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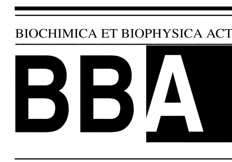
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Characterization of β-amyloid peptide precursor processing by the yeast Yap3 and Mkc7 proteases

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Abstract

Two proteases, denoted β- and γ-secretase, process the β-amyloid peptide precursor (APP) to yield the Aβ peptides involved in Alzheimer's disease. A third protein, α-secretase, cleaves APP near the middle of the Aβ sequence and thus prevents Aβ formation. These enzymes have defied identification. Because of its similarity to the systems of mammalian cells the yeast secretory system has provided important clues for finding mammalian processing enzymes. When expressed in *Saccharomyces cerevisiae* APP is processed by enzymes that possess the specificity of the α-secretases of multicellular organisms. APP processing by α-secretases occurred in *sec*1 and *sec*7 mutants, in which transport to the cell surface or to the vacuole is blocked, but not in *sec*17 or *sec*18 mutants, in which transport from the endoplasmic reticulum to the Golgi is blocked. Neutralization of the vacuole by NH_4Cl did not block α-secretase action. The time course of processing of a pro-α-factor leader-APP chimera showed that processing by Kex2 protease, a Golgi protease that removes the leader, preceded processing by α-secretase. Deletions of the genes encoding the GPI-linked aspartyl proteases Yap3 and Mkc7 decreased α-secretase activity by 56 and 29%, respectively; whereas, the double deletion decreased the activity by 86%. An altered form of APP-695, in which glutamine replaced Lys-612 at the cleavage site, is cleaved by Yap3 at 5% the rate of the wild-type APP. Mkc7 protease cleaved APP (K612Q) at about 20% the rate of wild-type APP. The simplest interpretation of these results is that Yap3 and Mkc7 proteases are α-secretases which act on APP in the late Golgi. They suggest that GPI-linked aspartyl proteases should be investigated as candidate secretases in mammalian tissues. © 1997 Elsevier Science B.V.

Keywords: β-Amyloid precursor processing; Yeast; Protease; Alzheimer's disease

1. Introduction

The β-amyloid peptide precursor (β-APP) isoforms comprise a family of transmembrane proteins that contain the amino acid sequences of the 40–42-residue Aβ peptides found in the cerebral amyloid deposits of Alzheimer's disease victims. Unidentified proteases cleave β-APP at three sites in or near its transmembrane sequence. Two of these cleavages (at the β- and γ-secretase sites) respectively yield the amino and carboxyl termini of Aβ. The α-secretase cleavage occurs near the middle of the Aβ sequence

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and thus prevents the formation of the amyloid peptides. The molecular biology and metabolism of β-APP has been reviewed by Haass and Selkoe [1].

α-Secretase converts β-APP from a cell-associated form to a secreted form [2]. β-APP resembles certain other transmembrane proteins in this property. Proteases also release transforming growth factor-α [3], colony-stimulating factor 1 [4], and angiotensin-converting enzyme [5], among other membrane-anchored proteins [6]. These processing enzymes remain unidentified; however, some recent evidence suggests that they share functional similarities [7].

The processing proteases of the secretory system have not readily yielded their identities, because they act in the environments of specific compartments and because when their isolation is attempted they become masked by other proteases released in cell lysates. The prohormone convertase that processes pro-insulin [8] was discovered only after the characterization of a yeast protease, Kex2 endopeptidase (Kex2p), which possessed the same substrate sequence specificity [9]. Sequence similarity searches and PCR amplification of the homologous mRNAs then revealed the coding sequences of the convertases for pro-insulin as well as for pro-opiomelanocortin [10] and other neuropeptide pro-hormones [11].

When transfected with a β-APP expression plasmid, certain yeast strains synthesize β-APP, translocate it to membranes, and secrete its ecto-domain [12,13]. The yeast protease cleaves β-APP at the same residue as mammalian α-secretase [12,13]. In this report we characterize the time-course of β-APP processing in various yeast strains and compare the rates of processing of wild-type β-APP and β-APP containing a mutation at the cleavage site. From these results we conclude that yeast α-secretase is a discrete enzyme that can act in the late Golgi, a compartment that is equivalent to a site of action of the mammalian enzyme [15,16]. We confirm the finding (Robert Fuller, private communication) that Yap3p and Mkc7p each possess α-secretase activity and we compared the relative activities of the two enzymes for an APP substrate altered at the cleavage site.

2. Methods and materials

2.1. Materials

2.1.1. Antisera

Antiserum R57 and monoclonal antibodies 6E10 and 4G8 were described in the previous publication [12]. Their epitopes are indicated in Fig. 1. Rabbit antiserum R104 was raised to native human secreted APP (sAPP) expressed in Sf9 cells infected with a recombinant baculovirus that expressed APP. Its epitope(s) have not been determined.

2.1.2. Yeast strains and cell culture

Yeast strains used in this study are listed in Table 1. Yeast transformation was carried out using an electroporation procedure [17]. Transformants were selected by growth on solid synthetic minimal medium without uracil (containing 1 M sorbitol). Transformed cells were precultured in Ura^-/Leu^- selective media (4% glucose) at 30°C. The cells were then either grown in YPD (2% glucose) at 30°C for APP expres-

Table 1
Yeast strains

Name	Genotype	Source or Ref.
JSC310	*MATa leu2 ura3-52 prb1-1122 pep4-3 prc1-407 GAP-ADR1::G418*	[12]
DS7	*MATα MFα1 / α2::LEU2 ade2 his3 leu2 trp1 ura3*	[31]
YBAD1	*MATα YAP3::HIS3 MFα1 / α2::LEU2 ade2 his3 leu2 trp1 ura3*	[31]
RSY269/*sec17-1*	*MATα ura3-52 his4-619*	R. Schekman
RSY271/*sec18-1*	*MATα ura3-52 his4-619*	R. Schekman
RSY299/*sec7-1*	*MATα ade2 his3-11 leu2-3, 112 trp-1-1 ura3*-1	R. Schekman
RSY782/*sec1-1*	*MATα ura3-52 his4-619*	R. Schekman
M200-6ck	*MATα KEX2::URA3 ura3 ade1 ilv3 sst1 sst2*	M. Whiteway
DE9	*MATα MFα1 / α2::LEU2 YAP3::HIS3 ade2 his3 leu2 trp1 ura3 MKC7::TRP1*	This study
DE12	*MATα MFα1 / α2::LEU2 MKC7::TRP1 ade2 his3 leu2 trp1 ura3*	This study

sion or in methionine-deficient synthetic medium (Met^-SM) (from Bio101, Vista, CA) 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).

2.2. Methods

2.2.1. Clone and mutant constructions

The construction of expression shuttle vector pBS24.1 harboring the APP-695 gene with the full-length pro-α-factor leader (pBS6α, Fig. 1) has been described previously [12]. In the construct, the inserted gene is controlled by the glucose-repressible ADH2/GAPDH hybrid promoter.

The substitution of Gln for Lys-612 was achieved by site-directed mutagenesis using PCR and the UDG cloning method devised by Rashtchian et al. [18]. *Sph*I-linearized plasmid pBS6α was used as the template. The internal mutagenic primers used were the following: CP1b (reverse, 5′CACCAAUUGAUGAUGAACTTCATA3′) and CP2b (forward, 5′CAUCAACAAUUGGUGUTCTTTGCAG3′). The flanking primers were: CP1a (forward, 5′CUACUACUACUAGACGGAGGAGATCTCTGAAGTG′) and CP2b (reverse, 5′CAUCAUCAUCAUGGCGTCGACTCACTAGTTC-TG3′). The mismatched position in each oligonucleotide is underlined. The two PCR fragments amplified from the APP-695 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 APP-695 mutant was subcloned into pBS6α by insertion of the pAMP2 *Bgl*II–*Sal*I restriction fragment into the *Bgl*II and *Sal*I sites of pBS6α. The sequence of the inserted fragment was verified by double-stranded sequencing of the plasmid templates (dsDNA cycle sequencing system, Gibco-BRL).

To confirm the absence of an intact *YAP3* gene in strain YBAD1, a Southern analysis of the genomic DNA from the cells was conducted. The DNA was isolated following the method of Philippsen et al. [19], 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′-CGTCAGGTTACCGCTAAAGGAAGC-3′ (1578–1601 of the *YAP3* sequence) [14]. DNA from the parent strain DS7 was run as a positive control.

2.2.2. MKC7 deletion

The *MKC7* gene was disrupted by the *TRP1* auxotrophic marker. The gene was PCR-amplified from yeast chromosomal DNA, using primers that introduced a 5′ *Sph*I site and a 3′ *Sma*I site. The PCR product was ligated into pGEM-T (Promega) and its identity was confirmed by restriction fragment analysis. The *TRP1* gene from YDp-W (kindly donated by Rolf Sternglanz, Stony Brook, NY, USA) was introduced as a *Sal*I/*Bam*HI fragment to replace the 680-bp *Sal*I/*Bam*HI fragment in the *MKC7* gene. This plasmid was digested with *Pvu*II and transfected into yeast strains YBAD1 and DS7 by electroporation. Colonies that grew on Trp^- medium were analyzed by PCR to confirm the presence of the *TRP1* insertion into *MKC7*.

2.2.3. 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 μCi of Tran [^{35}S] label (ICN) per OD_{600} unit cells. After pulse-labeling for 5–10 min, labeled proteins were chased for the indicated durations by addition of 1 mM cysteine, 5 mM methionine, 1 mM ammonium sulfate and 2% glucose. One-ml aliquots were collected into sodium azide (10 mM) on ice, and cell lysis and immunoprecipitation were performed as previously described [12]. 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 μl 2 × SDS sample buffer and subjected to Tricine–SDS–PAGE (7/11% discontinuous slab gel) described by Schagger and Jagow [20]. 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). The quantification of α-secretase activity in various yeast mutants (Table 2, Fig. 5) was performed on dried gels through the use of a Fujifilm BAS 1500 phosphorimager.

2.2.4. Effects of NH_4Cl on α-secretase activity

A culture of RSY271-pBS6α (*sec18*) was grown at 22°C overnight in methionine-dropout SM (4% glucose). The mid-log-phase yeast cells were then incubated in Met^-SM containing 0.05% glucose for 1.5 h at 35°C to induce both expression of APP fusion protein and blockage of protein transport at the endoplasmic reticulum. The cells (3 OD_{600} units/ml) were then grown in ammonium-free minimal medium (pH 7.7) with 1% proline as the nitrogen source (MV-pro) [21] for 30 min at 35°C. The cells were subsequently labeled for 30 min at 35°C, followed by treatment with 0.4 M NaCl or 0.4 M NH_4Cl for 20 min at the same temperature. The reversion of the blockage of the secretory pathway was initiated by the return of the labeled cells to 22°C, and they were chased for 3.5 h in the presence of the same concentrations of the two salts. Cell lysates were subjected to immunoprecipitation with R57 and Tricine–PAGE, and the gel was fixed, impregnated with EN^3HANCE (New England Nuclear), dried and exposed to film at −80°C for appropriate times.

2.2.5. Invertase assay

Sec mutants transformed with pBS6α were grown overnight in YPD (5% glucose) at 24°C to an OD_{600} of 0.9–2.6. Cells were transferred to fresh YPD with 0.1% glucose (0.8 OD_{600} units/ml) and incubated at 37 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. [22]. *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 [23]. One unit of activity is 1 μmol of glucose released per min.

2.2.6. Glycosylation studies

Yeast cells (RSY271/pBS6α; *sec18*) at early- to mid-log phase in methionine-deficient SM (3% glucose) were divided into two equal portions. Cells from one portion were resuspended in the same medium (3 OD_{600} units per ml) with tunicamycin (10 μg/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 de-

Table 2
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)
RSY271/*18-1*	0.11	2.94	1.93	62.0	1.66	0.60
RSY782/*1-1*	0.37	3.05	1.35	32.1	1.47	1.10
RSY299/*7-1*	0.10	2.13	1.63	71.8	1.45	0.62

[a] Units of invertase activity are μmol 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 = $(Ext_{37°C \to 25°C} - Ext_{37°C})/Int_{37°C} \times 100$.

scribed above. A control experiment without tunicamycin was similarly conducted. The effectiveness of tunicamycin on inhibition of N-linked glycosylation was confirmed by assaying α-factor secretion in the presence of tunicamycin as described by Julius et al. [24].

The Endo H digestion of radiolabeled APP was conducted according to the protocol by Genzyme Corp. R57-immunoprecipitated ^{35}S-labeled APP from RSY271/pBS6α were 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 1 × electrophoresis sample buffer (30 μl), heated in a boiling water bath for 2 min, and analyzed by Tricine–SDS–PAGE.

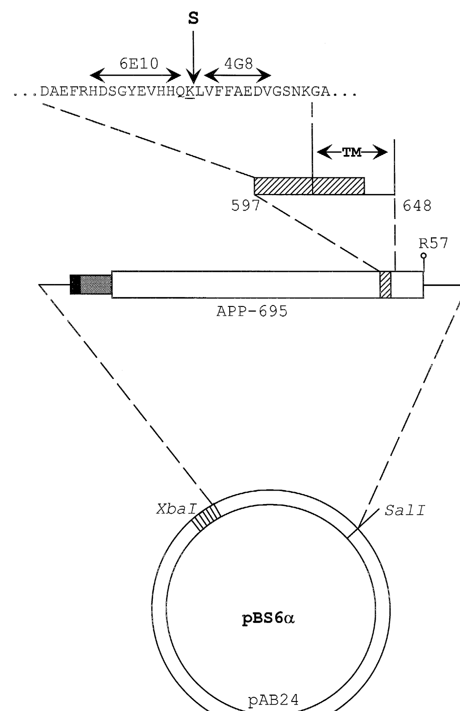

Fig. 1. Map of plasmid pBS6α and its pro-α-factor-leader-APP-695 insert. The β-peptide sequence (hatched box) extends into the transmembrane (TM) sequence. Polyclonal antibody R57 recognizes the seven residues at the COOH terminus. mAb 6E10 and mAb 4G8 recognize epitopes adjacent to the α-secretase cleavage 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 pro-α-factor leader.

3. Results

3.1. Time course of APP processing

Previous studies [12,13] had shown that yeast contains a functional homolog of mammalian α-secretase, which cleaves β-APP at lysine-612, 13 residues before the beginning of the transmembrane sequence (Fig. 1). To determine the time-course of processing of β-APP-695, a culture of JSC310 transfected with pBS6α (Fig. 1) was pulse-labeled with [^{35}S]methionine, and at timed intervals β-APP and its cleavage products were immunoprecipitated with antiserum R57 (specific for the C-terminal 7 residues) or R104 (raised to the ecto-domain). The immunoprecipitated proteins were separated by SDS–PAGE and visualized by autoradiography. β-APP-695 initially appeared as a single band of apparent mass about 105 kDa (Fig. 2A,B). The 7-kDa C-terminal fragment (CTF, Fig. 2A) and the 90-kDa extracellular domain (sAPP, Fig. 2B) generated by α-secretase began to appear after 10 min, as full-length APP coordinately disappeared. Two isoforms of sAPP (appearing as a double band around 90 kDa in Fig. 2B) were immunoprecipitated from the cell lysate in addition to full-length β-APP. The smaller form lacked the 6E10 epitope near the C-terminus of sAPP (data not shown). This form must have resulted from a subsequent cleavage of the α-secretase product, because R57 did not immunoprecipitate a C-terminal fragment complementary to it. The ca. 48-kDa translation product formed by cleavage in the vicinity of residue 300.

The pBS6α vector encodes a fusion protein in which APP-695 is preceded by the yeast pheromone pre-pro-α-factor leader (Fig. 1); however, in this experiment the fusion protein was not observed. The processing of the pro-α-factor leader by Kex2p occurs in less than 5 min [24], and had been completed before the cells were lysed at the first chase time point. In other experiments at higher levels of expression or lower temperatures (e.g. Fig. 4), the fusion protein was observed.

The time course of α-secretase cleavage was much slower than that of Kex2p, and it was biphasic (Fig.

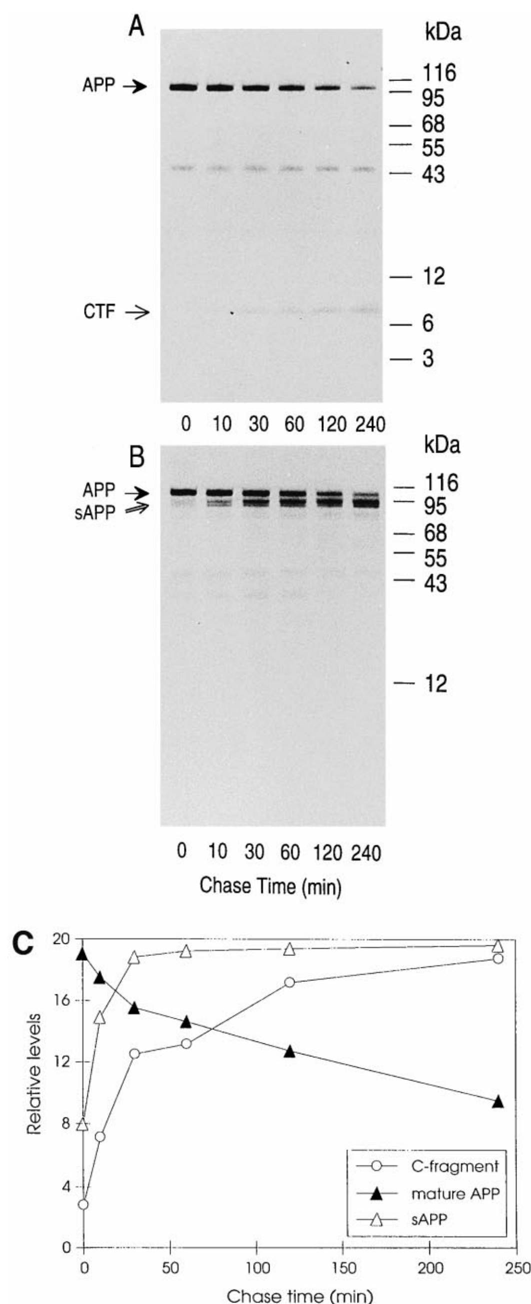

Fig. 2. Time-course of pulse-labeled APP cleavage by α-secretase. JSC310 transfected by pBS6α was labeled for 10 min with [^{35}S]methionine and cysteine. At the indicated intervals the peptides were immunoprecipitated with either R57 (panel A) or R104 (panel B) and subjected to electrophoresis and autoradiography. Panel C: densitometric analysis of the autoradiograms. CTF, C-terminal fragment; sAPP, secreted APP.

2C). The rapid phase has a half-time of about 18 min, while the slow phase proceeds with a half-time of about 2 h. The C-terminal fragments are relatively stable in JSC310, which lacks the major vacuolar proteases A and B [12].

The secretory form of APP accumulated within the cells over a period of 2 h (Fig. 2B). As in the previous study little sAPP appeared in the medium. In cells expressing APP for 2 days, about one-half of the sAPP becomes bound to the cell surface in a form that can be released by treatment with an alkaline thiol solution [12]; however, in 4 h of radiolabelling very little ^{35}S-labeled sAPP could be released by this procedure (data not shown).

3.2. Localization of α-secretase activity

Several steps of the yeast secretory process have been defined by conditional mutants that block secretion [22,25]. Strain RSY271 (*sec18*) contains a temperature-sensitive mutation that blocks ER to Golgi transport; whereas, in strains RSY299 (*sec7*) and RSY782 (*sec1*) mutations block transport from the Golgi to secretory vesicles, and from these vesicles to the plasma membrane, respectively. The *sec7* mutation also blocks transport to the vacuole [26]. To further identify the compartment in which α-secretase acts, we studied the processing of APP in these strains at permissive and non-permissive temperatures.

To confirm that our isolates each contained a *sec* mutation, we measured their capacities to synthesize and secrete invertase at 25 and 37°C. Each strain synthesized invertase at both 25 and 37°C, but could secrete the enzyme only at 25°C (Table 2). In addition, the mutants were thermoreversible [22]; upon return to the permissive temperature in the presence of a protein synthesis inhibitor, the accumulated invertase was secreted. This result indicates that, in all of the mutants, vesicular transport was not irreversibly damaged by the treatment.

Each strain was transfected with pBS6α and was pulse-labeled at either 25 or 37°C, and the extent of processing after 30 min was determined by the phosphorimaging procedure. The ratio of the radioactivity of the C-terminal fragment after a 30-min chase to that of full-length APP at zero time was calculated (Table 3). In *sec1* and *sec18* strains about 3% of the radioactive label originally incorporated into APP appeared in the C-terminal fragment after 30 min at 25°C (Table 3). Correcting for its relative methionine

content (0.2) about 15% of the initial APP was cleaved to the C-terminal fragment after 30 min. About 80–90% of the initial APP was turned over during the period. Other processes, particularly, vacuolar proteolysis, degrade both APP and the C-terminal fragment and lower the yield of the α-secretase product. A small fraction of APP escaped α-secretase and appeared on the cell surface (result not shown). At 37°C, α-secretase processing does not occur in the *sec18* mutant; however, processing is not much affected in the *sec1* strain, or in DS7, a strain that contains a wild-type secretory system. The small change in the yield of C-terminal fragment in DS7 indicates that α-secretase activity possesses a low temperature coefficient.

The *sec7* mutant in repeated experiments yielded higher extents of conversion to the C-terminal fragment. Part of the explanation for this behavior is that the *sec7* mutation, which blocks transport to the vacuole, prevents turnover of the C-terminal fragment. However, *sec7* also affects other steps in transit through the Golgi. Because of these complexities, and because this *sec7* strain is not isogenic with the other strains, direct quantitative comparisons are not warranted. In summary the results in Table 3 show that the *sec18* mutation blocks α-secretase action; whereas, *sec7* and *sec1* mutations do not significantly affect its action.

To confirm that α-secretase resides in the secretory system rather than the vacuole, we investigated APP cleavage in cells that had been treated with 0.4 M NH_4Cl, a concentration at which the proton gradient is eliminated, and the transport of proteins to the vacuole is blocked [27,28]. Since high concentrations of NH_4Cl interfered with the radio-labeling of proteins in yeast, we first labeled the proteins of *sec18*-pBS6α at 35°C, at which temperature the labeled proteins were retained in the ER, and subsequently treated the cells with 0.4 M NH_4Cl at the same temperature. As seen from Fig. 3 (left two lanes), under these conditions only APP fusion proteins existed, and no mature APP could be observed, which would require the transport of the fusion protein from the ER to the late Golgi to be processed by Kex2p.

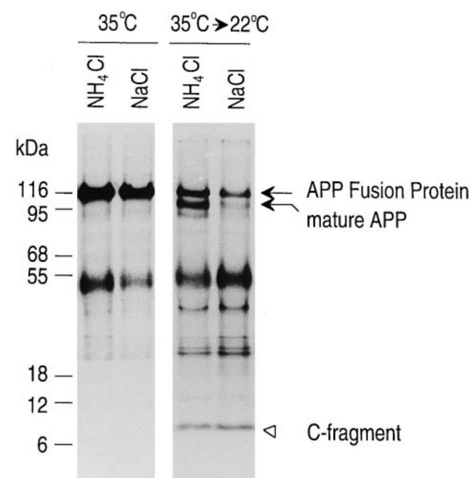

Fig. 3. APP cleavage by α-secretase in NH_4Cl-treated cells. RSY271 (*sec18*) harboring pBS6α was incubated at 35°C for 1.5 h to block protein transport at ER. The cells were then pulse-labeled with Tran [^{35}S] label at 35°C and treated with 0.4 M NH_4Cl or 0.4 M NaCl (as a control) at 35°C followed by incubation in 22°C waterbath to reverse the blockage of the secretory pathway. The APP processing was analyzed by immunoprecipitation of APP and its C-fragment, Tricine–PAGE and fluorography.

Table 3
Processing of β-APP in temperature-sensitive *sec* mutants [a]

Strain	Process blocked	CTF/APP_o		Ratio of 37/25°C
		25°C	37°C	
RSY271/*sec18-1*	ER → Golgi	0.035	0.000	0.0
RSY299/*sec7-1*	Golgi → secretory vesicles	0.100	0.067	0.7
RSY782/*sec1-1*	sec. ves. → plasma membrane	0.027	0.028	1.0
DS7	none	0.036	0.025	0.7

[a] *Sec* mutants transformed by pBS6α (3 OD_{600} units/ml) were incubated in SM free of methionine (0.05% glucose) at permissive (25°C) or non-permissive (37°C) temperatures for 2 h to induce APP expression and block the secretory pathway. The cultures were then radiolabeled and assayed as described in Section 2.2. Each PAGE gel was subjected to phosphorimaging, and from these values was calculated the ratio of the radioactivity of the C-terminal fragment (at a chase time of 30 min) to the radioactivity of full-length APP (at 0 chase time). The ratio of the amount of C-terminal fragment formed at 37°C to that formed at 25°C was then calculated.

Upon shifting to 22°C, however, protein transport was restored, which was indicated by the conversion of the fusion protein to mature APP (Fig. 3, right two lanes). NH_4Cl did not prevent the production of the 7-kDa C-terminal fragment and, accordingly, the α-secretase cleavage of APP. The vacuole, nevertheless, might be involved in other processing activities of APP. More mature APP remained intact in NH_4Cl-treated cells than in NaCl-treated cells, probably because NH_4Cl hampered the transport of mature APP to the vacuole and/or the processing of APP in the vacuole. To confirm that the NH_4Cl treatment eliminated the vacuolar proton gradient we showed (data not presented) that it prevented the pH-dependent vacuolar uptake of quinacrine [29].

3.3. Glycosylation of pro-α-APP

APP possesses two N-linked glycosylation sites to which polysaccharides could be attached in mammalian cells [30]. To determine whether these sites are glycosylated in yeast and whether their glycosylation affects the ability of α-secretase to cleave APP, we employed two independent methods of analysis. In untreated *sec18*/pBS6α labeled at 25°C, two predominant R57 immunoreactive full-length APP-containing proteins appeared (Fig. 4, lane 1, bands A and B). When the cells were labeled in the presence of tunicamycin, the predominant protein (starred band in lane 2) possessed a mass about 7 kDa less than band A and 8 kDa greater than band B. When the cells were labeled at 37°C to impose a secretion block 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 7 kDa 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. Since at 37°C the fusion protein cannot encounter Kex2p, the starred-bands 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. Since the α-leader region of pro-α-factor has three N-linked mannose-rich oligosaccharide chains which account for about 7 kDa of its molecular mass [24], the 7 kDa reduction of the molecular mass of tunicamycin-treated APP fusion protein must reflect the removal of all of those oligosaccharide chains

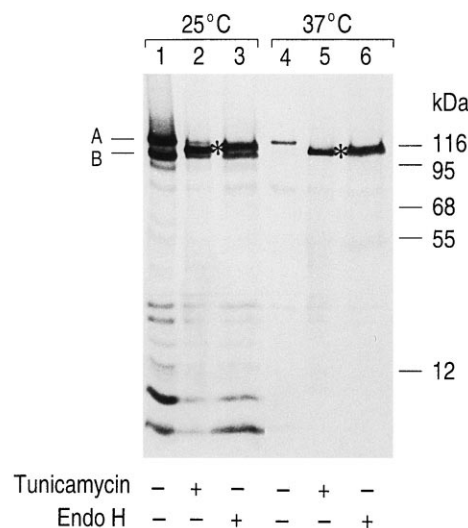

Fig. 4. Effect of glycosylation on α-secretase action. Strain RSY271 (*sec18*) transfected with pBS6α was labeled for 1 h with [^{35}S]methionine and cysteine in either the absence (lanes 1,3,4,6) or presence (lanes 2,5) of tunicamycin at either 25°C (lanes 1–3) or 37°C (lanes 4–6). Peptides containing the C-terminal epitope were immunoprecipitated with R57 and analyzed as described in Fig. 2A. Before electrophoresis, portions of the immunoprecipitate from untreated cells were digested with endoglycosidase H, as described in Section 2.2. The starred bands are discussed in the text.

from the α-leader portion of the fusion protein. Thus APP must not be subject to glycosyl modification in yeast. Indeed, both the mature α-leader-cleaved APP molecules with or without tunicamycin treatment have the same apparent molecular masses (compare bands B, lanes 1 and 2).

To confirm this conclusion, the material immunoprecipitated from *sec*18/pBS6α cells was exhaustively digested with the enzyme endoglycosidase H (endo H) to remove N-linked carbohydrate chains from the peptide backbone [31]. This treatment yielded two APP-related species similar to those formed in tunicamycin-treated cells, except that the larger species corresponding to the APP-α-leader fusion protein migrated slightly slower than the fusion protein (starred-band) from tunicamycin-treated cells (Fig. 4, lanes 3 and 5). A comparison of the relative densities of the bands in lanes 1 and 2 revealed that tunicamycin retarded the conversion of the fusion protein to mature APP. Tunicamycin induced a similar accumulation of unglycosylated pro-α-factor and

inhibition of α-factor maturation [24]. Thus efficient Kex2p cleavage requires glycosylation of pro-α-factor. The reduced amount of the 7-kDa C-terminal α-secretase product observed in lane 2 probably resulted from the reduced rate of transit of the fusion protein. In contrast, APP processing by yeast α-secretase does not require the glycosylation of APP.

3.4. APP processing in yap3, mkc7 and kex2 mutants

The aspartyl protease, Yap3p, can process pro-α-factor precursors at monobasic cleavage sites [14,32]. To investigate the role of Yap3p in the processing of APP, we expressed pBS6α in a *yap3* null mutant, YBAD1. The cells were labeled with [^{35}S]methionine and chased for 30 min. The extent of α-secretase cleavage of APP was then determined by immunoprecipitation of APP and the C-terminal fragment (CTF) followed by PAGE, autoradiography and phosphorimaging analysis. The extent of CTF formation

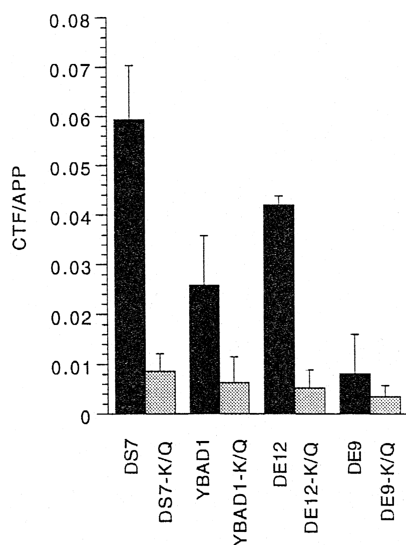

Fig. 5. Processing of wild-type APP-695 and APP(K612Q) in yeast strains bearing deletions in *YAP3* and *MKC7*. Cells were labeled for 10 min with Tran [^{35}S] label and chased for 30 min at 30°C. APP and C-terminal fragments were immunoprecipitated with R57 and subjected to SDS–PAGE. The radioactivity was quantified by phosphorimaging, and the fraction of radioactivity in APP (all forms) at $t=0$ that appeared in CTF at 30 min was calculated. The error bars indicate the standard deviations of two or three independent experiments. Strains: DS7, *YAP3*$^+$ *MKC7*$^+$; DE12, *YAP3*$^+$ *MKC7*$^-$; DE9, *YAP3*$^-$ *MKC7*$^-$; YBAD, *YAP3*$^-$ *MKC7*$^+$.

in the *yap3* mutant was about 44% of that of the DS7 parent strain (Fig. 5).

Mkc7 protease, an enzyme closely related to Yap3p, can also process pro-α-factor at basic residue pairs. When APP was expressed in the *mkc7* null mutant DE12 (Fig. 5) the extent of CTF formation was about 70% of the DS7 parent. In DE9, the *mkc7/yap3* double mutant, the extent of CTF formation was about 14% of the DS7 parent (Fig. 5). We compared the yields of secreted proteins from DE9 and DS7 by PAGE of proteins precipitated from the media from ^{35}S-labeled cultures (results not shown). Although there were some differences in the intensities of a few of the approximately 18 bands resolved, there was no systematic decrease in the yield of secreted proteins from DE9. Thus we concluded that neither deletion introduced a general defect into the secretory system.

To confirm the results of Zhang et al. [13] that α-secretase is not an alternative activity of Kex2p, we expressed pBS6α in a *kex2* mutant, M200-6C. As was previously reported, we found (results not shown) that in the absence of Kex2p the fusion protein was extensively cleaved to yield the C-terminal fragment.

3.5. Effect of pepstatin A on yeast α-secretase

Pepstatin A inhibits, to varying degrees, many aspartyl proteases in vitro as well as in tissue explants [33]. At 100 μM concentration it inhibited the cleavage of lipotropin by purified Yap3p to the extent of 85% [34]. Pepstatin A should inhibit the cleavage of APP by Yap3p, if the cleavage occurs in a low pH compartment accessible to the pentapeptide inhibitor. We attempted to inhibit the α-secretase cleavage of APP in intact yeast cells without success. We then prepared spheroplasts of DS7 expressing APP6α, incubated them with pepstatin A, and performed the pulse-labeling assay for α-secretase activity. When performed at pH 5.1 (Table 4), pH 6.6, or pH 7.4 (results not shown), the assay revealed no inhibition by pepstatin A at concentrations up to 80 μM. In all experiments the extent of α-secretase cleavage was only one-third of that observed in intact cells. This probably indicates that the spheroplasting procedure damaged the cells to some extent. The significance of these results are discussed in a later section.

Table 4
Effect of pepstatin A on α-secretase activity in DS7

Pepstatin A (μM)	CTF/APP$_o$	Relative CTF/APP$_o$
0	0.009	1.0
50	0.010	1.1
80	0.018	1.9

Cells from yeast strain DS7 expressing APP6α were converted to spheroplasts using lyticase as previously described [12]. Hypo-osmotic shock induced nearly complete lysis of the preparation. After incubation for 30 min with pepstatin A in spheroplast medium plus 50 mM Na citrate, pH 5.1, the spheroplasts were pulse-labeled with Tran [^{35}S] label for 10 min and chased for 30 min in the presence or absence of the inhibitor as indicated. The labeled APP and its C-terminal fragments were immunoprecipitated with R57, resolved by SDS–PAGE, and analyzed by phosphorimaging. CTF/APP$_o$ is the ratio of the amount of CTF after 30 min chase to the amount of APP (all forms) at 0 chase time.

3.6. α-Secretase processing of APP altered at the cleavage site

Mutagenesis studies have indicated that mammalian α-secretase can cleave the peptide bond between the 13th and 12th residues N-terminal to the transmembrane sequence, with little specificity for the nature of the 13th residue [35,36]. Other studies suggest that α-secretase may cleave at any of several residues in the vicinity if Lys-612 is not available [37]. We assayed the specificity of yeast α-secretase by determining its activity on APP-695 (K612Q), which was produced by introducing a point mutation into vector pBS6α.

When transfected into the parent strain DS7, APP-695 (K612Q) yielded about 15% of the amount of CTF from wild-type APP-695 (Fig. 5). To determine the effect of the lysyl-glutaminyl alteration upon the activities of Yap3p and Mkc7p, we analyzed the extent of CTF formation in single and double deletion mutants transfected with APP-695(K612Q). The yields of CTF from the mutant APP were small; consequently standard deviations of our measurements were relatively large (Fig. 5). Given this uncertainty, Yap3p appeared to be more specific for Lys-612 than Mkc7p. Yap3p cleaved the mutant APP at about 5% of the rate of wild-type APP; whereas, Mkc7 protease cleaved the mutant APP at about 20% of the rate of wild-type APP.

We did not determine whether Yap3p and Mkc7p actually cleaved the mutant APP at Gln-612. Although the phosphoimager bands from the Lys-612 and Gln-612 cleavage products appeared to be the same size, we were unlikely to discern size difference of less than five residues. We were able to immunoprecipitate a small amount of sAPP from the medium and to identify it with mAb 6E10, whose epitope includes the four amino acid residues amino-terminal to Lys-612 (not shown). Thus, in any case the cleavage occurred very close to Lys-612.

4. Discussion

The substantial deficit in α-secretase activity in the *mkc7* and *yap3* deletion mutants suggests that these proteases are the major yeast α-secretases, although the possibility exists that each activates a zymogen, which is the true α-secretase. The *mkc7/yap3* deletions do not affect the secretion of most endogenous proteins. We have not yet demonstrated that purified Yap3p can cleave APP at Lys 612; however, that yeast α-secretase prefers to cleave APP at a lysyl rather than a glutaminyl residue agrees with the specificity of Yap3p, which has been shown to cleave other substrates at single lysyl residues [14,32]. The specificity of Mkc7p has not been reported. Our results indicate that it is less highly specific for lysyl residues, since APP (K612Q) is cleaved to a somewhat greater extent in the *yap3* deletion than in the *yap3/mkc7* double deletion.

We initially suggested that yeast α-secretase was not likely to be a vacuolar enzyme, because the bulk of sAPP was found either in the medium or associated with extracellular membranes [12]. We demonstrated here that α-secretase processing proceeds in cells treated with 0.4 M NH_4Cl, which both blocks transport to the vacuole and increases the vacuolar pH. Other investigators [27–29] found that when yeast cells were treated with weak amines, bafilomycin A_1, or the proton ionophore carbonyl cyanide *m*-chlorophenyl hydrazone, the vacuoles were not acidified and did not accumulate the fluorescent amine quinacrine. More importantly, under these conditions the vacuolar enzymes, proteinase A and carboxypeptidase Y, were secreted rather than being translocated to their normal destination in the vacuole. These results suggest that in the absence of the

proton gradient proteins cannot be transported to the vacuole. We found that the treatment with 0.4 M NH_4Cl prevented the accumulation of quinacrine in vacuoles, but it did not diminish the activity of α-secretase processing of APP. Stevens et al. [26] reported that *sec7* mutants failed to transport carboxypeptidase Y to the vacuole; whereas, we found that α-secretase processing of APP is not diminished in a *sec7* mutant. In addition we also found that α-secretase processing occurred as rapidly in JSC310, which lacks proteases A, B and C, as in strains with wild-type vacuolar proteases. Although α-secretase cleavage does not occur in the vacuole, both uncleaved APP and the C-terminal fragment turn over more rapidly in WT cells than in vacuolar protease-deficient cells; therefore, they probably follow the default pathway for membrane proteins to the vacuole [38].

Both Yap3p and Mkc7p have been shown to be anchored to membranes through glycosyl phosphatidylinositol moieties, and they are thought to be primarily localized to the plasma membrane [39]. We found that APP is rapidly processed in *sec7* and *sec1* mutants, which block transport of APP to the plasma membrane. This result is susceptible to multiple interpretations. The *sec* mutations may trap both APP and Yap3p in the same compartment, where they might not normally have time to interact. Alternatively, in the *sec1* and *sec7* mutants at the non-permissive temperature another protease that normally resides in the late Golgi or in secretory vesicles may have enough time to cleave the trapped APP. Although these conjectures may not be invalid for the *sec7* mutant results, where increased APP processing was observed, they are not consistent with the *sec1* mutant results, where blockage of transport to the cell surface did not increase the extent of proteolysis over that observed in the DS7 control strain. The lack of inhibition of APP processing by peptstatin A is further evidence against the cell surface being the major site for APP processing by Yap3p.

The failure of pepstatin A to inhibit Yap3p/Mkc7p in vivo is not inconsistent with the reported characteristics of the inhibitor. Although it inhibits certain aspartyl proteases, notably pepsin and cathepsin D, at pH 4 with K_I values in the nanomolar range, it inhibits them poorly at a pH above 6 [40]. The pH of the compartment where α-secretase acts is unknown, but it is likely to resemble the pH of the mammalian Golgi which is about 6.45 [41]. Pepstatin A is a relatively poor inhibitor of Yap3p [34]; it inhibits purified Yap3p 85% at 100 μM, pH 4.6; its effect on Mkc7p has not been reported. Furthermore, if Yap3p cleaved APP in an acidic environment, one would have expected that NH_4Cl treatment would decrease the rate when it neutralized the acidic compartments; however, we found that the cleavage rate was not affected by NH_4Cl treatment. Whether inhibitory concentrations of pepstatin A can accumulate in the Golgi is unknown. For these reasons pepstatin A cannot be considered to be a general inhibitor of aspartyl proteases in vivo.

We propose the following model for the time-course of APP processing in yeast. Pro-α-APP is rapidly transported to the late Golgi, where it is cleaved by Kex2p within 5 min. Yap3p/Mkc7p then release sAPP within 18 min, in a later Golgi compartment. The minor fraction of sAPP formed in the slower phase may represent APP that avoided cleavage in the Golgi and escaped to cell surface, from which it may be recycled to the Golgi.

Since α-secretase cleavage of APP proceeds more slowly than the constitutive secretion of α-factor or invertase, APP must be selectively retained in the late Golgi. The transmembrane α-factor processing enzymes Kex2p, Kex1p, and dipeptidyl aminopeptidase are retained in the late Golgi by sequences in their cytoplasmic domains that may interact with clathrin adaptor proteins [42]. APP possesses a mammalian clathrin adaptor sequence in its cytoplasmic domain, which facilitates its processing in an endocytic compartment [43]. Possibly this sequence interacts with the yeast clathrin structure on the late Golgi.

As the APP-fusion protein passes through the ER and Golgi, its pro-α-leader sequence is glycosylated, like the endogenous pro-α-factor itself; however, the yeast enzyme does not appear to recognize the APP N-linked glycosylation sites. APP shares this property with several other mammalian proteins, for example, hepatitis B surface antigen, which, when expressed in yeast, is not glycosylated [44,45]. In CHO cells, only one of APP's potential N-linked glycosylation sites is modified [30]. In yeast, α-secretase processing does not require glycosylation of APP.

The function of yeast α-secretase remains to be identified. Although both Yap3p and Mkc7p were

discovered by their ability to suppress *kex2* mutations, their primary physiological functions may be related to their abilities to release membrane-associated proteins. We know of no other example in yeast where a membrane-associated protease releases the ecto-domain of a transmembrane protein. The juxtaposition of the active site of a membrane-bound protease and a membrane-bound substrate must introduce topological constraints that increase the specificity of cleavage.

In mammalian cells, α-secretase acts both on the cell surface and in a late-stage secretory compartment. CHO cells release cell-surface APP [33,43]; whereas, in PC12 cells, as in *S. cerevisiae*, α-secretase cleaves APP in a secretory compartment beyond the medial Golgi [15,16,46]. In MDCK cells, sAPP can be produced by cleavage both within a secretory compartment and on the cell surface [47].

Inhibitor studies of mammalian α-secretases have yielded equivocal results. Some of these studies have suggested that α-secretase may be a zinc metalloprotease [7,48]; whereas, others have shown no effects of zinc chelators [16]. In CHO cells the extensive cleavage of APP on the plasma membrane is sensitive to metalloprotease inhibitors [48]; however, it was not shown that this cleavage occurs at the α-secretase site. The protease that releases tumor necrosis factor from its precursor has recently been proved to be a zinc metalloprotease [49,50]; however, it differs from α-secretase in its peptide bond specificity and cleave distance from the plasma membrane.

It may be that mammalian cells contain several types of α-secretase. Cordell and co-workers [37] suggest that this would best explain the observed cleavage patterns of APP mutants. Such a redundancy has been reported among the pro-hormone convertases that process pro-opiomelanocortin in the pituitary. One pair of enzymes, PC1 and PC2, are subtilisin-related serine proteases [10]; whereas a third enzyme, PCE, is a Yap3-related aspartyl protease [51].

APP-like proteins have been found in all tested mammals and even in *Drosophila* [52]; however, no homologous protein has yet been reported in yeast. Yeast α-secretase thus may possess a function different from its mammalian counterpart. Other mammalian transmembrane proteins besides APP undergo proteolysis to secrete active ecto-domains. In mammalian cells certain growth factors, growth factor receptors, cytokines, cell adhesion molecules and ecto-enzymes exist in transmembrane and soluble extracellular forms. Yeast secretes a number of other enzymes into its periplasm as well as mannan, a glycoprotein component of its outer membrane [53,54]. Perhaps α-secretase processes one of these proteins. Alternatively, α-secretase might release the *ecto*-domain of a transmembrane signal transduction protein, thereby antagonizing its action. The identification of Yap3p and Mkc7p as secretases suggests the existence of a new biochemical process in yeast. It further suggests that mammalian secretases may be GPI-linked aspartyl proteases.

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