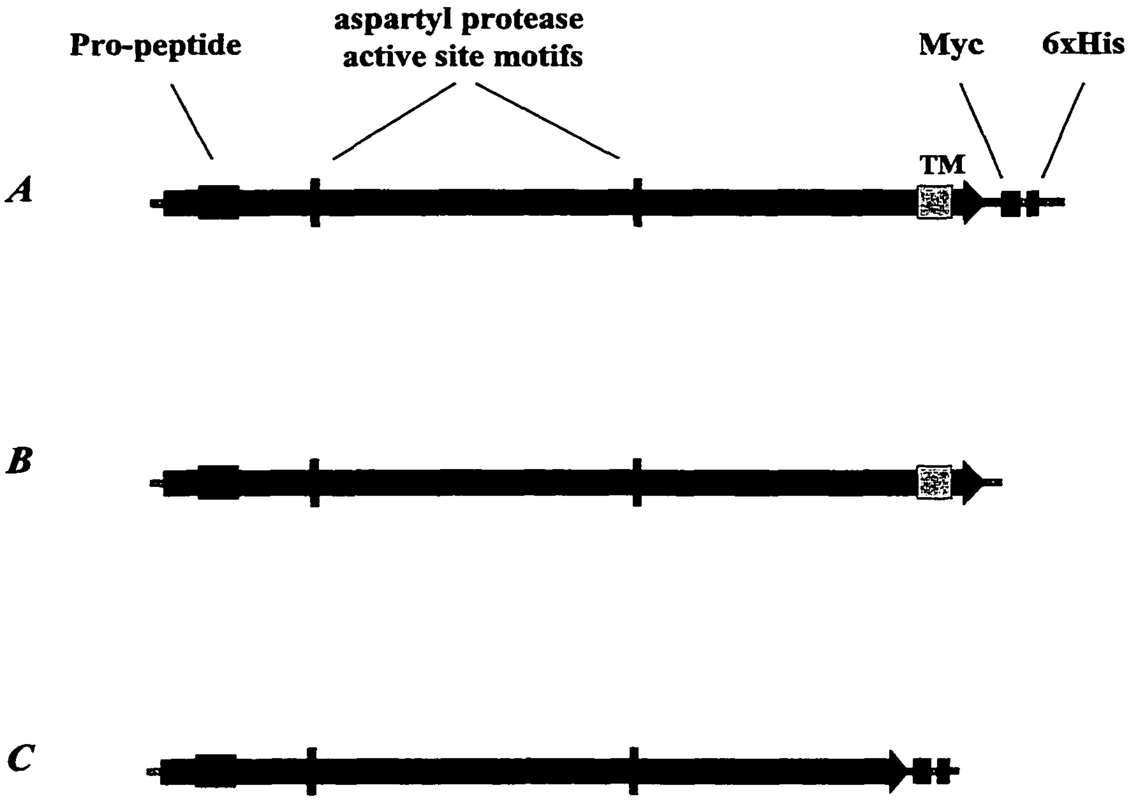


Figure 41: Expression of BACE1 and BACE2 in transiently transfected mammalian cells

A204 and COS-7 cells were transiently transfected with mammalian expression vectors carrying BACE1-myc-6xHis or BACE2-myc-6xHis. Figure shows Western blots from Triton-X100 cell extracts immunodetected with either anti-myc or antibodies directed towards the wild-type C-terminal regions of the proteins (R264 for BACE1 and R265 for BACE2). Endogenous expression levels of BACE1 and BACE2 are hardly seen.

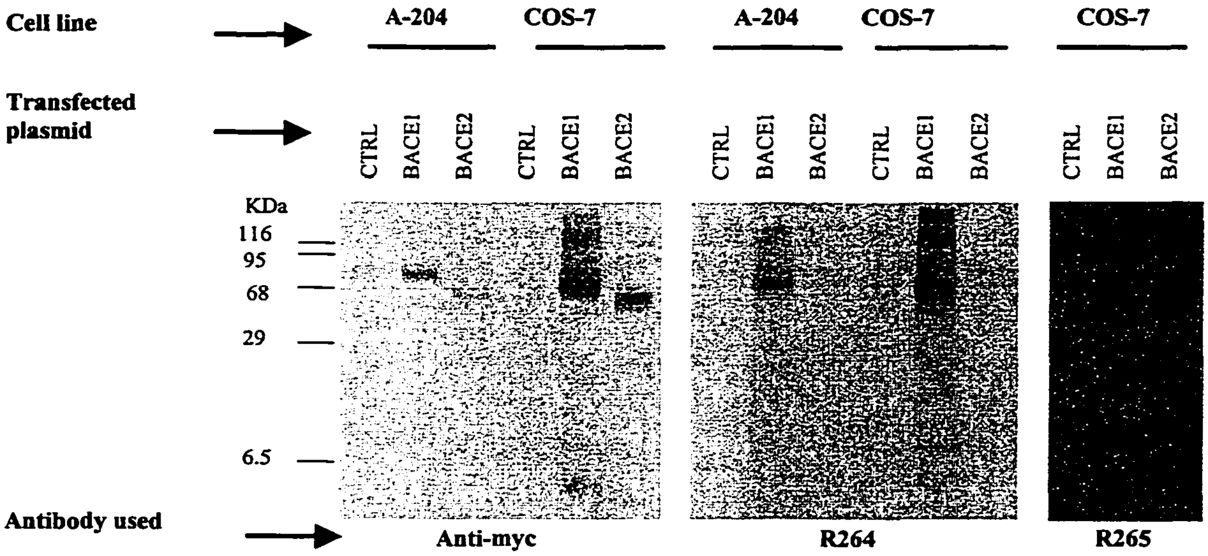
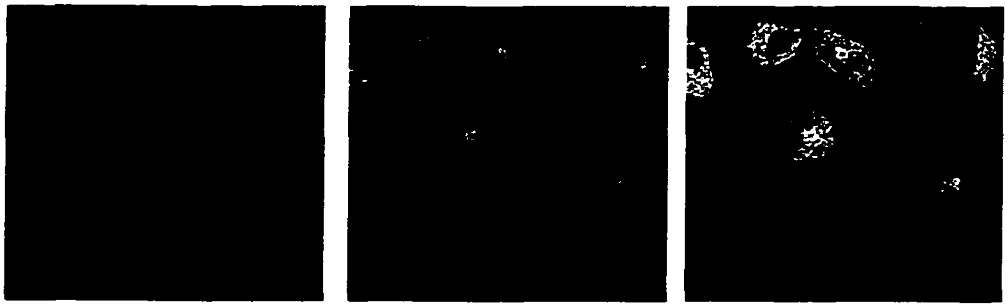


Figure 42: Images of BACE1-myc-6xHis and BACE2-myc-6xHis transiently transfected COS7 cells

COS-7 cells were transiently transfected with pDE327 (BACE1-myc-6xHis) or pDE328 (BACE2-myc-6xHis) and examined under confocal microscope after immunocytochemistry. R264 and anti-myc antibody were used to detect BACE1-myc-6xHis. Both antibodies seem to recognize the same protein, as seen when one overlays the images obtained for each of the antibodies independently. Anti-myc antibody was used to detect BACE2-myc-6xHis.

BACE1-myc-6xHis transfected



R264 **Anti-myc** **overlay**

BACE2-myc-6xHis transfected

Anti-myc

Figure 43: BACE1 and BACE2-myc-6xHis co-expression and localization in COS-7

BACE1 and BACE2-myc-6xHis were transiently co-transfected in COS7 cells and their intracellular localization visualized by immunocytochemistry and confocal microscopy. BACE1 was detected with immunopurified antibody R265, BACE2 was detected with anti-myc antibody (Invitrogen). Images from serial confocal planes (1μm apart) were captured. Secondary antibodies used: Alexa 448 (green) anti-rabbit IgG and Alexa468 (red) anti-mouse IgG. BACE1 (green) and BACE2 (red) seem to co-localize at Golgi-like structures. Co-localization with different organelle markers supports this observation (Figure 44).

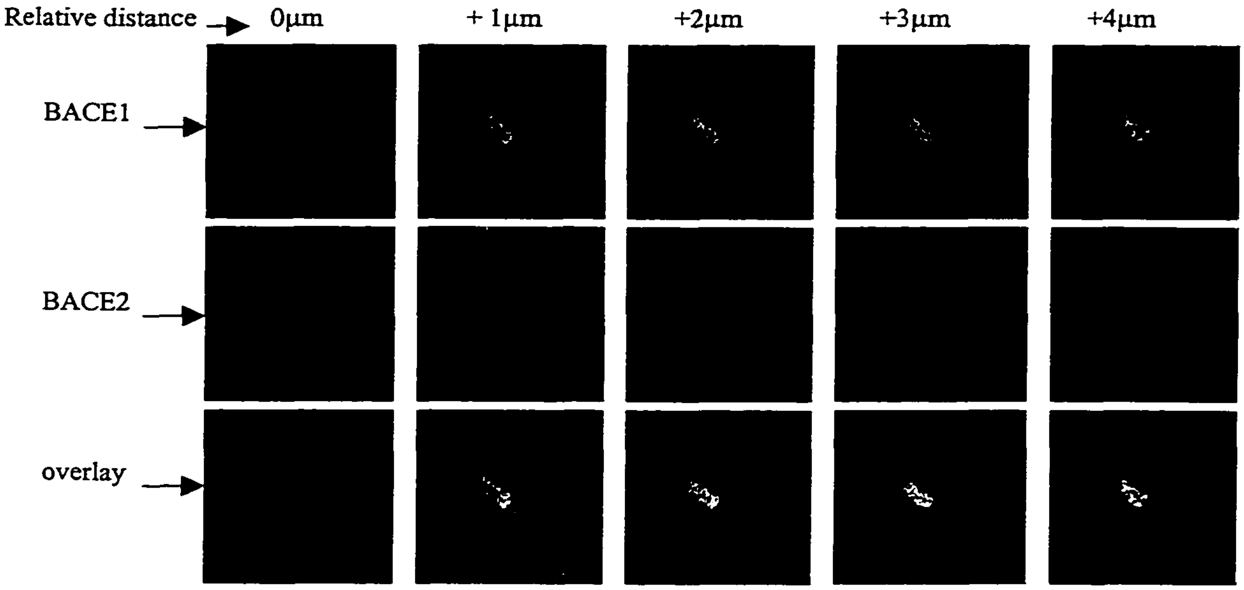
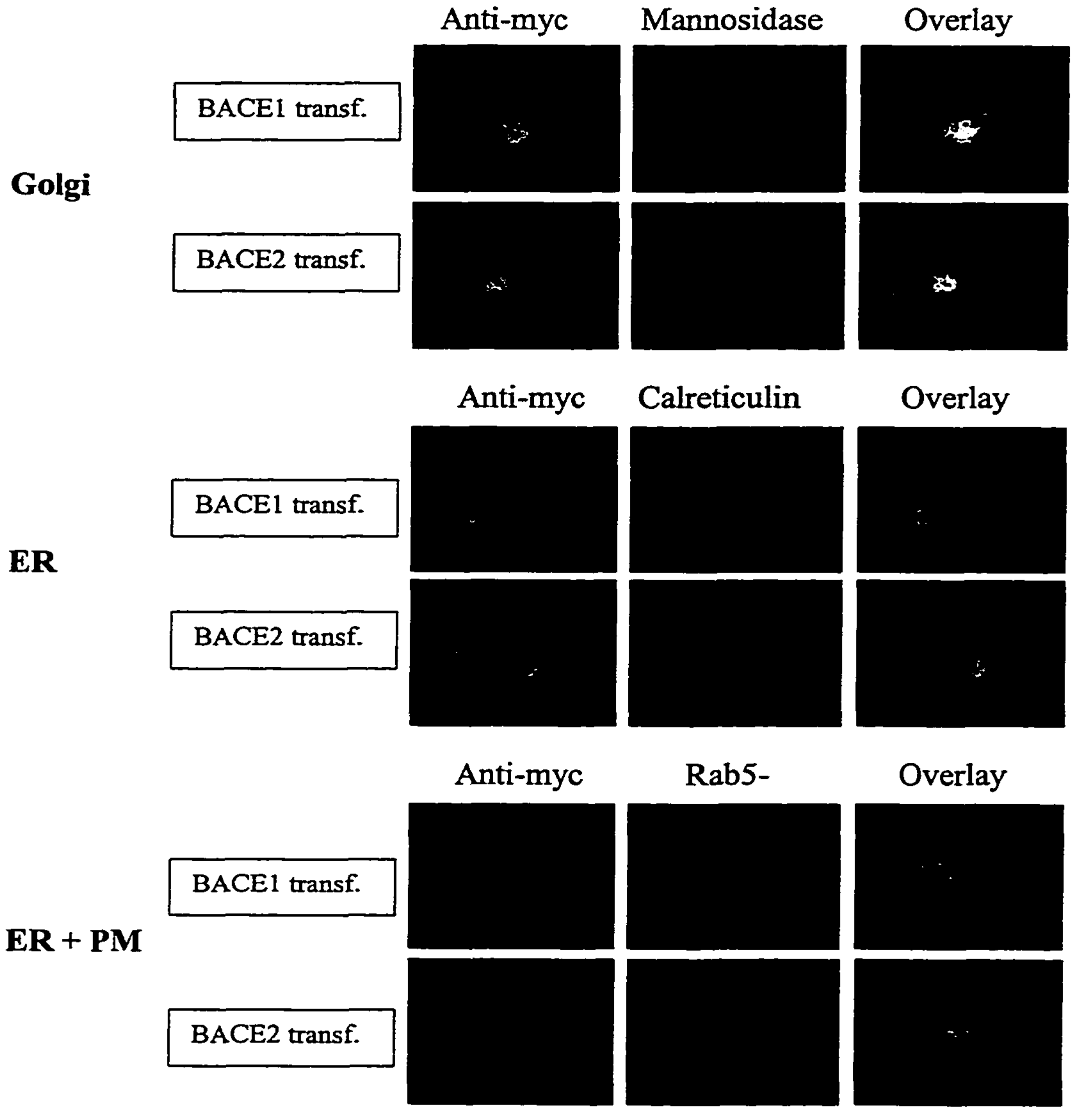


Figure 44: BACE2 localizes preferentially to ER

COS-7 cells were transiently transfected with either pDE327 (BACE1-myc-6xHis) or pDE328 (BACE2-myc-6xHis) and their localization was examined by co-immunostaining with antibodies against different organelle markers. A Rab5 fused to the green fluorescent protein (RAB5-GFP) was also co-transfected with either pDE327 or pDE328. BACE1 and BACE2 were detected with anti-myc antibody. Images were captured for BACE1 or BACE2 and organelle markers independently. Captured images from organelles in which BACE1 and BACE2 were localized are shown here.



6xHis tag, respectively). COS-7 cells were transiently transfected with pDE340 (wild-type BACE1), pDE341 (wild-type BACE2), or pDE288 (a plasmid control containing GFP). APP levels were determined by Western blotting of conditioned medium from these cells. Total secreted APP was detected with antibody R71. α-secretase cleaved APP (α-sAPP) was detected with antibody 6E10. Total sAPP was increased in both BACE1 and BACE2 but not control transfected cells (Figure 45 panel A). When relative amounts of α-sAPP and β-sAPP were determined, there was an apparent increase in β-sAPP in cells transfected with BACE2 (Figure 45 panel B). These preliminary experiments suggest that both BACE1 and BACE2 act as β-secretases (Figure 45). That BACE2 apparently yields a higher amount of β-sAPP is probably due to degradation of the epitope for the 6E10 antibody. This will give a bigger ratio when total sAPP/α-sAPP is calculated (Figure 45 panel B), a fact which will be clearer in the next paragraph. More experiments need to be done to determine the statistical significance of these observations.

When Aβ1-40 levels were determined in the same transfected cells, it was found that Aβ1-40 was greatly reduced in the BACE2 but not in BACE1 or control transfected cells (Figure 46A). To determine whether BACE2 transfected cells were degrading or re-internalizing Aβ, 115fmol/ml of synthetic $A\beta_{1\text{-}40}$ was added to cultures of cells transiently transfected with control, BACE1 or BACE2 expression vectors. $A\beta_{1\text{-}40}$ levels were then measured after 24 hours in conditioned media or cell pellets (Figure 46B).

Figure 45: Both BACE1 and BACE2 increase total secreted APP in transiently transfected COS7 cells

COS-7 cells were transiently transfected with pDE340 (wild-type BACE1), pDE341 (wild-type BACE2), or pDE288 (a plasmid control containing GFP). APP levels were determined by Western blotting of conditioned medium from these cells. Total secreted APP was detected with antibody R71. α-secretase cleaved APP (α-sAPP) was detected with antibody 6E10. Panel **A** represents the total amount of secreted APP in 24hr/10^6 cell. Panel **B** is a measure of secreted APP cleaved at the β-secretase site (β-sAPP), and it is expressed as the ratio of total sAPP / α-sAPP. **C** is an schematic representation of sAPP and location of epitopes of the antibodies used.

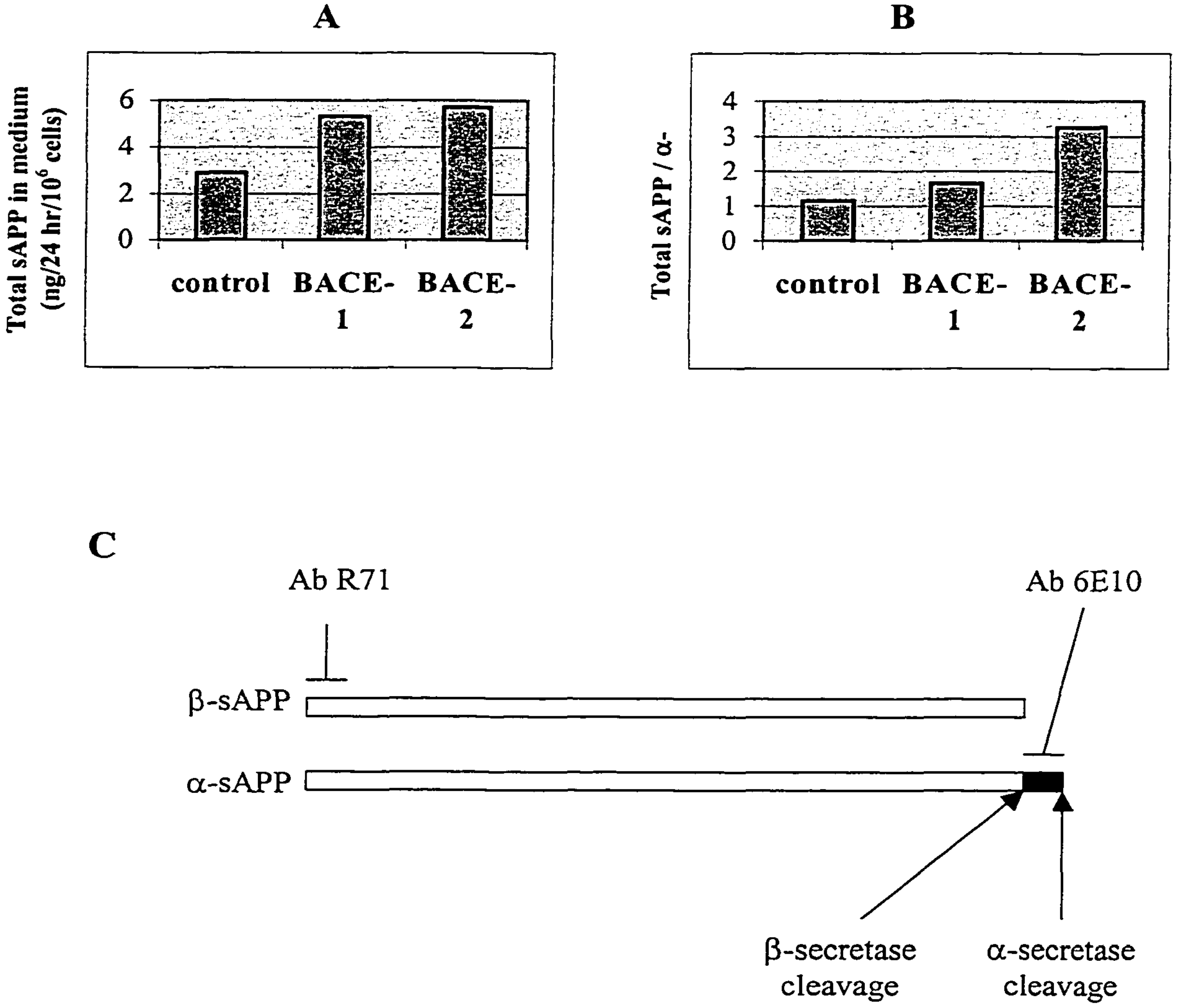
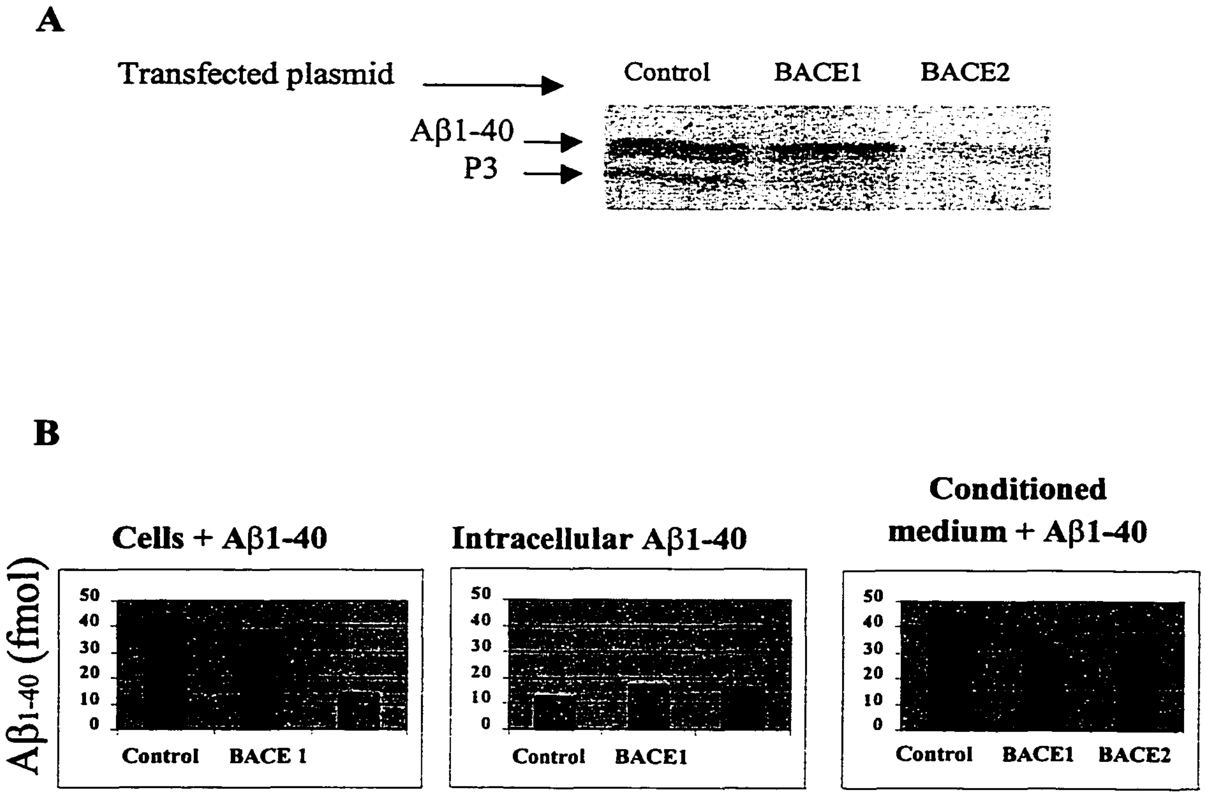


Figure 46: Aβ levels in BACE1 and BACE2 transiently transfected COS-7 cells.

Aβ levels were determined by Western blots of conditioned media from pDE340 (BACE1), pDE341 (BACE2) or pDE288 (plasmid control containing GFP) transiently transfected COS-7 cells. Aβ1-40 was detected with antibody R162. **A** conditioned media from transfected cell harvested after 24 hr post-transfection. **B** Aβ1-40 levels were measured 24 hr after adding 115fmol/mL of medium to cultures of transfected cells.



Remaining Aβ1-40 levels are greatly reduced in the conditioned medium of the cells transfected with the BACE2 plasmid (Figure 46B left panel). On the other hand, intracellular Aβ1-40 levels remained fairly similar among the samples (Figure 46B middle panel). This suggests that BACE2 transfected cells are able to degrade Aβ.

To determine if the Aβ degrading activity was cell-associated or was present in the conditioned medium of the BACE2 transfected cells, the same amount of Aβ1-40 added to the cultures above (115fmol/ml of synthetic Aβ1-40) was added to 24hr-conditioned media of cells transiently transfected with control, BACE1 or BACE2 expression vectors, and remaining Aβ1-40 levels were measured after 24 hours of incubation at 37°C (Figure 46B right panel). These preliminary results suggest that BACE2 cleaves at the β-secretase site and in addition it degrades Aβ, and that this degrading activity seems to be cell associated (Figure 46B). We have not identified the cleavage products, but Farzan et al. (2000) show that BACE2 is able to cleave a synthetic peptide spanning the β-secretase site between Met-1 and Asp1 of the Aβ sequence. Additionally, BACE2 cleaved another synthetic peptide, that spans residues 1-28 of the Aβ sequence, after Phe19 and Phe20 (Farzan et al. 2000). These results corroborate our findings. We think that in addition to the reported cleavage sites within the Aβ sequence, BACE2 is probably cleaving between the β- and α-secretase cleavage sites. This would explain the apparent loss of the 6E10 epitope when trying to determine secreted APP using antibody 6E10 (discussed above, Figure 45). BACE2 may play an important role in degrading Aβ *in vivo*, in tissues were it is expressed.

Presenilin 1 (PS1)

PS1 was expressed in yeast to determine whether it had any effect on APP processing, more specifically, if it had any secretase activity. Yeast expression vector pDE167 (wild-type PS1) was transfected into yeast strains DS7 and JSC310 (strain lacking major vacuolar proteases, see Materials and Methods). Additionally, pDE277 (Polish mutant PS1 P117L) was transfected in yeast strain DS7. Both PS1 wild type and the Polish mutant were successfully expressed in yeast (Figure 47). N- and C-terminal fragments (NTF and CTF, respectively) are clearly observed in DS7 but not too clearly in JSC310. Vacuolar proteases probably contribute to PS1 cleavage, as one compares the cleavage patterns in transfected DS7 and JSC310. The cleaved fragments have not been characterized yet, but the size of some fragments resemble those of PS1 CTFs seen in mammalian cells. Nevertheless, most of PS1 remains uncleaved, and little N- and C-terminal fragments are seen when vacuolar proteases are not present (Figure 47).

To determine whether PS1 exhibited any γ-secretase activity in yeast, it was co-transfected in *S. cerevisiae* DS7 with either full length APP695 (pDE195) or with C99 (APP C-terminal fragment after β-secretase cleavage – pDE264). Neither APP nor C99 processing seemed to be affected by PS1 expression (Figure 48). If PS1 is acting as a γ-secretase, a decrease in the amount of its substrates, C83 or C99, is expected. On the contrary, there is an apparent increase in the expression levels of APP and C99 when either PS1 wild type or PS1 Polish mutant are present. To determine if Aβ was being made, even though there was no an apparent change in APP or C99 processing pattern, conditioned media and cell extracts of cells co-expressing C99 and PS1 wild type or the Polish mutant were analyzed (Figure 49). No Aβ1-40 nor Aβ1-42 (not shown) was detected in the conditioned media or the cell extracts of yeast strains DS7 or yDE77 (DS7 carrying *yap3 mkc7* double deletion) (Figure 49 panels **C** and **D**). Processing of C99 by the yeast α-secretases (*YAP3* and *MKC7*) suggests that C99 is being correctly inserted into yeast membranes (Figure 49 panels **B**). Since there is no independent functional assay for PS1 activity, we cannot discard the possibility that indeed PS1 is a γ-secretase. PS1 may need other co-factors to be active, or it may not fold into its active conformation in yeast. These are issues that remain to be answered.

Cloning of the Tumor necrosis factor α(TNFα)-converting enzyme (TACE)

The tumor necrosis factor α converting enzyme (TACE) is the secretase that cleaves TNFα-precursor to release TNFα to the extracellular space. It was originally cloned by Black et al. (1997) and Moss et al. (1997), working independently, and it has been suggested to participate in the regulated (PKC activated) α-secretase cleavage of APP (Buxbaum et al., 1998).

To determine the role of TACE in α-secretase activity, human TACE was cloned in our laboratory from human spleen cDNA into the pCR3.1 vector (Invitrogen). TACE was subcloned into the yeast expression vector p413Gal1 to make pDE157. To facilitate detection of the protein, codons for the 3F4 PrP epitope (Bolton et al. 1991) were fused at the 3'end of the coding region to give a fusion protein. Later an antibody raised against

Figure 47: PS1 expression in yeast and mammalian cells.

Yeast expression vector pDE167 (wild-type PS1) was transfected into yeast strains DS7 and JSC310. Additionally, pDE277 (Polish mutant PS1 P117L) was transfected in yeast strain DS7. Upon induction of expression cell pellets were sonicated in the presence of 1% SDS buffer (see Materials and Methods) and subjected to SDS-PAGE. 7.5 μg of protein were loaded on 9% gels. Blots were detected with 2 N-terminus antibodies (R222 and X81) and 2 C-terminus antibodies (PS-1L and PS1-331). Extracts from rat primary neuronal culture (kindly provided by Dr. Jane Johnston) and COS-7 cells are included as controls.

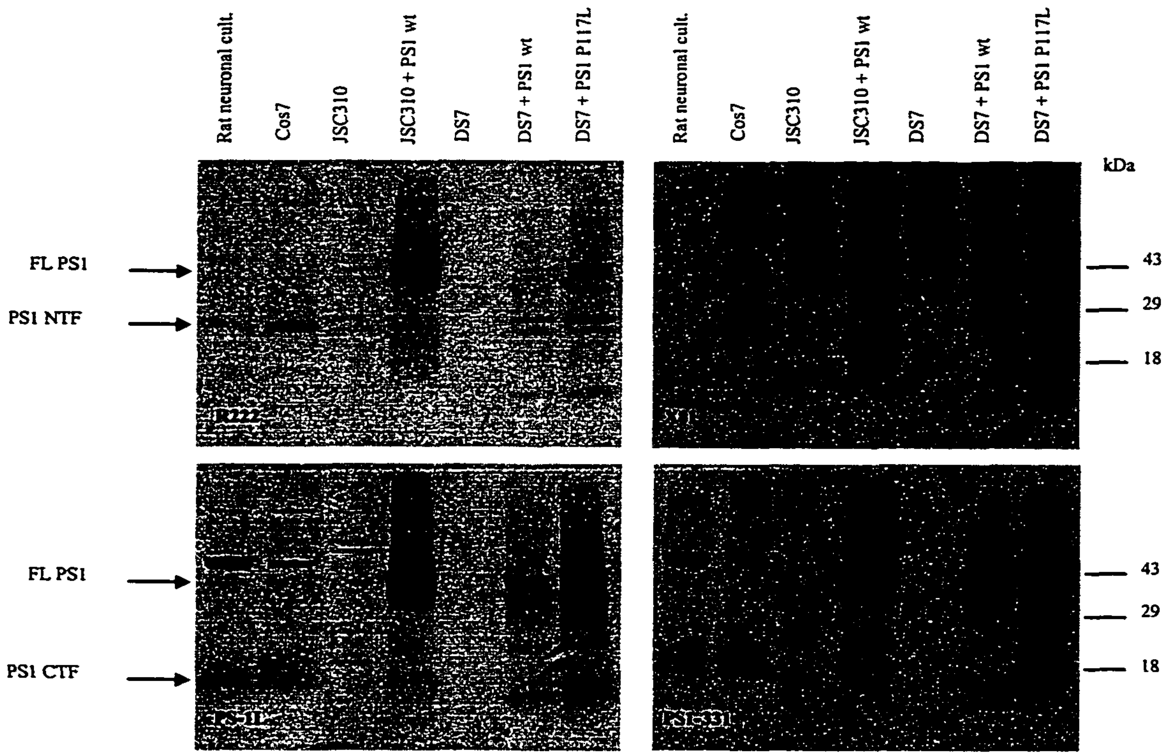


Figure 48: Cleavage pattern of APP or C99 in yeast does not seem to be affected by PS1 expression.

Mature APP695 and the last 99 C-terminal residues of APP (C99) were co-expressed with either wild type PS1 (pDE167) or PS1 P117L Polish mutant (pDE277). Cell pellets were extracted with 1% SDS buffer (see Materials and Methods) and subjected to SDS-PAGE and Western blotting. APP and C99 fragments were detected with antibody R57. Untransfected mammalian (COS-7) and yeast (DS7) cells are included as controls (panel A).

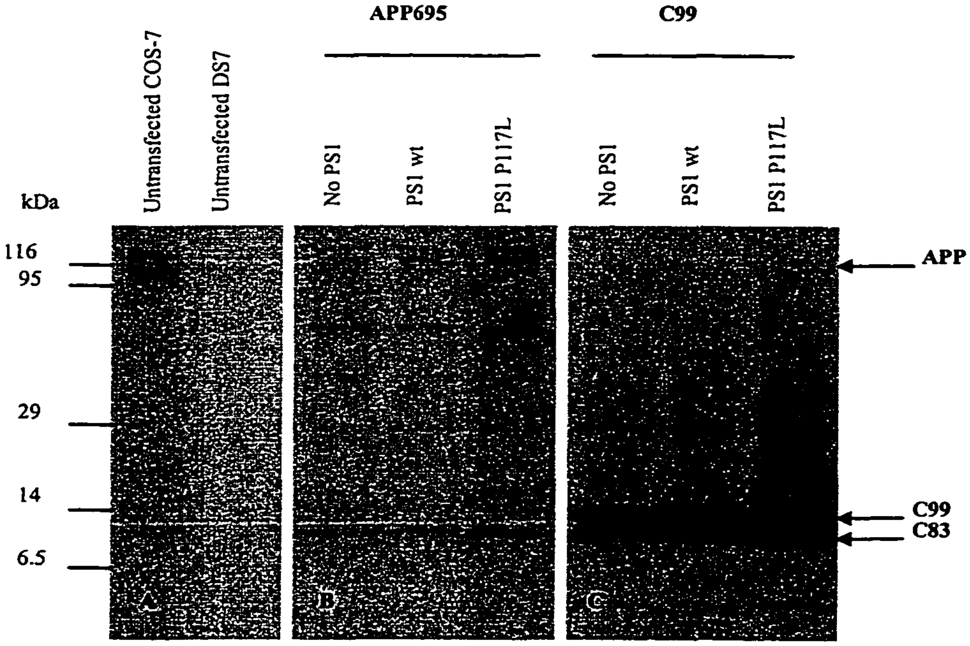
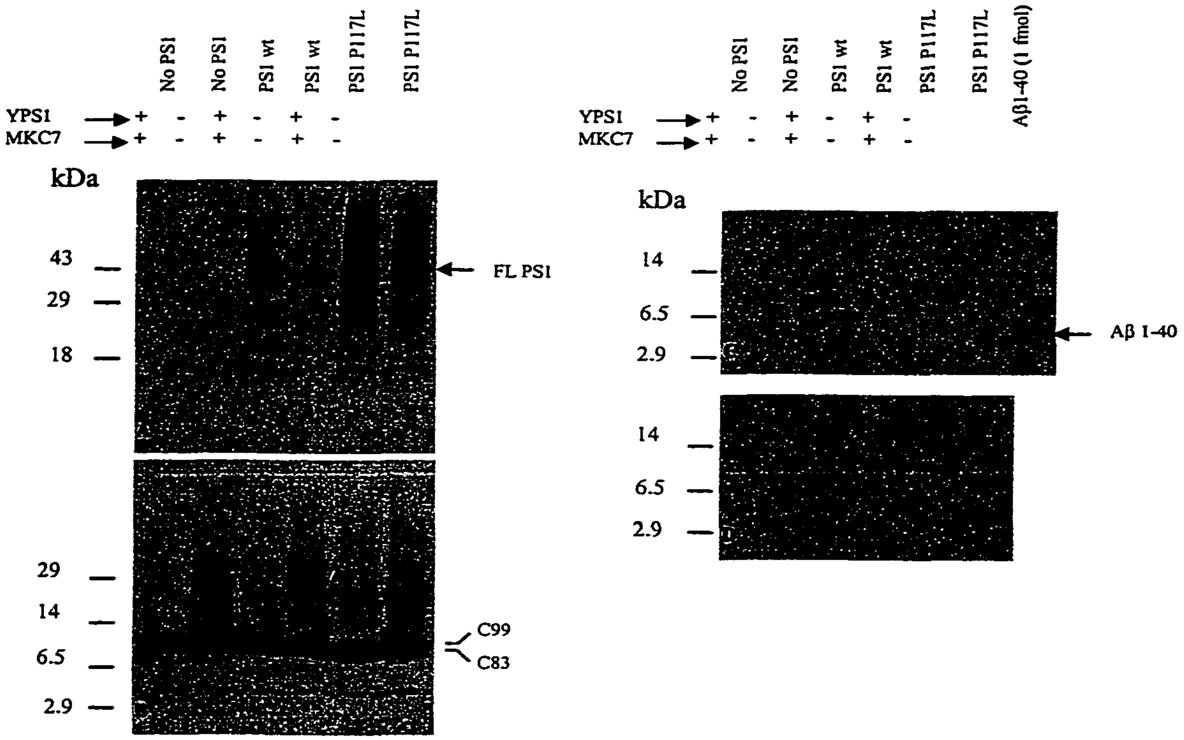


Figure 49: No Aβ detected in yeast co-expressing PS1 and C99.

Yeast strains DS7 and yDE77 (DS7 carrying *yap3/mkc7* double deletion) were transfected with pDE264 (C99 expression vector) and subsequently transfected with either pDE167 (PS1 wild type) or pDE277 (PS1 P117L Polish mutant). Conditioned media and cell pellets were harvested 8 hr induction of expression. Cell pellets were sonicated in the presence of 1% SDS buffer (see Materials and Methods). Conditioned media were cleared by spinning at 100K x g for 10 min. Cell extracts and conditioned media (concentrated about 8x) were subjected to SDS-PAGE and Western blotting. **A**, cell extracts detected with R222 (anti-PS1 N-terminus antibody); **B**, cell extracts detected with R57 (anti-APP C-terminus antibody); conditioned media (**C**) and cell extracts (**D**) detected with R162 (anti Aβ1-40 antibody). 1fmol of synthetic Aβ1-40 is included in **C** as control.



the C-terminus of TACE was obtained from Santa Cruz Biotechnologies. pDE157 was co-transfected with plasmid pDE179 (TACE substrate, pro-TNFα) in yDE77 (strain carrying the *yap3 mkc7* double deletion) (Figure 50). We were unable to detect TACE in western blots using the two antibodies we had available. We were also unable to detect released TNFα (indication of TACE activity) in the conditioned medium of the same samples shown in Figure 50 using an ELISA test (Oncogene Research). At this point, the TACE cDNA clone was sent to be sequenced again. Sequencing results indicated that the TACE cDNA clone had a STOP codon before the transmembrane domain. This mutation made a soluble protein, undetectable to the antibodies we had available, and probably not able to cleave its membrane bound substrate.

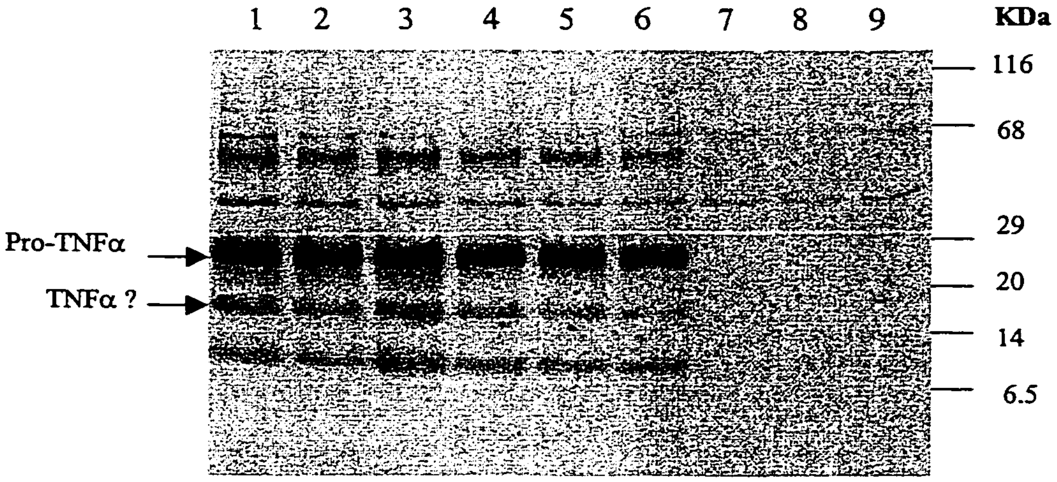
The STOP codon mutation was corrected by site-directed mutagenesis using Stratagene's QuikChange site-directed mutagenesis kit. DNA sequencing confirmed that STOP codon was corrected, but also that a second mutation appeared close to the first mutation. This time it was a one base deletion that shifted the reading frame. The protein produced by this plasmid was also a truncated form, lacking the transmembrane domain and the epitope for the two antibodies we had available.

TACE cDNA was PCR-amplified for a second time from spleen cDNA (Marathon Ready- Clontech). The PCR product was inserted directly into pcDNA3.1/CT-GFP-TOPO (Invitrogen). This mammalian expression vector allows to have a fusion protein

Figure 50: Expression of TACE and pro-TNFα in yeast

The yeast strain yDE77 (DS7 with the double *yap3 mkc7* deletion) was transfected with pDE179 (pro-TNFα) and/or pDE157 (human TACE). Conditioned media and cell pellets were harvested after 8 hr of induction of expression. Cell pellets were sonicated in 1% Triton X-100 buffer (see Materials and Methods) and subjected to SDS-PAGE and Western blotting. TNFα was detected with an antibody against the C-terminus of TNFα (Santa Cruz). TACE detection was attempted using the 3F4 monoclonal antibody (Bolton et al. 1991) and an antibody directed against the C-terminus of TACE (Santa Cruz Biotechnologies). The blot obtained with anti TACE did not show any protein besides background, and for this reason only the blot obtained with the TNFα antibody is shown here.

Lanes 1 - 2: yDE77 + pDE179 (pro-TNFα)
Lanes 3 - 6: yDE77 + pDE179 (pro-TNFα) + pDE157 (TACE)
Lanes 7 - 9: yDE77 + pDE157 (TACE)



with GFP (green fluorescent protein) at the C-terminus of TACE. Twelve clones that appeared correct by restriction analysis were transfected into COS-7 cells. Twenty four hours after transfection clones were examined for GFP fluorescence under the microscope. No fluorescence was visualized in any of the clones. Western blotting of cell pellets did not show TACE either. DNA of one of these clones revealed that it had the same STOP codon mutation than TACE cloned the first time. No more attempts to clone TACE have been done since then.

The pro and the catalytic domain of TACE have been expressed in yeast and this has proved to be active (Clarke et al., 1998). Although, the cloning procedure that the authors followed suggests that they may have also experienced cloning problems. Attempts to get a TACE clone from the group that cloned it have been unsuccessful.

Discussion

Yeast secretases

When human APP cDNA is introduced in the yeast *S. cerevisiae*, the protein is expressed, inserted into the yeast membranes and processed by yeast secretase(s) that have the same specificity as the human α-secretase (Hines et al., 1994; Zhang et al., 1994). The large N-terminus of APP after α-secretase cleavage (α-sAPP) can be detected in the conditioned medium of the transfected cells. The corresponding carboxyl terminal (αCTF) can also be detected inside the cell (Hines et al., 1994). Two yeast aspartyl proteases, Yps1 (formerly known as Yap3) and Yps2 (also known as Mkc7), seem to be responsible for most of the yeast α-secretase activity (Zhang et al., 1997; Komano et al., 1998) (Figure 6). Even though we were unable to detect any β- or γ-secretase activities in yeast (Hines et al., 1994; Zhang et al., 1997), Greenfield et al. (1999) reported the existence of Aβ-like containing peptides in a *S. cerevisiae* strain that lacks the Kex2 protease. This would suggest the existence of a β-secretase in yeast. Also, immunoprecipitation studies by Le Brocque et al. (1998) suggest that another yeast species, *Pichia pastoris*, possesses α-, β-, and γ-secretase activities. Although, α-secretase seems to be the predominant activity (Le Brocque et al., 1998). The apparent discrepancies in these studies may be due to the fact that different yeast strains or species (*S. cerevisiae* versus *P. pastoris*) were used. The lack of β- and γ-secretase activities in our yeast strain is advantageous in the sense that human secretase candidates can be screened by phenotypic complementation and, having depleted most of the α-secretase activity in our yeast strain, it allows us to screen for α-secretase candidates as well.

In this study, the role on α-secretase activity of two putative *S. cerevisiae* aspartyl proteases Yps3 (*YLR121c*) and Yps6 (*YIR039c*) that are highly homologous to the major yeast α-secretase, Yps1 (Table 3), were examined and found not to contribute significantly to the yeast α-secretase activity. Deletions of the genes encoding these two aspartyl proteases in a yeast strain carrying the yps1/yps2 double deletion was found not to reduce the residual α-secretase activity of this strain (Figure 21). Yps3 has been expressed under the control of the Gal1 promoter as a carboxy terminally truncated non-membrane anchored form (Olsen et al., 1999), and shown to be able to process APP at the α-secretase site to some extent. This suggests that over-expression of this protein may contribute to α-secretase activity in yeast, but endogenous levels do not significantly affect APP processing.

Yeast gene deletions

The techniques to disrupt genes in yeast generally involve the use of genetic markers, such as *URA3*, *TRP1*, *LEU2*, or *HIS3* that substitute a segment of the gene to be deleted. The selectable marker has to be flanked by 5' and 3' sequences homologous to the gene to be disrupted. After transformation with this construct, disruption of the gene of interest occurs by homologous recombination (Rothstein, 1991). Since the deletion of several genes in the same strain would result in the exhaustion of selectable markers, two techniques were considered to recycle these selectable markers after the disruption of the gene was achieved. Both methods take advantage of the relatively high frequency of recombination of homologous sequences in yeast, which is about 10^{-4} recombination events between direct repeat sequences (Alani et al., 1987). In the first method, reported by E. Alani et al. (1987), a deletion cassette is made by PCR-amplifying the cassette HisG-URA3-HisG (HisG is a *Salmonella* gene) from the plasmid pNKY51 (plasmid was obtained from Dr. David Kirkpatrick, Univ. of North Carolina) by using long PCR primers that include sequences homologous to the gene one wants to target. This method proved not to be efficient in our hands. No recombinant mutants were identified when we tried to delete the PHO5 and PHO3 genes. This may be perhaps due to the practical limitations in the length of the primers to be used (at least 50bp long homologous to the target sequence) and the low yield of the deletion cassette (PCR product to amplify, HisG-URA3-HisG cassette, is about 3845bp). The CRE/loxP system (Sauer, 1994) proved to be a more efficient way to target genes, perhaps because there is almost no limit in length of homologous sequence one can insert in the deletion cassette (the longer the homologous sequence the better the recombination). The presence of the CRE recombinase also increases the recombination events between the sequences flanking the marker gene, which facilitates marker recycling. For these reason the CRE/loxP system was the method of choice to make the deletions.

Yeast screening system

S. cerevisiae was used to make a screening system for the identification of human α-, β-, and γ-secretase(s) candidates from a human brain cDNA library. Since homologous and heterologous proteins are produced in yeast cytoplasm or directed to the secretory pathway (Moir and Davidow, 1991), and yeast is used routinely for the study of mammalian genes (Sherman, 1991), *S. cerevisiae* would be a good system to express proteins encoded by a human cDNA library. The choice of brain cDNA library is justified because the message for the secretases that act in the nervous system may be enriched in this tissue as compared to other tissues. APP fused to reporter proteins would facilitate mass screening of the library.

The yeast system consists of a yeast host strain with almost no secretase activity and a reporter molecule consisting of the major secreted yeast acid phosphatase, Pho5p, fused to the last 106 amino acids of the human APP Swedish mutant (Pho5p-APPsw). Additionally, all the genes that code for secreted phosphatases have been deleted in the host strain. Detection of any secreted phosphatase should come then from the cleavage of Pho5p-APPsw by secretase candidates. Pho5p-APPsw and a human brain cDNA library will be co-expressed in *S. cerevisiae* to screen for secretase candidates.

The yeast strain of choice to serve as the host cell for the screening system was a strain carrying the double yap3/mkc7 deletion. The deletion of the other yap3 homologues, ylr121c and yir039c, did not significantly reduce yeast α-secretase. Since the natural substrates for these aspartyl proteases are not known yet, a minimal disturbance of the host cell is desired.

Two forms of the green fluorescent protein (GFP) were also considered as reporter molecules to make the APP fusion proteins. GFP-APPsw fusion proteins could be detected in Western blots, but no fluorescence could be detected, above background levels, in the yeast cultures. This might be due to improper folding of the GFP part of the molecule, not allowing the fluorescent complex to form. However, when the cells expressing the GFP-APPsw fusion were examined under a fluorescence microscope, it was observed that only a few cells were fluorescent (Figure 24). Formation of the fluorescent complex may be favorable during some stages of the cell cycle, but this is just a speculation.

Other reporter genes considered to make the APP fusions were *SUC2* (a yeast gene that codes for invertase) and SEAP, a mutated gene coding for secreted human placental alkaline phosphatase (Berger et al., 1988). Detection of invertase is possible, but not as simple as the detection of phosphatases. The SEAP has been fused to APP before and expressed in mammalian cells (Pappolla et al. 1995). However, SEAP may not work in the yeast system because its optimum pH is around 10.0 (Chuang and Yang, 1990). It and would not be able to cleave phosphate ester substrates in yeast cultures (which we found may get as acidic as pH 2.0). On the other hand, Pho5p has a pH optimum between 3.0 and 4.5 (Barbaric et al. 1984). β-galactosidase was not considered for making a fusion protein because it is not secreted when expressed in yeast (Emr, 1983) and what we were looking for was a molecule able to be detected as a secreted product.

The last 105 amino acids at the carboxyl terminus of APP have been used before to make a fusion protein with SEAP and this fusion has been shown to be cleaved by the APP secretases (Pappolla et al., 1995). Since we wanted to introduce the Swedish mutation in our APP fusion protein, we thought that the inclusion of the 5 amino acids before the Swedish mutant residues (making a total of 106 amino acids of the APP C-term) would be also a good substrate for APP secretase candidates.

To minimize variability in expression levels of secretase candidates in the yeast screening system, yeast centromeric vectors were chosen instead of 2 µm-based vectors. Centromeric vectors have 1 or 2 copies inside the cell, while the number of 2-µm based vectors has a large variation (reviewed by Schneider and Guarente, 1991). Centromeric vectors are also much more stable than those based on the 2-µm circle origin; 97% of cells retained a plasmid with CEN3 after 20-30 generations (reviewed by Schneider and Guarente, 1991). Inserts in these vectors are under the control of the Gal1 promoter, and are constitutively expressed in the presence of galactose (but not glucose) in the growth medium.

The PHO5-C106sw reporter molecule was inserted into the yeast PHO5/PHO3 locus using the CRE/loxP system. This was done in order to have uniform expression levels of the reporter PHO5-C106sw and not to use up selectable markers, in case more than one plasmid needs to be co-expressed with PHO5-C106sw in the same cell. This fusion was shown to be behind the PHO5 promoter (Figure 32).

Mature APP695, with the α-mating factor pre-pro sequence in front, was inserted in some yeast strains behind the Gal1 promoter at the PHO5/PHO3 locus by the CRE/loxP system. This was done in order to minimize variability in expression levels and not to use selectable markers when APP needs to be co-expressed with other plasmids).

The yeast reporter system to screen for secretase candidates works when a yeast secretase is inserted in a plasmid. Phosphatase activity can be detected in the conditioned medium of cells expressing the PHO5-C106sw reporter and the yeast secretase yap3p (Figure 32, Figure 33). Selection can potentially be done directly from solid medium plates, by overlaying fluorogenic phosphatase substrates and/or by colony size. Cells expressing the PHO5-C106sw reporter and no secretases grow smaller than colonies that contain secretases. This difference in colony size can be better seen in growth curves of liquid cultures (Figure 34). This effect may be due to the re-internalization of the PHO5-C106sw molecules (APP portion contains re-internalization motif - Figure 12) and blockage of the secretory pathway, as has been reported when there is an intracellular accumulation of Pho5p in yeast cells.

Reported APP secretase candidates

Much progress has been made recently in the Alzheimer's disease field. A candidate for β-secretase in the brain, BACE1, has been found (Vassar et al., 1999; Sinha et al., 1999; Yan et al., 1999; Hussain et al., 1999; Lin et al., 2000), as well as its homologue on chromosome 21, BACE2 (Acquati et al., 2000; Hussain et al., 2000), whose role is not clear at this point. Members of the ADAM family of proteases, TACE (ADAM17) (Buxbaum et al., 1998) and ADAM10 (Lammich et al. 1999), and ACE (reviewed in Checler, 1995) seem to be involved in the α-secretase cleavage. The presenilins, associated with almost 50% of the familial AD cases, seem to form part of the γ-secretase complex, or perhaps they themselves are γ-secretases (Wolfe et al., 1999; Li et al., 2000; Esler et al., 2000). Cloning and expression of these secretase candidates was attempted in the present study to characterize their secretase activity in yeast and mammalian cells. They were also a good test for the yeast reporter system developed to screen for secretase candidates.

TACE cloning

Cloning of human TACE cDNA into yeast and mammalian expression vectors was unsuccessful. Its cDNA was twice PCR-amplified from human spleen cDNA and both times it contained the same STOP codon mutation before its transmembrane domain. It is not clear at which step the mutations appear. TACE was cloned by Black et al. (1997) from pig spleen. It is not clear if the same source of human spleen cDNA was used in both attempts to obtain a TACE clone by PCR-amplification in our laboratory, since the same mutation was identified the two occasions. Another source of cDNA may help to answer this question. Alternatively, the possibility exists that the mutation is being introduced by the bacterial strains used to propagate the TACE plasmids. We observed that *E. coli* strains DH5α and HB101 (Life Technologies) carrying the TACE plasmids grow very slowly. This was observed when growing them in either liquid or solid media. The fact that after correction of the STOP mutation a new mutation appeared supports the idea that the mutations did arise in bacteria. As was mentioned before, the pro- and catalytic domains have been expressed in yeast (Clarke et al. 1998) and shown to be active, even though their cloning procedure clearly reveals that the authors had cloning problems too. Since our goal was not just to have an active clone, but to test the yeast screening system with a full length human secretase candidate cDNA, no attempts were made to clone just the pro- and catalytic domains of human TACE.

Expression of TACE substrate, TNFα precursor, in yeast was successful (Figure 50). Pro-TNFα seems to be processed at some degree by yeast to produce a fragment of similar size as the one produced in mammalian cells (Figure 50). No secreted TNFα was detected with the methods used (ELISA kit from Oncogene Research).

Cloning of other APP α-secretase candidates, such as ADAM10 or ACE remain an option to test the yeast screening system.

BACE1 and BACE2 expression in yeast

All the evidence collected to date suggests that BACE1 is the major β-secretase for APPsw in the brain (Vassar et al. 1999; Yan et al. 1999). BACE2 seems to play a more important role in peripheral tissues. BACE2 is able to cleave APP at the β-secretase site, similarl to BACE1, but additionally it cleaves within the Aβ region (Farzan et al., 2000; this study).

In an attempt to characterize BACE1 and BACE2 in yeast and mammalian systems, their messages were cloned from human spleen cDNA. We attempted the expression of the full length cDNAs in yeast. At the limits of our detection, full length BACE1 and BACE2 do not seem to be expressed in yeast. BACE1 expression in yeast has been attempted by other laboratories, but it has been unsuccessful too (J. Shafer, personal communication). The pre-pro-α-mating factor sequence has been used before to target human APP to the secretory pathway (Hines et al. 1994). A similar approach was taken to express BACE1 and BACE2 in yeast. Their putative signal and pro-domain sequences were replaced with the signal and pro-domain sequences from the yeast α-mating factor. Expression of these fusion proteins can be detected by Western blotting (Figure 38, Figure 39). It is not clear yet whether these mature forms are active in yeast. Preliminary experiments are being done to answer this question. Since it has been reported that BACE1 is approximately 100X more efficient in cleaving substrates containing the APP Swedish mutation than substrates containing the wild-type sequence (Vassar et al. 1999), the mature forms of BACE1 and BACE2 are being co-expressed with PHO5-C105sw, as well as with full length APP wild-type and APPsw. The results obtained from these experiments would validate yeast as a model system to characterize APP secretases.

BACE1 and BACE2 in mammalian cells

To determine whether lack of BACE1 and BACE2 expression in yeast was intrinsic to the BACE1 and BACE2 constructs made, and to further characterize BACE1 and BACE2, they were expressed in COS7 and A204 cells. These proteins can be easily detected in transiently transfected cell either by Western blotting (Figure 41) or immunocytochemistry (Figure 42). Intracellular localization studies confirmed that BACE1 localizes to Golgi apparatus and early endosomes, as reported by Vassar et al. (1999). BACE2 also localizes to the Golgi and is similar to BACE1, and many other over-expressed proteins, in this sense. In contrast to BACE1, BACE2 localizes preferentially to the ER (Figure 44). This difference in localization would suggest different roles in cell metabolism. In fact, we confirmed that BACE1 increases β-sAPP and Aβ levels in transiently transfected cells. To our surprise, BACE2 increased β-sAPP, but decreased secreted Aβ levels in transiently transfected cells (Figure 45, Figure 46). BACE2 apparently is able to degrade Aβ, a finding that was later reported by Farzan et al. (2000).

The studies performed in mammalian cells indicated that we had functional BACE1 and BACE2 clones. Since mammalian expression vectors were derived from yeast expression vectors, this suggests that the expression problem in yeast is not intrinsic to the constructs made. It is possible that the protein is made but rapidly degraded because of improper folding. The fact that BACE1 and BACE2 can be expressed when they are behind the α-mating factor pre-pro sequence supports this idea, however, it has not been determined if any mRNA gets transcribed from expression vectors carrying full length BACE1 and BACE2.

Lack of processing of the pro-domain sequences in BACE1 has been shown not to be critical for activity. The activity of a secreted form of Pro-BACE when assayed with substrates corresponding to the wild type or Swedish mutant APP sequences were 74% and 43%, respectively, of BACE activity (Shi et al., in press J Biol Chem). For this reason, it is not expected that the lack of activity in yeast is due to the lack of pro-domain cleavage, in the event that the proteins are expressed but at non-detectable levels by our system.

Presenilin 1 / γ-secretase in yeast

Recent evidence suggests that the presenilins are involved in the γ-secretase cleavage of APP. Two aspartates located in two PS1 putative transmembrane domains of PS1 have been shown to be required for PS1 endoproteolysis and γ-secretase activity (Wolfe et al. 1997). In PS1 -/- mice γ-secretase is not present and several other proteins are not cut properly (St. George-Hyslop, 2000). PS1 was found to be part of a large detergent-soluble complex containing γ-secretase activity, and γ-secretase activity can be immunoprecipitated with anti PS1 antibodies (Li et al., 2000). Since there is no direct evidence that PS1 by itself has γ-secretase activity, PS1 and its substrates were co-expressed in the yeast system. Yeast offers the advantages of being an eukaryote that lacks PS1 and APP homologues. It is therefore a potentially useful system to determine whether PS1 participates directly in the γ-secretase cleavage.

When PS1 wild-type or PS1 P117L Polish mutant (linked to a very early onset of familial AD, Wisniewski et al. 1998) are expressed in yeast, they are processed by yeast enzymes to produce fragments that resemble in size the N- and C- terminal PS1 fragments seen in mammalian cells (Figure 47). Additional cleavages occur in yeast, which are probably products of yeast vacuolar proteases. These fragments have not been characterized, nevertheless, most of PS1 remains uncleaved.

PS1 wild-type and PS1 P117L were co-expressed in yeast with either APP695 or C99 (a γ-secretase substrate). There was no decrease in the γ-secretase substrates (C99 or C83) when either of the two PS1 proteins were expressed (Figure 48). Furthermore, Aβ1-40 formation was not detected either in the conditioned medium or cell pellets of these cultures (Figure 49). To the limits of detection of our system, PS1 does not appear to have γ-secretase activity in yeast. Since there is no independent assay for PS1 activity, we cannot discard the possibility that PS1 is γ-secretase. It is also possible that PS1 may need other co-factors to be active, or it may not fold into its active conformation. These are issues that need further studies.

Appendix

Glossary of terms

4α-PDD: a PDBu inactive analog.

ADAM: A Disintegrin And Metalloproteinase

AEBSF: 4-(2-aminoethyl) - benzenesulfonyl fluoride; a general serine protease inhibitor.

Amygdala: a structure in the limbic system involved in the transformation of recent memories into long-term memories.

APPsw: Naturally occurring mutation of APP695 at residues Lys595-Met596 to Asn-Leu.

Association cortex: parts of the frontal, temporal, and parietal lobes that seem to control the processes of thinking, planning and retaining memories.

BCIP: 5-bromo-4chloro-3-indolyl phosphate

Brefeldin A: inhibitor of Golgi processing. Promotes fusion of cis, medial and trans Golgi (but not TGN) with ER.

C99: the last 99 amino acids of APP. It starts at residue Asp1 of the Aβ sequence. It is considered a substrate for γ-secretase.

C106: The last 106 amino acids of APP. It starts 7 amino acids before Aβ sequence. It has shown to be processed by α-, β- and γ-secretases.

C. elegans : Caenorhabditis elegans (worm)

Calcineurin: protein phosphatase 2. A third major Ser/Thr protein phosphatase, which is activated by calcium/calmodulin

Calpains: calcium-activated proteases; they are intracellular cysteine proteases that are ubiquitously distributed in tissues of higher animals. They are composed of two subunits of 30 and 80 KDa, respectively. They are regulated by calcium ions but their precise biological function is not yet understood.

Calyculin A: a specific inhibitor of protein phosphatases 1 and 2A (IC50 ~ 0.1nM)

Carbonyl cyanide m-chlorophenyl hydrazone: a proton ionophore.

Chitin: is a polymer of N-acetyl-glucoseamine

Chloroquine: inhibitor of lysosomal activity and other compartments. It neutralizes lysosomes and other compartments such as endosomes and TGN (Higaki, J. 1995).

Computerized Axial Tomography (CAT): a special x-ray technique that produces images of the soft tissue of the body.

CTF: Carboxy terminal fragment.

Dementia: general term used to describe a chronic and substantial decline in two or more areas of cognitive function .

DiFMUP: 6,8-difluoro-4-methylumbelliferyl phosphate. A fluorogenic substrate for both acidic and alkaline phosphatase activity (Molecular Probes, Inc).

DIGs: detergent-insoluble, cholesterol and glycosphingolipid-rich membrane microdomains

E64: inhibitor of lysosomal activity - broad-spectrum cysteine proteinase inhibitor - irreversible.

FDP: fluorescein diphosphate. A fluorogenic phosphatase substrate (Molecular Probes, Inc).

Forskolin: a PKA effector (stimulates αAPP secretion in 293 cells)

Furin: This subtilisin-like endoproteinase removes paired basic residues to process precursor proteins. It contains several such sites near its amino end and may be autolytic.

GAL1: *Saccharomyces cerevisiae* galactokinase

GAL10: *Saccharomyces cerevisiae* galactokinase

GSK-3beta: glycogen synthase kinase-3beta

Glucan: glucose polymer (complex branched)

Hippocampus: a structure in the limbic system involved in the transformation of recent memories into long-term memories. The name comes from the Greek name 'seahorse' referring to the unusual shape of this brain structure.

HIS3: Yeast gene for imidazoleglycerolphosphate (IGP) dehydratase

Hydroxamate: Hydroxamic acids of structure RCON(OH)CH(2)CH(CH(2)C(6)H(5))CO(2)H

ICE: interleukin-1 (IL-1) β converting enzyme

IPTG: Isopropylthio-β-D-galactoside. Induces the synthesis of β-galactoside. Together with X-Gal allow blue/white screening of transfectants.

Kex2p: endoproteinase of late Golgi compartment; responsible for processing alpha factor precursor and M1 killer toxin precursor at Lys-Arg, Arg-Arg, and possibly Pro-Arg sites; unprocessed proenzyme is stable in the ER; cleaves C-terminal to a dibasic sequence. Gene located in S. cerevisiae chr XIV

Lactacystin: a more specific proteasome inhibitor than Z-IE(Ot-Bu)A-Leucinal

LEU2: yeast gene that encodes for β-isopropylmalate (β-IPM) dehydrogenase, which catalyzes the third step in leucine biosynthesis.

Leupeptin: inhibitor of lysosomal activity - cysteine proteinase inhibitor.

Limbic system: a group of brain structure involved in the control of emotions and memory.

London mutation: a V642I mutation in APP (695 numbering). The London mutation does not result in an overall increase of Aβ levels, but favors instead the production of the more amyloidogenic form

Mannan: complex polymer of mannose

MIG1: gene that codes for repressor responsible for repression of several genes (such as SUC, GAL, MAL, ADH) by glucose.

MIG2: gene that codes for repressor responsible for repression of several genes (such as SUC, GAL, MAL, ADH) by glucose.

MKC7: Multicopy suppressor of Kex2 Cold sensitivity (YPD)

MLD 28170: calpain proteinase inhibitor; reversible (just washing cells)

Monesin: inhibitor of Golgi processing. It is a carboxylic ionophore that blocks protein processing and transport within the distal Golgi and also disrupts endosome/lysosome function (Higaki, J. 1995).

Myc-6xHis tag: a sequence containing the c-myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) followed by 6 His residues. In the present work it was taken from commercially available vector pPIC-Z (Invitrogen)

N-ethylmaleimide: alkylating agent of free sulfhydryl groups.

NBT: Nitroblue tetrazolium

Neurofibrillary tangles: are nonmembrane-bound bundles of abnormal proteinaceous filaments, referred as paired helical filaments (PHF), that accumulate in the perinuclear cytoplasm of many cortical and limbic neurons

NICD: Notch intracellular domain

N-linked oligosaccharide: Asparagine linked oligosaccharide (only Asn in the sequences Asn-X-Ser or Asn-X-Thr becomes glycosylated; are by far the most common glycosylation found in proteins)

Nonsense mutation: any change in DNA that causes a termination codon to replace a codon representing an amino acid.

NT2 cells: human teratocarcinoma cells which irreversible differentiate into neurons and nonneuronal cells when exposed to retinoic acid.

NTF: Amino terminal fragment.

Null mutation: completely eliminates the function of a gene, usually because it has been physically deleted.

O-linked oligosaccharide: oligosaccharide linked to the hydroxyl group of Ser, Thr, or hydroxylysine; these are formed in the Golgi by mechanisms not fully understood)

Phorbol esters = plant products that bind to C-kinase and activate it directly.

PMA: Phorbol 12-myristate 13-acetate

Positron-emission tomography (PET): a medical technique that uses radioactive material to produce images of the brain cells in action.

Proteinase K: fungal serine protease.

RACE: 5'-rapid amplification of cloned ends.

SCAP: SREBP Cleavage Activating Protein

Senile plaques: structurally diverse array of spherical deposits of extracellular Aβ, dystrophic neurites, and activated microglia, among other components..

Senility: a lost of mental ability frequently considered as a normal part of aging.

SREBP = Sterol Regulatory Element Binding Protein

Swedish mutant: Naturally occurring mutation of APP695 at residues Lys595-Met596 to Asn-Leu.

TDH3: Glyceraldehyde-3-phosphate dehydrogenase 3, catalyzes the reversible oxidation and phosphorylation of D-glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate in glycolysis. mRNA and protein are very abundant (see references in Yeast Proteome Database)

TIMPs: tissue inhibitors of metalloproteinases.

TRP1: Yeast gene coding for N-C5'phosphoribosyl-anthranilate isomerase.

Type I integral membrane protein: single pass, with the C-term facing the cytoplasm, and N-term facing lumen of ER.

Type II integral membrane protein: single pass, with the N-term facing the cytoplasm, and C-term facing lumen of ER.

Type III integral membrane protein: multiple pass, any orientation.

URA3: Yeast orotidine 5'-phosphate decarboxylase gene

X-Gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Artificial substrate for β-galactosidase (X-Gal turns blue when metabolized by β-galactosidase). Together with IPTG allow blue/white screening of transfectants.

Yeast episomal plasmid (YEp): vectors based on the 2 μm plasmid and having as a selectable marker a yeast chromosomal gene. An episome is a plasmid that can replicate independently but can also be integrated into a chromosome. YEps are very effective in bringing about transformation, and occur in high copy number.

Z-IE(Ot-BU)A-Leucinal: a proteasome inhibitor

Table 12: Expression vectors used

A list of yeast and mammalian expression vectors used in this dissertation is given in this table. Centromeric vectors are single copy vectors. 2micron-based vectors are multicopy vectors, and are less stable than centromeric vectors (greater percentage of cells lose plasmid from generation to generation).

Name	Description
pBS6alpha	α pre-pro+APP695 yeast 2micron-based vector - with URA3/LEU2-d marker
p413Gal1	yeast centromeric vector with HIS3 marker - no insert
p423Gal1	yeast centromeric vector with URA3 marker - no insert
pDE041	PHO5-C106sw 2micron-based yeast vector - pBS6alpha backbone
pDE156	YAP3 centromeric vector with HIS3 marker
pDE167	h-PS1 yeast centromeric vector with HIS3 marker
pDE195	α pre-pro +APP695 yeast centromeric vector - with URA3 marker
pDE264	apha signal + APP C99 yeast centromeric vector - with URA3 marker
pDE277	PS1 P117L (Polish mutation) yeast centromeric vector with HIS3 marker
pDE284	h-PS1 yeast centromeric vector with URA3 marker
pDE316	BACE2 with myc-6xHis tag yeast centromeric vector with HIS3 marker
pDE326	BACE1 with myc-6xHis tag yeast centromeric vector with HIS3 marker
pDE327	BACE1 with myc-6xHis tag – mammalian vector (PCR3.1 Uni backbone)
pDE328	BACE2 with myc-6xHis tag – mammalian vector (PCR3.1 Uni backbone)
pDE332	wild type BACE1 yeast centromeric vector with HIS3 marker
pDE333	wild type BACE2 yeast centromeric vector with HIS3 marker
pDE340	wild type BACE1 mammalian vector (PCR3.1 Uni backbone)
pDE341	wild type BACE2 mammalian vector (PCR3.1 Uni backbone)
pDE358	α pre-pro-BACE1 with myc-6xHis tag yeast centromeric vector with HIS3 marker
pDE359	α pre-pro-BACE2 with myc-6xHis tag yeast centromeric vector with HIS3 marker
pDE401	Alpha leader+APP695sw yeast centromeric vector - with URA3 marker

Table 13: yeast strains used

Main yeast strains used in this dissertation are listed in the following table. DS7 (Y. Bourbonnais et al. 1993) was the parent strain to make all the deletions to develop the yeast screening system. JSC310 (Hines at al. 1994) lacks the major vacuolar proteases.

Name	Genotype
DS7	MATalpha MFalpha1/alpha2::LEU2 ade2 his3 leu2 trp1 ura3
JSC310	MATa leu2 ura3-52 prb1-1122 pep4-3 prc1-407 GAP-ADR1::G418
yDE077	DS7 with yap3 mkc7
yDE256	DS7 with yap3 mkc7 pho5/pho3::PHO5-C106sw/loxP pho11::loxP pho12::loxP
yDE276	DS7 with yap3 mkc7 pho5/pho3::Gal1p-APP695/loxP pho11::loxP pho12::loxP

Table 14: Summary of human cDNAs expressed in yeast centromeric vectors

The following table shows the different human cDNAs attempted to express in yeast. It also indicates the signal peptide and pro-domain, when applicable, sequences used in each case. Protein expression levels after 8 hr induction is also indicated, as detected by Western blotting.

Cloned cDNA	**Signal sequence**	**Pro–domain sequence**	**Protein expressed ?**
Human TNFα	own	own	+++
Human TACE	own	own	? (*)
Human APP695 wt	Yeast α-factor	Yeast α-factor	+++
Human APP751 wt	Yeast α-factor	Yeast α-factor	+++
Human APP695 sw	Yeast α-factor	Yeast α-factor	+++
Human APP751 sw	Yeast α-factor	Yeast α-factor	+++
APP C99	Yeast α-factor	none	
Presenilin 1 wild type	own	own	+++
Presenilin 1 mutants	own	own	+++
BACE1	own	own	-
BACE1	own	own	-
Mature BACE1	Yeast α-factor	Yeast α-factor	+ / -
Mature BACE2	Yeast α-factor	Yeast α-factor	+ / -

(*) cDNA with correct TACE sequence was not obtained.

+++	high protein expression levels
++	moderate protein expression levels
+	low protein expression levels
+ / -	very low protein expression levels
-	undetectable protein expression levels

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