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**LOCALIZATION AND PROCESSING OF BETA-AMYLOID PRECURSOR
PROTEIN IN THE RAT BRAIN AND YEAST *Saccharomyces cerevisiae***

by

WEI ZHANG

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

1996

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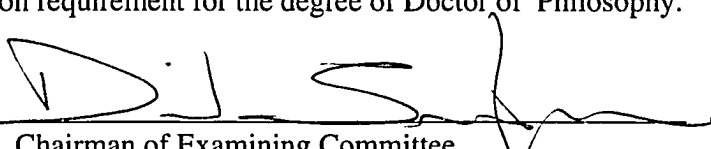
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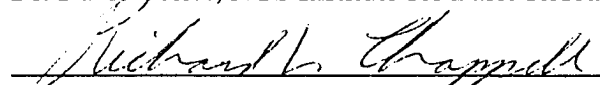
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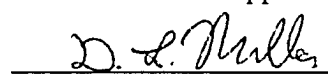
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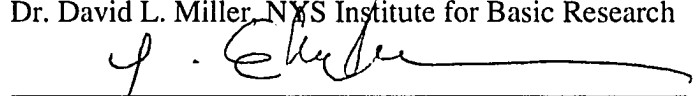
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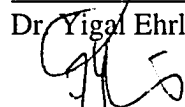

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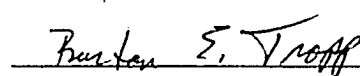
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Abstract

LOCALIZATION AND PROCESSING OF BETA-AMYLOID PRECURSOR
PROTEIN IN THE RAT BRAIN AND YEAST *Saccharomyces cerevisiae*

by

Wei Zhang

Adviser: Professor David Lee Miller

Mammalian cells can metabolize transmembrane β -amyloid precursor protein (APP), which is implicated in Alzheimer's disease, in several ways that result in the secretion of the ectodomain and amyloid β -protein ($A\beta$). We have investigated the subcellular locations of APP in rat brains. Immunoisolated APP-containing vesicles (APPV) show a concomitant enrichment of APP and synaptophysin (synaptic vesicle marker); a synaptic vesicle-enriched fraction (LP2) also reveals the co-existence of the two proteins. More importantly, purified synaptic vesicles contain 10-15% of APP in the brain. Therefore, APP is a constituent of synaptic vesicles and may regulate their functions.

Yeast contains precursor-converting enzymes homologous to their mammalian counterparts, and is susceptible to genetic manipulation. As a first step toward identifying APP-processing enzymes (secretases), we have studied the processing of human APP in transformed yeast (*Saccharomyces cerevisiae*). The cells express high levels of APP (1% of total protein), and, like mammalian cells, yeast can cleave APP within the $A\beta$ region to generate a soluble C-terminal truncated fragment (sAPP), while a complementary C-terminal fragment (CTF) accumulates in the membrane. Amino acid

sequencing demonstrates that the secretory cleavage site of APP is 12 residues away from the membrane-spanning sequence and is identical to “ α -secretase site” in mammalian cells. A pulse-chase study reveals that the half-life of APP turnover is 30-40 min; the parallel appearance of sAPP and CTF further confirms that they are the products of same protease(s). Unlike its mammalian counterpart, yeast α -secretase is basic amino acid-specific; however, processing of APP occurs efficiently in yeast mutants defective in Yap3 and Kex2 peptidases, both of which cleave at basic amino acids. Yeast α -secretase is not a vacuolar protease because APP cleavage proceeds undiminished in both a vacuolar protease-deficient strain and cells treated with a lysosomotropic agent, NH_4Cl . Using temperature-sensitive secretion mutants, we located the yeast α -secretase in the Golgi, possibly in a distinct late Golgi region distal to the compartment containing Kex2/Kex1. We conclude that yeast possesses a unique protease that resembles mammalian α -secretase; therefore, yeast can serve as a model for understanding mammalian APP secretase.

Acknowledgments

I express my deepest gratitude and appreciation to my mentor, Dr.David Miller, who has shepherded me, with tremendous patience, through the long and difficult journey of my doctoral training, and has set an example of being creative, rigorous, and yet detached in doing science.

I also wish to offer my heartfelt thanks to my father, Hua-tang Zhang, and my mother, Li-li Yan, for their deep love and support, which is the basis for everything I do.

Special gratitude is due to Dr.Yu-Wen Hwang, who gave me invaluable advice on my research and enthusiastically guided me into the computer world.

In addition, I, with pleasure, acknowledge the efficient, selfishless and tireless (but never tiresome) help of Dr.Robert Denman, who elegantly assisted me in engineering my first recombinant DNA.

To my teacher, Dr.David Soifer, goes my sincere thanks for providing generous support in many ways.

I am also grateful to Dr.Michael Quinne for his critical assistance in dissecting rat brains, to Dr.Anna Potempska for teaching me the techniques of electrophoresis and immunoblotting, and to Dr.Victoria Hines for providing the yeast transformants.

I am indebted to Dr.Julia Currie, Dr.David Bolton and Dr.Narayanarao Ramakrishna for offering stimulus and comments on my work.

Special thanks also goes to my English teacher, Professor Hack, who taught me how to organize a chapter and to write concise, interesting and coherent sentences.

I sincerely appreciate the friendship and encouragement of Dr.Latika Chanderkar, Dr.Ying-Ju Sung, Ms.Patricia Casiano, Ms.Diane Coccozza, and my good friends Dongjie Liu and Hui Deng.

Finally, I express my appreciation to many others who helped me, though they are not identified herein.

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Abbreviations

A β	amyloid β -protein
α C	α -leader- α -factor-C-terminal fragment chimera
ALP	alkaline phosphatase
APP	amyloid β -protein precursor
APP6 α	α -leader-APP695 fusion protein
APPV	APP-containing vesicle
BSA	bovine serum albumin
CPY	carboxypeptidase Y
CTF	C-terminal fragment of APP
DPAP	dipeptidyl aminopeptidase
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
endo H	endoglycosidase H
ER	endoplasmic reticulum
HPLC	high-performance liquid chromatography
kD or kDa	kilodalton
KEX1	Kex1 carboxypeptidase gene
KEX2	Kex2 endopeptidase gene
leu/ura SM	synthetic medium deficient in leucine and uracil
LP2	synaptic vesicle-enriched fraction
MDCK	Madin-Darby canine kidney cell
met-SM	synthetic medium deficient in methionine
pBS	yeast shuttle vector
PMSF	phenylmethanesulphonyl fluoride

PNS	post-nuclear supernatant
sAPP	secreted or soluble APP
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
<i>sec</i>	temperature-sensitive secretion mutant
SM	yeast synthetic medium
SV	synaptic vesicle
Tricine	N-tris (hydroxymethyl) methylglycine
Vps10	receptor for vacuolar carboxypeptidase Y
Yap3	yeast aspartyl endopeptidase

INTRODUCTION

Alzheimer's disease (AD) is a human cerebral degeneration disorder characterized by gradual loss of memory, reasoning, orientation and judgment, correlated with the hallmark amyloid deposits in the hippocampus and association cortex of AD brain. The amyloid deposits occur extracellularly as parenchymal (neuritic) plaques and cerebrovascular amyloids; the former exist as a continuum from plaques with no associated neuronal and glial alteration (diffuse plaques) to those with profound cytopathology (mature plaques) (Selkoe, 1994). Both neuritic plaques and vascular amyloids contain a small peptide of 39-42 amino acids, referred to as the amyloid β -protein ($A\beta$). $A\beta$ is generated by proteolytic cleavage of an amyloid β -protein precursor (APP), a 100-140 kDa integral membrane protein encoded by a gene on human chromosome 21 (Kang et al., 1987; Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988; Miller et al., 1993). Alternative splicing of a single APP pre-mRNA generates three major isoforms containing 695, 751 or 770 amino acids (Lemaine, 1989; Yoshikai, 1990). APP751 and APP770 harbor an additional 56-residue insert homologous to the Kunitz-type serine protease inhibitor (KPI) (Muller-Hill, 1989) that inhibits trypsin, chymotrypsin, γ subunit of NGF, and other serine proteases *in vitro*. The APP gene has been highly conserved throughout evolution. It is expressed in a variety of tissues; highest levels are found in brain, lung, kidney, muscle and spleen (Tanaka, 1989; Neve, 1988). The APP695 isoform is predominantly expressed in mammalian brains (Neve, 1988) and is regulated during brain development (Loffler, 1992).

APP Maturation and Processing Through Complicated Pathways

APP is a transmembrane protein (type I) with a small cytoplasmic carboxyl domain of 47 residues, a single transmembrane region and a large extracellular N-terminal domain (Fig.1). The 4-kDa $A\beta$ fragment comprises 28 residues in the ectodomain plus the first 11-15 residues of the transmembrane region. Like other

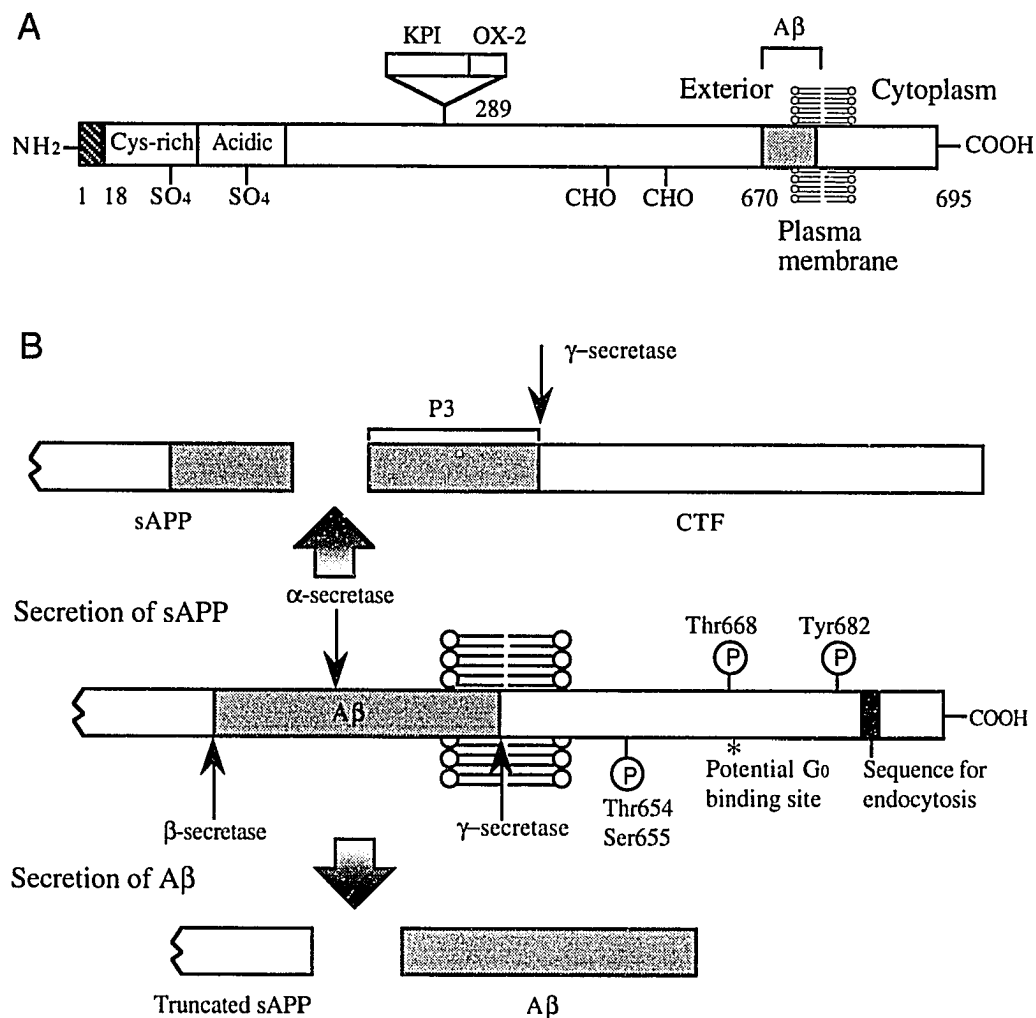


Figure 1. Schematic diagram of the amyloid β -protein precursor (APP) and its major metabolic pathways. Panel (A) depicts the APP695 isoform. Other isoforms contain a Kunitz-type protease inhibitor domain (KPI, 56 residues) (APP751, APP770) and a sequence homologous to MRC OX-2 antigen (APP770). The β -amyloid peptide region (A β) is shown as a solid box. A 17-residue signal peptide (striped bar) occurs at the N-terminus. Two N-glycosylation (CHO) sites are found at the extracellular domain. The A β -containing region is expanded in Panel (B). Potential phosphorylation sites at the C-terminal domain are labeled. The arrows indicate the sites of proteolytic cleavage made by unidentified proteases that generate various APP intermediates. α -secretase cleaves APP into a secreted form of APP (sAPP) and an 83-residue membrane-bound C-terminal fragment (CTF) that is speculated to give rise to P3 peptide; In an alternative pathway, β -secretase cleaves at the N-terminus of the A β region to generate a truncated sAPP and a longer C-terminal fragment that may be further processed by γ -secretase to form A β .

membrane proteins, newly synthesized APP is transported through the ER and Golgi *via* bulk flow along the secretory pathway, where a series of post-translational modifications occur. APP has tyrosine-sulfated residues and N- and O- linked carbohydrates (Wiedemann et al., 1989; Pahlsson, 1992). APP also exists in the core of a chondroitin sulfate (cs) proteoglycan secreted by glial cells (Shioi et al., 1992). APP phosphorylation occurs, but the phosphorylation sites may depend on cell type. The exogenous addition of protein kinase C (PKC) or a proline-directed protein kinase (cdc2 kinase) to detergent-permeabilized PC12 cells phosphorylated cytoplasmic residues serine655 and threonine668, respectively (Suzuki et al., 1992; Suzuki et al., 1994). In contrast, APP was phosphorylated only on a serine residue in the extracellular domain (ectodomain) in transfected human embryonic kidney 293 cells (Hung and Selkoe, 1994). The relationship between phosphorylation and APP metabolism will be discussed in detail below.

Most APP is concentrated in the Golgi complex (Caporaso et al., 1994). At least a portion of newly-synthesized APP has been shown to move anterogradely in a rapid phase of axonal transport (Koo et al., 1990; Sisodia et al., 1993) *via* a kinesin-based motor (Ferreira et al., 1993), possibly after APP exits the Golgi complex. Studies with cultured hippocampal neurons (Ferreira et al., 1993), in brains of rat, human and bovine (Sapirstein et al., 1994), and in optic nerve (Morin et al., 1993) show that, during its translocation down the axon, APP is deposited at sites in the axolemma and the periaxolemmal domain of the myelin complex. A portion of APP finally reaches the synaptic sites, based on the co-localization of APP and synaptophysin (a synaptic vesicle marker) at central and peripheral synaptic sites (human and rat brains and neuromuscular junctions) (Schubert et al., 1991). In addition, APP was also found in purified rat synaptic plasma membrane, indicating that some APP are targeted to the nerve terminal and inserted into the membrane (Shimokawa et al., 1993). However, some workers did not find the concentration of APP in synaptic sites using either

immunoelectron microscopy (Caporaso et al., 1994) or immuno-light microscopy (Ferreira et al., 1993). On the other hand, non-neuronal cells like microglia and astrocytes have very little APP inserted at the cell surface (Haass et al., 1991).

After reaching the plasma membrane *via* the constitutive secretory pathway, a small portion of mature APP is internalized and transported to endocytic compartments. APP has been found in neuronal secondary lysosomes in human brains (Benowitz and Rodriguez, 1989) as well as in late endosomes of cultured hippocampal neurons (Ferreira et al., 1993). APP co-localized with the lysosomal marker Igpl20 in lysosomotropic drug chloroquine-treated CHO cells overexpressing the APP gene (Caporaso et al., 1994). The internalized APP can recycle back to the cell surface within 5-10 min (Yamazaki et al., 1993), and it is eventually degraded in lysosomes (Estus et al., 1992; Glode et al., 1992; Haass et al., 1992). APP internalization is a receptor-mediated process *via* cytoplasmic sequence motifs of APP. One sequence, GYENPTY, which is related to the low density lipoprotein receptor (LDLR) internalization signal FDNPVY, plays a major role in APP endocytosis (Lai et al., 1995) (Fig.1B). The internalized full-length APP is apparently processed into a complex set of 8-12 kDa C-terminal derivatives that include potentially amyloidogenic forms with the entire A β at or near their N-termini (Estus et al., 1992; Golde et al., 1992; Haass et al., 1992). The corresponding complementary N-terminal fragment (truncated sAPP), which ends at methionine670 just prior to the start of A β , has been detected in the medium of APP-transfected cells, though at a very low level (Seubert et al., 1993) (Fig.1B). Because A β accumulates pathologically in AD and Down's syndrome alike, and because the C-terminus of A β is embedded in the hydrophobic environment of the membrane, it had been postulated that A β could only be produced from cells under abnormal circumstances, *i.e.* membrane damage. However, a spate of more recent evidence has unequivocally demonstrated constitutive A β secretion and the formation of a C-terminus-truncated A β (P3 fragment) from cultured cells (Haass et al., 1992b;

Seubert et al., 1992; Shoji et al., 1992) (Fig.1B). A β has also been isolated from human and rodent cerebrospinal fluid (CSF) (Seubert et al., 1992; Shoji et al., 1992). The secreted 4-kDa peptide isolated from these cells begins principally at the asparagine1 residue that is the major N-terminus of the A β purified from AD brain, but the peptide also displays N-terminal heterogeneity (Haass et al., 1992b). The N-terminus of P3 corresponds to leucine17 of A β region, the residue C-terminal to the α -secretase cleavage site (see below), which suggests that P3 may be generated by both α -secretase and γ -secretase.

In many cell types, APP proteolytic cleavage results in the constitutive secretion of a 90-100 kDa soluble N-terminal fragment (sAPP) (Schubert et al., 1989; Weidemann et al., 1989) and a 8.7-10 kDa C-terminal fragment that remains in the membrane (Sisodia, 1990; Anderson, 1991) (Fig.1B). This cleavage, mediated by an unidentified proteinase(s) termed " α -secretase", occurs within the A β region (between lysine16 and leucine17 of A β) (Esch et al., 1990; Sisodia et al., 1990; Wang et al., 1991); thus, amyloid formation is precluded.

The relative utilization of these two pathways appears to differ among cell types (Haass et al., 1991; Hung et al., 1992). Both glial and neuronal cells produce very low levels of the secreted derivative (sAPP) and the 10 kDa C-terminal fragment compared to non-brain cells. The balance of these competing metabolic pathways is thought to be crucial for amyloid formation. The identification of several missense mutations, within or immediately flanking the A β region of APP gene in certain kindreds with familial, autosomal dominant, early-onset AD (FAD) (Mullan and Crawford, 1993), strongly suggests that APP metabolism is central to the disease mechanism (Fig.2). Accordingly, much effort has been directed to elucidating APP intracellular trafficking and processing and how these processes are regulated.

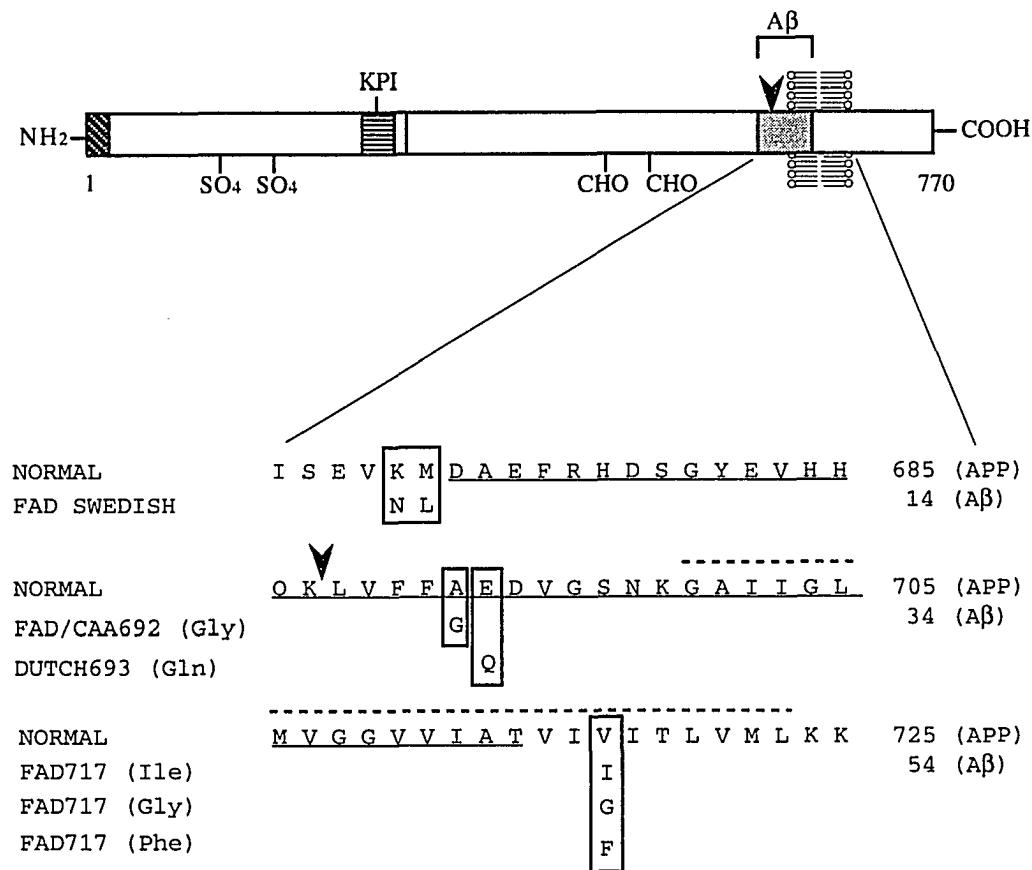


Figure 2. APP missense mutations associated with familial Alzheimer's disease (FAD) or closely related β -amyloidotic disorders. The underlined residues represent the A β ₁₋₄₃ peptide. The broken line indicates the location of the transmembrane domain. The boxed residues represent the missense mutations. Dutch: hereditary cerebral hemorrhage; CAA: congophilic amyloid angiopathy phenotype. Arrow head marks the α -secretase cleavage site. (Selkoe, 1994)

POSSIBLE LOCATIONS FOR A β GENERATION

At present, the cellular mechanism of A β generation is unknown. The following lines of investigation suggest that A β formation does not occur in the endosome-lysosome pathway: (a) no effect of lysosomal inhibitors (chloroquine, NH₄Cl, leupeptin) on A β secretion, despite of the accumulation of the C-terminal fragment containing the entire A β region upon the treatment (Knops et al., 1992; Busciglio, 1993), (b) the absence of A β in purified lysosomes (Haass et al., 1993), and (c) the normal A β secretion from I-cell fibroblasts with severe lysosomal deficiency (mucopolipidosis II) (Podlisny et al., 1993).

One recent study, however, has implicated the endocytic pathway in A β formation. Vesicular pH alteration was found to inhibit A β and P3 production (Koo et al., 1994); blocking surface APP endocytosis by either deleting YEVPTY sequence in the cytoplasmic tail, or removing the entire cytoplasmic domain, or depleting cellular potassium levels dramatically reduced A β secretion in transfected CHO cells (Koo et al., 1994; Haass et al., 1993). In addition, after selective cell surface radio-iodination, radiolabeled A β was recovered in the medium from their cultures; this indicates that the cell surface APP was a direct precursor of A β . Thus, it is postulated that one A β formation pathway uses surface APP internalization into early endosomes followed by excision and release of A β (Koo and Squazzo, 1994).

CHARACTERIZATION OF α -SECRETASE(S)

APP proteolytic cleavage within A β region permits constitutive secretion of the N-terminal domain of APP (sAPP). Although lysine16 seems to be the preferred cutting side, natural alternative sites have been reported. Zhong and his colleagues (1994) demonstrated that CV-1 monkey fibroblasts overexpressing APP751 cleaved A β region after phenylalanine19 (40%) as well as lysine16 (60%). Interestingly, fibroblasts from Down's syndrome individuals showed a collection of cleavage sites inside the A β region, but not at lysine16. The specificity of mammalian α -secretase has been

examined by a series of point-mutations and deletions around the major cleavage site between lysine16 and leucine17. The initial results suggested that α -secretase activity was independent of the sequence around the major cleavage site; instead, α -secretase appears to require only an α -helical conformation at certain distance (12-13 residues) from the membrane (Maruyama et al., 1991; Sisodia, 1992; Sahasrabudhe et al., 1992). However, more detailed biochemical analysis revealed that at least two deletions (4 to 6 residues), downstream of lysine16, yielded two additional cleavage sites (glutamate11 and histidine13). This indicates that α -secretase has a degree of sequence specificity. These data suggest that APP secretion may involve multiple proteinases, collectively referred to as α -secretase.

α -Secretase seems to act in multiple subcellular locations. Direct evidence for the cell-surface as a source of α -secretase came from the observation that biotin- or sulfate- or iodine-labeled cell surface APP was cleaved and released to the medium (Haass et al., 1992a; Sisodia et al., 1992; Koo and Squazzo, 1994; Haass et al., 1993; De Strooper et al., 1993; Jacobsen et al., 1994; Lai et al., 1994). Similarly, blocking surface APP endocytosis did not impair sAPP secretion, but rather enhanced its production; the elevation of pH in acidic compartments by a specific inhibitor of the vacuolar H^+ -ATPases, bafilomycin A1 (baf A), also did not inhibit sAPP release in human 293 cells and primary human mixed brain cultures. These data suggest that intracellular acidic organelles probably are not important in APP processing in these cell types (Knops et al., 1995). Thus, one major α -secretase activity seems to reside on the cell surface. However, there is some evidence for an intracellular location of α -secretase as well. For example, sAPP was found inside cells (De Strooper et al., 1993; Sambamurti et al., 1992).

It appears that the location of α -secretase action is cell-type specific; both cell surface and intracellular compartments contain α -secretase in some cells. Since most of the studies about APP trafficking and processing have been performed with non-

polarized cells, recent efforts have been directed toward studying APP processing in polarized cells; these cells are more relevant to the protein transport and metabolism that occur in neurons and endothelial cells of brain vascular system. In Madin-Darby canine kidney cells (MDCK), which have distinct basolateral and apical plasma membranes, 80-90% of sAPP is released from the basolateral surface (Haass et al., 1994). Most of the cell-surface APP on the basolateral membrane gave rise to sAPP, supporting the idea of a cell-surface-localized α -secretase. However, when the cells were treated with ammonium chloride, polarized sAPP secretion was abolished, and sAPP was released from both basolateral and apical membranes. Moreover, sAPP could be detected in the membrane vesicles purified from MDCK cells. Therefore, in polarized cells, α -secretase cleavage might also occur in an intracellular compartment, and the resulting sAPP might be transported in acidic vesicles whose traffic can be regulated by pH.

REGULATION OF APP METABOLISM

APP metabolism is controlled by many factors. Protein kinase C (PKC) activation by phorbol esters enhanced α -secretase cleavage of APP. Thus, sAPP secretion and accumulation of C-terminal fragment increased; whereas, A β production decreased (Buxbaum et al., 1990; Buxbaum et al., 1993; Hung et al., 1993). Many extracellular signals (first messengers) and depolarizing agents exert their positive effects on α -secretase activity by regulating the phospholipase C/PKC pathway. Carbachol stimulation of muscarinic acetylcholine receptor subtypes m1 or m3 led to increased sAPP secretion and a concomitant decrease in A β formation. These effects were blocked by staurosporine, a protein kinase C inhibitor (Buxbaum et al., 1992; Nitsch et al., 1992; Hung et al., 1993). Electrical stimulation of hippocampal slices had the same effect on sAPP secretion (Nitsch et al., 1993); therefore, neuronal activity and neurotransmitters may regulate APP metabolism. Likewise, stimulating metabotropic glutamate receptors accelerated APP processing into sAPP *via* the phosphatidylinositol

4,5-bisphosphate/PKC cascade (Lee et al., 1995). Although initial thought on the mechanism for this regulation focused on the direct effect of PKC activation of APP phosphorylation, recent evidence has shown that the process does not involve elevated APP phosphorylation. Furthermore, mutating the potential APP phosphorylation sites did not affect cleavage (Hung and Selkoe, 1994). These results argue that the regulation of sAPP production is independent of APP phosphorylation. Rather, the target(s) for regulation in the PKC activation might be a regulator of α -secretase, components controlling protease access to APP, or α -secretase itself.

α -Secretase cleavage of APP is also regulated in a cell cycle-dependent manner (Suzuki et al., 1994). At the G2/M phase of PC12 cells, the level of secreted sAPP decreased, and that of the complementary membrane-bound C-terminal fragment increased. This alteration of APP metabolism, which closely correlated with the enhanced APP phosphorylation at threonine668 in the C-terminal domain of APP695, may be due to activated cdc2 kinase. Cell-cycle dependent-APP processing may represent an important mode of APP metabolism in normal dividing cells in the brain, i.e. glial cells, as well as in cells of non-nervous tissues. It is conceivable that aberrations in this regulation may contribute to abnormal A β formation in glial cells, which are a major source of A β in normal brains (Busciglio et al., 1993).

Altered calcium homeostasis may play a pivotal role in AD (Mattson, 1992). Indeed, intracellular calcium elevation by calcium ionophores substantially increased A β production, without affecting sAPP secretion (Nitsch et al., 1992; Querfurth and Selkoe, 1994); the increase in A β induced by the calcium surge was mediated by a cAMP- and PKC-independent mechanism (Querfurth and Selkoe, 1994).

The indirect regulation of α -secretase by PKC is further supported by a genetic study on mechanism of transforming growth factor α (TGF- α). APP belongs to a large family of membrane proteins that undergo cleavage to release their ectodomains into extracellular medium in a regulated manner (Ehlers and Riordan, 1991). The group

includes membrane proteins involved in various aspects of cell communication and regulation, i.e. TGF- α , colony-stimulating factor (CSF-1), tumor necrosis factor α (TNF- α) receptors and APP. A CHO cell mutant defective in TGF- α cleavage also lost its ability to cut other proteins in the family, including APP, in response to a PKC activator PMA. This discovery suggests that the regulated cleavage and secretion of APP, TGF- α , and other membrane proteins in the family is likely to be mediated by a common component(s) downstream of the signal pathway, which can be activated by various substances (Arribas and Massague, 1995). This postulate is consistent with the idea that β APP is not the direct target in PKC-stimulated sAPP production.

SPECULATION ON APP FUNCTIONS

Several physiological roles of APP and its derivatives have been suggested. Soluble APP has been found to promote neurite outgrowth by influencing the release of extracellular matrix-bound growth factors (i.e. FGF) (Small, 1992) and lowering intracellular calcium levels (Mattson, 1994). In addition, sAPP can mediate neural or non-neuronal cell adhesion (Schubert et al., 1989; Milward et al., 1992). Secreted sAPP with KPI insert, referred to as protease Nexin2 (NP2), can inhibit serine proteases *in vitro* (Van Nostrand et al., 1989), and may be involved in the regulating the coagulation cascade (Smith et al., 1990; Van Nostrand et al., 1990). Secreted APP may also serve as a scavenger for histones that could be released from damaged cells (Potempska et al., 1993). Recent studies showing the interaction of APP with a heterotrimeric GTP-binding protein, G_0 (Nishimoto et al., 1993), and the high affinity of APP for transforming growth factor β suggest that cell-surface holo-APP may function as a receptor to transduce extracellular signals,

Although $A\beta$ is constitutively secreted into cerebrospinal fluid and plasma of normal individuals, it is not clear whether it has specific function or simply represents a byproduct of proteolytic degradation. Recent studies strongly suggest that freshly solubilized, monomeric $A\beta$ is relatively inert; whereas, aggregated, oligomeric and

polymeric A β (aged), which are formed by *in vitro* incubation of the peptides, is cytotoxic (Pike et al., 1993; Busciglio et al., 1993). The detection of natural oligomers of secreted A β in cell culture would facilitate an understanding of the processes of A β aggregation and its effect on cells under physiological conditions (Podlinsky et al., 1995).

PROBLEMS AND STRATEGIES

A. Intracellular Localization of APP in rat brains

The intracellular distribution of APP may give clues to its functions and processing sites. Despite the latest surge in the information regarding the localization of APP in various neuronal and non-neuronal cells *in vitro* and *in vivo*, most of the data were obtained from immunohistochemical and immunoelectron microscopical studies. The sensitivity and specificity of these morphological techniques may hamper the precise definition of APP distribution in subcellular compartments, and could be responsible for the contradictory conclusions found in the literature. To overcome the obstacles inherent in morphological studies, we have tried to biochemically determine intracellular location of APP by identifying the nature of APP-positive vesicles purified from rat brains, using antibodies directed to markers of various subcellular organelles. The implicated organelle was then isolated for further study. In **PART I**, we first demonstrate *in vivo* that rat brain APP is inserted into intracellular compartments, in a defined orientation that agrees with previous *in vitro* data and the predicted structure of APP determined from its cDNA (Kang et al., 1987). The APP C-terminal tail protrudes from the vesicles in brain homogenates and is accessible to antibodies against APP's C-terminus. Secondly, analyses of immunoisolated APP-containing vesicles (APPV) and highly purified synaptic vesicles suggest that a small portion of brain APP is located in synaptic vesicles and may play a role in synaptic vesicle-related events.

B. A Yeast Model For APP Trafficking and Processing

Although it has been known for several years that the constitutive sAPP secretion pathway precludes A β formation and, thus, may play a pivotal role in amyloid formation, the α -secretase responsible for this has eluded identification. In an attempt to identify the α -secretase, we initiated studies on the APP trafficking and processing in yeast, the unicellular eukaryote.

The choice of yeast *Saccharomyces cerevisiae* as a model was based on several considerations. First, the secretory system in yeast basically resembles that in mammalian cells. Newly synthesized yeast proteins traverse the endoplasmic reticulum (ER) and the Golgi complex, where modifications and processing occur, until they reach the *trans* side of the Golgi, an equivalent of mammalian trans-Golgi network (TGN). In the Golgi, the proteins are sorted either to the vacuole or to the cell surface. Although yeast secretion is a constitutive process compared to neuronal transmitter release that is tightly regulated by calcium, the molecular machinery for membrane budding and fusion are the same for both types of cells. So are the underlying mechanisms for retention of resident proteins in the ER and Golgi. Therefore, insights derived from APP trafficking in yeast may be applied to mammalian cells. Secondly, genetic approaches in yeast have proved fruitful in elucidating the complex mechanisms of vesicle-mediated protein transport and processing. One of the problems in defining the location(s) of APP-processing proteinases, i.e. α -secretase, is the rapid protein transport through the secretory pathway in mammalian cells. In this regard, the availability of yeast *sec* mutants, whose secretory pathway is blocked at specific stages at nonpermissive temperatures, should be particularly useful for determining the locations of activities of the APP-processing proteases. Finally and most importantly, yeast has enzymes that are homologous to those found in mammalian cells. Yeast contains a prohormone-converting enzyme (*KEX2* gene product), a member of the subtilisin family of serine proteinases, that is both functionally and structurally related to its mammalian

counterparts (Julius et al., 1984; Smeekens, 1991). Furthermore, a recent study indicated that yeast could faithfully cleave mammalian pro-somatostatin into mature somatostatin-28. In addition, a yeast gene encoding an aspartyl protease which plays a role in this precursor processing has been cloned (Bourbonnais, 1993). Since the APP processing pattern in the insect cells overexpressing APP751 cDNA was similar to that in mammalian cells (Ramakrishna et al., 1991), the proteases for APP processing may well be conserved throughout eukaryotes. Following this reasoning, we predicted that yeast cells may have proteases that are capable of generating an APP processing profile similar to that in mammalian cells. The identification of these yeast proteases may, in turn, help identify their mammalian counterparts in the future.

In **PART II**, we provide evidence that yeast indeed possesses proteinase(s) that cleave in the middle of A β region of APP with a specificity identical to that of mammalian α -secretase. In addition, the yeast α -secretase activity was narrowed down to a late Golgi compartment. Finally, by constructing a point mutation near the cleavage site we have demonstrated that the yeast α -secretase requires a basic amino acid for its activity.

PART I

Localization of Alzheimer β -amyloid Precursor Protein in Synaptic Vesicles of Rat Brains

MATERIALS AND METHODS

Antibodies

Antibodies against various regions of APP695 were raised in adult female New Zealand white rabbits (prepared by Dr. Pankaj Mehta, NYS Institute for Basic Research): antibody R57 for the C-terminus (residues 672-695, epitope: 689-695); R3 for a membrane-adjacent portion of the C-terminus of APP (649-671); R71 for a sequence near the N-terminus (46-62). Mouse anti-synaptophysin (SY38) and anti- β -tubulin monoclonal antibodies were purchased from Boehringer Mannheim. Rabbit anti-cathepsin D polyclonal antibody and goat anti-Na/K ATPase polyclonal antibody were purchased from Calbiochem. Mouse anti-human factor VIII-related antigen antibody was from DAKOPATTS a/s in Denmark. For antibody binding assays, immunoglobulin G (IgG) was isolated from R57 and preimmune rabbit serum on a Bakerbond ABX column (40 μ m particle size) according to the manufacturer's instruction. The purity of the IgG fraction was checked by SDS-PAGE. The C-terminus specific antibody (R57) was further isolated from total IgG by peptide affinity chromatography. The titer of the affinity purified R57 was determined by ELISA.

Preparation of Membranous Vesicles from Rat Brains

The procedure was performed at 0-4°C. Half of a frozen rat brain (Pel-Freez Biologicals) was homogenized in a buffer composed of 0.25M sucrose, 10 mM Tris-HCl, pH 7.4 (10% W/V) first in an Ultra-Turrax homogenizer (Tekmar Company) with one or two 5-s bursts, and then in a motorized Potter-Elvehjem homogenizer (1,255 rpm, clearance 0.17 mm) with 8 strokes (up and down). The nuclei and unbroken cells were removed by centrifugation at 1300 x g for 10 min in Sorval RC-5 (SS-34 rotor). The postnuclear supernatant (PNS) was sedimented at 100,000 x g for 1 h in a Beckman L8-80M centrifuge. The resulting membranous vesicle pellet (P1) was resuspended in homogenization buffer and aliquots were stored at -70°C.

Alkaline Extraction of Membrane Proteins

The vesicle fraction (P1) from rat brain homogenate was subjected to alkaline extraction as described previously (Wessels et al., 1991). The vesicle fraction was diluted with 100 mM Na₂CO₃ (pH 11.5), and the pH was adjusted with 0.1N NaOH to 11.5. After a 10 min-incubation on ice, the samples were loaded onto a 100 µl cushion of 0.2 M sucrose, 60 mM HEPES (pH 11), 150 mM potassium acetate, 2.5 mM magnesium acetate and 1 mM DTT in ultracentrifuge eppendorf tube and centrifuged at 100,000 x g for 60 min at 4°C in a Beckman TL100 centrifuge. The entire supernatant, including the cushion, was removed and precipitated with an equal volume of 20% TCA. The pellet fraction and the TCA pellet of the supernatant were dissolved in SDS-PAGE sample buffer, and analyzed by 10% SDS-PAGE and western blot with R57 as primary antibody and goat anti-rabbit antibody as second antibody.

Protease Protection Assay

For trypsin digestion, a procedure previously described was followed (Wessels et al., 1991). Fraction P1 was first treated with 3 mM tetracaine for 5 min at room temperature to stabilize the vesicle membranes, and then cooled on ice. The digestion was initiated by adding freshly prepared TPCK-treated trypsin to the samples to a final concentration of 50 µg/ml in a total 180 µl reaction volume. Alternatively, vesicle membrane proteins were incubated with proteinase K (0.02-1.0 mg/ml) in a digestion buffer (100 mM potassium acetate, 2.5 mM magnesium acetate, and 50 mM triethanolamine) for 1 h at room temperature; subsequently, the digestion was stopped with 2 mM PMSF. In a parallel experiment, 2% non-ionic detergent Triton X-100 was included in the digestion mixture to show the inherent sensitivity of APP to the proteases. All the protease-treated membrane proteins were separated by 10% Tricine SDS-PAGE; subsequently gel was immunoblotted and then probed with R57 and R71 antibodies.

Assay of Sequence-Specific Antibody Binding to Vesicles

Vesicle fraction (P1, 150 μ l) was incubated with horse serum (20 μ l) for 10 min on ice to block the nonspecific binding sites for the antibody. The binding assay was carried out by mixing the blocked vesicles with a series dilutions of affinity purified R57 in a total volume 200 μ l for 1.5 h on a shaker at room temperature. As a control, the same amount of preimmune rabbit serum was incubated with the vesicle fraction. The unbound antibodies were removed by centrifugation at 40,000 rpm in a Beckman TL100 centrifuge for 20 min after addition of 800 μ l homogenization buffer to the above mixture. The pellet was washed two more times, and resuspended in 150 μ l of the same buffer. The vesicles with bound antibodies were dissolved in Laemmli sample buffer and subjected to 10% Tricine SDS-PAGE followed by immunoblot analysis using goat anti-rabbit antibody to detect any bound antibody. The recovered antibody was quantitated by densitometry of the IgG heavy chain band on the blot with reference to the standard concentration curve of R57 (Shimadzu dual-wavelength flying-spot scanner, cs-9000).

Competitive ELISA

The specificity of R57 for binding to vesicles was measured by a competitive solid phase enzyme-linked immunosorbent assay (Competitive ELISA). In a set of microtiter wells pre-blocked with 5% BSA/PBSA, R57(0.34 pmole IgG) in blocking buffer (0.2% BSA/PBSA, 0.05% Tween-20) was incubated for 2 h at room temperature with increasing amounts of brain vesicle preparation (P1) (0-32 μ l; equivalent APP input: 0-1.6 pmole), which was pretreated with 10% horse serum for 10 min, in the presence or absence of various concentrations of pre-immune rabbit IgG (0.17, 0.34, 0.68 pmole). The mixtures were then transferred to another set of wells coated with a peptide (corresponding to residues 672-695 of APP695) encompassing the R57 epitope (3.8 pmole/well), incubated for 2 h at 37°C and then washed 3 times in PBSA. The amount of antibody bound in the wells was determined colorimetrically by incubating

100 μ l of a secondary antibody (alkaline phosphatase conjugated goat anti-rabbit antibody, 1:3,000) for 1 h and then adding 100 μ l of 1.0 mg/ml p-nitrophenyl phosphate in substrate buffer (0.001% MgCl_2 , 10% diethanolamine, pH 11). Signal quantitation was measured by absorbance at a wavelength of 405 nm (Biomek 1000 automated laboratory workstation).

Preparation of a Synaptic Vesicle-Enriched Fraction (LP2)

A subcellular fraction enriched in synaptic vesicles (LP2) was prepared from the rat brain according to the procedure described by Huttner et al. (1983). Briefly, one fresh brain of a male Sprague-Dawley rat (Harlan Sprague-Dawley, Inc., Madison WI) was homogenized with a Teflon-glass homogenizer in 10 volumes of ice-cold homogenization buffer (HB: 0.32 M sucrose, 10 mM HEPES-NaOH, pH 7.4, 0.1 mM EDTA, 1 mM PMSF, 1 μ g/ml leupeptin). The homogenate was centrifuged at 800 x g for 10 min, and then the supernatant was centrifuged at 9,000 x g for 15 min. The resulting pellet (P2) was resuspended in 20 ml HB, centrifuged at 10,200 x g for 15 min to form a synaptosome-rich pellet (P2'). P2' was resuspended in 1.6 ml HB, mixed with 10 volumes of H_2O , immediately transferred to a Dounce homogenizer and subjected to 6 up and down strokes. The above sample was then mixed with 120 μ l 1M HEPES-NaOH (pH 7.4) and kept on ice for 30 min. After homogenization and passing through a 25-gauge needle, the lysed P2' (L) fraction was subjected to centrifugation at 25,000 x g for 20 min. The supernatant (LS1) was centrifuged again at 165,000 x g for 2h. The resulting pellet, LP2 (a highly synaptic vesicle-enriched fraction), was resuspended in either 2.5 ml of 40 mM sucrose (in 10 mM HEPES, pH 7.4) for equilibrium centrifugation or 1.2 ml of HB for immunoisolation of SV.

Equilibrium Discontinuous Sucrose Gradient Centrifugation of LP2

Two and one half milliliters of LP2 fraction was passed through a 25-gauge needle several times to break any aggregation before it was layered on the top of a 50 mM to 1 M equilibrium discontinuous sucrose gradient (30 ml) in an Ultra-ClearTM

centrifuge tube (Beckman). After a 5 h-spin at 65,000 x g in a Beckman SW27 rotor, 1.5 ml fractions were collected, and the proteins were acetone precipitated and redissolved in Laemmli sample buffer. The proteins in each fraction were analyzed by 10% Tricine SDS-PAGE followed by immunoblotting with anti-synaptophysin (SY38) and anti-APP (R57) antibodies.

Immunobead Isolation of Organelles

Immunoisolation of organelles was carried out at 4°C.

A. Isolation of APP-containing vesicles (APPV). Immunoisolation of APPV was performed by incubating P1 fraction (5 mg) from the rat brain homogenate (described above) and affinity purified R57 (3.2 µg IgG) in an immunoisolation buffer (II buffer: 0.32 M sucrose, 1% BSA/PBSA, 1 mM EDTA, 1 µg/ml leupeptin, 1 mM PMSF, 1 µg/ml pepstatin) plus 20% horse serum in a total volume of 700 µl for 4 h. The mixture was centrifuged to remove unbound antibodies on a discontinuous sucrose gradient (0.8 M to 2 M), and the resulting membrane band on the gradient interface was collected, mixed with 700 µl II buffer and centrifuged again for 30 min at 100,000 x g. The membrane pellet containing both free vesicles and vesicle-antibody complexes was resuspended in 700 µl II buffer with the aid of 1-ml mini glass homogenizer (Wheaton). The sample was then incubated with 100 µl magnetic beads crosslinked with sheep anti-rabbit IgG(M-280; 50% slurry; Dynal) overnight with constant rotation. The beads were retrieved using a magnetic particle concentrator (Dynal), washed with II buffer five times, and treated with 50 µl Laemmli sample buffer. The eluted proteins were then subjected to 7/11% Tricine SDS-PAGE, silver staining and immunoblotting.

B. Isolation of synaptic vesicles. A modified immunoisolation procedure (Burger et al., 1989) was used. Coupling of anti-synaptophysin antibody (SY38) to the magnetic beads was achieved by mixing 0.4 ml of SY38 (4 µg IgG) overnight with 120 µl of magnetic beads coated with sheep anti-mouse antibody (M-280; 50% slurry; total 12 µg sheep IgG; Dynal). The beads were then washed four times in PBS containing 10 mM EGTA

and 0.1% BSA (pH 7.4), and resuspended in 50 μ l of the same buffer. The SY38-coupled beads were incubated for 4 h at 4°C with LP2 fraction (160 μ g protein), which had been pretreated with 10% sheep serum to saturate non-specific antibody binding sites. The final total reaction volume was 0.5 ml in II buffer plus 10% sheep serum. The beads were retrieved and processed for analyses as described for APPV isolation. As a control, an identical amount of anti-human factor VIII IgG (4 μ g), preabsorbed on the magnetic beads, was incubated with LP2 under the same conditions. Another control was conducted by incubating SY38-coated beads with a membrane fraction of a rat kidney cortex which was prepared by centrifugation of the PNS at 100,000 x g.

Electrophoresis and Immunoblot

Proteins were resolved by either conventional 10% SDS-PAGE (Laemmli, 1970) or 7/11% Tricine SDS-PAGE (Schägger and Jagow, 1987). The proteins on the gel were detected by Coomassie Blue staining or silver staining. For immunoblotting, the proteins separated by electrophoresis were transferred electrophoretically to nitrocellulose membranes (0.45 micron, Bio-Rad) at constant 100 v for 1 h in transfer buffer (20 mM Tris, 150 mM glycine, 15 % methanol). Nonspecific binding sites were blocked by incubating the membrane in blocking buffer (0.2% BSA/PBSA, 0.05% Tween-20) for half an hour. The membranes were then incubated with varying dilutions of antibodies in blocking buffer overnight, washed, and incubated with an appropriate secondary antibody conjugated to alkaline phosphatase for 1 h. The color was developed by adding 0.4 mM nitro blue tetrazolium (NBT) and 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in alkaline phosphatase buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M MgCl₂, pH 9.0). The intensity of protein staining was quantitated by densitometry using a Mirror 600 color scanner and the computer software IPLab Gel (Signal Analytics).

RESULTS

APP Is an Integral Membrane Protein in the Rat Brain

Under strongly alkaline conditions ($\text{pH} > 11$), biological membranes have been shown to be disrupted to open sheets, and to be stripped of peripheral proteins (Fujiki, 1982). We used this method to distinguish whether brain APP is an integral protein, a soluble protein or a peripheral protein. In the vesicle fraction (P1) from rat brains, there were two protein bands of 97 kD and 110 kD that reacted with APP C-terminal specific antibody (R57) (Fig.3, lane C). Occasionally, a third slightly longer APP band was also seen (Fig.6, lane 2). APP695 is the major isoform expressed in the brains of adult rats (Higgins et al., 1990). The two prominent APP bands might represent full-length APP695 with different post-translational modifications. Upon alkaline extraction, nearly all APP was recovered in the membrane pellet (Fig.3, lane P), whereas only a small fraction of APP was present in the supernatant (Fig.3, lane S). The result indicates that APP molecules exist as integral membrane proteins after their synthesis on ribosomes on the rough endoplasmic reticulum.

Protease Sensitivity of the Vesicular C-Terminal Domain of APP

Because vesicles are closed structures with a defined cytoplasmic side out orientation, proteins and polypeptide domains exposed on the outside of vesicles can be selectively digested by proteases, while the protein domain inside vesicles are protected from digestion. Proteinase K was selected as a probe to study APP topology. Proteinase K cleaves proteins at aromatic and hydrophobic aliphatic amino acid residues. Due to the relatively frequent and uniform distribution of these residues in most parts of the C-terminal domain of APP, digesting APP with proteinase K should result in the loss of 41 amino acids from this domain if it resides in cytosol. As shown in the immunoblot (Fig.4), the C-termini of APP species were very sensitive to the proteinase K digestion, and disappeared after 1 h treatment with 0.2 mg/ml of

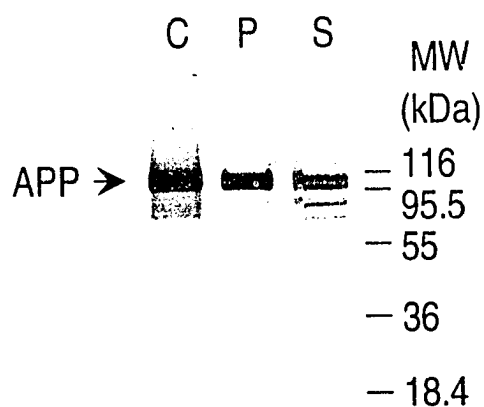


Figure 3. Alkaline extraction of APP within vesicle membranes of the rat brain. Vesicle fraction (P1) from rat brain homogenate was treated with Na_2CO_3 alkaline solution (pH 11.5). The extracted proteins (lane S) and the proteins remaining in the vesicle membrane (lane P) were separated by centrifugation, subjected to 10% SDS-PAGE and immunoblotted with antibody for C-terminus of APP (R57). Untreated P1 fraction was served as a control (lane C). The position of the two APP bands are indicated by *arrow*.

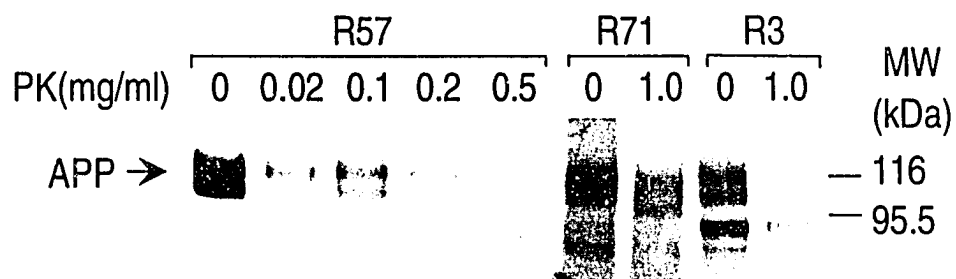


Figure 4. Proteinase K protection assay. A vesicle fraction (P1) from the rat brain was digested with proteinase K at different concentrations for 1 h at room temperature. The proteins from the treated vesicles were then resolved by 7/11% discontinuous Tricine SDS-PAGE, transferred to a nitrocellulose membrane and detected with antibodies against different regions of APP: R57 for the last seven residues of C-terminus; R71 for N-terminus and R3 for a membrane-adjacent C-terminal region. Note the increased mobility of the proteinase K-digested APP with respect to the intact APP (compare the two lanes probed by R71).

proteinase K, which was indicated by the lack of immunoreactivity of the digested APP to an antibody (R57) directed to the last seven amino acids of the C-terminal domain. However, the N-termini, as detected with antibody R71, were relatively intact even after the digestion with proteinase K with 5 times the amount of the enzyme that effectively cleaved the C-terminal tail (Fig.4). In the presence of the non-ionic detergent Triton X-100, the N-terminal domain of APP was removed by proteinase K (data not shown), indicating the inherent sensitivity of this domain to the proteinase. In addition, the APP in the proteinase K-treated vesicle preparation also lost the epitope for antibody R3 which recognizes a C-terminal region adjacent to the membrane (residues 649-671 of APP695) (Fig.4). Thus, most of the C-terminal domain of vesicular APP was removed by proteinase K. The resulting molecular weight shift of APP was apparent as demonstrated by the electrophoretic mobility change of APP (Fig.4, compare the two lanes probed with R71). This 4 kD apparent molecular weight reduction suggested that at least 40 amino acids were removed from the 47 amino acid long-C-terminal tail.

The existence of only one APP fragment with molecular mass 4 kD lower than intact APP after proteinase digestion also excludes the possible multiple-fold spanning of APP because more digested APP fragments with much smaller molecular weights would be generated if APP should cross membrane more than once. Trypsin digestion of the membrane fraction from rat brains produced similar results except that the C-terminal tail of the lower APP band (97 kD) was less sensitive to the proteinase than the upper APP band (110 kD) (data not shown). Thus, brain APP is inserted into the membrane with its N-terminus inside the vesicles equivalent of the extracellular space or lumen of intracellular organelles because of its resistance to protease digestion, and with its C-terminus facing outside of vesicles equivalent to cytosol *in vivo* because of its sensitivity to proteinases.

Accessibility of the C-Terminal Tail of Native APP in Vesicles for Sequence-Specific Antibody Binding

The localization of the C-terminus of APP on the outside of the vesicle membrane from rat brain homogenate made it possible to isolate vesicles containing APP or/and its C-terminal fragments (APPV) using the C-terminus-specific antibody (R57). Analysis of these vesicles should provide valuable information on APP trafficking and processing in the brain. We thus conducted the following assay to characterize the antibody binding to APPV. C-terminus specific antibody (R57) was incubated with rat brain vesicles, and then the unbound antibodies were washed out. Quantitative analysis (Fig.5A) showed that the amount of bound antibody increased with the input amount of antibody. In contrast, pre-immune rabbit serum, serving as a control for nonspecific binding, bound to the vesicles very weakly at concentrations below 110 pmole per assay volume. But the preimmune IgG binding increased quickly at concentrations above 500 pmole pre assay volume. In order to determine the specificity of R57 in the binding assay, we used a competitive ELISA, in which the peptide encompassing R57 epitope was bound to microtiter wells, and incubated with R57 in the presence of APP-containing vesicles. If the vesicles bound R57 specifically, they should compete with the peptide for antibody binding. As the amount of native vesicular APP incubated with R57 increased, less antibodies were left bound to the peptide on the microtiter plate (Fig.5B). Thus, R57 recognizes a competing epitope, presumably the very end of the C-terminus of native APP, in the membrane vesicles from the rat brain. To exclude the possibility that the inhibitory effect of APP-containing vesicles on the binding of antibody to the immobilized peptide was caused by nonspecific binding of the vesicle membranes, pre-immune serum from the same rabbit used for generation of R57 was included in the competitive ELISA. Theoretically, the pre-immune IgG should displace any R57 from the nonspecific

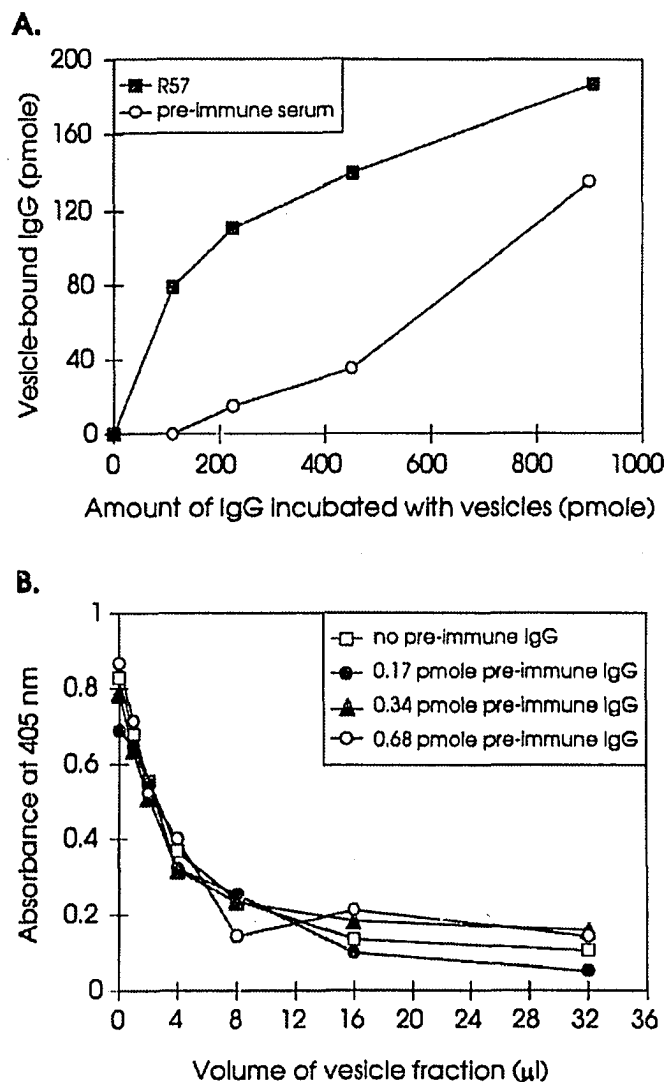


Figure 5. Sequence-specific antibody binding to APP-containing vesicles. (A) Vesicle binding profile of the C-terminus-specific antibody (R57). Different concentrations of R57 were incubated with vesicles (P1 fraction, estimated 32.2 pmole APP) from rat brains as described in MATERIALS AND METHODS (PART I). The vesicle-bound antibody was detected on immunoblots with a goat anti-rabbit antibody and quantitated by densitometry. As a control, the assay was also performed with the pre-immune serum from the same rabbit that generated R57. (B) Competitive ELISA with a synthetic peptide and native vesicular APP. A peptide corresponding to a portion of the C-terminal tail of APP encompassing the epitope for R57 was immobilized in microplate wells (3.8 pmole/well), and incubated with R57 (0.34 pmole IgG) in wells containing increasing amounts of vesicles (P1) from rat brain, in the presence or absence of pre-immune serum IgG. After 2 h, the microplate wells were emptied and processed by ELISA. The peptide-bound IgG was quantified by measuring the absorbance at 405 nm.

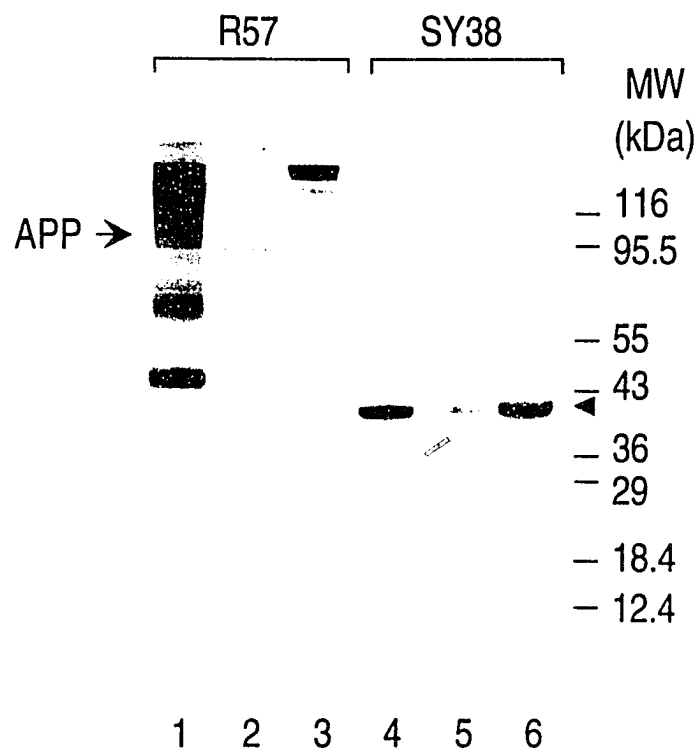


Figure 6. Synaptophysin in APP-containing vesicles. Immunoisolation of APP-containing vesicles (APPV) was performed by incubation of P1 fraction (5 mg) from rat brains and R57 (3.2 μ g) for 4 h at 4°C followed by centrifugation to remove unbound R57. Magnetic beads crosslinked with anti-rabbit IgG were used to retrieve the R57/APP complex. The APPV bound to the beads were analyzed by 7/11% Tricine SDS-PAGE and immunoblotting with R57 and anti-synaptophysin antibody SY38, as marked at the top of the blot. Serving as a control, the same amount of magnetic beads was incubated with P1 without R57. Lanes 1,4: APPV (7.8 μ g); lanes 2,6: P1 fraction (55 μ g); lanes 3,5: control beads without R57 input. *Arrow head* indicates synaptophysin. The smear on the top of the APP bands in lane 1 is due to APPV-bound R57 which crossreacted with the secondary antibody.

TABLE 1. Enrichment of Synaptophysin and APP During Purification of APPV and SV^a

	Fraction	Synaptophysin	APP
APPV isolation	P1	1.0	1.0
	APPV	34	21
SV isolation	PNS	1.0	1.0
	LP2	10	3.0
	SV	110	5.0

^a Subcellular fractions were obtained and are designated as described in Materials and Methods (Part I) and in the text. The amount of synaptophysin and of APP in each fraction was determined by densitometry of the corresponding band after PAGE-SDS and immunoblotting. The value of enrichment factor reflects relative ratio of synaptophysin or APP to the total protein in each fraction, and is set arbitrarily here.

binding sites on vesicles, and more free R57 would be available to the peptide. As shown in Fig.5B, even when the concentration of the pre-immune IgG was twice that of R57, the pre-immune IgG clearly could not reduce the inhibitory effect of APP-containing vesicles on the binding of R57 to the peptide.

The sequence-specific antibody binding assay and the competitive ELISA establish three points. First, there is an epitope on the vesicles that is recognized by R57 and is competitive with the peptide; the binding of the antibody to the vesicles is quite specific. Second, since R57, which is selectively directed against the last seven amino acids of the APP C-terminus, binds to the vesicles, the C-terminus must be exposed to cytosol; this corroborates the conclusion from the proteinase protection assay. Third, the last seven amino acids of the APP C-terminus are not folded or buried in a secondary or tertiary structure, and are recognized by R57 and are accessible for the antibody binding.

Synaptophysin in APP-Containing Vesicles

Based on the finding that C-terminus-specific antibody (R57) can recognize these vesicular APP C-termini in their native conformation with high specificity as

described above, APP-containing vesicles (APPV) were isolated from the rat brain homogenate using R57. The antibody was immobilized on the magnetic beads during the immunoisolation of APPV. This method avoids the potential contamination caused by other sedimentable components because organelles are separated by the magnetic property of the beads rather than sedimentation (Howell, 1989). An immunoblot of the proteins of the isolated APPV probed by R57 showed the typical two to three APP bands of the rat brain (Fig.6, lane 1). The high background is due to the R57 bound to the APP-containing vesicles, which reacted with the second antibody used to stain the immunoblot. Because of the low protein loading, the intensity of the APP bands in lane 1 appears weak; however, when calculated in terms of APP content per total protein, APP was enriched 22-fold in the APPV fraction compared to the starting P1 fraction (Table 1). In contrast, the magnetic beads themselves bound negligible amounts of APPV (Fig.6, lane 3). This means that the beads bound specifically to APP-containing vesicles or their derived membrane. When the proteins of APPV were probed with an antibody (SY38) against synaptophysin (a major synaptic vesicle resident protein), a prominent immunoreactive band of 38 kDa appeared on the blot, indicating the presence of synaptophysin in APPV (Fig.6, lane 4). Compared with the starting material P1 fraction, synaptophysin was enriched 34-fold in APPV (Table 1). At steady state, synaptophysin is mainly found in neurotransmitter-containing and electron-lucent synaptic vesicles (SV) at presynaptic sites and neuromuscular junctions (Wiedenman et al., 1985; Navone et al., 1986). These data suggest that synaptic vesicles might correspond to part of APP-containing vesicles.

Coexistence of APP and Synaptophysin in the Synaptic Vesicle-Enriched Fraction

We further investigated the relationship between synaptic vesicles (SV) and APP by analyzing a synaptic vesicle-enriched fraction (LP2) obtained from rat brain. This SV-enriched fraction was subjected to an equilibrium discontinuous sucrose density gradient centrifugation (50 mM to 1 M). This procedure further separated SV in LP2

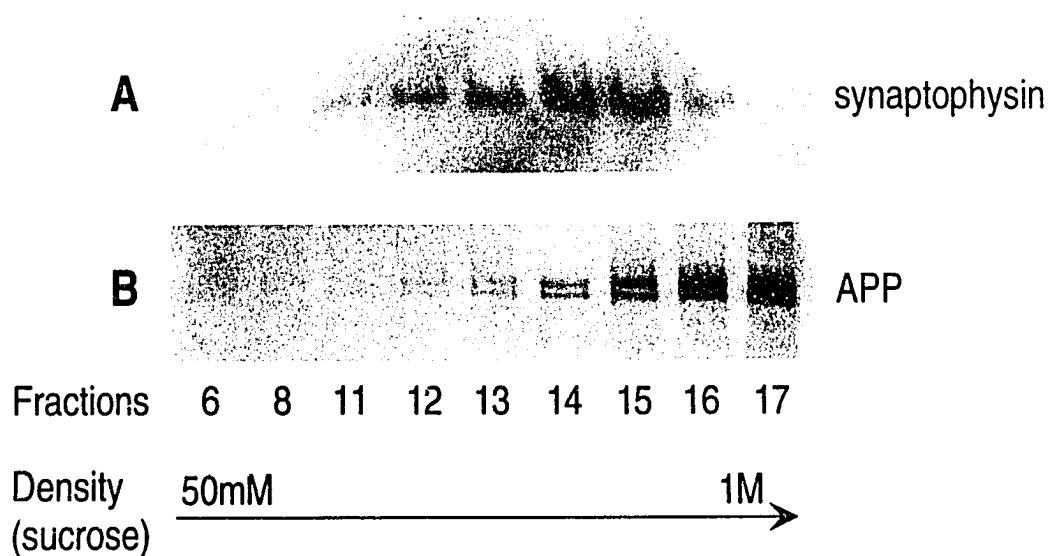


Figure 7. Coexistence of APP and synaptophysin in the synaptic vesicle-enriched fraction. A synaptic vesicle-enriched fraction (LP2) was prepared from the homogenate of one rat brain. LP2 was subject to an equilibrium discontinuous sucrose gradient (50 mM-1 M), and 17 fractions (1.5 ml each) were collected. The proteins in each fraction were acetone precipitated, analyzed by 10% Tricine SDS-PAGE, and immunoblotted with (A) anti-synaptophysin antibody SY38, and (B) anti-APP antibody R57. The fractions are numbered from the top to the bottom of the gradient. The peak of synaptophysin can be seen in fractions 14 and 15 in which APP also co-exists.

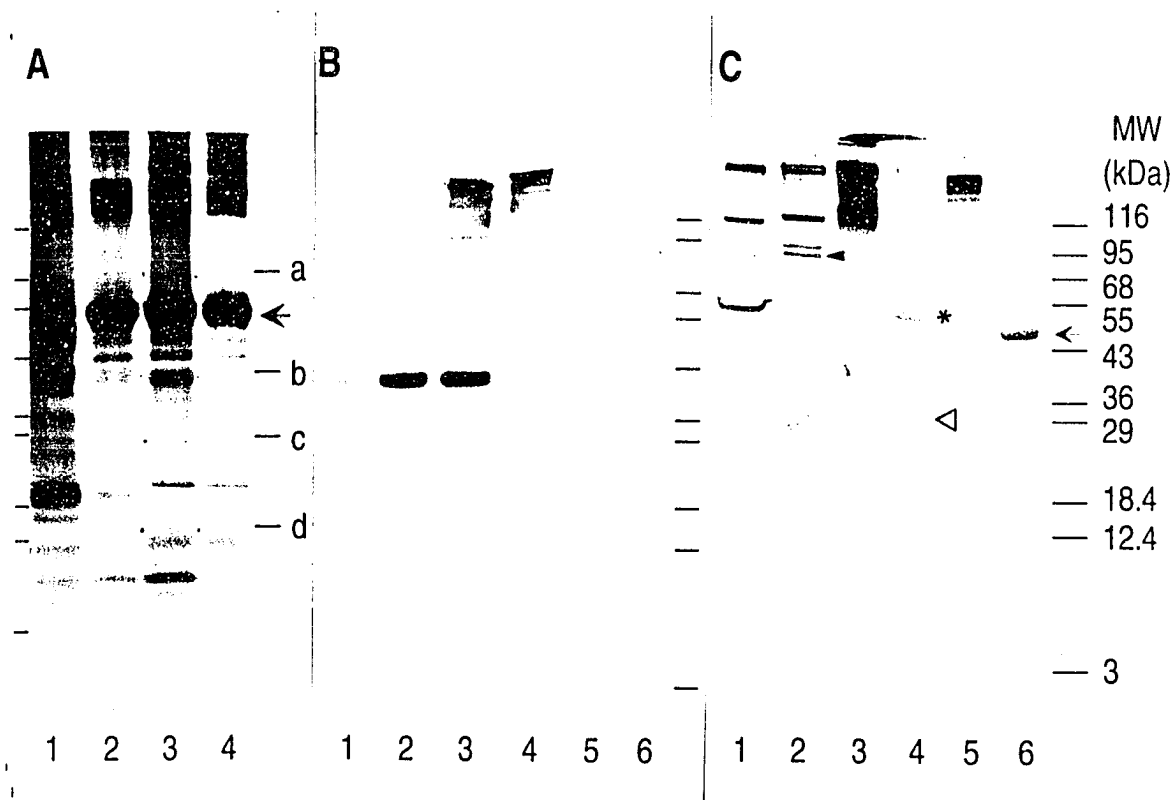


Figure 8. Immunoblotting of synaptic vesicles from rat brains. Synaptic vesicles were immunoprecipitated from the LP2 fraction of rat brains using SY38-coupled magnetic beads. Beads coated with anti-human factor VIII IgG (control beads) were also used for the isolation under the same conditions. The proteins of the vesicles bound on the beads were separated by 7/11% Tricine SDS-PAGE followed by various analyses. The protein profile of the isolated SV (1 µg) was visualized by silver staining (A lane 3) and immunoblotting stained for synaptophysin with SY38 (B lane 3). For comparison, the protein profiles of the two starting materials PNS (20 µg; A,B: lanes 1) and LP2 (6 µg, A,B: lanes 2), as well as that of the control beads (A,B: lane 4) were included. The positions of bands corresponding to the known SV proteins are indicated (A): a, synapsin Ia/b; b, synaptophysin; c, p29; d, synaptobrevin. Arrow in (A) points to the position of the sheep serum albumin present in the immunoprecipitation buffer. As controls, the proteins from either the SY38-coated beads that were incubated with a vesicle fraction from a rat kidney cortex (B: lane 5) or the kidney vesicle fraction (30 µg; B: lane 6) were also analyzed by immunoblotting with SY38. (C): Evaluation of the contamination of various subcellular organelles in the isolated SV with antibodies directed for Na⁺/K⁺ATPase (plasma membranes, lanes 1,2), Cathepsin D (lysosomes, lanes 3,4), and β-tubulin (microtubules, lanes 5,6). SV: lanes 1,3,5; PNS: lanes 4,6; LP2: lane 2. Arrowhead points to the position of ATPase; Asterisk and open triangle indicate pro-Cathepsin D and subunit (34 kDa) of active Cathepsin D respectively; Arrow shows the position of β-tubulin. The strong bands in lanes 3 and 5 are due to the antibodies bound to the beads.

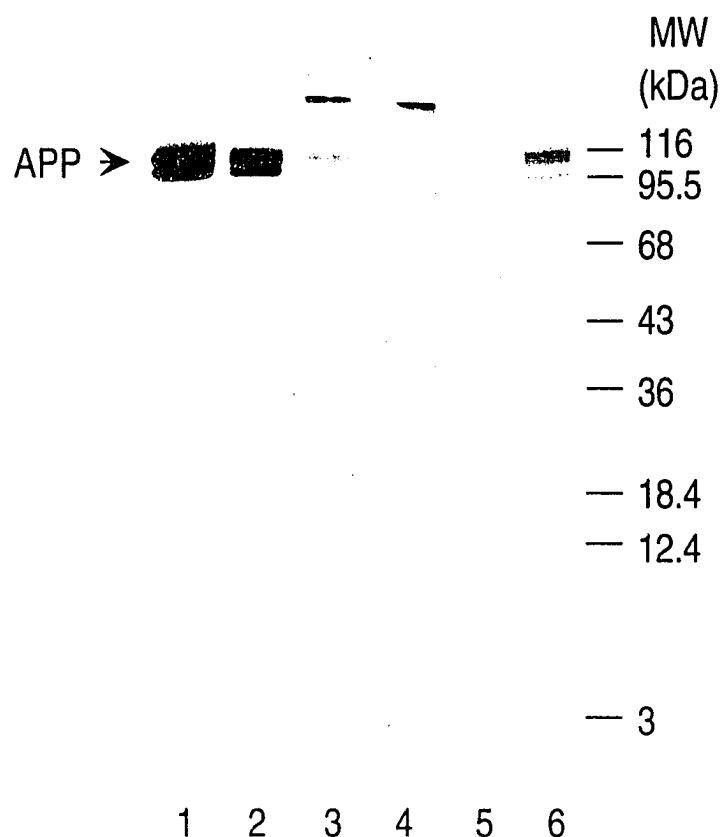


Figure 9. APP in immunoisolated synaptic vesicles. The immunoisolated SV described in Fig.6 was subjected to 7/11% Tricine SDS-PAGE followed by immunoblotting with anti-APP antibody R57. For comparison, other fractions were used with the same arrangement as in Fig.6B. 1. PNS (20 µg); 2. LP2 (6 µg); 3. SV (1 µg); 4. control beads; 5. SY38-coated beads incubated with a vesicle fraction from the rat kidney cortex; 6. a vesicle fraction from the rat kidney cortex.

from other organelles. The fractions collected from the gradient were checked with anti-synaptophysin antibody (SY38), and showed that the SV marker protein was mainly localized in fractions with density of 0.4-0.6 M sucrose (Fig.7A); this agrees with the buoyant density of SV reported in the literature (Huttner et al., 1983). Staining the fractions with anti-APP antibody (R57) revealed that a portion of membrane-associated APP co-localized with synaptophysin, although most APP appeared to reside in higher density fractions (1M sucrose) (Fig.7B). The co-existence of APP and synaptophysin in the same fractions supports the notion that APP might be a synaptic vesicle protein. However the presence of APP in other vesicles with the same density as that of SV can not be ruled out by this experiment.

APP in Immunoisolated Synaptic Vesicles

To determine definitively whether APP is present in SV or not, highly purified SV were isolated from SV-enriched fraction (LP2) with an immunoisolation technique similar to that used for isolation of APPV. The technique is based on the fact that anti-synaptophysin antibody (SY38) can specifically recognize and bind to the cytoplasmic domain of synaptophysin in SV and thus it is possible to immunoisolate SV by SY38 bound to the magnetic beads. The protein profile of the purified SV was clearly different from those of the post-nuclear supernatant (PNS) and LP2 (Fig.8A: lanes 1 and 2, respectively). Most of the major SV proteins (synapsin, synaptophysin, synaptobrevin and p29) were recognized by silver staining that results in a protein pattern similar to that described previously for SV isolated by conventional procedures (Huttner et al., 1983). The immunoisolated SV preparation was 10-fold more enriched in synaptophysin than LP2 (Fig.8B and Table 1). Contamination by other organelles like lysosomes, microtubules and plasma membrane could not be detected by staining the immunoblot with the antibodies against marker proteins for these subcellular organelles (Fig.8C). No binding of synaptophysin-positive vesicles to the beads conjugated to a control IgG (anti-human factor VIII monoclonal antibody) (Fig.8A,B,

lane 4) or to unconjugated beads (data not shown) was observed. These data indicate that nonspecific binding to the magnetic beads used in this study was minimal at best, and that the immunisolated SV were extremely pure and virtually free of contamination by other subcellular organelles.

Immunoblots of the proteins from SV with anti-APP R57 revealed an APP-immunoreactive band (Fig.9, lane 3); this supports the results from the APPV study that SV contained APP. Interestingly, only the 110 kD APP appeared in the SV preparation, whereas the smaller 95 kD APP was hardly detected (compare lanes 2 and 3 in Fig.9). This might reflect the differential localization of different APP species in subcellular compartments; alternatively, if the 110 kD species is mature APP and the 95 kD immature, only the mature APP should be expected to enter SV. Due to the limited amount of anti-synaptophysin antibody SY38 (4 μ g IgG) used for SV immunoisolation, the isolated SV only yielded 1 μ g detergent-soluble protein for analysis by SDS-PAGE. The low protein loading on the gel thus accounts for the weak staining of SV-located APP by anti-APP antibody R57. However, when the enrichment factor (APP/total protein) is calculated, APP was enriched 5-fold over the starting material PNS (Table 1).

To exclude the possibility that the APP bound on the immunobeads came from non-SV APP-containing vesicles, the beads coated with anti-synaptophysin (SY38) were incubated with the post-nuclear supernatant (PNS) prepared from a rat kidney cortex. The kidney cortex contains moderate levels of an APP isoform (116 kD) (Fig.9, lane 6), and undetectable amount of synaptophysin (synaptic vesicles) (Fig.8B, lane 6). The beads could not pull out any protein immunoreactive with R57 from the kidney cortex (Fig.9, lane 5). This indicates that APP bound on the beads that were incubated with LP2 fraction must represent the APP in SV. In addition, the beads coated with the control IgG (anti-human factor VIII) failed to bind either APP (Fig.9, lane 4) or SV (Fig.8, lane 4), further confirming the high specificity of SY38-coated beads used in

SV isolation. Taken together, our data strongly suggest that a small proportion of brain cellular APP pool (10-15%) is present in the synaptic vesicles in the rat brain.

DISCUSSION

Instead of direct isolation and analysis of all major subcellular organelles, we investigated the APP intracellular localization by analysis of APP-containing vesicles (APPV) immunoisolated by a sequence-specific antibody. In order to employ this strategy, we first determined the APP topology in rat brains. By alkaline extraction and proteinase protection assay, we demonstrated that most of the APP in rat brains were integral membrane proteins, which were inserted as type I membrane proteins, with their N-termini in the lumen of the secretory system and their C-termini in the cytosol. This confirms the topology prediction from the hydrophilicity profile of APP (Kang, 1987) and the results from an *in vitro* translation study (Dyrks, 1988). Our data also support the findings of Schubert et al. (1991), who claimed that the major APP form in rat brain is membrane bound. However, our results argues against the conclusion of Loffler and Huber (1992) that most of β -APP forms in rat brain are soluble proteins.

Both antibody binding assays and a competitive ELISA showed that the C-terminal tail of APP was in a relaxed conformation accessible to antibodies directed to the tail. The recognition and binding of the APP C-terminal tail-containing vesicles (APPV) by the antibody R57 were highly specific, which limited contamination by other APP-negative vesicles. The presence of synaptophysin, a synaptic vesicle marker, in APPV purified from rat brains, prompted us to further analyze the relationship between synaptic vesicles and APP. The co-existence of APP and synaptophysin in a SV-enriched fraction and the presence of APP in highly purified SV unambiguously pointed out that some APP molecules are targeted to SV. For the following reasons, it

is unlikely that the localization of APP in SV could result from the fusion between APP-containing vesicles and SV during SV preparation, or contamination of SV fraction by other subcellular organelles bearing APP: (1) Physiologically relevant membrane fusion needs appropriate temperature and energy (Colombo et al., 1992). The conditions used in the immunoisolation prevents possible membrane fusion incurred by manipulation of the brain tissue; (2) The anti-synaptophysin antibody (SY38) has been well characterized regarding its binding specificity and it has been widely used in immunoisolation of SV (Wiedenmann and Franke, 1985; Burger et al., 1989; Walch-Solimena et al., 1993); (3) We used magnetic beads as a matrix for immobilizing SY38 to avoid contamination caused by sedimentation of non-SV vesicles, thereby further increasing the purity of our SV preparation. Although we have not evaluated the isolated SV morphologically by electron microscopy, the various biochemical assays confirmed the high purity of SV.

It seems likely that these APP are resident SV proteins. Compared to some resident SV proteins such as synaptophysin, which has a half-life of about 1 day (Regnier vigouron et al., 1991), APP in rat brains has a half-life of 10 h (Potempska et al., 1991). However, it is noteworthy that the estimated turnover rate of rat brain APP is an average value from different APP populations both in individual cells and in heterogeneous brain cells including microglia and astrocytes ($t_{1/2}$ of 30-45 min) (Haass et al., 1991). Hence, a subpopulation of brain APP should have a much longer life time, comparable to that of synaptophysin; these long-life APP might be the APP residing in SV.

Ten to fifteen percent of brain APP was found to reside in SV. Given the fact that the total brain APP comes from both neuronal and non-neuronal cells, such as astrocytes and microglia that express substantial amounts of β APP, the SV-residing APP should constitute a higher percentage of APP within neurons. Nevertheless, each synaptic vesicle is likely to contain a very low amount of APP, in contrast with multiple copies of the major SV membrane proteins like synaptophysin and synapsin, which contribute each ~7% of synaptic vesicle proteins and 0.3% of brain proteins in rats (Jahn and Südhof, 1993). SVs are among the smallest membranous organelles known, with an average diameter of 50 nm. It was estimated that the phospholipid/protein mass ratio in SV is 3:1, and that the membrane of one SV cannot contain more than 10-15 different protein species in addition to the proton pump (Jahn and Südhof, 1993). Taking into consideration that there are a dozen synaptic vesicle proteins already discovered and most of them are oligomers, any other SV protein must be far less abundant. Therefore, being a large membrane protein, it is perhaps not surprising that APP could be present only as a minor constituent of SV.

In addition, we speculate that APP would be present preferentially in SV of disparate groups of neurons throughout the rat brain. APP is differentially expressed in the various regions of the brain, being most abundant in the hippocampal pyramidal cell layer, amygdala, parahippocampal gyrus, cerebellum and the cerebral cortex (Card et al., 1988; Mita et al., 1989; Löffler and Huber, 1992). Moreover, the relatively small amount of brain APP (0.04% total rat brain protein, Potempska et al., 1991) compared to the vast amount of synaptic vesicles (10 μ M in human brain) (Jahn and Südhof, 1993)

precludes the possibility that APP is a ubiquitous component of SVs in all types of neurons. Rather, APP might serve as a modifier of SV in the neurons expressing high levels of APP within the disparate brain regions, in order to allow subtle adaptation of these neurons to special tasks (discussed below).

α pp shares several features with the known major SV proteins: isoforms from alternative splicing, post-translational modifications like glycosylation and phosphorylation, and evolutionary conservation of its primary structure from *Drosophila* to humans. However, APP, at variance with SV proteins, has a widespread distribution in a variety of tissues besides the nervous system and endocrine tissues (Neve et al., 1988; Tanaka et al., 1989), which suggests that it may have multiple functions. The relative importance of this difference is, at present, unknown because a synaptophysin-like protein was also found in nonneuronal cells (Zhong et al., 1992).

Our finding contrasts with several earlier reports. Morin et al. (1993) have shown that APP and synaptophysin are in two different populations of rapid-transport vesicles in the rabbit optic nerve. Likewise, Ferrira et al. (1993), using an immunohistochemical technique, observed that APP did not colocalize with synaptic sites in 30-d-old hippocampal cultures. Using immunofluorescence, laser confocal microscopy and immunoelectron microscopy with simultaneous detection of APP and synaptophysin, Schubert et al. (1991) reported a preferential localization of APP at synaptic sites of human and rat brain and neuromuscular junctions. However, an EM study conducted by the same authors pointed to different locations for APP and synaptophysin at presynaptic sites. Although these morphology studies are helpful for

defining the APP distribution within cells, the low sensitivity of the technique may compromise the conclusions drawn from the studies. Considering that only a minor proportion of intracellular APP pool is in SV, and each SV may contain one copy of APP, the APP signal in SV must be extremely weak. In addition, both APP and synaptophysin are particularly sensitive to glutaraldehyde fixation (Schubert et al., 1991; Hoog et al., 1988; Currie et al., 1991), so that the labeled structures in the fixed brain sections and cultured neurons probably represent only the sites of high antigen concentration, whereas lower concentrations of the antigens might have escaped the detection. It is also not unusual for anti-APP antibodies to bind nonspecifically to various structures in brain sections, making it difficult to interpret the data (Dr. J.R. Currie, personal communication). Direct isolation and biochemical analysis of subcellular membrane structures, such as SV and APPV, circumvented the problems inherent in the histochemical studies, allowing more sensitive and accurate detection of intracellular APP. On the other hand, rat synaptic vesicles had been prepared from density equilibrium and found to contain little APP (Shimokawa et al., 1993). However, we consistently observed immunoreactive APP bands in SV. We attribute our detection of APP in SV to the high sensitivity of anti-APP antibody employed in our studies.

APP has been proposed to have an intracellular function besides serving principally as a cell surface protein and a precursor for soluble APP derivatives or A β (Haass et al., 1991). The appearance of APP in SV raises the question about its function regarding SV biology. Synaptic vesicle membranes are recycled for use at least 20 times, and possibly many hundreds of times before being removed from the nerve

terminal (Ceccarelli and Hurlbut, 1980). The small number of protein molecules in SV membrane requires the protein sorting to be highly accurate during recycling. It was discovered that several SV proteins did not float freely in the membrane but rather formed a loose sorting complex as a single entity during the SV cycle (Bennett et al., 1992b; Johnston and Sudhof, 1990). APP also undergoes recycling. It has been shown to be localized in purified synaptic plasma membrane (Shimokawa et al., 1993). Furthermore, APP binds to a trimeric G protein subunit G_O (Nishimoto et al., 1993) which may be involved in a post-endocytotic sorting of membrane proteins into SV in neurons (Bomsel et al., 1992). It is thus tempting to postulate that APP is part of the superstructure of SV proteins and could serve as a sorting signal mediated by G_O . We tested this hypothesis by trying to immunoprecipitate the putative APP-synaptophysin complex from mild detergent-solubilized SV proteins (CHAPS, octylglucoside or Triton X-100), using anti-APP or anti-synaptophysin antibody. However, no such complex was observed (data not shown). This suggests that APP does not belong to the multimeric protein complex, and, hence, APP plays no role for the formation or function of the sorting complex as synaptophysin does (Johnston and Südhof, 1990).

So, why do special groups of neurons need APP in their SVs? Although SVs are generally homogenous with respect to protein composition, there is protein isoform variety in SVs of different groups of neurons (Jahn and De Camilli, 1991). In electric fish brains, the SV2 protein of SV contains a unique modification on its keratan chains that is specific for electromotor neurons; this neuron type-specific glyco-modification may be involved in nerve terminal-extracellular matrix adhesive interaction after SV

exocytosis (Scranton et al., 1993). Likewise, SV-located APP in neurons of discrete brain regions might regulate SV activities and, thus, fine-tune these neurons to perform on some special tasks (a sort of neuronal individuality), i.e. learning and memory in the hippocampus. Indeed, APP has been implicated in such a function because behavioral abnormalities in *Drosophila* resulting from mutations of a APP-like protein were corrected by inserting the human APP gene into the mutant flies (Luo et al., 1992). Evaluating behavioral changes in the available APP-knock-out transgenic mice would clarify APP's role in learning and memory as well as in SV recycling.

Soluble A β is secreted by normal cells (Haass et al. 1992b; Seubert et al., 1992) and is possibly produced in a non-lysosomal acidic compartment (Shoji et al., 1992). The synaptic vesicle possesses a vacuolar proton pump, the V-ATPase, to generate an electrochemical proton gradient for transport of neurotransmitters to the vesicles (Maycox, 1990), and thus the acidic lumen of SV makes it a possible cellular compartment for A β formation. So far, it is unclear whether APP might undergo proteolysis in SV because no C-terminal fragments of APP or A β has been detected in our SV preparations.

Our study also suggests that most cellular APP may be localized in other membrane compartments besides SV. Fig.4 shows the appearance of large quantity of APP in high density fractions besides the SV-containing fractions. Subcellular localization of APP in secretory granules of salivary gland and anterior pituitary has been reported (Catteruccia et al., 1990). We speculate that the rat brain APP in the high density fractions might resemble APP in large dense core vesicles (LDCVs) of neurons,

the counterparts of the secretory granules of exocrine and endocrine organs. We hope to show this directly in the future.

In conclusion, we have demonstrated that a minor portion of Alzheimer β -amyloid precursor protein is located in synaptic vesicles of rat brains. The SV-located APP are probably restricted to neurons in discrete brain regions bearing high concentrations of APP, which are also the neuronal population vulnerable for neuritic plaque formation in human AD. Accordingly, APP expression and turnover might be linked to synaptic vesicle formation and degradation and neuronal plasticity in selected brain areas. Under the pathological conditions of AD, abnormal APP metabolism might lead to damage of synaptic vesicles and disruption of synapses in these brain regions (Masliah et al., 1989).

PART II

The Expression and Processing of Human β -amyloid Peptide Precursors in *Saccharomyces cerevisiae*: Evidence for A Novel Endopeptidase in the Yeast Secretory System

Materials and Methods

Antibodies were raised to synthetic peptides that corresponded in sequence to various regions of β -APP-695 (Fig. 10A): rabbit antiserum R71, residues 46-62; rabbit antiserum R57, 672-695 (epitope, 689-695); mouse monoclonal antibody (mAb) 6E10, 597-611 (epitope, 601-611); mAb 4G8, 597-619 (epitope, 613-619) (Kim et al., 1988). R104 was raised to the extracellular domain of β -APP-751 (18-667) produced by a recombinant baculovirus procedure (Ramakrishna et al., 1991). Its epitope(s) have not been determined. The synthetic peptide α -factor-K, which is α -factor bearing a C-terminal lysyl residue, was kindly provided by Prof. Fred Naider (Chemistry Dept., College of Staten Island).

Yeast and E. coli strains Yeast strains used in this study are listed in Table 2. APP expression and its processing were mainly examined in strain JSC310. This strain is a derivative of the protease-deficient strain BJ2168 (Jones, 1991). Most significantly, JSC310 contains an integrated copy of the ADR1 gene under the control of the glyceraldehyde-3-phosphate-dehydrogenase promoter. Increased levels of ADR1, the transcriptional activator of the alcohol dehydrogenase promoter, have been shown to increase heterologous protein expression levels (Denis, 1987). Plasmids were propagated in *E. coli* strain HB101 or MAX EFFICIENCY DH5 (GIBCO BRL). Transformations, plasmid isolations and DNA manipulations were performed as described by Sambrook, et al. (1988) or manufacturers' protocols. YPD medium is 1% yeast extract, 2% peptone and 2% glucose. Yeast synthetic medium (SM) is 0.67% yeast nitrogen base without amino acids (Difco), 0.05% complete supplement mixture (CSM, Bio-101) and 4 % glucose.

Expression vector constructions and mutagenesis Four plasmid constructs were made to study the expression of APP in *S. cerevisiae* (Fig.10B). The structural genes for APP695 and APP751 were isolated from a human retina cDNA library (Ramakrishna et al., 1991). To express the full-length gene product, the APP695 and APP751 coding

TABLE 2. Yeast Strains

Name	Genotype	Source or Ref.
JSC310	<i>MATα leu2 ura3-52 prb1-1122 pep4-3 prc1-407 GAP-ADR1::G418</i>	Hines et al. (1994)
DS7	<i>MATα MFα1/α2::LEU2 ade2 his3 leu2 trp1 ura3</i>	Bourbonnais et al. (1993)
YBAD1	<i>MATα YAP3::HIS3 MFα1/α2::LEU2 ade2 his3 leu2 trp1 ura3</i>	Bourbonnais et al. (1993)
RSY269/sec17-1	<i>MATα ura3-52 his4-619</i>	R. Schekman
RSY271/sec18-1	<i>MATα ura3-52 his4-619</i>	R. Schekman
RSY299/sec7-1	<i>MATα ade2 his3-11 leu2-3,112 trp1-1 ura3-1</i>	R. Schekman
RSY782/sec1-1	<i>MATα ura3-52 his4-619</i>	R. Schekman
M200-6ck	<i>MATα KEX2::URA3 ura3 ade1 ilv3 sst1 sst2</i>	M Whiteway
SY1854	<i>MATα bar1::LEU2 pFUS!::HIS3 ura3-52 leu2-3,112 ade1</i>	P. Kinsey

sequences were amplified by PCR, with an added 5' Nco I site and added 3' Sal I site, using the following primers: forward - 5'GCTCCACCATGGTGCCCGGTTTGGC3' and reverse -5'GGCGTCGACTCACTAGTTCTGCATCTGC3'. The NcoI-SalI fragment containing the APP gene was ligated to a derivative of the yeast/*E.coli* shuttle vector pAB24 (Brake, 1990) cut with BamH I/Sal I. Transcriptional control to allow APP synthesis only in the absence of glucose was achieved by fusing the structural gene with a BamH I/Nco I fragment containing the glucose-repressible ADH2/GAPDH hybrid promoter (Cousens et al., 1987). The resulting vector also contains LEU2-d and URA3 genes for selection of yeast transformants under leucine- or uracil-deficient conditions, and pBR322 sequences for amplification in *E.coli*. In the final expression vector, the expression "cassette" is flanked by the ADH2/GAPDH promoter and the α -factor terminator sequences (Barr et al., 1988). This cloning generated APP6F (APP-695 full length gene) and APP7F (APP-751 full length gene). To generate a construct fused to the full-length alpha-factor leader, the forward primer 5'GCTCTCTAG-

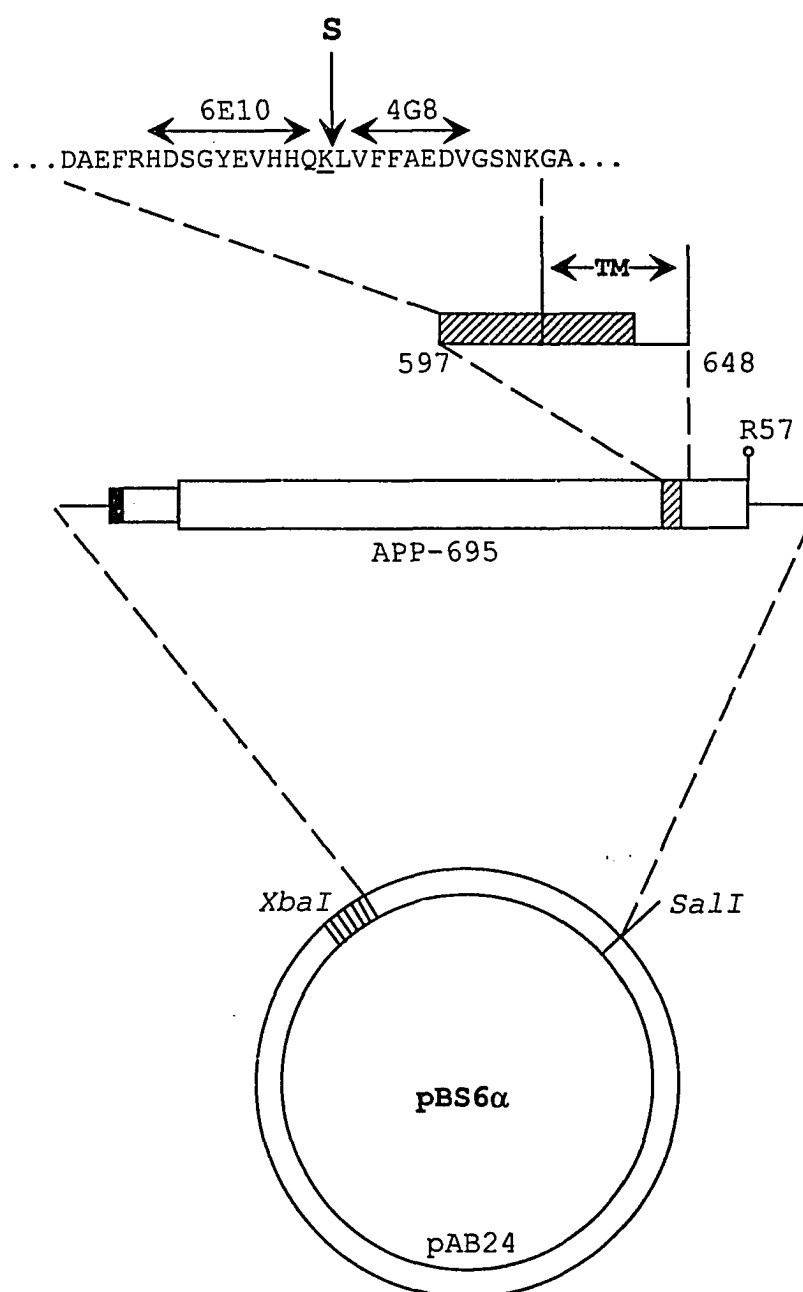
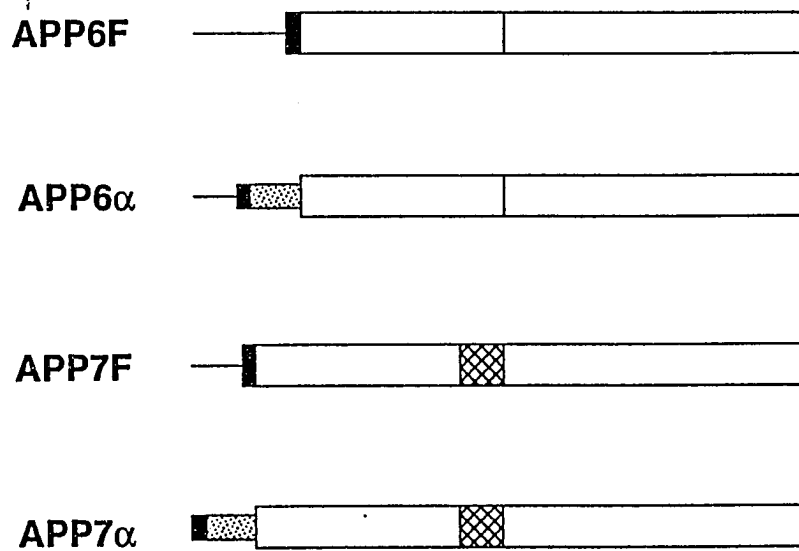


Figure 10. A. Map of plasmid pBS6α and its APP insert and locations of APP epitopes. The Aβ sequence (hatched box) extends into the transmembrane (TM) sequence. Polyclonal antibody R57 recognizes the 7 residues at the COOH terminus. mAb6E10 and mAb 4G8 recognize epitopes adjacent to the α-secretase cleavage site (S). The underlined Lys-612 was changed to Gln in plasmid pBS6α(K/Q). The dark and stippled boxes respectively denote the secretory signal sequence and the leader sequence of yeast prepro α-factor. (*continued*)



(Figure 10, *continued*) **B. The APP constructs inserted into pAB24.** Dark segments are signal sequences. Stippled segments are α -leader sequences. Hatched regions are the KPI domain.

ATAAAAGACTGGAGGTACCCACTGATGG3' was used together with the reverse primer described above. The Xba I/Sal I DNA fragment formed contained a Lys-Arg KEX2 cleavage site followed by the start of the mature APP coding sequence (L E V...). This fragment was ligated into pBS24.1 together with a BamH I/Xba I fragment containing the ADH2/GAPDH promoter and the signal-leader sequence of prepro α -factor precursor (Brake, 1990). This cloning generated APP6 α (mature APP695 fused to the prepro-alpha factor leader) and APP7 α (mature APP751 fused to the prepro alpha factor leader).

The substitution of Gln for Lys-612 (Fig.10A) was achieved by site-directed mutagenesis using PCR and the UDG cloning method devised by Rashtchian et al. (1992). Sph I-linearized plasmid pBS6 α was used as the template. The internal mutagenic primers used were the following: CP1b (reverse, 5'CACCAAUUGAUGA-UGAACTTCATA3') and CP2b (forward, 5'CAUCAACAAAUUGGUGUTCTTTGC-AG3'). The flanking primers were: CP1a (forward, 5'CUACUACUACUAGA-CGGAGGAGATCTCTGAAGTG') and CP2b (reverse, 5'CAUCAUCAUGGC-GTCGACTCACTAGTTCTG3'). The mismatched position in each oligonucleotide is underlined. The two PCR fragments amplified from the APP695 gene were treated with uracil DNA glycosylase (UDG), annealed to plasmid pAMP2 (GIBCO BRL) and introduced into MAX EFFICIENCY DH5 competent cells (GIBCO BRL). Subsequently, the APP695 mutant was subcloned into pBS6 α by insertion of the pAMP2 Bgl II-Sal I restriction fragment into the Bgl II and Sal I sites of pBS6 α . The sequence of the inserted fragment was verified by double-stranded sequencing of the plasmid templates (dsDNA cycle sequencing system, GIBCO BRL).

To generate a construct (α C) that contains α -factor fused to the C-terminal fragment of APP (Fig.11), a BamHI/XbaI fragment harboring the ADH2/GAPDH promoter and α -leader sequence was amplified from pBS6 α using the following primers: ADH1 (forward 5'CCAAGGATCCTTCAATA-TGCG3') and ADH2 (reverse

5'CTTTTATCTAGAGATACCCCT3'). A XbaI/SalI fragment was also generated by PCR, which contained the α -factor followed by lysine and APP's C-terminal fragment starting at α -secretase cleavage site. The following primers were used: AFVFOR (forward 5'GCTCTCTAGATAAAAGATGGCACTGGTTGCAATTGAAGCCAGGT-CAACCAATGTACAAATTGGTGTCTTTGCAGAA3') and AFVREV (reverse 5'GGCGTCGACTTATCTTTTGTACATTG-GTTGACCTGG3'). Both fragments were ligated to pBS24.1 at BamH I and Sal I sites to generate pBS α C.

To confirm the absence of an intact *YAP3* gene in strain YBAD1, a Southern analysis of the genomic DNA from the cells was performed. Yeast DNA was isolated following the method of Philippsen et al. (1991), and hybridized to a probe corresponding to a region contained in the *YAP3* gene but not in the homologous *BAR1* gene. The sequence of the probe was 5'CGTCAGGTTACCGCTAAAGGAAGC3' (1578 to 1601 of the *YAP3* sequence according to Egel-Mitani et al., 1990). DNA from the parent strain DS7 was run as a positive control.

Expression of APP and other chimeric proteins Plasmids were transformed into various yeast strains by electroporation (Becker and Guarente, 1991). Positive transformants were selected on uracil deficient solid synthetic medium (containing 1 M sorbitol). For expression studies, transformed cells were precultured in uracil/leucine dropout synthetic medium (ura-/leu- SM, 4% glucose) at 30°C. The cells were then either grown in YPD (2% glucose)(1:50 dilution) at 30°C for APP expression or in methionine-deficient synthetic medium (met-SM) containing 3% glucose for radiolabeling. Temperature-sensitive mutants (*sec* mutants) were grown at 22°C (permissive temperature) and assayed at 37°C (non-permissive temperature).

Analysis of APP expression Cell extracts for the initial characterization of APP expression were prepared by glass-bead extraction. The cells were grown for 48 h, harvested by centrifugation and washed once with cold lysis buffer (50 mM Tris/HCl, pH 7.5, 2 mM EDTA, 50 mM NaCl, 1 mM PMSF) and recentrifuged. The cell pellet

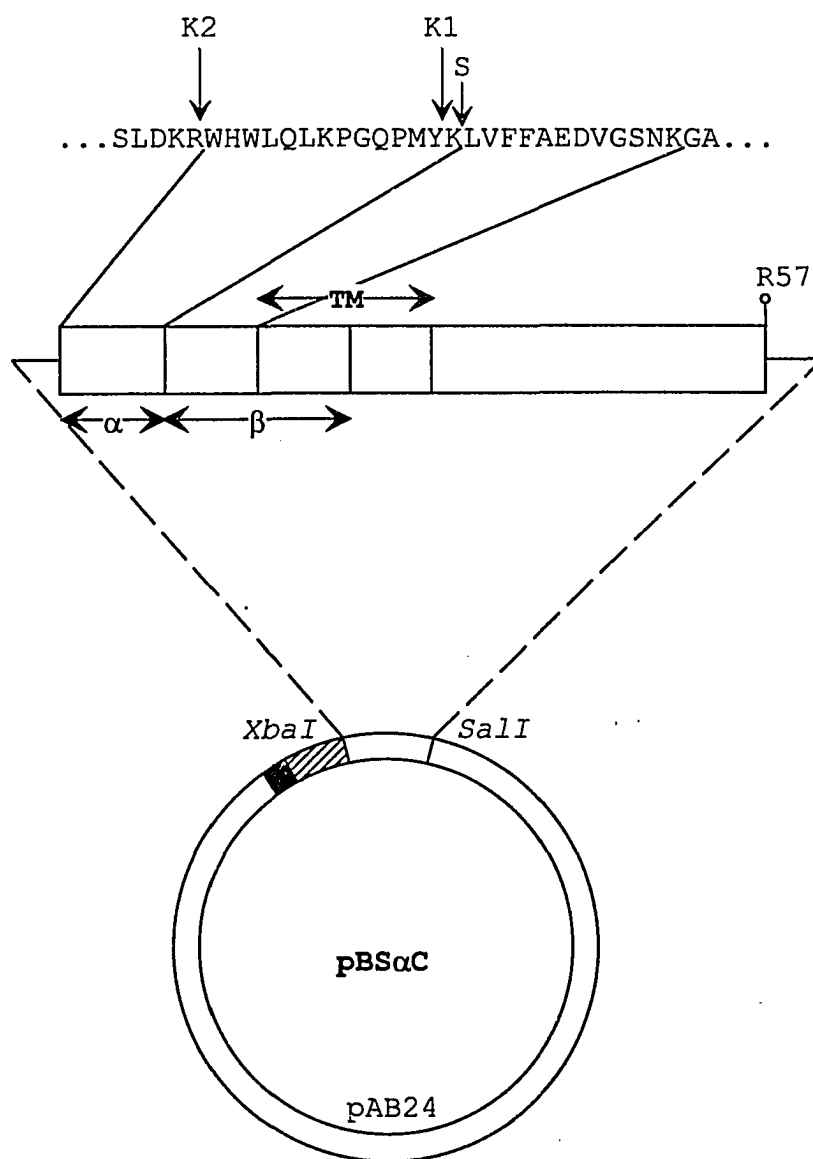


Figure 11. Schematic illustration of chimeric protein (αC) αC protein consists of the leader sequence and α-factor (α) fused to a C-terminal fragment that includes part of Aβ (β) extending into the transmembrane region (TM). The two pieces are separated by a lysine residue. Yeast α-secretase cleavage site is indicated by "S". The processing sites of Kex2p (K2) and Kex1p (K1) are shown.

was resuspended in 1 ml of lysis buffer and 0.5 g acid-washed glass beads (425-600 μ , Sigma) were added. The samples were vortexed 5 min at 4°C. The extract was removed and the glass beads were washed once with 1 ml of lysis buffer. The wash and extract were pooled and were subjected to a brief centrifugation at 1000 x g to remove nuclei and unbroken cells. The membranes were sedimented at 100,000 x g for 30 min. Both the supernatant (cytosol) and sedimented fractions were subjected to SDS-PAGE and immunoblot analysis.

For assaying the solubility of membrane-bound APP in Triton X-100, the membrane pellet prepared as above from 3.5×10^8 yeast cells was suspended in 1 ml extraction buffer (1% Triton X-100, 0.5 M KCl, 50 mM Tris-HCl, pH 7.4, and a cocktail of proteinase inhibitors, 1 mM EDTA, 1 mM pepstatin, 1 mM PMSF, 4 μ g/ml leupeptin) and incubated for 1 h at 4°C. The supernatant was obtained by ultracentrifugation at 100,000 x g for 30 min, and the proteins were precipitated by SDS-quinine sulfate. Both the precipitates of the detergent extract and the Triton X-100-insoluble material were dissolved in SDS sample buffer (2% SDS, 10% glycerol, 20 mM DTT, 62 mM Tris-HCl, pH 6.8, 0.01% bromphenol blue) and subjected to SDS-PAGE.

Immunoprecipitation of cellular APP and its C-terminal fragments A yeast cell pellet (5×10^8 cells) was dissolved in 400 μ l lysis buffer (2% SDS, 50 mM Tris-HCl, pH 7.4, and the cocktail of protease inhibitors) by agitation at 0°C with glass beads on a vortex mixer at full speed at 30 sec intervals for 4 min. The sample was heated in a boiling water for 3 min and cooled. The supernatant solution was collected, and the beads were washed once with 2 ml buffer (1.25% Triton X-100, 190 mM NaCl, 60 mM Tris-HCl, pH 7.4), and the wash was pooled with the extract. The yeast protein extract was cleared of cell debris by centrifugation at 100,000 x g at 4°C.

Immunoprecipitation of yeast cellular APP and its C-terminal fragments was performed according to a published procedure (Franzoso et al., 1991) with some

modifications. Two hundred microliters of yeast cell extract (560 µg protein, prepared according to the preceding paragraph) was combined with 600 µl of IP buffer (1% Triton X-100, 0.2% (w/v) SDS, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4) and preadsorbed with 30 µl protein A-beads (50% protein A-Trisacryl, Pierce, Inc.). The resulting suspension was incubated with 40 µl of affinity-purified R57 overnight at 4°C. Forty microliters of protein A-beads were added, and the mixture was rotated for 2 h at 4°C. The beads were washed twice with IP buffer and twice with detergent-free wash buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4). The sample was then eluted with 60 µl SDS sample buffer and subjected to SDS-PAGE. Immunoprecipitation controls were prepared from fresh rabbit cerebral cortex by homogenization in a buffer composed of 0.3 M sucrose, 10 mM HEPES-NaOH, pH 7.4, and the cocktail of proteinase inhibitors. The post-nuclear supernatant obtained from the homogenate by centrifugation at 800 x g for 10 min was ultracentrifuged at 100,000 x g for 1 h at 4°C. The resulting membrane pellet was suspended in the homogenization buffer at a concentration of 14 mg protein/ml. Five hundred microliters of the membrane preparation was treated with 1 ml of a buffer composed of 1% Triton X-100, 1% Na deoxycholate, 100 mM NaCl, 50 mM Tris-HCl, pH 7.4, and the preceding protease inhibitor mixture. APP and its C-terminal fragments from rabbit cerebral cortex were immunoprecipitated similarly except that the IP buffer did not contain SDS.

Immunoprecipitation of secreted APP A 1 ml aliquot of cell-free broth from a 48 h culture was preadsorbed with 30 µl protein A beads (50% protein A-trisacryl, Pierce) for 15 min at 4°C, and clarified by a brief centrifugation in a bench-top centrifuge. The supernatant was incubated with 40 µl anti- APP R104 serum for 4 h at 4°C. The optimal amount of primary antiserum was determined in a preliminary experiment. The immune complexes were adsorbed by incubation with 40 µl protein A beads overnight at 4°C and processed for Tricine-SDS-PAGE as described below. Control experiments with purified APP produced in a baculovirus system (Ramakrishna et al., 1991)

indicated that APP695 was nearly quantitatively immunoadsorbed from the medium. Additional experiments showed that APP was not degraded in the broth.

SDS-PAGE and Immunoblotting Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in discontinuous 7/11% slab gels according to the method described by Schagger and Jagow (1987). Proteins resolved by Tricine-SDS-PAGE were electrophoretically transferred to nitrocellulose in 150 mM glycine, 20 mM Tris-base, and 15% methanol. The nitrocellulose filters were incubated first with 1% nonfat dry milk in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 0.1% sodium azide, pH 7.4) for 3 h at 37°C, and then rinsed 3 times with blocking buffer (0.5% BSA in PBS, pH 7.4). Strips cut along the lanes from the filters were probed with antibodies toward different regions of APP. Incubation with the primary antibody was carried out for 10 h at 4°C. After washing with 1% dried milk in PBS and blocking buffer sequentially, the membranes were incubated with secondary antibody conjugated to alkaline phosphatase (goat anti-rabbit IgG or goat anti-mouse IgG, Tago) for 1 h. The blots were washed 5 times with blocking buffer for 5 min. each, and the color was developed in 0.4 M nitro blue tetrazolium (NBT) and 0.4 M 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in alkaline phosphatase buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M MgCl₂, pH 9.0). The intensity of staining was quantified by photodensitometry, using varying amounts of APP to produce the standard curve.

Protein Radiolabeling Yeast cells were first grown in leu/ura deficient synthetic medium (4% glucose) at 30°C, and then in the synthetic medium free of methionine (Met-SM) with 3% glucose at 30°C until early-to-mid exponential phase ($OD_{600}=0.5-1$). The cells were collected by centrifugation, resuspended in fresh Met-SM containing 0.05% glucose (3 OD_{600} units/ml), and incubated at 30°C for 2 h to induce APP expression. Labeling was initiated with 25 μ Ci of Tran³⁵S label / OD_{600} unit cells. After pulse-labeling for 5-10 min, labeled proteins were chased for the indicated times

by adding 1 mM cysteine, 5 mM methionine, 1 mM ammonium sulfate and 2% glucose. One milliliter aliquots were collected into sodium azide (10 mM) on ice, and cell lysis and immunoprecipitation were performed as previously described (Hines et al., 1994). Both polyclonal rabbit anti-C-terminus antibody (R57) and anti-sAPP (R104) were used to immunoprecipitate the labeled APP from the cell lysate. The final immunocomplex immobilized on protein A beads (Pierce Inc.) was resuspended in 30 μ l 2 x SDS sample buffer and subjected to Tricine-SDS-PAGE (7/11% discontinuous slab gel) described by Schagger and Jagow (1987). The gels were dried and exposed to Kodak X-Omat AR films at room temperature for appropriate periods of time. Densitometric analysis of the autoradiogram was carried out with a scanning densitometer (Mirror 600 color scanner) and the computer application IPLab Gel (Signal Analytics).

Proteinase K protection Assay The topology and extent of membrane association of APP was carried out using a modification of the method of Wessels et al., 1991. Membrane fractions were prepared by 2 procedures, both of which gave similar results. Procedure 1: Spheroplasts were prepared with Zymolyase (Campbell and Duffus (eds.), 1988). The spheroplasts were disrupted by 10 strokes in a loose-fitting glass homogenizer into 20 mM Hepes, pH 6.0, 1 M sorbitol, 1 mM PMSF, and nuclei and unbroken cells were removed by centrifugation at 2,000 x g for 5 min. A crude membrane fraction was obtained by the centrifugation of the post-nuclear supernatant at 12,000 x g for 10 min. Procedure 2: Cells were broken in homogenization buffer (1.2 M sorbitol, 10 mM potassium phosphate, pH 7.2) by agitation with an equal volume of acid-washed glass beads on a vortex mixer at maximal force for 5 periods of 30 sec each with 20 sec intervals on ice. The glass bead-free supernatant was spun at 600 x g for 10 min, and was further centrifuged at 100,000 x g for 20 min at 4°C. The membrane pellet was resuspended in homogenization buffer.

Aliquots of either vesicle preparation were incubated with 1 mg/ml proteinase K in digestion buffer (100 mM potassium acetate, 2.5 mM magnesium acetate, 50 mM

triethanolamine, pH 8.5, and 320 mM sucrose) for 1 h at room temperature with total volume of 60 μ l. The digestions were terminated by the addition of 1 mM PMSF. In a separate set of digestions, 1% Triton X-100 was included in the mixture to show the inherent sensitivity of APP to the protease. The digested vesicles were solubilized in SDS sample buffer and were immediately boiled.

Effect of NH_4Cl on α -secretase activity. A culture of RSY271-pBS6 (*sec18*) was grown at 22°C overnight in methionine-dropout SM (4% glucose). The midlog phase yeast cells were then incubated in met-SM containing 0.05% glucose for 1.5 hr at 35°C to induce both expression of APP fusion protein and blockage of protein transport at the endoplasmic reticulum. The cells (3 OD₆₀₀ units/ml) were then grown in ammonium free-minimum medium (pH 7.7) with 1% proline as the nitrogen source (MV-pro) (Stevens et al., 1986) for half an hour at 35°C. The cells were subsequently pulse-labeled for 30 min at 35°C, and then treated with 0.4 M NaCl or 0.4M NH_4Cl for 20 min at the same temperature. Reversion of the secretory pathway blockage was initiated by re-incubating the labeled cells at 22°C; they were chased for 3.5 hr in the presence of the same concentrations of the two salts. Immunoprecipitation of APP and its C-terminal fragments with R57 and Tricine-PAGE were described as before. The gel was fixed, impregnated with EN³HANCE (New England Nuclear), dried and exposed to film at -80°C for appropriate times.

Quinacrine uptake The neutralization of yeast vacuoles by NH_4Cl was assessed by quinacrine uptake (Weisman et al., 1987). Yeast cells that were treated with the salts under the same conditions as described above (*Effect of NH_4Cl on α -secretase activity*) were incubated for 5 min at room temperature in the presence of 200 μ M quinacrine in MV-pro medium (pH 7.7) containing either 0.4 M NH_4Cl or 0.4 M NaCl. The cells were washed once in the same medium without the dye, resuspended in the medium, and mounted on ConA-coated slides for phase-contrast optics and fluorescence microscopy (FITC filter).

Invertase Assay. *Sec* mutants transformed with pBS6 α were grown overnight in YPD (5% glucose) at 24°C to an OD₆₀₀ of 0.9-2.6. Cells were transferred to fresh YPD with 0.1% glucose (0.8 OD₆₀₀ units/ ml) and incubated for 37°C or 24°C for 1.5 h to induce invertase expression. An aliquot (1 ml) was then removed and treated with sodium azide (final concentration 10 mM) at 0°C. Another aliquot (1 ml) from the culture at 37°C was further incubated at 22°C for 3 h in the presence of 1 mg of glucose and 0.1 mg of cycloheximide to measure the secretion of accumulated intracellular invertase. Samples from each condition were washed with 10 mM sodium azide and resuspended in 0.5 ml of the same solution. To assay internal invertase, spheroplast lysates were prepared following the modified procedure of Franzusoff et al. (1991). N-ethylmaleimide was added to eliminate residual DTT. External and internal invertases were assayed at 37°C by the glucose oxidase and peroxidase system (Sigma) as described by Goldstein and Lampen (1975). One unit of activity is 1 mole of glucose released per min.

Glycosylation studies Yeast cells (RSY271/pBS6 α ; *sec18*) grown in methionine-deficient SM (3% glucose) to early-to-mid log phase were divided into two equal portions. Cells from one portion were resuspended in the same medium (3 OD₆₀₀ units per ml) with tunicamycin (10 μ g/ml). The culture was preincubated for 30 min at 25°C to deplete the intracellular pool of dolichol-linked oligosaccharides, and again subdivided into two equal portions. Cells were resuspended in the Met⁻ SM with 0.05% glucose under the same conditions. One portion was shifted to 37°C; while the other was kept at 25°C. After 1 h at the indicated temperatures, cells were labeled for 40 min., and analyzed by immunoprecipitation with R57, as described above. A control experiment without tunicamycin was similarly conducted. The effectiveness of tunicamycin on N-linked glycosylation inhibition was confirmed by assaying α -factor secretion in the presence of tunicamycin as described by Julius et al. (1984b).

Endo H digestion of radiolabeled APP was conducted according to the protocol by Genzyme Corp. R57-immunoprecipitated ^{35}S -labeled APP from RSY271/pBS6 α was removed from protein A beads by heating in 1% SDS (20 μl) in a boiling water bath, diluted by the addition of 180 μl 50 mM sodium citrate (pH 5.8), heated again for 1 min, and clarified by centrifugation. The supernatant was incubated in the presence of Endo H (50 mU/ml; Genzyme) at 37°C for 5 h. The sample was dried using a centrifugal concentrator (Savant Speed-Vac), redissolved in 30 μl of 1x electrophoresis Laemmli sample buffer (0.06 M Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 10 mM dithiothreitol, 0.025% Bromphenol Blue), heated in a boiling water bath for 2 min, and analyzed by Tricine SDS-PAGE.

Sequencing of yeast secreted APP IgG was purified from 2 ml R104 serum on a Bakerbond ABX column (J.T. Baker) and coupled to CNBr-activated Sepharose 4B (2g, Pharmacia) according to the manufacturer's instructions. The extent of the coupling was estimated by SDS treatment of the beads and western blotting with anti-rabbit IgG antibody. For affinity purification of yeast secreted APP, 10 ml of yeast broth from a 48 h cell culture was adjusted to 1% Triton X-100, 150 mM NaCl and a cocktail of protease inhibitors included as described in the section *Analysis of APP expression*. The R104-Sepharose column was prewashed with binding buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) and incubated with the broth overnight on a shaker at 4°C. The column was washed with binding buffer and phosphate buffered saline (PBS) sequentially. The bound proteins were eluted from the column with 50% ethylene glycol in 100 mM triethylamine (pH 11.5). Ethylene glycol was removed from the eluate by dialysis in PBS containing 0.1% NaN_3 . The proteins in the eluate were then precipitated by the quinine sulfate-SDS method, dissolved in 2x Laemmli sample buffer (with DTT) and subjected to 7/11% Tricine SDS-PAGE. The proteins were electro-transferred to a ProBlott membrane (Applied Biosystems) according to the manufacturer's procedure. The Coomassie Blue stained APP bands were excised and

microsequenced using an Applied Biosystems Model 470A microsequencer as previously described (Yang et al., 1994). The amino acid sequence was picked by the Applied Biosystems Model 610A data analysis system and was confirmed by manual analysis.

Isolation and sequencing of the APP membrane-bound COOH-terminal fragments

A 1 liter culture of JSC310/6 α was grown in YPD medium for 48 h at 30°C. The cells were harvested, resuspended in 180 ml of lysis buffer (0.7 M sorbitol, 10 mM potassium phosphate, pH 7.2 and a cocktail of protease inhibitors) and broken by passing through a French pressure cell five times at 18,000 psi. The cell lysate was spun for 10 min at 1,000 x g, and the resulting supernatant was centrifuged for 30 min at 50,000 x g. The membrane pellet was solubilized with 50 ml of extraction solution (1% Triton X-100, 0.2% SDS, 10 mM potassium phosphate, pH 7.2 and protease inhibitors) followed by centrifugation at 50,000 x g for 1 h at 4°C. The cleared solubilized membrane protein solution (30 ml) was further concentrated to 15 ml using an Amicon stirred ultrafiltration cell (YM1 membrane). The concentrated membrane extract was chromatographed over a Sephacryl HR S-100 column (2.6 by 100 cm) that had been equilibrated in 0.1 M NH₄HCO₃ and 0.1% SDS. Fractions containing the C-terminal fragments were pooled, lyophilized and rechromatographed on the same column. The fractions containing the 7-12 kDa C-terminal fragments but lacking the full-length APP were pooled, adjusted to 1% Triton X-100 and pH 8.0, and passed through a Sepharose 4B column coupled with rabbit anti-rat albumin IgG that had been prewashed with 1% Triton X-100 in PBS. The pass-through solution was further adjusted to 150 mM NaCl, and incubated with 3 ml of R57 IgG coupled Sepharose for 4 h at 4°C. The column was washed with 15 volumes of 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4 followed by several volumes of PBS. The bound proteins were finally eluted with 1% SDS in 50 mM Tris-HCl, pH 7.4, and subjected to protein precipitation, electrophoresis, electroblotting and microsequencing as described for the secreted APP.

Partial Purification and Kex1 digestion of α -Factor and its derivative DS7pBS α C was grown in 100 ml of leu/ura SM (1% glucose) for two days at 30°C, and the purification was performed according to the procedure by Ciejek et al. (1977). The chilled cell-free medium (pH 2.7) containing 1 mM PMSF and 1 mM leupeptin was percolated at 4°C through a bed (2 ml) of Bio-Rex70 equilibrated with 0.1 N acetic acid. After washing with 2 bed volumes of 0.1 N acetic acid and 50% ethanol consecutively, the peptide was eluted with 2.5 bed volume of 0.01 N HCl in 80% ethanol. The collected eluate was vacuum-dried, resuspended in 200 μ l H₂O, neutralized by 1 M NH₄OH and dried again. The final partially purified peptide was dissolved in 20 μ l digestion buffer (25 mM BisTris/HCl, pH 6.0). As a positive control, α -factor was also purified from the medium of *sec18* culture.

Kex1 Δ p digestion was conducted as described by Latchinian-Sadek and Thomas (1994). Four microliters of purified α -factor was incubated with 0.5, 1.0, and 1.5 μ l of Kex1 Δ p (a gift from Dr. Brian Shilton, Biotechnology Research Institute, National Research Council, Canada) for 1 hr at 30°C (7.2 ng/ μ l in digestion buffer). The digestion was stopped by chilling the sample with ice.

Halo Assay The halo assay was conducted basically following the procedure by Bourbonnais et al. (1993). The tester strain SY1854 lacks the barrier protease to degrade α -factor and thus is supersensitive to the pheromone. Following an overnight growth at 30°C in YPD medium, 5 μ l of SY1854 culture were mixed with 10 ml of warm YPD medium containing 0.7% agar (42°C) and overlaid onto YPD plates. After agar had solidified, the purified or synthetic α -factor solution (4 μ l) was then spotted onto the agar, and the plates were incubated overnight at 30°C. To test the secretion of α -factor by DS7pBS α C and *sec18* cells, synthetic medium (leu/ura deficient, 1% glucose, 0.7% agar) was used instead of solid YPD.

HPLC Analysis of Secreted Radio-labeled α -Factor A culture of DS7 harboring the α C gene (3 OD₆₀₀ units/ml) in met-free SM (0.05% glucose) was labeled with Tran³⁵S-

label (25 $\mu\text{Ci}/\text{OD}600$ unit) for 5 hr at 30°C. The cell-free medium with proteinase inhibitors (1 mM PMSF, 1 mM leupeptine and 1 mM pepstatin) was adjusted to pH 2.7, and subjected to chromatography as described above, and the partially purified ^{35}S - α -factor derivative was resuspended in 200 μl of H_2O . One tenth of the sample was further resolved by 7/11% Tricine SDS-PAGE. The band comigrating with synthetic α -factor was cut out; the proteins were extracted by mixing the gel slice with 1 ml of 50% acetonitrile for 5 hr at 4°C and then vacuum-dried and resuspended in 100 μl H_2O . HPLC analysis of the extracted peptides was performed according to the procedure by Latchinian-Sadek and Thomas (1993). Synthetic α -factor and α -factor-K were included in the sample as internal standards. The eluates were collected every 20 sec, and their radioactivities were counted on a LKB ligand scintillation counter.

RESULTS

Rationale

Because yeast, the simplest eukaryotic cell, possesses many subcellular structures equivalent to those of a mammalian cell and is amenable to genetic manipulation, it has become a favorite vehicle for studying the modification and processing of many mammalian proteins. We thus expressed human APP gene in yeast in order to determine whether the organism would mimic the pattern of the mammalian processing of the protein, and hence could serve as a simple system for the identification of α -secretase. One major concern was to make sure that the human APP entered yeast secretory system. Many heterologous proteins have been expressed in yeast *Saccharomyces cerevisiae*, including epidermal growth factor (hEGF) (Brake et al., 1984), β -endorphin, interferon α 1, and proinsulin (Cousens et al., 1987; Zsebo et al., 1986). However, most of the heterologous proteins require the leader sequence of prepro α -factor mating pheromone for proper secretion and processing in yeast. α -factor mating pheromone, which is secreted by α -mating type yeast, promotes the mating response. This peptide is derived from the prepro- α -factor precursor and consists of an 83-residue leader sequence and four repeats of the mature α -factor peptide, each preceded by a spacer Lys-Arg-(Glu/Asp-Ala)₂₋₃ (Kurjan and Herskowitz, 1982). Precursor processing involves four different proteinases, one of which, the endoprotease Kex2p, cleaves on the carboxyl side of the Lys-Arg sequence present in the spacer; this is followed by the removal of the two basic residues by the carboxypeptidase Kex1p (Fig.12). We, therefore, constructed plasmids in which the human APP gene was joined to the sequence encoding the α -leader region of prepro- α -factor, and expected that the α -leader would allow efficient APP transport through the secretory pathway in the transfected yeast.

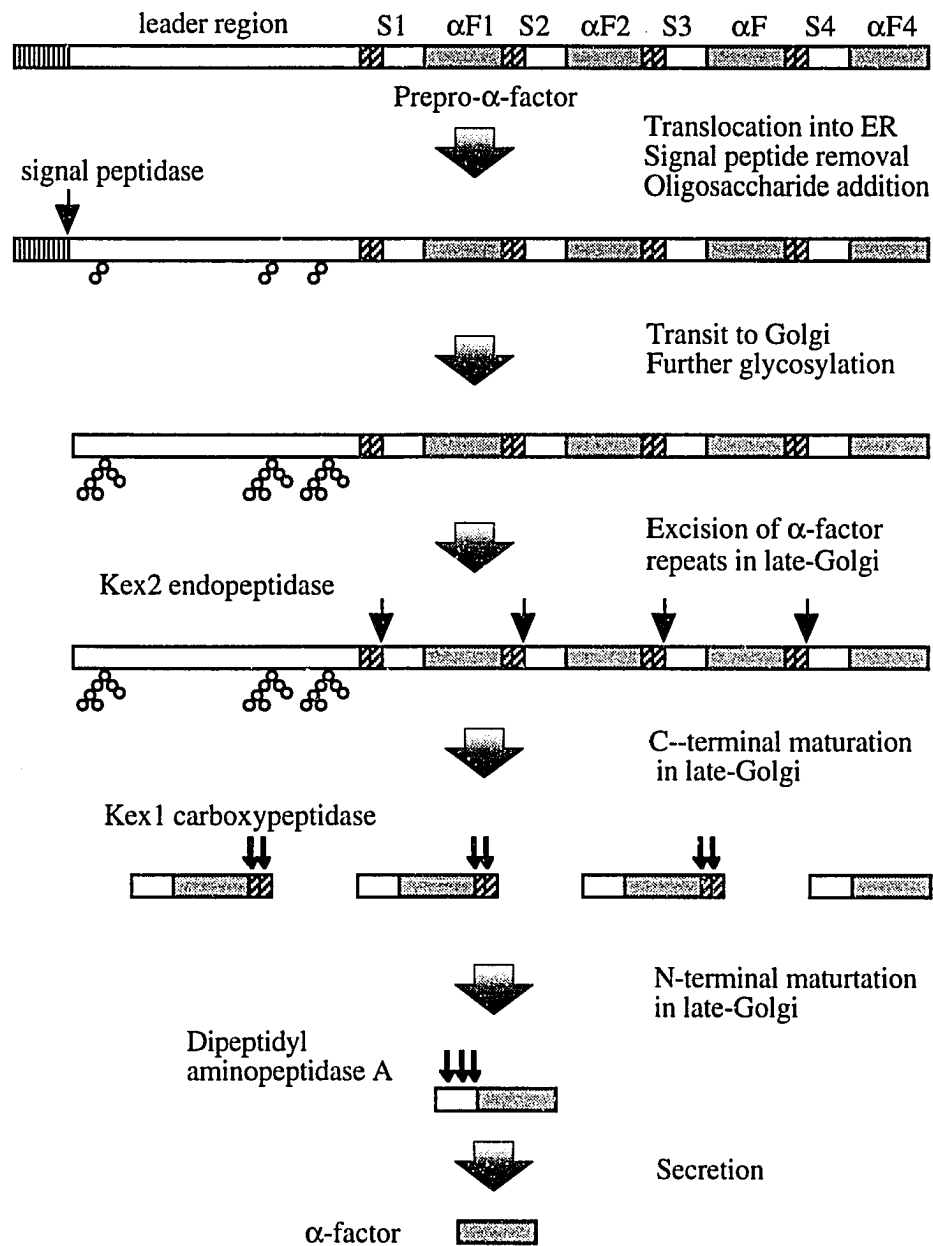


Figure 12. Diagram for processing of prepro- α -factor in yeast. The four copies of α -factor peptide are indicated by solid rectangles, and the signal sequence by a vertically crosshatched rectangle. Spacer regions consist of Lys-Arg (two diagonally crosshatched boxes) followed by two or three Glu-Ala or Asp-Ala pairs (open boxes). The clusters of small circles represent glycosylation sites at the leader region. The sequential proteolytic events are also shown.

Expression of α -leader-APP Fusion Protein in Yeast

The cDNAs encoding human APP695 and APP751 isoforms were placed into a yeast expression shuttle vector pBS24.1 behind the leader sequence of prepro- α -factor (pBS6 α , pBS7 α) or the authentic human signal peptide (pBS6F, pBS7F) (Fig.10B). Expression is controlled by a glucose-repressible hybrid promoter, which contains the regulatory region of the alcohol dehydrogenase II promoter and the 3' end of a glyceraldehyde-3-phosphate dehydrogenase promoter (ADH2/GAPDH) (Cousens et al., 1987). Transient expression is expected to minimize the potential toxic effect of high levels of heterologous APP on the normal cell physiology of the transfected yeast. The constructs were introduced into a variety of yeast strains, and APP protein expression was visualized by immunoblotting with a battery of polyclonal and monoclonal antibodies, whose epitopes are denoted in Fig.10A. Figure 13 shows the expression in the transfected JSC310 strain. Each of the four recombinant vectors expressed high levels of APP immunoreactive with anti-C-terminus antibody R57. By quantitative immunochemical staining, using purified rat brain APP(Potempska et al., 1991) as a standard, we estimated that APP comprised 1% of the total yeast proteins.

Because the cells transfected with vector pBS24.1 alone contained no detectable immunoreactive proteins, immunodetection was APP-specific (Fig.13, lane 1). The single 95-105 kDa APP695 band must encompass the full-length APP695 based on its reaction with antibodies directed to the epitopes near the NH₂-terminus (R71) as well as the C-terminus (R57) (Fig.15A,B: lanes 2 and 5). In addition, judging by their sizes on the gel, these bands may represent mature APP free of α -leader sequence. APP751 expressed by pBS7 α had a more complex array of bands around 95 kDa region; these may contain both fusion protein and mature APP (Fig.13, lane 3).

For the later pulse-chase studies of APP processing, we determined the APP induction as a function of glucose concentrations. As shown in Fig.14, while no APP was detected in the transfected cells before the glucose level dropped to zero, the APP

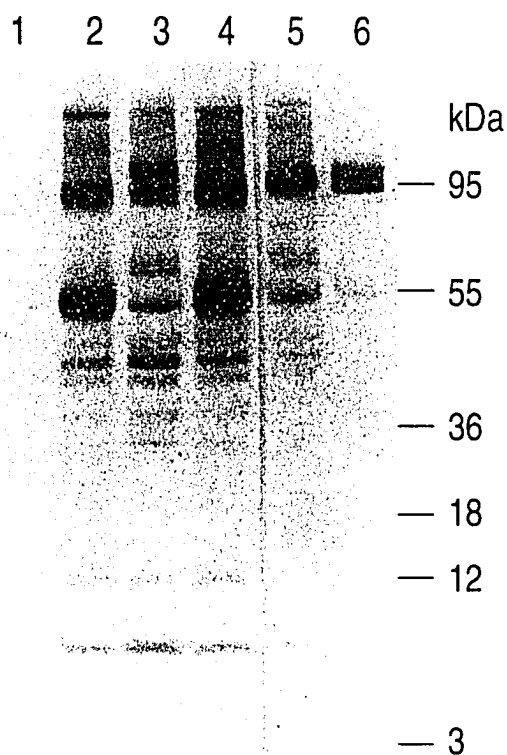


Figure 13. Expression of human APP genes in yeast strain JSC310. Yeast cells expressing APP constructs defined in the text were homogenized with glass beads. The homogenates were subjected to SDS-PAGE and were then immunoblotted with antibody R57 specific for the C-terminus of β -APP. pAB24 vector (lane 1); APP6 α (lane 2); APP7 α (lane 3); APP6F (lane 4); APP7F (lane 5); rat brain APP (lane 6). The APP bands have apparent molecular weights of 95-105 kD. All four constructs yield additional strongly immunoreactive bands of 50 kD.

KINETICS OF APP INDUCTION AND GLUCOSE LEVEL

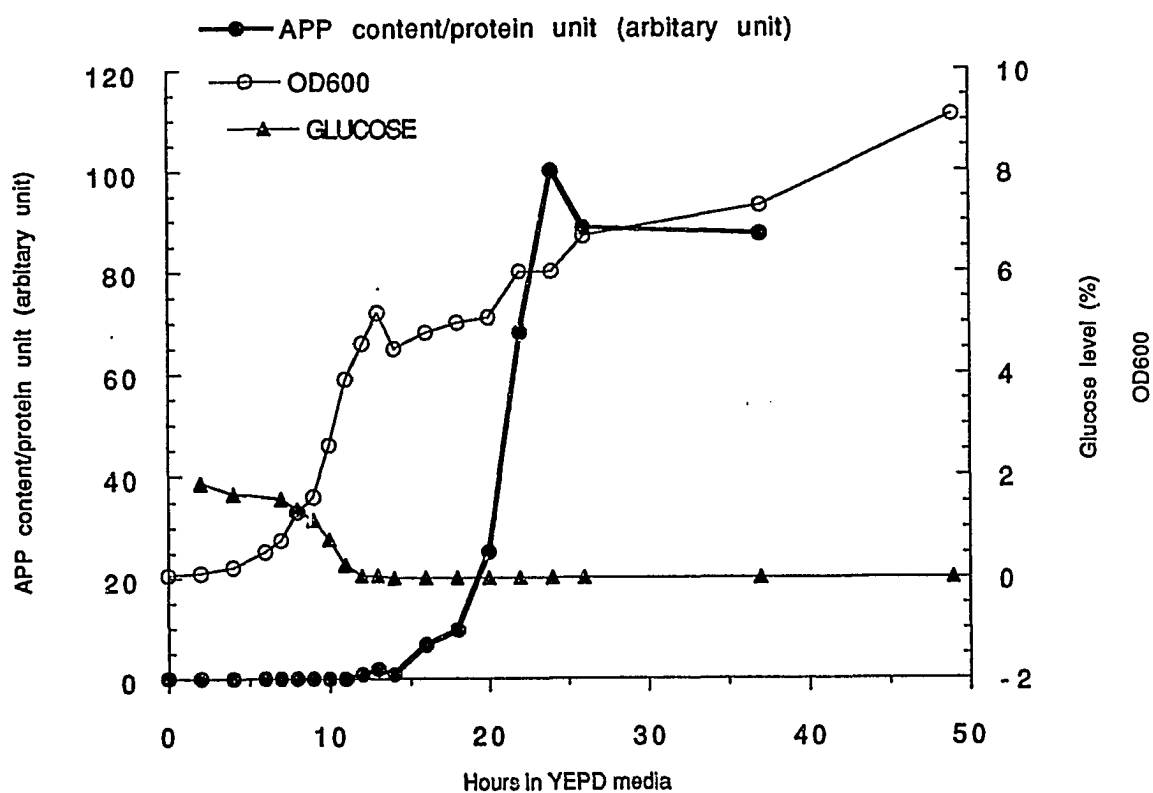


Figure 14. APP expression as a function of glucose levels. Yeast JSC310 with APP6 α was first grown in leu/ura selective medium (4% glucose) and then changed to YPD. Aliquots were collected over a 49-h period. The glucose level in the broth of each aliquot (triangle) was determined using Sigma diagnostic kit based on the o-toluidine method. All data were corrected for non-glucose reducing substances in YPD that lacked glucose. Total cellular APP (solid circle) was detected by immunoblotting the cell lysate with R57 and quantified by photodensitometry. The cell density (open circle) was measured by absorbance at 600 nm (OD₆₀₀).

expression was quickly induced when all glucose was consumed, reaching a maximum level after 24 h. Figure 14 also shows that there were two hours of delay between the depletion of glucose and APP expression; this may be due to the time needed to turn on the APP gene and the protein synthesis. These data showed that yeast APP expression system can be readily controlled by glucose as predicted from the nature of ADH2/GAPDH promoter.

APP Enters Yeast Secretory System With Correct Orientation

APP is a type I membrane protein with a cytoplasmic C-terminal domain (50 residues) and an extracellular (intraluminal) N-terminal domain (625 residues) separated by a single membrane region. To define the topology of APP in yeast, we subjected membrane vesicles prepared from yeast cells transfected with pBS6 α to proteinase K digestion, and used various antibodies to detect the carboxyl and amino portions of APP. If APP695 is inserted into yeast ER with the correct orientation, its C-terminal domain should be vulnerable to exogenous proteinase digestion, whereas its N-terminal portion should be protected from such an attack.

Figure 15A shows the result of such an assay on yeast cells expressing APP695 behind the α -leader sequence (APP6 α). Membrane vesicles were prepared from the yeast cells under optimal conditions so that most of the vesicles retained their original orientation in the secretory system (not inside-out form). The epitope on the C-terminal domain disappeared upon proteinase K digestion, as judged by the loss of immunoreactivity with antibody against C-terminus (R57) (compare lanes 1 and 2 in Fig.15A); on the other hand, the exogenous proteinase did not cleave the N-terminal epitope recognized by antibody R71 (Fig.15A, lane 3). The smaller size of the protease-treated band stained by R71 reflects the loss of most of the 45 C-terminal residues. In contrast, protease treatment of the membrane vesicles in the presence of 1% Triton X-100 (Fig.15, lane 4) completely digested the epitope on the N-terminal

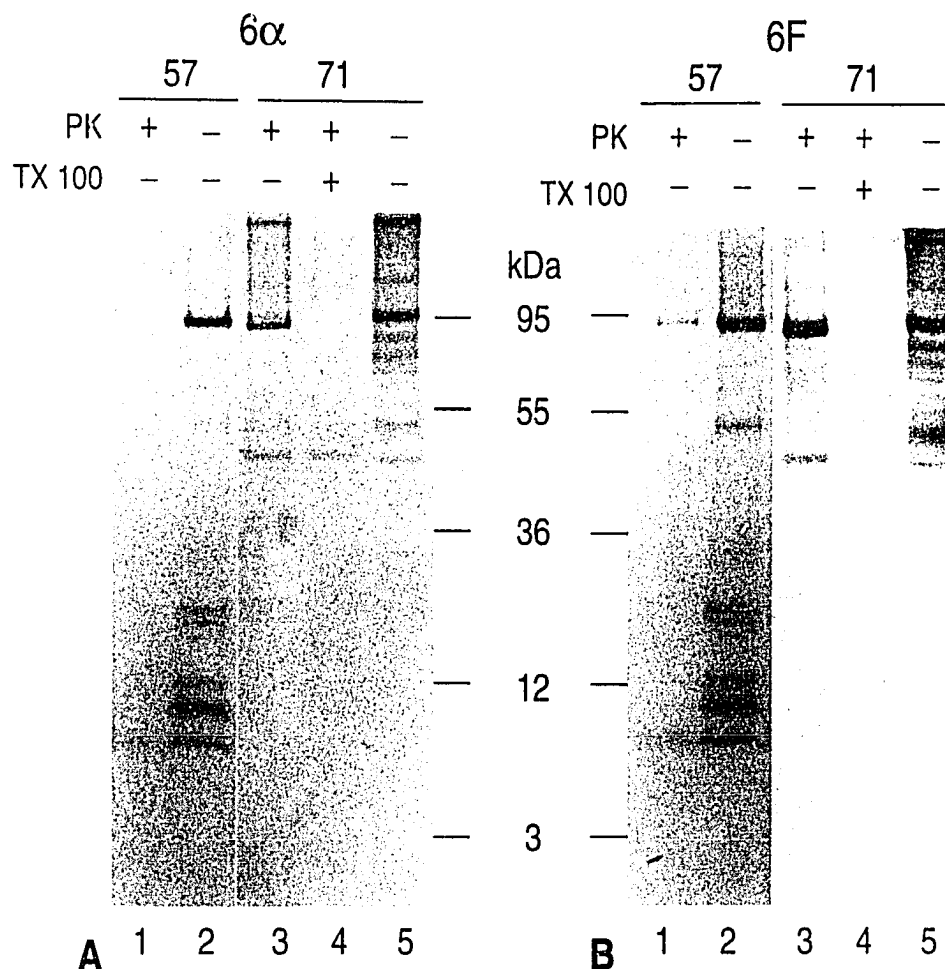


Figure 15. Protease protection study of membrane-associated APP. Yeast cells expressing APP6 α or APP6F were homogenized with glass beads. The membrane fraction obtained from the post-nuclear supernatant by centrifugation at 100,000 x g was digested with 1 mg/ml proteinase K, either in the presence (lane 4) or absence (lanes 1,3) of Triton X-100. The digested products were resolved by 7/11% Tricine-SDS-PAGE and immunoblotted with antibodies directed to the N-terminal domain (R71) and C-terminal domain (R57). Lanes 2 and 5 contain controls lacking proteinase K. Panel A: the membranes obtained from the expression of APP6 α in JSC310. Panel B: the membranes obtained from the expression of APP6F in JSC310.

APP EXPRESSION AND SOLUBILITY IN TRITON X-100

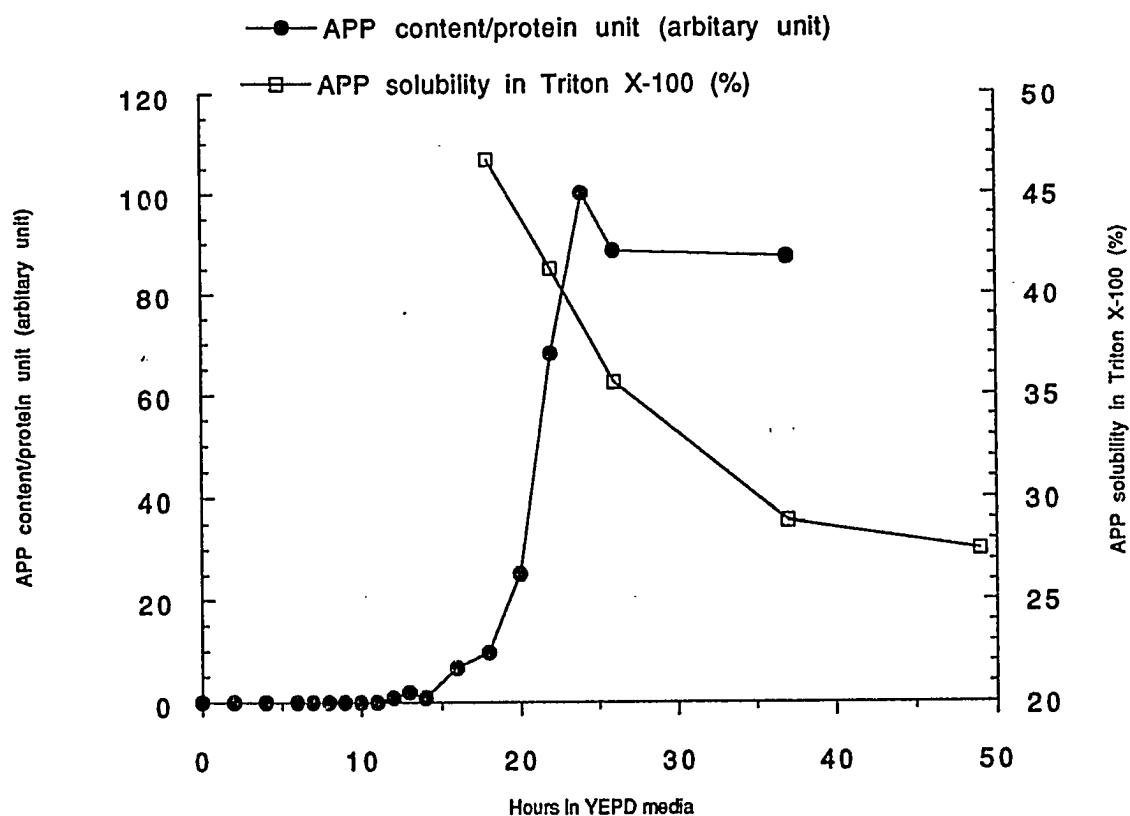


Figure 16. APP expression levels and its solubility in Triton X-100. Cell membrane fractions from yeast JSC310-6 α grown for various periods of time in YPD were subjected to 1% Triton X-100 extraction. The amount of extracted APP was measured following SDS-PAGE. The level of APP expression is represented by APP content/total protein (*circle*). APP solubility in Triton X-100 is indicated by percentage of extracted APP over total APP (*square*).

domain; this excludes the possibility that the digestion resistance was due to the intrinsic insensitivity of this region to the proteinase K.

By these criteria the majority of APP molecules were inserted into the lumen of the endoplasmic reticulum (ER) in the correct orientation instead of being retained in the yeast cytosol. APP695 expressed behind the human signal sequence (APP6F) produced similar results (Fig.15B), indicating that the yeast translocation machinery recognized the human signal sequence.

Many overexpressed heterologous proteins in yeast or bacterial cells tend to form aggregates within the cells. To evaluate the conformation of APP in the transfected yeast cells, we conducted non-ionic detergent extraction experiments. Mechanical rupturing of cells expressing APP6 α with glass beads released undetectable amounts of APP in the cytosol fraction (data not shown). When extracted with 2% Triton X-100 at a protein concentration of 4 mg/ml, the membrane fraction released 25-50 % of APP, depending on the age of the culture (Fig.16). Greater extraction efficiency was observed at shorter growth times and lower levels of expression. Comparable experiments on other yeast strains of diverse genetic backgrounds produced similar results. This procedure releases most of the APP from mammalian cell membrane (Potempska et al., 1991). Taken together, these data indicate that most APP is successfully inserted into the secretory membranes; however, 50-75% of the protein aggregates in the secretory pathway. These results are consistent with the conclusion from the protease protection experiment.

Secretion of Extracellular Domain of APP

Yeast strain JSC310, expressing pBS6 α , secretes a 85-90 kD APP-related protein into the medium. The secreted protein was immunoprecipitated from the conditioned medium by antiserum R104 directed to the extracellular domain of APP. The protein reacted strongly with monoclonal antibody 6E10, but not with m4G8 that recognizes the carboxyl side of α -secretase cleavage point (Fig.17, compare lanes 1

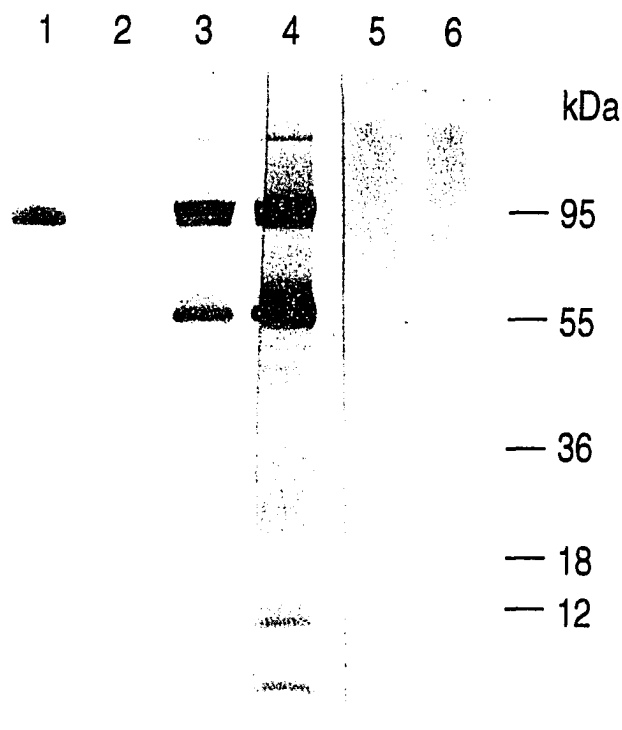


Figure 17. Secretion of APP by JSC 310 cells transformed by pBS6 α . The cell-free media from JSC310 transfected with pBS6 α (lanes 1,5) or with pBS24 as control (lanes 2,6) were subjected to immunoprecipitation with antibody R104 specific for the extracellular domain of APP, and the precipitated proteins were resolved by 7/11% Tricine-SDS-PAGE. The gels were immunoblotted with monoclonal antibodies 6E10 (lanes 1,2) and 4G8 (lanes 5,6). A membrane extract of the recombinant yeast cells (lane 3 probed with 6E10; lane 4 probed with R57) was used here for comparison of the relative positions of membrane-bound and secreted APP.

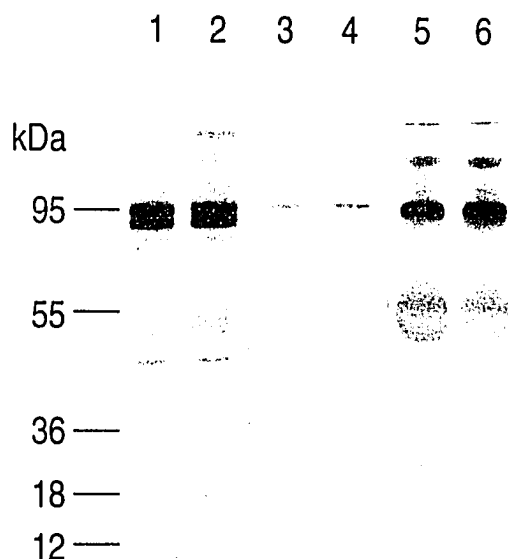


Figure 18. Retention of the secreted form of APP695 in yeast cells. A membrane fraction was prepared from yeast JSC310/6 α grown for 48 h at 30°C. This fraction was divided into two portions and was subjected to either 2% SDS or 1% Triton X-100 extraction. After centrifugation at 50,000 x g for 30 min, the Triton-insoluble material was further dissolved in 2% SDS. Both SDS extracts of total membrane proteins and of the Triton-insoluble material (150 μ g protein each) were immunoprecipitated with antibody R104. The immunocomplexes retrieved by protein A beads were resolved on 7/11% Tricine SDS-PAGE, transferred onto nitrocellulose membrane and probed with mAb6E10, mAb4G8 or R57. SDS extract of total membrane proteins: lanes 1,3,5; Triton X-100-insoluble proteins: lanes 2,4,6. 6E10: lanes 1,2; 4G8: lanes 3,4; R57: lanes 5,6.

and 5). No substance was immunoprecipitated from the broth of the control yeast expressing the vector pBS24.1 (Fig.17, lanes 2 and 6). The immunoreactivity profile of this protein is consistent with its being the extracellular domain of APP formed by cleavage at the α -secretase site (residues 16-17 of A β region). Secretion of APP was also observed in JSC310 harboring pBS7 α , though at much lower level; no APP, however, was secreted by the recombinants containing the human signal sequence (pBS6F and pBS7F).

We further identified the secreted mAb 6E10-reactive APP by microsequencing the protein isolated by an immunoaffinity column crosslinked with R104. The amino-terminal sequence LEXXTDGNAG, deduced with the aid of the Applied Biosystems data analysis algorithm, is compatible with the APP amino terminal sequence, LEVPTDGNAG.... This result confirms the identity of the immunoreactive protein and indicates that the Kex2 endopeptidase had cleaved off the α -factor leader sequence as expected. By measuring the mAb 6E10 immunoreactive bands of both intracellular and secreted APP, about 1% of the total APP695 appears in the medium after two-days of growth.

The low level of APP secretion in yeast prompted us to investigate whether the secreted form of APP (sAPP) was trapped within the cells. Total APP695 as well as the Triton-insoluble fraction of APP695 were immunoprecipitated with R104, resolved by Tricine SDS-PAGE, and revealed by multiple antibodies (Fig.18). mAb 6E10 (lanes 1 and 2) reacted with two bands in the 95-100 kDa region. In contrast, mAb 4G8 (lanes 3 and 4) and R57 (lanes 5 and 6) reacted only with the upper band. The lower band exhibited the same antibody specificity and electrophoretic mobility as the secreted form of APP695 (Fig.17); therefore, it must represent the intracellular sAPP. From the intensities of the two bands (Fig.18, lane 1), we concluded that about one-half of APP695 had encountered yeast α -secretase; however, a larger proportion had been retained by the cells than had appeared in the medium. The cellular retained sAPP was

also observed in ^{35}S -methionine-labeled cells after immunoprecipitation with the antibody R104 (Fig.21B). The cell-associated sAPP could be either secreted but stuck with the cell walls, or it had aggregated within the secretory system.

To distinguish the two possibilities, we treated the cells with 5 mM DTT at pH 10.5 for 30 min (Scott and Schekman, 1980); about one-half of the cell-associated, α -secretase-cleaved APP695 (sAPP) was secreted into the medium without releasing any of the intact intracellular APP695 (Fig.19). Thus, most of the secreted sAPP existed as a disulfide-crosslinked aggregate bound to the exterior of the cells.

Membrane-bound C-terminal Fragments

Yeast cells expressing APP have two membrane-bound C-terminal fragments that reacted with R57 directed to APP's C-terminal epitope (Fig.13; Fig.20, lane 5). Host cells or cells transfected with the vector (pBS24) alone had no such fragments (Fig.13, lane 1). These small fragments were further characterized by immunoprecipitation, PAGE and immunoblotting with a battery of antibodies (Fig.20). The 12 kD band, labeled as "a", reacts with mAb 6E10 as well as mAb 4G8 (Fig.20, lanes 1 and 3), suggesting that it may contain intact $\text{A}\beta$. On the other hand, the 7 kD band, denoted "b", contains only the mAb 4G8 epitope, which resides at the carboxyl side of α -secretase cleavage site (Fig.20, compare lanes 1 and 3). A 7 kD C-terminal fragment also appears in the rabbit brain extract (Fig.20, lane 6), and probably represents the α -secretase cleavage product. The "b" band in the yeast extract may represent the same byproduct of the protease activity. Rabbit brain also contains a somewhat smaller mAb 6E10-reactive peptide (Fig.20, lanes 2 and 6) than yeast "a" band. C-terminal fragments of similar sizes have been found in other mammalian cells (Golde et al., 1992; Haass et al., 1992b).

The identity of "b" band was further investigated by sequencing. Peptide "b" (Fig.20) was purified from a membrane preparation of JSC310 expressing pBS6 α by consecutive steps of size fractionation, immunoabsorption (with purified R57), and

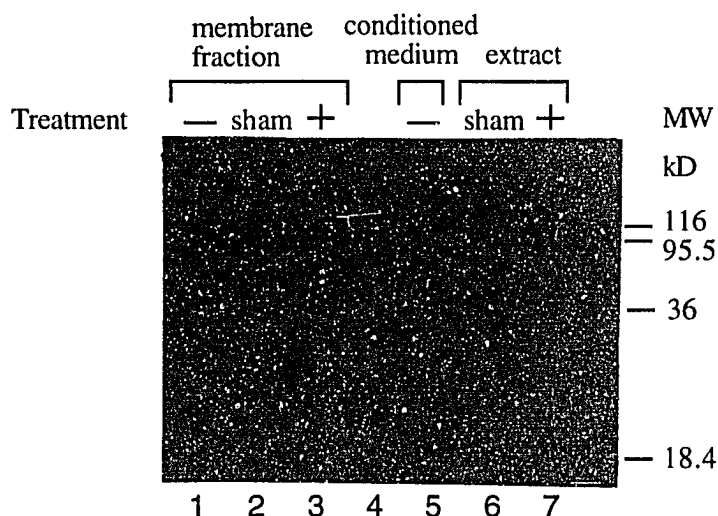


Figure 19. Release of cell-associated sAPP from the surface of yeast cells. JSC310 cells expressing APP6 α that had been grown to mid-log phase were resuspended in fresh YPD (pH 10.5) containing 5 mM DTT and 0.05% NaN₃, and incubated for 30 min at 30°C. After centrifuging the cell mixture, the cell-free supernatant (extract) was collected, and a membrane fraction was prepared from the treated cells. The proteins from both the extract (lane 7) and the SDS-dissolved membrane fraction (lane 3) were immunoprecipitated with the antiserum R104, separated on SDS-PAGE and blotted to a nitrocellulose membrane which was probed by monoclonal antibody 6E10. The sAPP secreted into the medium from the cells (conditioned medium) is also shown (lane 5).

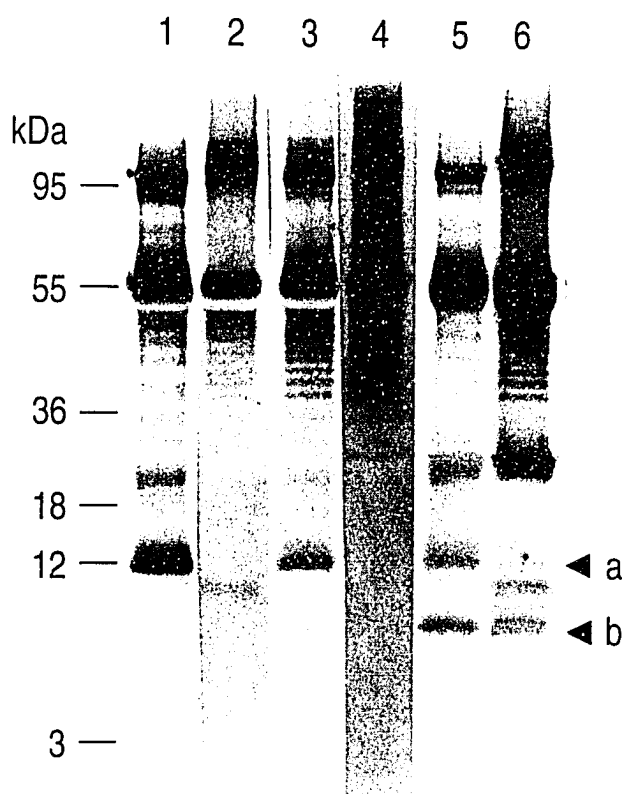


Figure 20. Formation of C-terminal fragments of APP in transfected yeast strain JSC310. Membrane protein extracts were prepared from yeast JSC310 transformed with pBS6 α and from rabbit brain using an SDS lysis buffer and then immunoprecipitated with R57. The proteins were separated on 7/11% Tricine-SDS-PAGE, blotted onto nitrocellulose, and analyzed for the presence of C-terminal fragments of β -APP using antibodies 6E10 (lanes 1,2), 4G8 (lanes 3,4) and R57 (lanes 5,6). lanes 1,3,5: yeast; lanes 2,4,6: rabbit brain. The two major C-terminal bands of 7 and 12 kDa are indicated by *arrow heads a* and *b*. The artifactual staining at ca 50 kDa and 25 kDa results from the cross-reactivity of rabbit IgG chains with the second antibody. The lanes were purposely overdeveloped to intensify the 7 and 12 kDa fragments.

SDS-PAGE. After electrophoretic transfer to a PVDF membrane, the peptide was excised and sequenced. The sequence of the first 15 residues of peptide "b" turned out to be LVFFAEDXGXNKGAI..., which corresponds to residues 17-31 of A β (LVFFAEDVGSNKGAI...). The 8th residue was lost due to power failure. The 10th residue, Ser, would have been converted to PTH-dehydro-Ala at a level below the limits of detectability in our system. Hence, the amino-terminus of this fragment corresponds exactly to that of the C-terminal fragment resulting from mammalian α -secretase cleavage. Therefore, the detection of both amino-terminal extracellular part of APP and the membrane-bound C-terminal fragment demonstrates that yeast possesses a distinct protease that resembles mammalian α -secretase.

Pulse-Chase Analysis of APP Processing in Yeast JSC310 Expressing APP695 with α -Leader

To determine the kinetics of the yeast α -secretase cleavage, yeast JSC310 expressing APP6 α were pulse-labeled with Tran ³⁵S-label for 10 min and chased for various periods. Radio-labeled APP and its intermediates containing the C-terminal domain were immunoprecipitated with anti-C-terminus antibody R57 and resolved by SDS-PAGE. With pulse-labeling as short as 5-10 min, all of the fusion proteins were converted to mature APP, free of α -leader sequence at the 0 chasing time (Fig.21A) based on its apparent molecular weight. The rapid cleavage of α -leader from the fusion protein is similar to the processing of the endogenous α -factor precursor, in which proteolytic removal of the α -leader region is accomplished within 10 min and no precursor could be detected (Julius et al., 1984). Thus, the APP6 α fusion protein and α -factor precursor may share the same transport route until they encounter Kex2 endoprotease residing in the late-Golgi. Other strains of yeast transfected with pBS6 α showed somewhat slower rates of Kex2p cleavage, and both the fusion protein and the mature APP were present at the beginning of the chase (Fig.22, sec18). The newly formed α -leader-free full length APP disappeared over the chase (Fig.21C); meanwhile,

there was a concomitant accumulation of a 7 kD R57-immunoreactive C-terminal fragment which was identified previously as yeast α -secretase cleavage product (CTF) (Fig.21A). Consistent with the previous observation of intracellular accumulation of the secreted form of APP (sAPP), a radio-labeled R104-immunoreactive band (90 kD), which corresponds to sAPP, also appeared after 10 min of chasing and built up in the following 4 hr in the cells (Fig.21B). However, the labeled sAPP that was trapped within the cells was resistant to high pH (10.5) and DTT treatment which was shown to be effective to release cell surface-bound sAPP from 2-day-cultured cells described above (data not shown). Nor did trypsin digestion of whole yeast cells released any APP in the medium (data not shown). Surprisingly, although soluble APP was readily detected in the medium from long-term cell culture (2 days) (Fig.17), no radio-labeled sAPP could be found in the medium under the pulse-chase paradigm. Considering that during the pulse-label period, only a small pool of APP was labeled and that 1% of the labeled APP could accumulate in the medium, we speculate that the amount of labeled sAPP in the medium was too small to be detected by our methods.

The synchronous appearance of the C-terminal fragment and the secreted form of APP (Fig.21C) further supports the idea that they are the cleavage products of a single proteinase--yeast α -secretase. The reciprocal kinetics of the mature APP versus its C-terminal fragment and sAPP clearly indicates a precursor-product relationship. If this is true, the APP cleavage by the α -secretase must occur after Kex2p removes the α -leader from the APP6 α fusion protein.

Another salient feature of APP processing in JSC310 cells is the long turnover rates of mature APP ($t_{1/2}$ >3 hr) and its C-terminal fragment ($t_{1/2}$ >8 hr) (Fig.21C). Since the JSC310 strain has defective vacuoles, APP and its metabolites might have been delivered to the vacuole but failed to be digested. Most significantly, the use of this particular strain suggests that yeast vacuoles may not contain α -secretase activity.

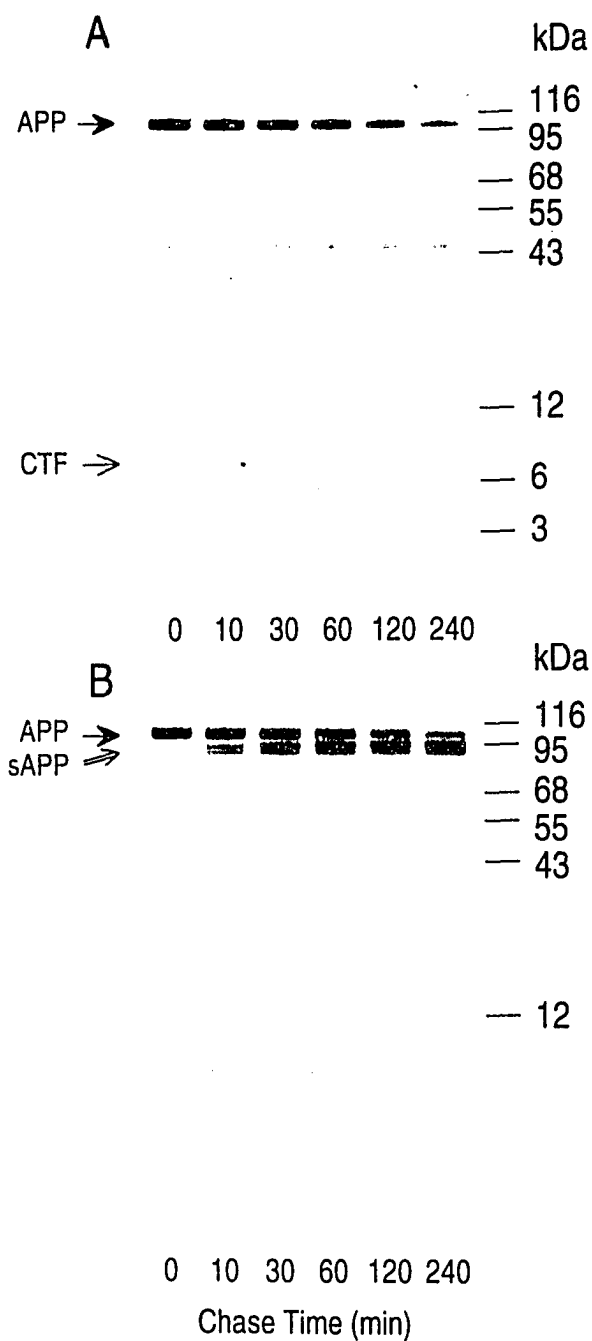
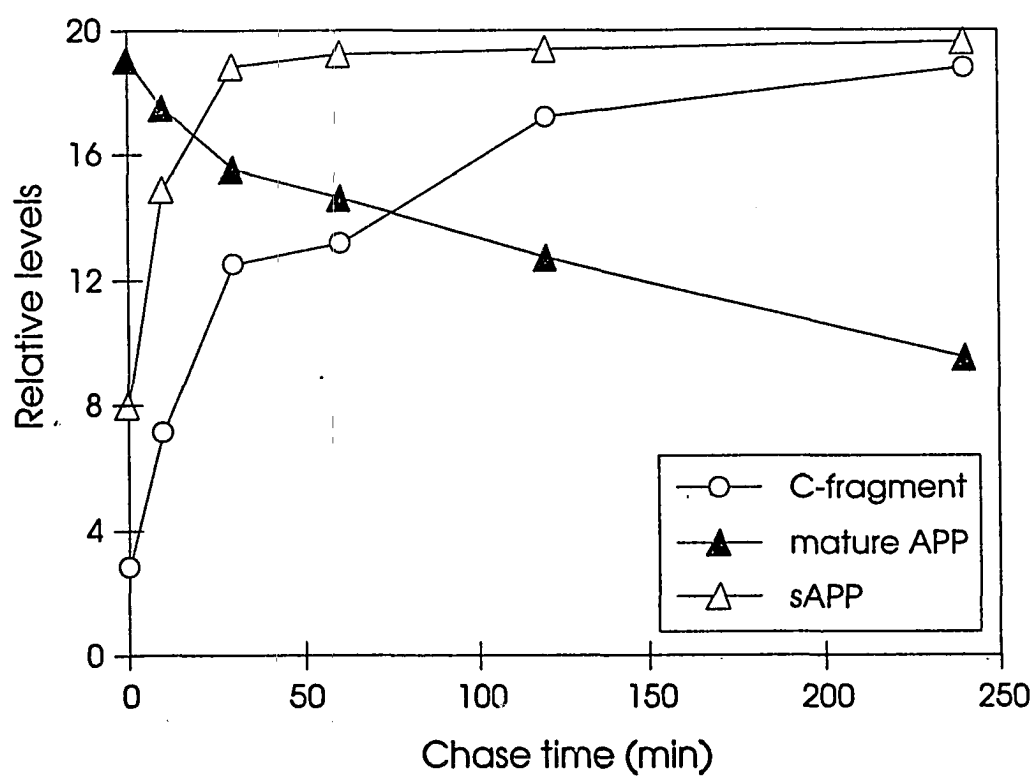


Figure 21. Time-course of pulse-labeled APP cleavage by α -secretase. JSC310 transfected by pBS6 α was labeled for 10 min with [^{35} S]-methionine and cysteine. At the indicated intervals the peptides were immunoprecipitated with either R57 (panel A) or R104 (panel B) and subjected to electrophoresis and autoradiography. (*continued*)

C

(Figure 21, *continued*) Panel C: densitometric analysis of the autoradiograms.

TABLE 3. Processing of APP in the Temperature-Sensitive Secretion Mutants (*sec* Mutants)^a

Mutant	ts Sec Block	<u>7 kD C-fragment/β-APP %</u>	
		24°C	37°C
RSY271/ <i>sec18-1</i>	ER→Golgi	44.70	0
RSY299/ <i>sec7-1</i>	Golgi→secretory vesicles	39.87	32.14
RSY782/ <i>sec1-1</i>	sec. ves.→plasma membrane	26.85	19.30

^a The processing of the α -leader-APP fusion protein in the *sec* mutants were analyzed as described in Figure 22. Each autoradiograph was subjected to photodensitometry, and from these values the ratio of the density of the C-terminal fragment (at a chase time of 30 min) to the density of full-length APP (at 0 chase time) was calculated. The ratios are in some cases greater than expected based upon methionine contents, because during the 10-20 min labeling time an appreciable amount of cleavage had occurred.

Proteolytic Processing of APP by Yeast α -secretase Occurs Late in the Secretory Pathway

The structure of APP6 α fusion protein requires cleavages by both Kex2 endoprotease and α -secretase to form the secreted form of APP. The kinetic study suggested that the α -secretase acts after Kex2 protease. Because Kex2 is present in the late Golgi, yeast α -secretase probably resides in or beyond the late Golgi compartment. To unambiguously define the location of yeast α -secretase, we initiated studies of the processing of APP6 α fusion protein in temperature-sensitive secretion mutant strains (*sec* mutants). The rationale is that failure to deliver APP6 α protein to the α -secretase site would result in accumulation of the protein; thus, by blocking the secretory pathway at specific stages, it should be possible to identify the location of the yeast α -secretase. The availability of *sec* mutants offers a big advantage over the use of pharmacological agents to block vesicle transport because these drugs may either incompletely block the

transport (leakage) or affect parameters of proteolytic processes (non-specificity). The results with four *sec* mutants are shown in Fig.22 and Table 3. *Sec 17* and *sec 18* cells are defective in transport of secretory proteins from the ER to the Golgi at nonpermissive temperature (37°C) (Novick and Schekman, 1983), whereas *sec7* and *sec1* cells express temperature-sensitive defects in protein transport from the Golgi to secretory vesicles, and from secretory vesicles to plasma membranes, respectively. Each mutant strain was transformed with pBS6 α , and the expression of the fusion protein and the secretion block was induced at 37°C. After pulse-labeling by Tran ³⁵S-label for 10–20 min, the cells were chased for up to 4 h at 37°C. The results revealed that blocking protein exit from the ER led to the accumulation of the fusion protein without generation of α -leader-cleaved full-length APP and the 7 kD-C-terminal fragment (Fig.22 *sec18*: lanes 4-7). On the other hand, if the block was imposed after transport through the Golgi (*sec7*), APP6 α fusion proteins were processed into mature APP by Kex2p, suggesting that it had reached the late Golgi. In addition, both 7 kD C-terminal fragment (CTF) and cell-associated sAPP appeared over the chase despite the block of transport at the late Golgi stage (Fig.22 *sec7*: lanes 4-7). The same processing pattern as that of *sec7* was observed when transport from secretory vesicles to plasma membranes was blocked (*sec1*) (Fig.22 *sec1*: lanes 4-7). These data were quantitated and summarized in Table 3. Taken together, these results indicate that APP α -secretase cleavage is likely to take place in the Golgi compartment.

We confirmed the efficient secretory blockage in the *sec* mutants by assaying the invertase secretion (Novick et al., 1980) under the same conditions used in the preceding experiment (Table 4). At the nonpermissive temperature, all *sec* mutants showed reduced levels of invertase secretion and accumulation of intracellular invertase. When the temperature was shifted from 37°C to 25°C, the accumulated invertase was secreted into the medium. Therefore the secretion blockage was

TABLE 4. Invertase Secretion and Accumulation by *sec* Mutants

Strain	Units/mg Dry Weight ^a					
	External (90 min 37°C)	Internal (90 min 37°C)	External ^b (90 min 37°C→ 3 h 25°C)	%Release ^c	External (90 min 25°C)	Internal (90 min 25°C)
RSY299/7-1	0.10	2.13	1.63	71.8	1.45	0.62
RSY782/1-1	0.37	3.05	1.35	32.1	1.47	1.10
RSY271/18-1	0.11	2.94	1.93	62.0	1.66	0.60

^a Units of invertase activity are μ mole of glucose released per min.

^b Cells were incubated at 25°C in the presence of 0.1 mg/ml cycloheximide as described in the text.

^c % release = (Ext 37°C→25°C–Ext 37°C) / Int 37°C x 100

complete and reversible in all the *sec* mutants; the failure of processing of APP in the *sec* mutants was solely due to a transport block instead of damage to other cellular processes. Although not involved in α -secretase activity, the ER seems to slowly degrade the accumulated fusion protein in its lumen at 37°C (Fig.22 *sec18*: lanes 4-6).

α -Secretase Is Not a Vacuolar Protease

The vacuole of yeast contains a variety of hydrolytic enzymes; the pH optima of most of these enzymes is acidic (Wiemken et al., 1979). To further investigate the role of the yeast vacuole in APP cleavage by α -secretase, we analyzed APP processing in cells treated with ammonium chloride, a lysosomotropic agent that blocks the vacuolar delivery of proteinases and increases the vacuolar pH as well (Rothman et al., 1989).

Because ammonium chloride inhibited the radio-labeling of cellular proteins (possibly attributable to interrupted transport of ³⁵S-methionine into the cells), we used *sec18* mutants to circumvent the problem. The proteins were first labeled with Tran³⁵S-label at the restrictive temperature in the absence of ammonium chloride to accumulate

the labeled proteins inside the ER. Then the labeled proteins were allowed to traverse the secretory pathway in the presence of 0.4M ammonium chloride at the permissive temperature. As seen in Fig.23A, before the temperature shift, all of the newly-synthesized fusion protein APP6 α was retained in the ER; none reached the Golgi, as judged by the lack of mature APP (lanes 1 and 2). When the temperature was shifted to 22°C, protein transport resumed, as evidenced by the appearance of mature APP (lane 3). However, both cells treated with ammonium chloride and with control levels of sodium chloride generated same amount of C-terminal fragment.

We also noted the slow maturation of APP; more APP6 α fusion proteins remained in NH₄Cl-treated cells after 3.5 hr chase than those in control cells (compare lanes 3 and 4, Fig.23A). This probably reflects the toxic effect of the high concentration of NH₄Cl on either the protein transport or Kex2p function. Nevertheless, α -secretase activity seems not drastically perturbed by NH₄Cl on the basis of generation of C-fragment.

The neutralization of acidic pH of the vacuole by NH₄Cl treatment was confirmed by a quinacrine uptake assay (Fig.23B). The ammonium chloride-treated cells showed no fluorescence in vacuoles, reflecting the blockage of acidic pH-dependent vacuolar accumulation of quinacrine and thus an increase of vacuolar pH. Both the short (20 min) and long-term (4 hr) treatments with the salt produced the same results. In contrast, sodium chloride-treated cells exhibited strong fluorescence in vacuoles, indicating the acidic nature of these lumens.

These observations demonstrate that inactivating the vacuole has no effect on APP cleavage by α -secretase, and, therefore, vacuolar proteinases do not account for the α -secretase activity; these results are consistent with the results from the *sec* mutant study.

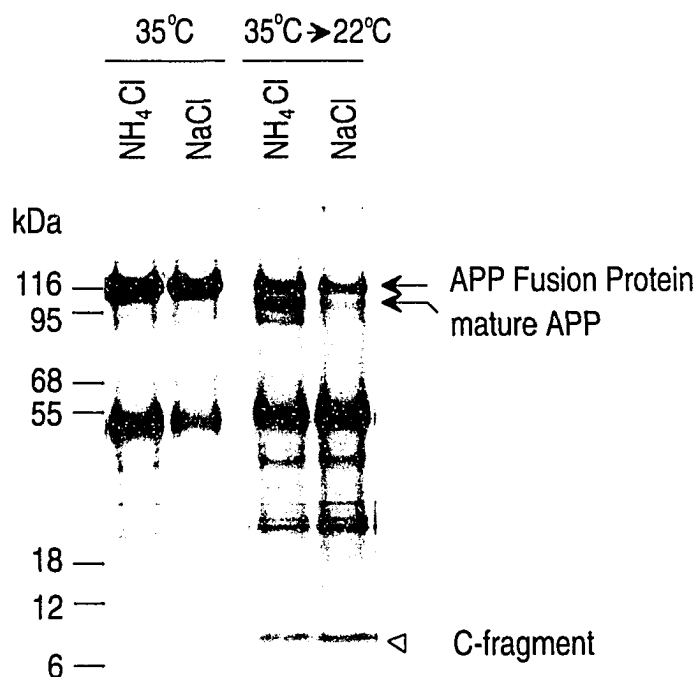
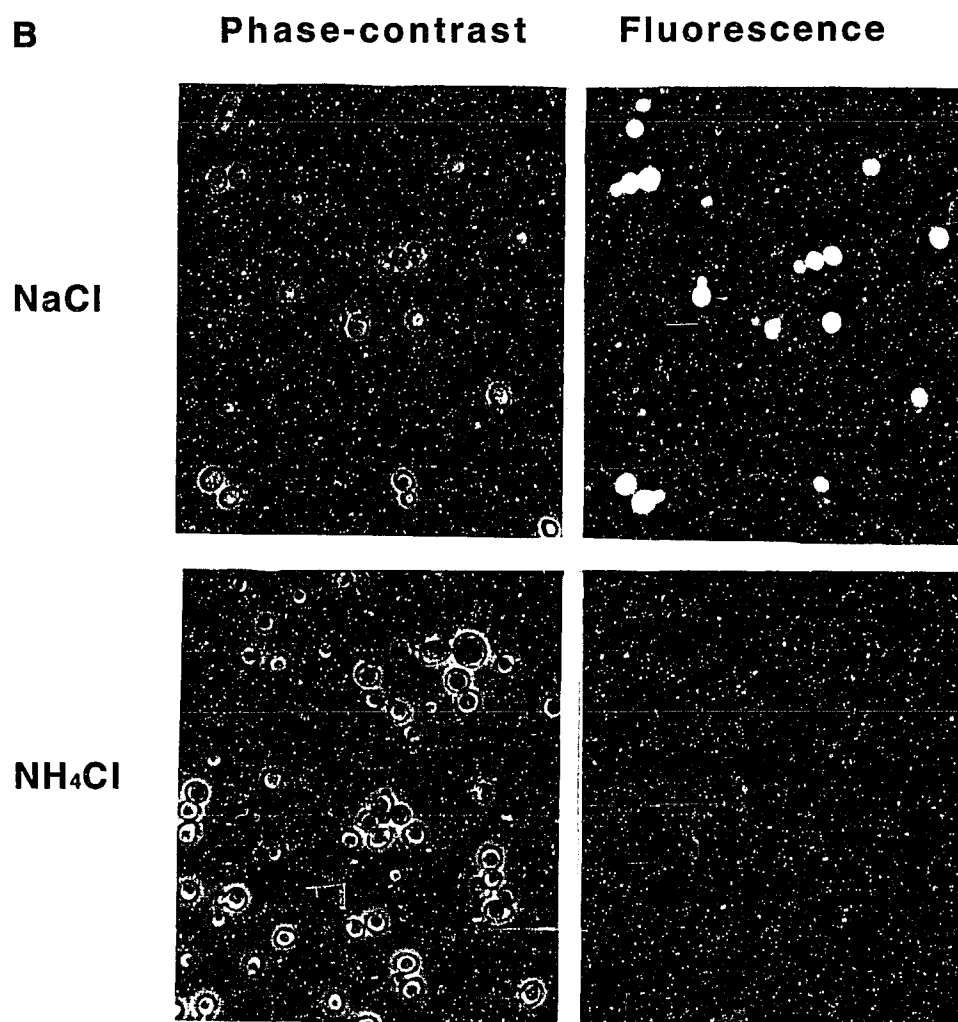
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Figure 23. APP cleavage by α -secretase in NH₄Cl-treated cells. (A) RSY271 (*sec18*) harboring pBS6 α was incubated at 35°C for 1.5h to block protein transport at ER. The cells were pulse-labeled with Tran ³⁵S-label at 35°C and treated with 0.4 M NH₄Cl or 0.4 M NaCl (as a control) at 35°C, and then incubated at 22°C to reverse the secretory pathway block. APP processing was analyzed by immunoprecipitating APP and its C-fragment, Tricine SDS-PAGE and fluorography. (*continued*)



(Figure 23, *continued*) (B) Blocking vacuolar uptake of quinacrine in NH_4Cl -treated cells. Strain RSY271 was treated with quinacrine in the presence of 400 mM sodium chloride (NaCl) or ammonium chloride (NH_4Cl), and viewed by phase-contrast optics (left) and fluorescence (right) microscopy.

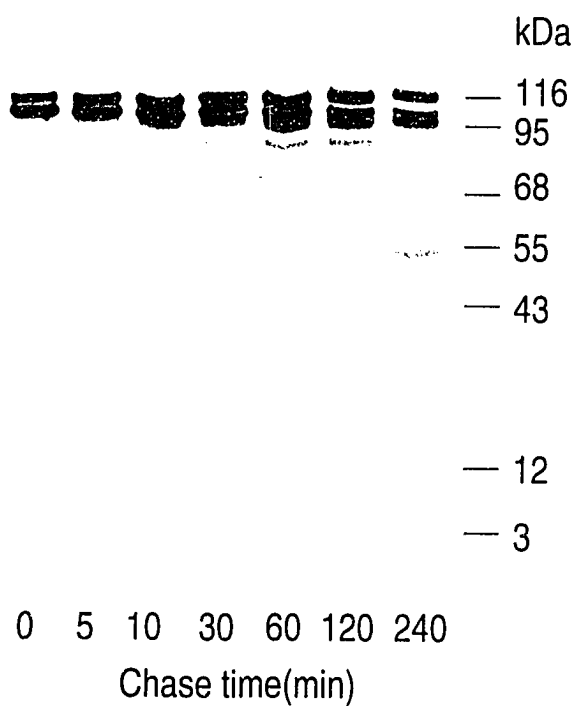
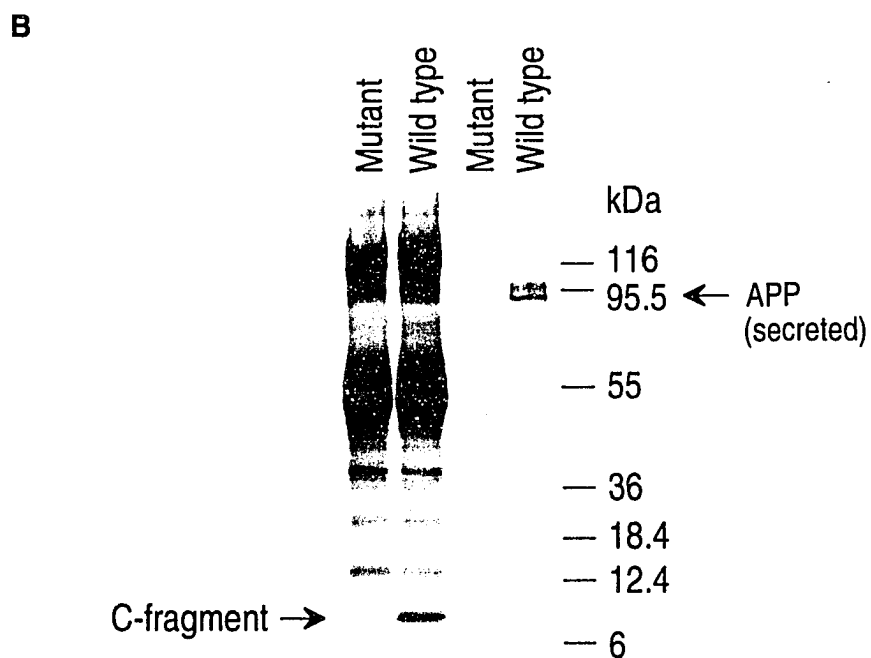
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Figure 24. α -Secretase activity on APP(K612Q). (A) Strain JSC310 was transfected with pBS6 α (K612Q), and the time-course of cleavage of the APP variant was followed as described in Figure 18. The labeled proteins were immunoprecipitated by antiserum R57. (*continued*)



(Figure 24, *continued*) (B) The transfected cells were cultured for 24 hr, and the cells and media were analyzed for α -secretase cleavage products by Western blotting. The cell lysates (left 2 lanes) were assayed by R57, and the media (right 2 lanes) were assayed with mAb6E10.

Yeast α -secretase is sequence-dependent

Mutagenesis studies demonstrated that mammalian α -secretase is capable of cleaving many different bonds at a constant distance (12-13 residues) N-terminal to the membrane segment (Sisodia, 1992). To assess the specificity of yeast α -secretase, we introduced a point mutation into pBS6 α by substituting a glutamine for a lysine residue, the 13th residue from the transmembrane domain, (K612Q) (Fig.10A). Yeast cells (JSC310) were transformed with the modified vector and metabolically labeled and chased. The mutation greatly reduced the rate of the 7-kD C-terminal fragment formation (Fig.24). An alternative cleavage slowly produced a 13 kDa C-terminal fragment (Fig.24A), but this fragment was also detected in western blots of JSC310 cells expressing WTAPP695 (Fig.24B, compare lanes 1, 2). When the altered gene was expressed for longer period (24 hr), a small amount of 7 kDa fragment was produced, but it only accounts for a small fraction of the amount produced by WT APP695 (not visible in Fig.24B). A small amount of sAPP was secreted from long-term cultures expressing the mutant protein, but it was again a small fraction of that secreted by cells expressing WT APP695 (Fig.24B, lanes 3 and 4). Although no sequencing has been done to determine the exact cleavage site, it must lie within six residues of the mutated residue, since the secreted mutant protein bound mAb 6E10 but not mAb 4G8 (data not shown). Therefore, unlike its mammalian counterpart, yeast α -secretase apparently requires specific sequences near the cleavage site for its activity, specifically the basic residue lysine 612.

Functional Separation of α -Secretase From Kex1p Activities

In order to isolate yeast mutants defective in α -secretase, we tried to use α -secretase-dependent α -factor secretion as a screening method. We constructed a chimeric gene (α C) that encodes the α -leader and one copy of α -factor connected to the C-terminal fragment of APP (CTF) with lysine, which is indispensable for specific α -secretase cleavage as described above (Fig.11). We reasoned that the α -leader would

direct the fusion protein to the late Golgi, where Kex2p would free the N-terminus of α -factor. Next, α -secretase would cut off the C-terminal fragment, and then Kex1p would remove the lysine residue on the C-terminus of α -factor. Subsequent secretion of α -factor as monitored by the halo-assay should indicate the presence of α -secretase activity. The halo-assay is a simple, sensitive method to detect α -factor: the pheromone secreted by α -cells inhibits the growth of a tester cell (a type), which is supersensitive to α -factor, resulting in a clear zone (halo) surrounding the secreting cells (Sprague, 1991).

The α C chimera described above was expressed in α -cells that carried nonfunctional copies of the *MF α 1* and *MF α 2* genes (strain DS7); thus, secretion of bioactive α -factor requires the proper processing of the fusion protein. A pulse-chase study showed that the fusion protein first appeared as a 18 kD protein and then gradually disappeared; concomitant accumulation of a C-terminal fragment, which migrated with the CTF of α -secretase product (Fig.25), indicated that α -secretase cleaved the fusion protein around the junction between CTF and α -factor portion.

To our surprise, however, when the DS7 transformant expressing α C was grown on a lawn of supersensitive a-cells, a zone of inhibition (halo) was not observed (Fig.26B). This suggested that bioactive α -factor was not secreted from DS7. Amino acid substitution studies have shown that except for a few N-terminal residues, changing amino acids and the length of native α -factor peptide drastically compromises its bioactivity (Masui et al., 1979; Eriotou-Bargiota et al., 1992). It is possible, then, that Kex1p had not removed the lysine residue at the C-terminus of the α -factor being secreted from the transformant. To test this hypothesis, the α -factor secreted from DS7/pBS α C was partially purified from the medium by an ion-exchange column, and digested with a soluble Kex1 protease (Kex1 Δ p, C-terminus-truncated). This enzyme has been shown to efficiently process α -factor-Lys-Arg to mature active

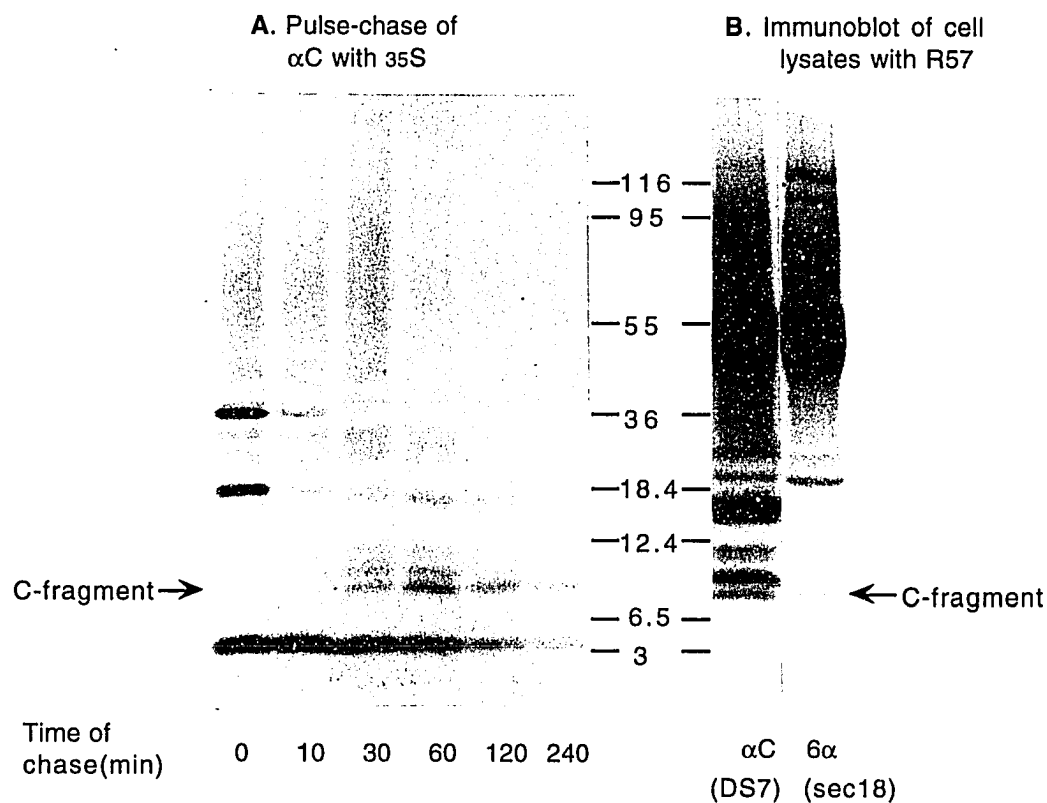
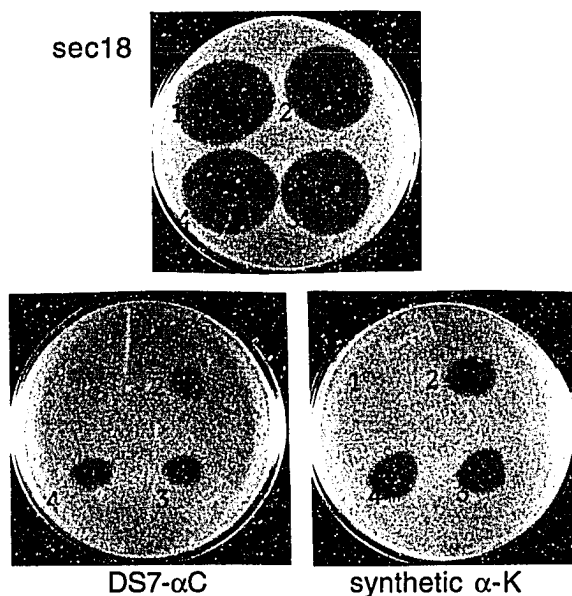


Figure 25. Processing of α C fusion protein in DS7 strain. (A) Pulse-chase study. DS7 cells carrying pBS α C construct were pulse labeled with ^{35} S-methionine for 10 min and chased for various periods of time. The labeled proteins were immunoprecipitated using R57, and analyzed by SDS-PAGE and autoradiography. (B) Immunoprecipitation of the C-terminal fragment of APP from the lysate of DS7 harboring pBS α C that had been grown for two days at 30°C. *Sec18* cells expressing APP6 α serve as a positive control for the C-terminal fragment (7 kD).

A. Kex1p digestion of purified α -factors



B. Determination of activity of Kex1p in DS7

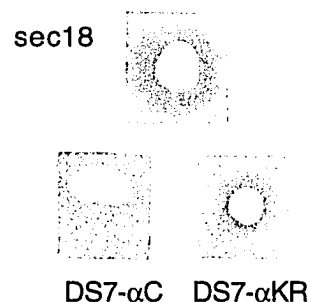


Figure 26. Release of α -factor from α -factor intermediate. (A) Activation of the α -factor secreted from DS7/pBS α C by Kex1p digestion. The α -factor secreted from DS7/pBS α C was partially purified by ion-exchange chromatography as described in Methods and Materials (Part II). The sample was treated with Kex1p (soluble form) at different concentrations (ng/ μ l): 0 (1), 0.67 (2), 1.4 (3), 1.8 (4). One microliter of the treated samples was spotted onto plates seeded with SY1854, a mutant strain that is supersensitive to α -factor. The halos are caused by inhibition of growth of the SY1854 cells by α -factor. The Kex1p digestion was also performed with the purified α -factor from *sec18* cells (containing wild type *MF α 1* and *MF α 2* genes) and the synthetic C-lysyl- α -factor (α -K). (B) Determining the activity of endogenous Kex1p in DS7. DS7 expressing α -factor-CTF (DS7/pBS α C) or α -factor-Lys-Arg (DS7/pBS α KR) were grown in YPD medium (2% glucose) for two days; the cell suspensions were spotted onto SY1854-seeded plates in YPD (0.05% glucose). *Sec18* cells served as a positive control of secretion of bioactive α -factor.

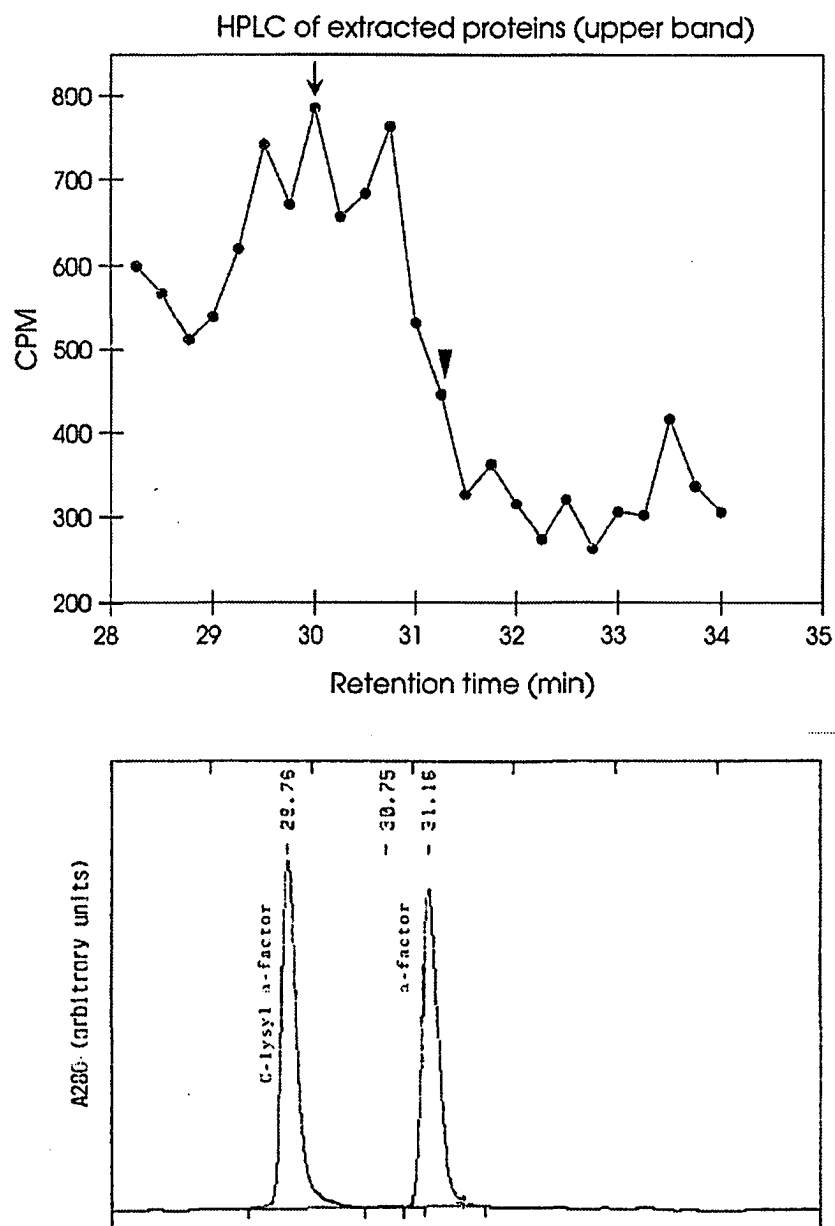


Figure 27. HPLC analysis of secreted α -factor derivative from DS7/ α C. The α -factor derivative that was secreted by [^{35} S]-methionine-labeled DS7/pBS α C was purified by Bio-Rex70 ion-exchange column and 7/11% Tricine SDS-PAGE, and analyzed by HPLC as described by Materials and Methods (PART II). The lower panel shows the elution profile of the internal standards, synthetic α -factor peptide and C-lysyl α -factor peptide, which were detected at 280 nm. Their corresponding retention times are also marked in the upper panel (arrow: α -factor; arrowhead: C-lysyl α -factor). The eluates of the samples were monitored by radioactivity.

α -factor (Latchinian-Sadek and Thomas, 1994). When the Kex1 Δ p-treated α -factor was tested, it elicited halos (Fig.26A). Hence, Kex1p in the transfected cells failed to process the lysyl α -factor.

This conclusion was further supported by HPLC analysis of [³⁵S]-methionine-labeled α -factor secreted by the transfected cells. The labeled peptide was purified by consecutive sizing columns and Tricine SDS-PAGE. The peptides extracted from the gel band which comigrated with synthetic α -factor, were analyzed by HPLC. Although multiple peaks were obtained, the retention time of the major peak corresponded to that of the synthetic α -factor-Lys (internal standard); whereas, no peak matched the synthetic α -factor (Fig.27); this indicates that α -factor-Lys was the major species secreted by DS7/pBS α C.

To determine whether DS7 contained active Kex1p, a shuttle vector harboring pro- α -factor-Lys-Arg was introduced into the cells. Halo-assays demonstrated that the cells secreted bioactive α -factors that arrest the growth of the tester cells (Fig.26B, DS7- α KR). Thus, DS7 has an active Kex1p that can remove basic residues at C-terminus of α -factor.

Since yeast carboxypeptidase Kex1p can only cleave basic amino acids on a free C-terminus, we speculate that α -secretase and Kex1p are likely to reside in different locations of the late Golgi region; thus, Kex1p can not encounter and process the α -factor-K generated by α -secretase.

Inhibiting N-linked Glycosylation Does Not Affect Yeast α -Secretase Activity

Like the processing of precursors of many secretory proteins, the maturation of α -mating factor from prepro- α -factor requires glycosylation (Julius et al., 1984). APP has two N-glycosylation sites in its ecto-domain (Weideman, 1989; Pahlsson, 1992). To determine whether N-linked glycosyl modification of APP occurs and whether the glycosylation affects the α -secretase cleavage in yeast cells, two independent methods

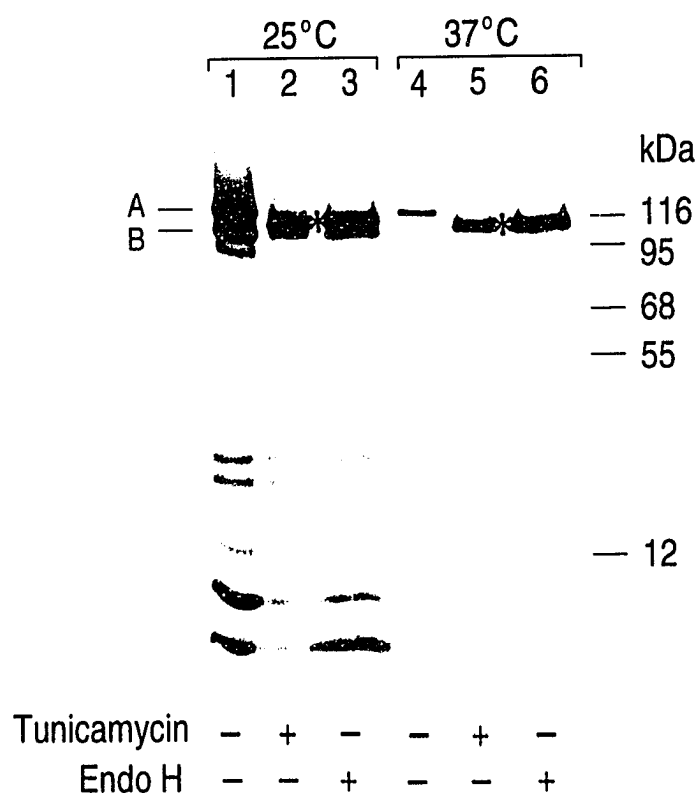


Figure 28. Effect of glycosylation on α -secretase action. Strain RSY271 (*sec18*) transfected with pBS6 α was labeled for 1 hr with [^{35} S]-methionine and cysteine, in either the absence (lanes 1,3,4,6) or the presence (lanes 2,5) of tunicamycin at either 25°C (lanes 1-3) or 37°C (lanes 4-6). Peptides containing the C-terminal epitope were immunoprecipitated with R57 and analyzed as described in Figure 18. Before electrophoresis, portions of the immunoprecipitate from untreated cells were digested with endoglycosidase H, as described in Materials and Methods (Part II). The starred bands are discussed in the text.

of analysis were employed. In untreated *sec18/pBS6 α* labeled at 22°C, two predominant APP-containing proteins appeared (Fig.28, lane 1: bands A and B). When the cells were labeled in the presence of the drug tunicamycin, which specifically blocks the biosynthesis of N-linked glycosyl chains (Mahoney and Duskin, 1979), a major APP-related species appeared (lane 2: starred band) that possessed an apparent molecular weight 7 kD smaller than A band, and 8 kD greater than B band. When a secretion block was imposed in the transport from the ER to the Golgi, a single R57-immunoreactive species was detected in the presence of tunicamycin (lane 5), which was again 7 kD smaller than the glycosylated APP fusion protein retained in the ER (lane 4), and had a mass identical to that of the starred band at 25°C (lane 2). Since at 37°C, the fusion protein cannot encounter Kex2p, the starred band at 25°C must correspond to the unglycosylated APP fusion protein. Band A is then the glycosylated fusion protein, and band B is the full-length APP free of α -leader. As the α -leader region of the α -factor precursor has three N-linked mannose-rich oligosaccharide chains which account for about 7 kD of its molecular mass (Julius et al., 1984), the 7 kD reduction of the molecular weight of tunicamycin-treated APP fusion protein must reflect the removal of all those oligosaccharide chains from the α -leader portion of the fusion protein, and, accordingly, the absence of glycosyl modification of APP itself. Indeed, both the mature α -leader-cleaved APP molecules with or without tunicamycin treatment have the same apparent molecular weight (compare bands B in lanes 1 and 2, Fig.28).

To confirm this conclusion, the material immunoprecipitated from *sec18/pBS6 α* cells was exhaustively digested with the enzyme endoglycosidase H (endo H) to remove N-linked carbohydrate chains from the peptide backbone (Tarentino et al., 1974). This treatment yielded two APP-related species similar to those formed in tunicamycin-treated cells except that the larger species corresponding to APP- α -leader fusion protein migrated slightly slower than the fusion protein (starred band) from tunicamycin-

treated cells (Fig.28, lanes 2 and 3). As in the tunicamycin treatment, endo H-digested mature APP had the same mass as untreated APP, further supporting the notion that APP is not modified by glycosyl chains in yeast. Taken together, both tunicamycin treatment and endo H digestion indicates that, in contrast to APP in mammalian cells, APP in yeast cells does not undergo N-linked glycosylation.

Tunicamycin greatly inhibited the 7-kD C-terminal fragment formation by yeast α -secretase (Fig.28, lane 2). The reduced amount of the 7 kDa C-terminal fragment of α -secretase product probably resulted from the lower rate of transit of the fusion protein and may be attributed to the drug treatment. Similar accumulation of unglycosylated pro- α -factor and inhibition of maturation of α -factor after treating yeast cells with tunicamycin has also been documented (Julius et al., 1984).

Processing of APP Is Not Affected In *yap3* and *kex2* Null Mutants

The aspartyl protease YAP3 can process α -factor precursors at monobasic cleavage sites (Egel-Mitani et al., 1990; Bourbonnais et al., 1993). In an attempt to investigate the role of Yap3 protease in APP processing, we expressed APP6 α in a *yap3* null mutant (YBAD1). The cells were labeled with Tran ³⁵S label and chased for various time periods. As shown in Fig.29A, the disappearance of the full-length APP and appearance of the 7-kD C-terminal fragment in the mutant was similar to that in JSC310 cells. In addition, soluble APP secretion was not hampered in the *yap3* mutant, since soluble APP was immunoprecipitated by R104 from the medium of the mutant cell culture (Fig.29B, lane 3). The secreted APP lacked the APP C-terminus because R57 failed to bind to it. Furthermore, it bound to mAb 6E10 but not mAb 4G8. These immunoreactivities indicate that APP was cleaved at the same cleavage site in both mutant cells and wild type cells. By southern blotting, we confirmed that our mutant culture still maintained the *YAP3* gene deletion (Fig.29C). We conclude that Yap3 protease is not yeast α -secretase.

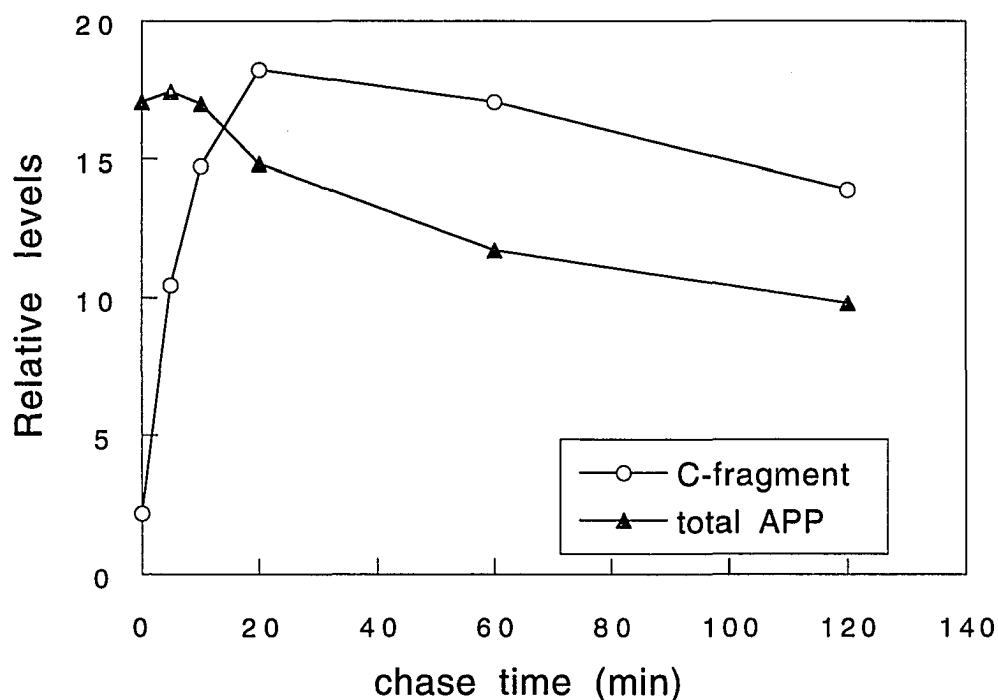
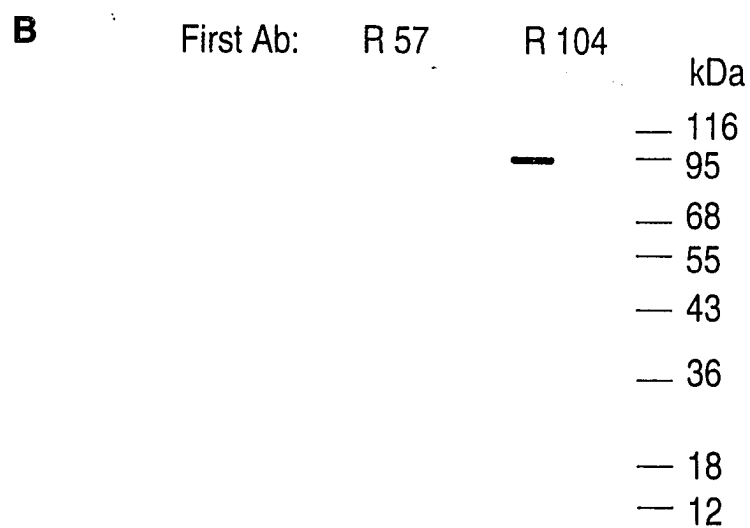
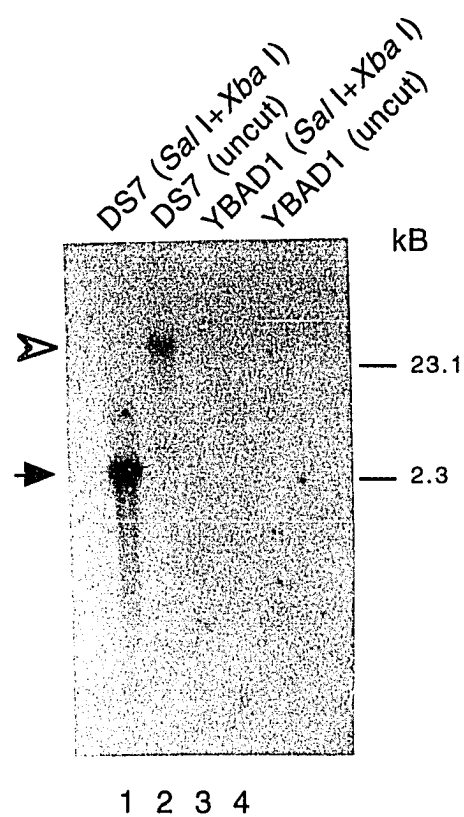
A

Figure 29. Turnover of APP in a *yap3* mutant strain. (A) Strain YBAD1 (*yap3* mutant) transfected with pBS6 α was pulse-labeled and chased, and the peptides containing the C-terminal epitope of APP were immunoprecipitated with R57 and analyzed densitometrically. (B) Immunoprecipitation and analysis of sAPP from the medium of the YBAD1 culture. The medium was treated with either R57 or R104 and the immunoprecipitated peptides were subjected to Western blot analysis using mAb6E10 or mAb4G8. (C) Southern analysis of the genomic DNA from the *yap3* mutant strain. The DNA isolated from the cells was hybridized to a probe corresponding to a unique region in *YAP3* gene. The DNA from the parent strain DS7 was run as a positive control. Note that both the genomic DNA from DS7 (lane 2) and its *Sal* I-*Xba* I restriction fragment (lane 1) hybridized to the probe, but those from strain YBAD1 did not. Hybridization was performed at 55°C overnight. (*continued*)



Second Ab: 6E10 4G8 6E10 4G8

C



(Figure 29, *continued*)

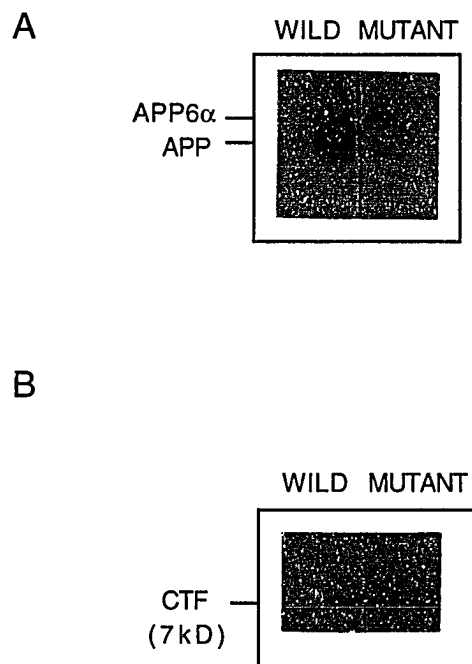


Figure 30. APP processing in a *kex2* null mutant. Yeast M200-6C (*kex2* null mutant) expressing APP6α were grown in YPD (2% glucose) for two days, and the cell lysate was subjected to western blotting using the antiserum R57. Yeast YBAD (wild) that contains a wild-type *KEX2* gene is shown on the blot as a positive control. (A) Failure to remove the α-leader from APP6α fusion protein in the mutant cells due to the lack of the Kex2 endopeptidase. (B) Uncompromised production of the C-terminal fragment of APP in the mutant cells.

We also investigated APP processing in a *KEX2*-deficient mutant (M200-6C) expressing APP6 α . As previously reported (Zhang et al., 1994), APP cleavage by yeast α -secretase proceeded in the absence of Kex2p; this ruled out Kex2 as the yeast α -secretase (Fig.30).

DISCUSSION

Human APP undergoes a complicated multi-pathway processing in mammalian cells. One processing that might be affected in AD is the secretion of the extracellular part of APP by α -secretase cleavage in the middle of β -peptide region, a process that prevents amyloid formation. However, identification of mammalian α -secretase has proved to be arduous. In many ways, the yeast secretory system resembles its mammalian counterpart (Table 5); Like mammalian proteins, newly-synthesized yeast proteins enter the yeast endoplasmic reticulum and are transported through the early- and mid- Golgi, along which modifications of proteins occur; upon reaching the late-Golgi, they are sorted to the vacuole, the plasma membrane and the cell wall, or the extracellular space (Pryer et al., 1992 ; Nothwehr and Stevens, 1994). Yeast and mammalian secretory systems seem to share a common vesicle docking/fusion mechanism, and many proteins that are involved in these processes in both cells are homologous and even interchangeable, such as small GTP-binding proteins; NSF and Sec18p; SNAPs and Sec17(α)p; VAMP, Bet1p and Sly2p; and Syntaxin, Sed5p and Pep12p (Pryer et al., 1992; Sollner et al., 1993; Bennett and Scheller, 1994). The similarities between the secretory systems in yeast and mammalian cells, as well as the discovery of yeast homologs of the enzymes responsible for maturation of hormones, make yeast *Saccharomyces cerevisiae* a good model to investigate mammalian biochemical processes. The aim of this work is to establish that yeast is a suitable model for studying APP processing. The present study demonstrates that the secretory system of yeast contains proteases with specificities similar to the mammalian α -secretase, and that yeast α -secretase resides in late-Golgi.

Transport and Processing of APP in Yeast Secretory Pathway

Human APP, with a pro- α -leader or with a human signal peptide, was inserted into the yeast ER membrane in the correct orientation and transported within the secretory system. Our studies show that yeast can process the heterologous APP

TABLE 5. Comparison Between Yeast and Mammalian Secretory Systems

	Yeast cells	Mammalian cells
<u>Membranous structures involved in protein transport</u>		
ER	yes	yes
Golgi	early, mid, late	cis-Golgi network, Golgi apparatus (cis, medial, trans), trans-Golgi network (TGN)
hydrolytic compartment	vacuole	endosome/lysosome
transport vesicles	yes	yes
<u>Protein secretion</u>	constitutive	constitutive (most cells), regulated (neurons and endocrine cells)
<u>Endocytosis</u>	yes	yes
<u>Protein sorting</u>		
to lysosomes/vacuoles	transmembrane proteins: default; soluble proteins: signal-mediated	transmembrane proteins: signal-mediated; soluble proteins: mannose-6-phosphate modification
to plasma membranes secretion of soluble proteins	signal-mediated default	default (constitutive secretion); signal-mediated (regulated secretion)
<u>Retrieval/retention of ER resident proteins</u>		
retention signal	HDEL (soluble proteins) KKXX (membrane proteins)	KDEL (soluble proteins) KKXX (membrane proteins)
receptor for signals	Erd1p/Erd2p for HDEL	Erd2p homolog for KDEL (human)
<u>Vesicle docking/fusion machinery</u>	Sec18p-Sec17(α)p-SNAREs complex (homologous to mammalian counterpart)	NSF-SNAP-SNAREs complex ^a

^a NSF: N-ethylmaleimide-sensitive fusion protein; SNAP: soluble NSF attachment proteins; SNAREs: SNAP receptor

into a membrane-bound 7 kD C-terminal fragment and a soluble N-terminal portion that is partially secreted. Immunoblotting using antibodies that recognize various APP epitopes and direct sequencing analysis demonstrated that the cleavage site is identical to that of mammalian α -secretase. We refer to this novel endopeptidase as yeast α -secretase.

Although the α -leader is essential for correct transport and processing of other heterologous proteins expressed in yeast (Brake et al., 1984; Zsebo et al., 1986; Bourbonnais et al., 1991), the necessity of the α -leader in processing APP appears not to be an absolute one, because cleaving of APP by α -secretase occurred in both yeast expressing α -leader-APP (6 α) and yeast expressing APP with an authentic signal sequence (6F) (data not shown). However, the α -leader seems to be required for sAPP secretion, since only yeast expressing α -leader-APP secreted sAPP into the medium..

In the JSC310 strain, the hybrid APP6 α protein was rapidly cleaved by Kex2p to remove the α -leader segment so that only mature APP was observed even with very short labeling times. Since mature Kex2p resides in the late Golgi, APP6 α fusion proteins should have reached that Golgi region. However other possibilities remain: the fusion protein might be processed by newly synthesized Kex2p in the ER or early Golgi compartments. Several lines of evidence argue against this possibility. Experiments using tunicamycin and Endo H demonstrate that the hybrid protein was modified by oligosaccharide chains on the α -leader region, and thus must traverse through the Golgi where the glycosylation occurs. In addition, pulse-chase studies often show an intermediate APP-related species that migrates more slowly than the mature APP and can be chased into a species with an apparently higher molecular weight (Fig.22, *sec1*, chase at 24°C). It is believed that this species reflects the process of glycosyl modification of unglycosylated or core-glycosylated fusion proteins. These data support the idea that the fusion protein reaches the late Golgi, where it is cleaved by Kex2p. Our conclusion is consistent with the previous discovery that the late Golgi

was the only place for Kex2 protease-dependent processing of several hybrid proteins; for example, a vacuole-targeted hybrid with a Kex2p cleavage site (Graham and Emr, 1991) and trimeric proteins with Kex2 cleavage site separating Sec12p (or Sec22/Sly2p or Bet1/Sly12p) and invertase (Boehm et al., 1994), all indicate that the cleavage by Kex2p is a suitable indicator for the passage of proteins through the late Golgi compartment.

Kex2p is involved in the processing of yeast endogenous soluble α -factor precursor. Our study points out that transmembrane proteins (i.e. APP- α -leader hybrid) that reach the late Golgi compartment can also be cleaved by Kex2p as efficiently as soluble proteins.

The late Golgi is a protein sorting point. However, because yeast has a much less elaborate secretory system (the yeast Golgi structure is not visible unless under special conditions, such as at nonpermissive temperature in the case of *sec* mutants), the sorting machinery at the late Golgi can be easily saturated by overexpressed APP; this leads to an accumulation of the protein, possibly in an aggregated form, and may explain why only 25-50% of APP achieves a Triton X-100 extractable conformation.

Yeast α -secretase cleavage of APP proceeds in two phases, with a 18 min first half-life and a 120 min second-half-life (Fig.21C). This pattern of processing is comparable with that of mammalian α -secretase, in which most sAPP is recovered in the first 15 min and substantially lower amounts are in the subsequent 45 min (i.e. in transfected CHO cells) (Koo and Squazo, 1994). In yeast cells, newly synthesized sAPP is detected after 5-10 min of chase similar to that in several mammalian cell lines expressing endogenous or heterologous APP (Haass et al., 1993; Oltersdorf et al., 1990; Weidemann et al., 1989).

In both yeast and mammalian cells, only a minor proportion of APP undergoes α -secretase cleavage and appears in the extracellular medium. Assuming that the Triton X-100-extractable APP is the only substrate for α -secretase, one calculates that 50% of

the APP appears to be cleaved, 10% of which is secreted to the medium and 40% is associated with cells in a disulfide crosslinked form, possibly in the periplasmic space. Following this reasoning, α -secretase activity is quite high in yeast cells. Mammalian cells exhibit varying amounts of α -secretase activity. Transfected Hela cells secrete about 30% of recombinant APP (Weidemann et al., 1989), whereas neurons produce a small amount of sAPP (Wertkin et al., 1993), and microglia and astrocytes secrete none (Haass et al., 1991).

Yeast α -Secretase Resides in the Golgi

Yeast contains a large number of proteinases that are localized in various compartments including the cytosol, vacuole, mitochondria, the ER, and Golgi complex, and in membranes (vacuole, the ER, the Golgi, and the plasma membrane of the cell) (Jones et al., 1991). Cytosol proteinases are apparently not responsible for APP processing since APP is inserted into the ER membrane and the α -secretase cleavage site within the lumen is not accessible to these proteinases.

The yeast vacuole is an acidic organelle containing hydrolytic enzymes, and is considered to be equivalent to the lysosome of animal cells (Wiemken et al., 1979; Rothman and Stevens, 1988). Yeast transmembrane proteins are transported to the vacuoles by default to be degraded. Extensive structure analysis of two vacuolar membrane proteins (type II) alkaline phosphatase (ALP) and dipeptidyl aminopeptidase (DPAP) suggest that apart from being attached to the membrane, no structural information is necessary for these proteins to reach the vacuole. Therefore, it is conceivable that APP- α -leader hybrid or mature APP could be delivered to the vacuole where sAPP and C-terminal fragments could form. Furthermore, since proteins in vacuoles usually cannot be secreted, the production of sAPP in the vacuole may explain the intracellular retention of majority of sAPP in yeast. We think that the vacuole is unlikely to be the site of action of α -secretase for the following reasons: First, α -secretase shows high activity in yeast strain JSC310, which lacks the major vacuolar

proteases. JSC310 strain has mutated *PRB1*, *PRC* and *PEP4* genes, which results in a lack of active proteinase B, carboxypeptidase Y and proteinase A, respectively. The *pep4* mutation also causes a reduction or elimination of most vacuolar proteinase activities simultaneously (Jones, 1991). However, when the APP hybrid proteins were expressed in these cells, production of the 7 kD C-terminal fragment and sAPP proceeded rapidly; second, the lysosomotropic agent ammonium chloride, which causes mislocalization of vacuolar enzymes (e.g. proteinase A) to the plasma membrane and disrupts the acidification of the vacuole, leading to the malfunction of the organelle (Rothman et al., 1989), does not affect α -secretase cleavage of APP. Perhaps the most convincing evidence arguing against the involvement of the vacuolar proteinases in the sAPP production came from the study of *sec* mutants.

By blocking APP transport at specific stages of the secretory pathway using *sec* mutants at a restrictive temperature, we were able to locate the α -secretase to the Golgi. While the APP- α -leader fusion protein accumulated in the ER did not undergo α -secretase cleavage in *sec18* cells, sAPP and the 7 kD C-terminal fragment production occurred in the *sec7* cells, where protein transport was blocked in the Golgi.

The issue of the localization of mammalian α -secretase is still controversial. In most APP-expressing cells, sAPP can be detected extracellularly; blocking APP internalization enhances sAPP secretion. On the other hand, sAPP appears inside cells of certain types (i.e. human neuroglioma H4 cells, Kuentzel et al, 1993), and sAPP accumulation within cellular compartments precedes, and is proportional to, its appearance in the extracellular medium. In polarized MDCK cells, α -secretase can act both on the plasma membrane and inside cells (Haass et al, 1994). These findings are consistent with the notion that mammalian α -secretase can either reside on the cell surface or inside cells, or both depending on cell types. The intracellular site of α -secretase may be a late compartment of the default constitutive secretory pathway (De Strooper et al., 1993), for example, the trans-Golgi network/post-Golgi vesicles

(Sambamuri et al., 1992). However, the lack of ways to manipulate the protein secretion in mammalian cells prevents drawing definitive conclusions from these studies. In contrast, yeast α -secretase appears to act in a single compartment. Although we can not rule out the possibility that the cell surface could serve as additional place for the endoprotease, by using *sec* mutants and other constructs, we provided direct evidence that yeast α -secretase is located in the late Golgi region.

sAPP delivery from the late Golgi to cell surface in yeast cells seems to be a slow process, which may account for the failure in detection of cell-surface sAPP after a short chase period but the ready release of the surface-bound sAPP from cells expressing APP for two days after the alkaline-thiol treatment. In contrast, yeast constitutively secrete α -factor or invertase with high speed. According to the current model of yeast secretory transport, soluble proteins are delivered and secreted in the default bulk flow. At present, we do not have a satisfying explanation for the differential rates of the vesicular transport of sAPP and endogenous soluble proteins.

Dose Yeast Have a Distinct α -Secretase-Containing Golgi Compartment?

Studies with *sec7* alone could not determine the exact location of α -secretase within the Golgi. The yeast Golgi apparatus, while lacking the distinct morphological equivalent of the mammalian stack of cisternae (Franzusoﬀ and Schekman, 1989), has been functionally defined by the reactions involving glyco-modifications and proteolytic processing. In general, the yeast Golgi can be separated into early, mid and late compartments with marker enzymes α -1,6-mannosyltransferase (early), α -1,3-mannosyltransferase (mid) and Kex2p (late). The Kex2p-containing compartment is postulated to be at the extreme *trans* side of Golgi, the functional equivalent in yeast of the mammalian *trans*-Golgi network, where secretory and vacuolar proteins are sorted (Bryant and Boyd, 1993). It was found that the defect in protein transport imposed by *sec7* involves multiple points of transit through the Golgi apparatus, and the blockage at these points are incomplete (leaky) (Franzusoﬀ and Schekman, 1989). Leaky blockage

is also true for our *sec7* mutant, in which, in spite of the non-permissive temperature, the APP- α -leader hybrid traverses the *cis* and mid Golgi and reaches the late Golgi, as evidenced by the removal of the α -leader sequence from the hybrid protein and the appearance of mature APP. Accordingly, the exact location of α -secretase within the Golgi cannot be determined by *sec7* mutant; for example, α -secretase cleavage of APP might be continuous throughout the Golgi as the fusion protein is transported in the Golgi lumen.

Two experiments shed light on the possible intra-Golgi location of yeast α -secretase. The kinetic study of APP processing in JSC310 demonstrated that APP6 α fusion proteins were rapidly converted into mature APP by Kex2p followed by slow accumulation of C-terminal fragment and sAPP resulting from α -secretase cleavage of APP. The simplest explanation for this is that α -secretase acts after Kex2p. If this is true, α -secretase should reside in the Kex2p compartment or in a more distal Golgi compartment. Evidence supporting this hypothesis came from the study of the processing of a hybrid protein (α C gene product) consisting of α -leader and one copy of α -factor fused to the C-terminal fragment of APP. Kex2p and α -secretase cleaved away α -leader and the C-terminal fragment from the hybrid respectively to produce α -factor-K, but Kex1p, a Golgi carboxypeptidase, which removes the dibasic residues KR at the C-terminus of α -factor during α -factor maturation, failed to clip the lysine to generate bioactive α -factor. Therefore, α -secretase and Kex1p activities are topologically separated. A recent immunoisolation study has revealed that Kex1p resides in the same Golgi compartment as Kex2p (Bryant and Boyd, 1993); thus, α -secretase activity should also be separated from Kex2p activity. In addition, the *sec* mutant study located the α -secretase in the Golgi. Therefore, although we can not exclude the possibility that α -secretase is co-localized with Kex1p and Kex2p in a single Golgi compartment, each occupying a differing micro-environment, it is more likely that α -secretase resides in a

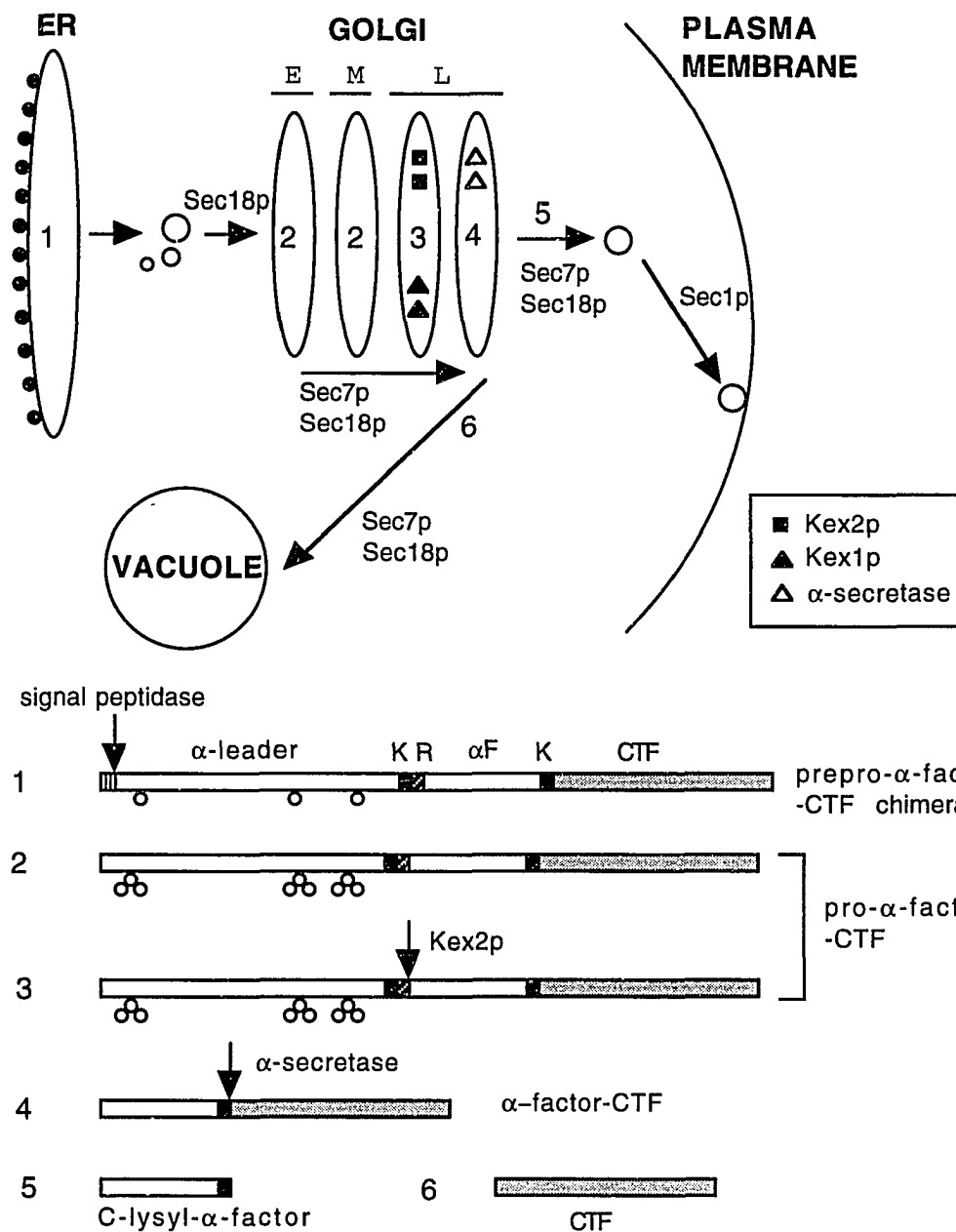


Figure 31. Model for the compartmental organization of posttranslational modification and processing of prepro- α -factor-CTF (α C) and protein sorting events in the yeast Golgi Complex. The intermediates of the fusion protein that are generated in each compartment are shown. The Golgi is conceptualized as three regions (early, E; Mid-, M; Late, L), but their boundaries need not be interpreted as representing physically distinct entities; one or more functional units (i.e. Kex1p and α -secretase) may reside differentially within a single cisterna. The proteins that may function in vesicular trafficking but are defective in the *sec* mutants used in our experiments are also indicated. K: Lys; R: Arg; α F: α -factor; CTF: C-terminal fragment of APP.

distinct Golgi compartment distal to the late Golgi compartment which contains Kex2p and Kex1p. According to this model (Fig.31), the hybrid protein α C traverses the ER, early and mid Golgi compartments, where protein glycosylation occurs, and arrives at the late Golgi, where the α -factor-C-terminal fragment is generated by removing the hybrid's α -leader region by Kex2p; since the lysine residue at the C-terminus of α -factor is not free, Kex1p does not cut it. The α -factor-CTF finally reaches the more distal α -secretase-containing compartment, where it is cleaved to yield α -factor-K that is subsequently secreted along the bulk flow pathway, and the membrane-bound APP C-terminal fragment that goes to the vacuole to be degraded.

This model for the yeast Golgi architecture is different from the current one, since it places the α -secretase-containing compartment near the most distal limit of the Golgi, and suggests that this compartment, rather than the Kex2p/Kex1p-containing compartment, serves as a sorting point for secretory and vacuolar proteins. This new Golgi compartment could not be revealed by the previous studies on the processing and transport of CPY and α -factor precursor within the Golgi complex (Graham and Emr, 1991) because α -secretase did not modify the two reporter proteins. Confirmation of this model awaits the cloning of yeast α -secretase and its direct localization to the Golgi.

Yeast α -Secretase is a Unique Mono-Basic Specific Endopeptidase

Based on the variation of APP cleavage sites both among different cell lines and in the same fibroblasts from Down's syndrome individuals, Zhong et al. (1994) proposed that mammalian cells may have a family of α -secretases with multiple specificities. The existence of multiple α -secretases may explain why the substitution of lysine by glutamate or any other amino acid around the mammalian α -secretase cleavage site did not prevent cleavage (Sisodia, 1992). In contrast, yeast α -secretase cleaves APP at lysine of the A β region in preference to glutamine, although this preference is not absolute. We found that no mutant protein (K612Q) cleavage occurred

in the pulse-chase experiment; however, in cells grown for longer periods of time, the 7 kD C-terminal fragment was detected at one-tenth the level of wild type APP695. We have not identified the exact cleavage site of the mutant protein. On the other hand, since the cleavage happens only at one of several lysines within APP, the structural context of the cleavage site must also play an important role in determining site selection. We observed that altering the sequence N-terminal to lysine had no effect on the cleavage; this was evident in the undiminished processing of α C fusion protein (Fig.25); however, the sequence at carboxyl side of the α -secretase cleavage site may determine the enzyme's specificity. Mutagenesis of this region should clarify this question. We conclude that, unlike mammalian cells, yeast has one major type of α -secretase with a cleavage selectivity in favor of a mono-basic residue. Other types of α -secretases might exist, but they play minor roles in the APP processing.

Yeast endoproteases Yap3p and Kex2p resemble α -secretase in that they all can recognize and cleave at basic amino acids within a polypeptide; Yap3p is mono-basic specific, and Kex2p dibasic specific. Furthermore, Kex2p belongs to a family of subtilisin-related endoproteases that process protein precursors at basic site in the general sequence, (K/R)XXR↓ or (K/R)R↓ (Smeekens, 1993). However, the sequence around the cleavage site of APP predicts that yeast α -secretase is unlikely to be a member of this family. To determine whether the two proteinases contribute to α -secretase activity, pBS6 α was introduced into a *yap3* null mutant and a Kex2-deficient mutant. Both cells display normal α -secretase activity. Thus, Yap3p and Kex2p are not yeast α -secretase. The same result with the *kex2* mutant was reported by Zhang and his colleagues (1994).

APP in yeast was found not to undergo glycosyl modification. This may reflect the different consensus sequences for carbohydrate addition in yeast and mammalian cells. This finding also implies that yeast α -secretase does not require substrate glycosylation for its function. Similarly, APP glycosylation is not important for

mammalian α -secretase because sAPP production and secretion were not altered when a calcium ionophore inhibited APP O-glycosylation (Querfurth and Selkoe, 1994). Secretion of some other proteins such as human protein C are also not dependent on complete Golgi processing (McClure et al., 1992).

The mammalian APP falls within a group of proteins with membrane-bound and soluble isoforms, including angiotensin-converting enzyme (ACE) and transforming growth factor- α (TGF- α). α -Secretase-like endoproteinases convert the membrane forms into soluble forms; however, the proteinases themselves remain largely uncharacterized (Ehlers and Riordau, 1991). Surprisingly, despite the diverse structures and functions of proteins with dual forms, the α -secretase-like proteinases seem to cleave the proteins in the group through a common mechanism, whose components respond to multiple stimulators, i.e. phorbol esters and calcium ionophores (Arribas and Massague, 1995). Yeast α -secretase may not be regulated by the same activators, because yeast protein kinase C is not stimulated by phorbol esters (Antonsson et al., 1994). Interestingly, our preliminary data indicate that α -factor pheromone seems to enhance the yeast α -secretase activity in a dose-dependent manner (Fig.32). The binding of α -factor to its receptor triggers a trimeric G protein-mediated signal transduction that involves multiple kinases, leading to expression of new genes--a mating response (Cairns et al., 1992). Yeast α -secretase, therefore, may be regulated by this signal cascade and play a role in a cellular event necessary for yeast conjugation.

Nonspecific Degradation of APP and Its Fragments

Although vacuoles do not contain α -secretase activity, the relative stability of APP and the C-terminal fragment in JSC310 with defective vacuoles demonstrates that the vacuole is the main place where APP and its intermediates are degraded. How APP and the C-terminal fragment arrive at the vacuole is poorly understood. The pathway from the Golgi to the vacuole may be direct, or it may involve transport to the plasma

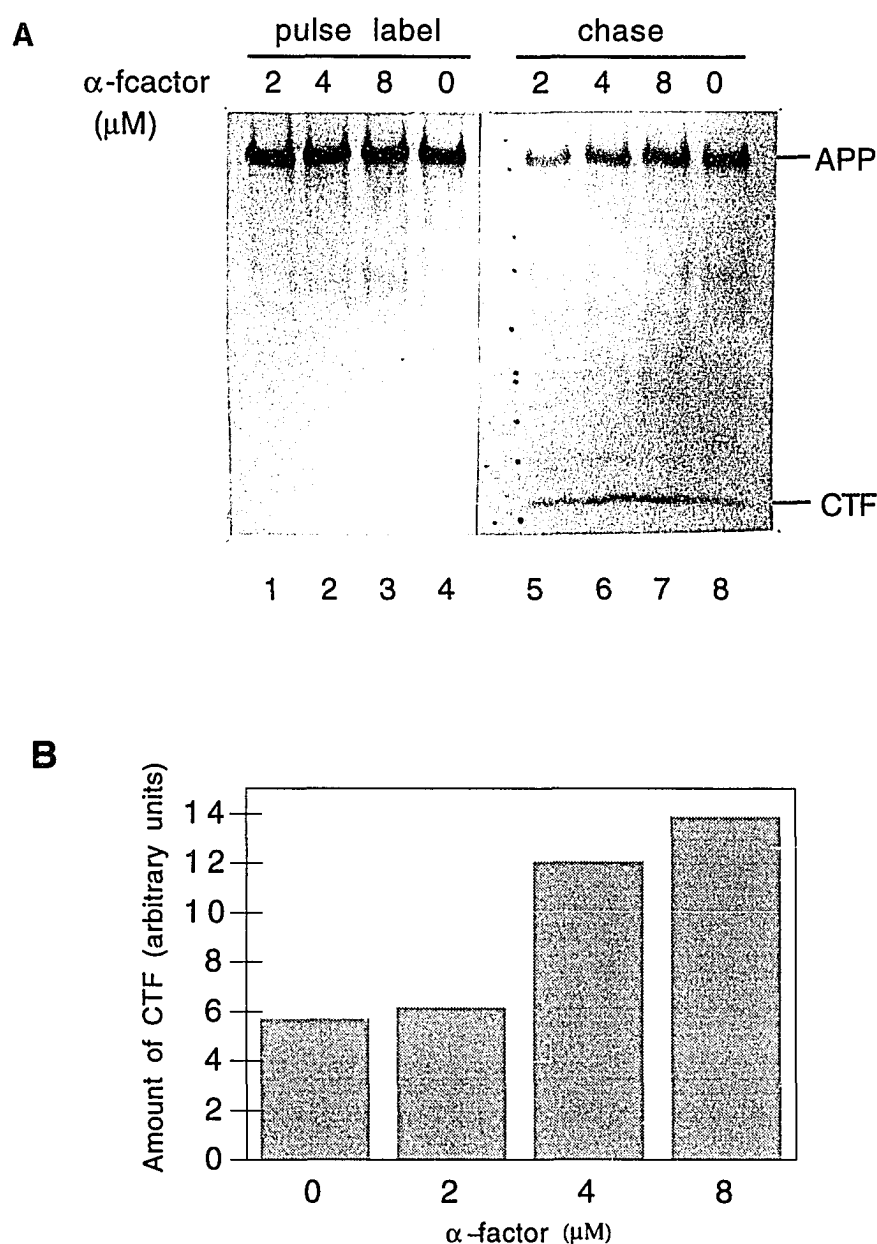


Figure 32. Effect of α -factor on activity of α -secretase. JSC310 cells harboring pBS6 α were incubated for 10 min with synthetic α -factor of 0, 2, 4 and 8 μ M in methionine-deficient SM. The cells were then labeled with Tran ^{35} S-label for 10 min and chased for one hour in the presence of the α -factor peptide at the indicated concentrations. The labeled cells were processed and analyzed as described in Figure 18, using R57 as the antibody for immunoprecipitation. (A) Autoradiograph of the labeled APP and its C-terminal fragment (CTF) produced by α -secretase cleavage. (B) Quantitation of the relative amounts of CTF that appeared on the autoradiograph.

membrane and subsequent delivery to the vacuole by an endocytic mechanism (Roberts et al., 1992; Wilcox et al., 1992; Nothwehr et al., 1993; Cereghino et al., 1995). The APP processing in the *sec1* mutant (Fig.22 and Table 3) showed that when vesicular traffic to the plasma membrane was blocked, degradation of APP and its C-terminal fragment still occurred. Therefore, vacuolar delivery of APP and its C-terminal fragment is likely to be direct from the late Golgi. In animal cells, however, APP and its C-terminal fragment are transported to the lysosome *via* endocytosis (Selkoe, 1994) as well as directly from an intracellular secretory compartment (Lai et al., 1995). A similar direct vacuolar transport of endogenous yeast membrane proteins has been documented for some late Golgi resident proteins such as Kex2p (Roberts et al., 1992).

It has been shown that a substantial amount of immature APP is cleaved in a non-lysosomal compartment, possibly a pre-Golgi compartment (Kuentzel et al., 1993). The ER is known to possess proteinases that selectively degrade abnormal newly-synthesized proteins caused by defective modifications or mis-folding or mutations as a quality control mechanism in addition to the lysosomal degradation (Fra et al., 1993; McCracken et al., 1993). Similarly, yeast also seems to have a non-lysosomal proteolytic compartment for APP degradation, most likely within the ER, as judged by the gradual disappearance of APP-6 α fusion protein trapped in the ER during the chase period in the *sec18* mutant (Fig.22). Nevertheless, the significance of the ER degradation of APP is uncertain. ER-accumulated APP may be abnormally folded exposing potential degradation targeting signals, as has been observed in the pre-Golgi degradation of IgM (Fra et al., 1993). On the other hand, in wild type cells, most of APP is inserted into the ER membrane in a correct conformation and quickly exits the ER so that the ER proteinases can neither recognize nor have the time to bind to APP. In short, the yeast vacuoles play a bigger role in disposal of APP than the ER under normal conditions.

Speculations on APP Function

The yeast secretory system differs from its mammalian counterpart, in that transport of integral membrane proteins to yeast vacuole occurs by default, while yeast plasma membrane proteins may require signals for their localization (Nothwehr and Stevens, 1994). Thus, APP and its C-terminal fragment, both being type I membrane proteins and presumably lacking the yeast signal for plasma membrane targeting, may be sorted to the vacuole at the late Golgi and eventually degraded. In this regard, it is interesting to note that APP seems to contain a consensus signal for yeast late Golgi retention. The aromatic residue-rich retention signal (Y/F)X(Y/F), present in the cytoplasmic tails of the late Golgi membrane proteins such as Kex2p, Vps10p (a sorting receptor for the vacuolar CPY) and dipeptidyl aminopeptidase A (DPAP A), is essential for retrieving these proteins from a post-Golgi/prevacuolar compartment back to the late Golgi apparatus in a clathrin-dependent manner, after the proteins escape or transport ligands to the vacuole, thus maintaining the proper protein composition and function of the Golgi (Nothwehr and Stevens, 1994; Cereghino et al., 1995). APP contains not only a clathrin-binding sequence, YENPTY, but also the late Golgi retention motif, YKF, in its cytoplasmic domain (Table 6), suggesting that APP may reside at the late Golgi and shuttle between the Golgi and a prevacuole compartment as the other late-Golgi residents do. One would argue that if this is so, APP in the transfected yeast should be quite stable. However, compared to Vps10p (half-life >3 h), APP is metabolized much faster ($t_{1/2}$ = 30 min). This instability is probably a result of APP overexpression and the saturable nature of the retrieving apparatus. Consistent with this explanation, overexpressing Kex2p, Kex1p and Vps10p drastically decreased their stability, and the proteins are found on the vacuolar membrane (Roberts et al., 1992; Wilcox et al., 1992; Cooper and Bussey, 1992; Nothwehr and Stevens, 1995). In mammalian cells,

TABLE 6. Comparison of Primary Sequence Motifs From the Cytoplasmic Tails of Yeast DPAP, Kex2p with Human APP and Bovine CI-MPR

	Protein	Sequence ^a
Yeast	DPAP	PRES <u>FQ</u> <u>END</u> I ₉₀
	Kex2p	RAET <u>Y</u> <u>E</u> <u>F</u> <u>D</u> I I ₇₁₈
	Vps10p	F <u>Y</u> <u>V</u> <u>E</u> ₁₄₅₈
Human	APP695	ENPT <u>Y</u> <u>K</u> <u>F</u> <u>F</u> <u>E</u> Q ₆₉₂
Bovine	CI-MPR	ANV <u>S</u> <u>Y</u> <u>K</u> <u>Y</u> <u>S</u> <u>K</u> <u>V</u> ₂₃₆₅
Consensus		---- Φ - Φ ----

^a Residues that are underlined and in boldface have been shown by mutagenesis to be necessary for proper localization in the late Golgi (Nothwehr and Stevens, 1994). Residues underlined only are predicted to be necessary for retention in the late Golgi. **Φ** represents aromatic amino acids.

overexpressed APP also tends to be unstable ($t_{1/2}$:30-60 min) (Selkoe, 1994) compared with the endogenous APP in cultured neurons ($t_{1/2}$: 3 h) (Wertbin et al., 1993); in the rat brain, the half-life of APP can reach 10 h (Potempska et al., 1989).

It is conceivable that APP might also shuttle between the trans Golgi network and the endosome in animal cells. Based on its deduced primary sequence, APP has long been suspected to be a receptor protein. The majority of APP has been localized in TGN both in cultured cells and in brains (Caporaso et al., 1994), whereas a small fraction of APP appears on the cell surface. The pattern of APP distribution inside cells resembles that of mammalian cation-independent mannose phosphate receptor (CI-MPR). As a Golgi membrane protein, CI-MPR binds soluble lysosomal enzymes modified by mannose 6-phosphate in the trans-Golgi and transports the ligands to endosomes, where the ligands dissociate from the receptor and the receptor returns to the Golgi. CI-MPR also appears on the plasma membrane and delivers exogenous lysosomal enzymes to lysosomes *via* the endocytic pathway (Klumperman et al., 1993). Interestingly, the putative Golgi-retention signal of APP is also similar to the sorting motif of CI-MPR (Canfield et al., 1991) (Table 6). Therefore, like CI-MPR, APP

might function as a receptor that transports endogenous ligands from the Golgi to the endosome/lysosome, or scavenges exogenous ligands back to cells. Future experiments with deletion of the Golgi-retention signal in APP should help to clarify APP's receptor function.

Future Prospects

Our discovery that yeast *Saccharomyces cerevisiae* possesses proteases with specificities similar to the mammalian α -secretase, represents the first major step toward identifying the mammalian α -secretase. While we have sketched the outline of the location of yeast α -secretase, a detailed, high resolution map of the distribution of the protease awaits the sequencing of the gene. By analysis of immunisolated Golgi membrane vesicles using antibodies against different intra-Golgi markers, we will put our Golgi model to test, and thus have a deeper understanding of the compartmentation of yeast Golgi apparatus, which lacks the stacks of cisternae. We are currently screening for yeast mutants defective in α -secretase activity in order to finally identify the gene by genetic complementation. Given the fact that yeast does not have a protein homologous to APP, such mutants should also be invaluable for clarifying the biological role(s) of the yeast α -secretase.

Recent studies have shown that yeast and higher eukaryotic cells share a common type of endoproteases for the maturation of prohormones with diverse functions and structures. The striking similarity between yeast and mammalian α -secretase activities further supports the notion that yeast contains proteases that are preserved evolutionarily in the more advanced eukaryotic cells. It is thus tempting to speculate that both α -secretases of yeast and mammalian cells may contain conservative regions vital to their function. If this is true, mammalian α -secretase could be searched more efficiently rather than randomly by standard molecular biological techniques once the sequence of the yeast α -secretase is available. The ultimate identification of mammalian α -secretase will greatly enhance our knowledge about normal APP

processing and help to find out the molecular defects leading to the amyloidogenic cascade in Alzheimer's disease.

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