Processing of the Alzheimer's Disease Amyloid Precursor Protein in *Pichia pastoris*: Immunodetection of α-, β-, and γ-Secretase Products†

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ABSTRACT: βA4 (Aβ) amyloid peptide, a major component of Alzheimer's disease (AD) plaques, is a proteolytic product of the amyloid precursor protein (APP). Endoproteases, termed β- and γ-secretase, release respectively the N- and C-termini of the peptide. APP default secretion involves cleavage within the βA4 domain by α-secretase. To study the conservation of APP processing in lower eukaryotes, the yeast *Pichia pastoris* was transfected with human APP_{695} cDNA. In addition to the full-length integral transmembrane protein found in the cell lysate, soluble/secreted APP (sAPP) was detected in the culture medium. Most sAPP comprised the N-terminal moiety of βA4 and corresponds to sAPPα, the product of α-secretase. The culture medium also contained minor secreted forms detected by a monoclonal antibody specific for sAPPβ (the ectodomain released by β-secretase cleavage). Analysis of the cell lysates with specific antibodies also detected membrane-associated C-terminal fragments corresponding to the products of α and β cleavages. Moreover, immunoprecipitation of the culture medium with three antibodies directed at distinct epitopes of the βA4 domain yielded a 4 kDa product with the same electrophoretic mobility as βA4 synthetic peptide. These results suggest that the α-, β-, and γ-secretase cleavages are conserved in yeast and that *P.* pastoris may offer an alternative to mammalian cells to identify the proteases involved in the generation of AD βA4 amyloid.

The principal component of Alzheimer's disease (AD)[1] amyloid plaques is the 40−43 amino acid peptide βA4 (or Aβ) (*1*, *2*) which is proteolytically derived from a type I integral membrane protein, the amyloid precursor protein (APP) (*3*). βA4 is a normal, but minor, cellular secretory product (*4*) and is found in many biological fluids (*5*). The βA4 peptide contains part of the APP transmembrane domain and exists as several forms with varying solubility. The predominant form secreted in cerebrospinal fluid and in cell culture media is βA4 [1−39/40], whereas the longer forms, βA4 [1−42/43], which are less souble and more prone to aggregate, constitute the nucleating seeds for amyloid deposition (*6*). The discovery of a tight genetic linkage between mutations in the βA4 region of APP and the occurrence of a subset of early-onset familial AD cases (reviewed in ref *7*) strongly suggest that APP processing and βA4 release play a pre-eminent role in AD. Indeed, the disease-causing point mutations are clustered near the cleavage sites of the α-, β-, and γ-secretases, the proteases involved in the release of APP from the membrane and in the generation of βA4. Additionally, familial AD mutations recently identified in the presenilin genes have been shown to alter APP processing and to elevate the ratio of long:short forms of βA4 (*8*), again indicating a possible misprocessing of APP in AD.

A series of major processing events occur near the transmembrane domain of APP, resulting in the release of APP ectodomain and the generation of membrane-associated C-terminal fragments (CTFs). Numerous studies involving mammalian cell cultures have shown that the major APP secreted form (sAPPα) is generated in the late secretory pathway by cleavage within the βA4 domain (*9*−*11*) (for a review see ref *12*). An alternative cleavage due to β-secretase releases the amino terminus of βA4 and produces another APP ectodomain, sAPPβ (*5*). Although β-secretion corresponds to a minor APP trafficking pathway in most cultured cells, it has been shown to constitute the preferred pathway in neurons (*13*). β-Secretase cleavage may take

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[1] Abbreviations: βA4 (or Aβ), 4 kDa peptide, constituent of Alzheimer's disease amyloid; ACE, angiotensin-converting enzyme; AD, Alzheimer's disease; APP, amyloid precursor protein; CTF, carboxyl-terminal fragment; EDTA, ethylenediaminetetraacetic acid; FAD, familial Alzheimer's disease; Ig, immunoglobulin; IP, immunoprecipitation; mAb, monoclonal antibody; PBS, phosphate saline buffer; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate; TGFα, transforming growth factor α; TNFα, tumor necrosis factor α; Tris, tris(hydroxymethyl)aminomethane; WB, Western blot.

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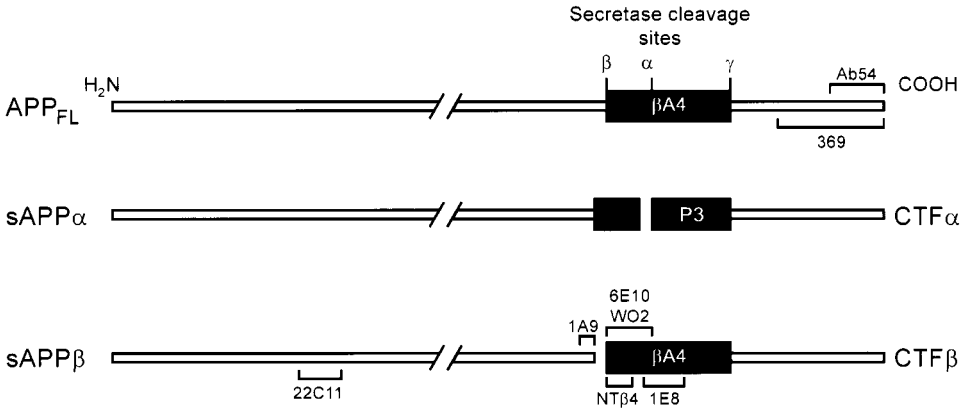

FIGURE 1: Schematic representation of APP, the secretase cleavage products, and the various antibody epitopes. APP is represented as an open bar, and the secretase cleavage sites are indicated (α, *β*, *γ*). *β*A4 peptide, which is the proteolytic product of *β*- and *γ*-secretase, is displayed as a black bar. p3 peptide, which is the product of α- and *γ*-secretase cleavages, is part of *β*A4. APP_{FL} indicates APP full-length (or integral form); sAPPα is the ectodomain released by α-secretase; sAPP*β* is the ectodomain released by *β*-secretase; and CTFα and CTF*β* are the C-terminal fragments produced by α- and *β*-secretase cleavages, respectively. The epitopes of the various antibodies are indicated.

place in the secretory pathway (*14*−*16*) or/and in the endocytic pathway (*17*). Cleavages by α- and *β*-secretase generate membrane-associated C-terminal fragments (CTFα and CTF*β*, respectively) which may subsequently be processed by *γ*-secretase to produce the p3 and *β*A4 peptides (*18*,−*19*). There is now evidence that in mammalian cultured cells the soluble and aggregating forms are produced by different proteases or proteolytic pathways, since some inhibitors of *β*A4 formation are more effective at inhibiting the release of *β*A4 [1−40] than *β*A4 [1−42] (*20*,*21*). Recent reports suggest that *β*A4 [1−42] is generated in the endoplasmic reticulum (*22*−*24*) while *β*A4 [1−40] is released in the trans-Golgi network (*24*).

None of the three secretases has been conclusively identified, although extensive studies with transfected and untransfected cell systems, derived from mammalians and invertebrates, have allowed partial characterization of the properties of the proteases involved (reviewed in ref *12*). α-Secretase activity has been shown to be structure-dependent rather than sequence-dependent (*25*−*26*) and to cleave preferentially an α-helix at a certain distance from the membrane. This resembles the release of other integral membrane proteins, such as transforming growth factor α, interleukin-6, or the angiotensin-converting enzyme (*27*−*29*), and which is attributable to cell surface membrane-inserted metalloproteases. *β*-Secretase cleavage may generate several *β*A4 N-termini possibly resulting from the activity of alternative proteinases (*30*) but the major *β*-secretase activity is known to cleave APP specifically at the N-terminus of *β*A4 and to have a greater specificity for APP FAD Swedish mutant than for APP wild type (*31*, *32*). *β*-Secretase is altered by specific inhibitors of serine proteases (*33*). The generation of *β*A4 peptides with various C-termini suggests that *γ*-secretase has a relaxed specificity. Recent data raise the possibility that *γ*-secretase cleavage is not due to a specific protease (*34*−*35*), supporting previous reports which suggested that *γ*-secretase may be part of a general cellular degradation system, such as a cathepsin D-like lysosomal enzyme (*36*−*38*) or the proteasome (*39*−*41*).

Studies of APP expression with non-mammalian cells have shown that these cells are capable of processing membrane-associated APP and releasing sAPPα (*42*−*44*). However, there is no report to date of APP processing by *β*- and/or *γ*-secretases in these systems. We have studied the processing of APP_{695} in the methylotrophic yeast *Pichia pastoris*, a system which offers high levels of expression of APP with neurotrophic and biochemical properties similar to brain-derived APP (*45*, *46*). We show immunological evidence for *P. pastoris* processing APP by *β*- and *γ*-secretase cleavages. This suggests that *β*- and *γ*-secretase activities are also present in a primitive eukaryote, a system which is eminently tractable to genetic manipulation.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals. Nonidet P-40, Triton X-100, casein, glass beads, and the protease inhibitors antipain, aprotinin, pepstatin A, and phenylmethyl-sulfonyl fluoride (PMSF) were purchased from Sigma (Castle Hill, NSW, Australia), yeast extract and peptone from Oxoid Australia Pty Ltd (Heidelberg, Vic, Australia), and yeast nitrogen base from Difco Laboratories (Detroit, MI). ECL kit and Rainbow protein molecular weight markers were from Amersham Corporation (Castle Hill, NSW, Australia). RPMI cell culture media were from ICN/Flow (Seven Hills, NSW, Australia). Protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit from Pierce (Rockford, IL).

Antibodies. Rabbit antiserum Ab54 was raised to APP_{695} synthetic peptide [676−695]. Mouse monoclonal antibodies (mAb) 1E8 and 1A9 were raised to, respectively, *β*A4 [13−27] and APP [591−596] (APP_{695} numbering). mAb 6E10 (*β*A4 residues [1−17]) was purchased from Senetek (Maryland Heights, MO). Polyclonal antibody 369 (*47*) (anti-APP [656−695]) was a kind gift from Dr. Sam Gandy (New York University, Orangeburg, NY). mAb NT*β*4 (raised to *β*A4 [1−10]) which was kindly provided by Dr. Austen (St George Hospital Medical School, London, UK) has been shown previously to recognize specifically the free N-terminus of *β*A4 (*48*)). mAb WO2 (raised to *β*A4 residues [1−16] (*49*)) and mAb 22C11 (APP N-terminal region (*10*)) have been described before. The various antibody epitopes are depicted in Figure 1.

P. pastoris Expression Clones. DNA encoding the APP_{695} gene, from the N-terminal lysine (amino acid 18) to the

carboxyl-terminal asparagine (residue 695 of APP695, according to Kang et al.(*3*)) was amplified by the polymerase chain reaction (PCR). The forward oligonucleotide 5′CCCCGGGATGCTGGAGGTACCCACTGATGG3′ and the reverse oligonucleotide 5′CCCCGGGGGTCTAGTTCTGCATCTG3′ included an *Xma*I site for cloning into the *P. pastoris* expression vector pHIL-S1 (Invitrogen Corporation). The 3′ primer has an in-frame stop codon preceding the *Xma*I site. pHIL-S1-APP_{695} plasmid DNA was linearized with *Nsi*I (Promega, Rozelle, NSW, Australia) and transfected into the *P. pastoris* strain GS115 by spheroplasting. APP expressing clones were identified by Western blotting the culture supernatants with 22C11. The highest APP_{695} expressing clone was used for this study and is called pp695F. Cloning of $sAPP_{770}\alpha$ and $sAPP_{770}\beta$ into *P. pastoris* was reported previously (*46*). Cloning of C-97 (which corresponds to the last 97 C-terminal amino acids of APP, including βA4, minus residues Asp^1 and Ala^2) has been described previously (*37*).

Yeast Culture, Lysis, and Extraction. Clone pp695F was inoculated into 400 mL of BMGY medium (1% yeast extract, 2% peptone, 100 mM KH_2PO_4, 1.34% yeast nitrogen base, 0.4 mg/L biotin, 1% glycerol) and cultured in a 2 L baffled flask for 48 h. The cells were collected by sedimentation, resuspended in 400 mL of BMMY induction medium (0.5% methanol replacing 1% glycerol in BMGY), and cultured for another 48 h. The cells were resuspended in cold breaking buffer with protease inhibitors (BBPI) (50 mM NaH_2PO_4, pH 7.4, 1 mM EDTA, 5% glycerol, 5 μg/mL antipain, 5 μg/mL aprotinin, 0.5 μg/mL pepstatin A, 1 mM PMSF) and lysed by vortexing with glass beads. Centrifugation at 23400*g* for 10 min yielded a supernatant referred to as lysate supernatant. The corresponding pellet was resuspended in BBPI containing 1% Triton X-100 and extracted by gentle inversion for 1 h at 4 °C. Centrifugation at 23400*g* for 10 min yielded a supernatant referred to as Triton extract.

Characterization of sAPP Products from IMR-32 Cell Culture. IMR-32 neuroblastoma cells were grown to confluence in DMEM/Hams F12 (1:1) medium containing 10% bovine fetal serum. The cells were washed twice with PBS and incubated in serum-free DMEM/Hams F12 for 16 h. The conditioned medium was concentrated 100-fold by ultrafiltration at 4 °C, using a Centriprep-10 (Amersham), fractionated by SDS−PAGE on a 6% Tris-Glycine gel, and blotted, as described below. To demonstrate binding specificity, 1A9 was preincubated for 16 h at 4 °C with peptides CISEVKM, GSNKGPIILM, or ISEVKMD, at 60 μM, prior to immunodetection.

Immunoprecipitation and Western Blotting. The precipitating antisera or monoclonal antibodies were absorbed to protein A-Sepharose (an anti-mouse Ig linker was used for immunoprecipitations with monoclonal antibodies), and samples were added in a total volume of 1 mL of 50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 7.4, containing 0.2% Nonidet P-40 (STEN). For sAPPβ and βA4 immunoprecipitations, the protein A-sepharose absorbed antibodies were added directly to 5 mL of culture medium. After overnight incubation at 4° C, the immunoprecipitates were subjected to three washes with STEN buffer containing 0.1% SDS, one wash with STEN containing 0.5 M NaCl, and one wash with Tris buffer, without detergent. The samples were denatured and resolved on Tris-glycine gels followed by transfer to 0.4 μm PVDF. For the βA4 immunoblots, proteins were electrophoresed on 10%−15% discontinuous Tris/Tricine gels and transferred to 0.2 μm nitrocellulose, according to Ida et al. (*49*). The blots were probed for 2−3 h with primary antibody and incubated with a horseradish peroxidase secondary antibody conjugate (Dako, Botany, NSW, Australia), and then developed by the enhanced chemiluminescence ECL detection system (Amersham).

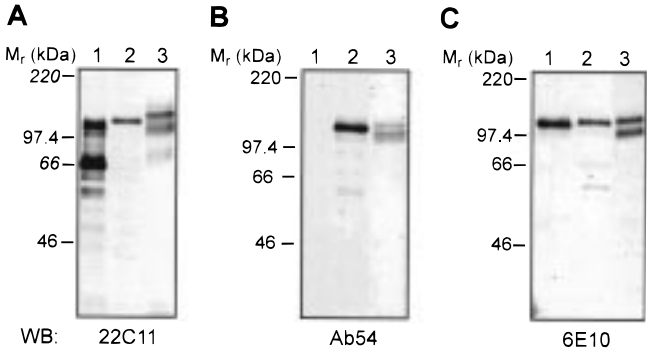

FIGURE 2: Immunocharacterization of APP integral and secreted forms in *P. pastoris* transfected with APP695 cDNA. Samples from the yeast conditioned medium and cell lysate were resolved on SDS 8.5% acrylamide gels and immunoblotted using APP site-specific antibodies. Lane 1 corresponds to the culture supernatant; lane 2 corresponds to 1% Triton X-100 extract of the membrane pellet of the cell lysate; and lane 3 corresponds to human brain membrane-associated APP (semi-purified "P" fraction prepared by Q-Sepharose chromatography, according to Moir et al. *(70)*). Blots were probed with mAb 22C11, directed to the amino-terminal region of APP (panel A), antiserum Ab54, directed to the C-terminus of the cytoplasmic domain (panel B), and mAb 6E10, directed to βA4 residues [1−17] (panel C).

RESULTS

Characterization of APP secretion by Pichia pastoris. A DNA fragment encompassing the APP_{695} gene minus the signal peptide was expressed in the yeast *P. pastoris* by cloning into the secretion vector pHIL-S1. The APP protein was fused to the acid phosphatase signal peptide for secretion through the yeast secretory pathway. Since *Saccharomyces cerevisiae* has been shown to secrete sAPP via an α-secretase activity, we screened for APP expressing clones by immunoblotting culture supernatants with mAb 22C11. A number of expressing clones were identified (data not shown), and the highest expressing clone, termed pp695F, was selected for this study.

Analysis of the pp695F culture supernatant with anti-APP N-terminal mAb 22C11 (Figure 2A, lane 1) revealed APP secreted products as two bands of 115 kDa (major species) species and 105 kDa (minor species). The apparent molecular weights of these bands are consistent with those of APP species from human brain (Figure 2A, lane 3) and from various eukaryotic cell systems (*10*). Polyclonal antibody Ab54 (raised to the C-terminal APP residues 676−695) did not detect any APP in the culture medium (Figure 2B, lane 1), confirming that secretion was accompanied by truncation of the cytoplasmic domain. Additional bands of 65 kDa and lower molecular weight were also detected and represent breakdown products. Analysis of the yeast membrane-associated fraction with 22C11 showed a band of 120 kDa (Figure 2A, lane 2), close to the size detected for membrane-associated human brain APP (Figure 2A, lane 3). This 120

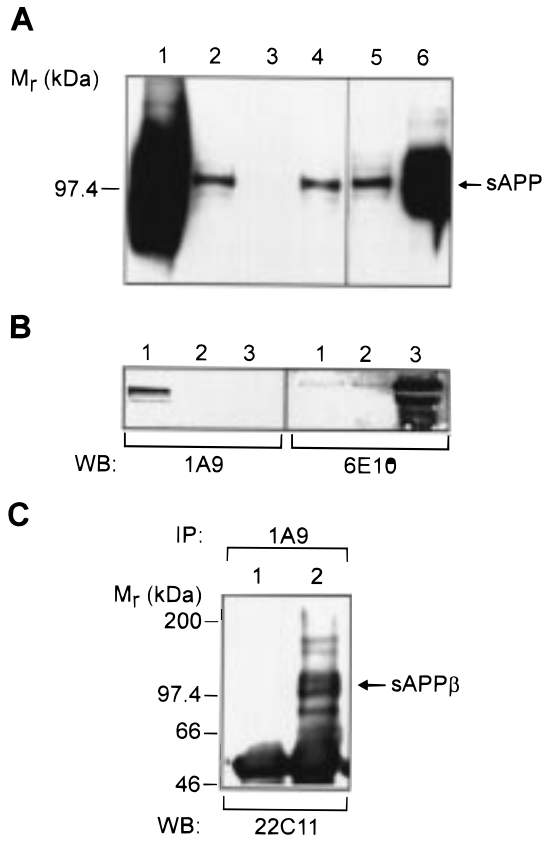

FIGURE 3: Characterization of sAPPβ specific mAb 1A9 and detection of sAPPβ from the culture supernatant of *P. pastoris* transfected with APP_{695}. (A) Immunoblots of APP species secreted in concentrated medium from IMR-32 neuroblastoma cells, detected with antibodies 22C11 (lane 1), 1A9 (lane 2), 1A9 preabsorbed with the peptide immunogen CISEVKM (lane 3), 1A9 preincubated with unrelated APP peptide GSNKGPILM (lane 4), 1A9 preincubated with ISEVKMD (lane 5), and antibody βA4 [1−10] *(71)* (lane 6). (B) Immunoblots of recombinant proteins $sAPP_{770}\beta$ (lanes 1) and $sAPP_{770}\alpha$ (lanes 3), developed with mAb 1A9 and 6E10. Lanes 2 correspond to a pHIL-S1 vector control yeast. (C) Immunoprecipitation of sAPPβ from culture supernatant of *P. pastoris* transfected with APP_{695} using mAb 1A9, followed by immunoblotting with mAb 22C11. Culture media (5 mL) from control vector yeast (lane 1) and from yeast transfected with APP_{695} (lane 2) were used for immunoprecipitation.

kDa band was also detected by Ab54 (Figure 2B, lane 2) and thus corresponds to the full-length molecule, with an intact cytoplasmic domain.

To characterize the secretion cleavage site, *P.* pastoris medium was analyzed with monoclonal antibody 6E10 which is directed to the N-terminal moiety of βA4 (residues [1−17]). The 115 kDa secretory product was detected (Figure 2C, lane 1) and thus contains the N-terminal portion of βA4. This data is consistent with cleavage occurring at the α-secretase site. 6E10 also detected full-length membrane-associated APP (Figure 2C, lane 2).

Secretion of sAPPβ by Pichia pastoris. To determine whether APP secretion in *P. pastoris* also involves cleavage at the β-secretase site, we used monoclonal antibody 1A9 which recognizes specifically the C-terminus of sAPPβ. Specific binding of 1A9 to sAPPβ was first demonstrated by Western blotting of secreted APP from IMR-32 neuroblastoma cells (Figure 3 A). Antibodies 22C11 (Figure 3A, lane 1) and βA4 [1−10] (Figure 3A, lane 6) detected a strong band of approximately 110 kDa molecular weight which corresponds to the product of α-secretion, sAPPα. 1A9 detected a band with a similar electrophoretic mobility but of much lower intensity (Figure 3A, lane 2). The signal was abolished when 1A9 was preincubated with the immunizing peptide CISEVKM, which corresponds to the carboxyl-terminus of sAPPβ (Figure 3A, lane 3). There was no competition with peptide GSNKGAIIGLM, which represents APP residues 621−631 and also has a C-terminal methionine (Figure 3A, lane 4), or with peptide ISEVKMD, which corresponds to the 1A9 epitope but includes an extra C-terminal residue (Figure 3A, lane 5).

The specificity of 1A9 was also analyzed using recombinant sAPP proteins. The APP_{770} gene terminating at either β or α cleavage sites was expressed in *P. pastoris*. The recombinant proteins were analyzed for reactivity with both mAb 1A9 and 6E10. mAb 1A9 recognized the $sAPP_{770}\beta$ protein (Figure 3B, lane 1) but not the $sAPP_{770}\alpha$ protein (Figure 3B, lane 3). As expected, 6E10 antibody reacted with $sAPP_{770}\alpha$ (Figure 3B, lane 3) but not with $sAPP_{770}\beta$ (Figure 3B, lane 1). The control yeast, transfected with pHIL-S1 vector, was not reactive with either of the two antibodies (Figure 3B, lanes 2). These data, together with the peptide competition experiments, indicate that 1A9 is specific for the free C-terminus of sAPPβ and does not cross-react with sAPPα.

To test whether *P. pastoris* secretes sAPPβ, the medium from the yeast transfected with APP_{695} was immunoprecipitated with mAb 1A9 and the immunoprecipitate analyzed by Western blotting with mAb 22C11 (Figure 3C, lane 2). A doublet of 100−110 kDa was observed, which was not immunoprecipitated from the control medium of yeast transfected with pHIL-S1 control vector (Figure 3C, lane 1). The size of the 110 kDa band is consistent with that of sAPPβ secreted from IMR-32 cells. The 100 kDa band of the doublet (Figure 3C, lane 2) and the 80 kDa species may correspond to forms with less glycosylation whereas the weaker bands observed in the 150−170 kDa range may correspond to highly glycosylated forms, as often observed when expressing proteins in yeast. All of these bands were absent from the control yeast medium (Figure 3C, lane 1).

Analysis of Carboxyl-Terminal Fragments Derived from Secretase Conversion. To characterize further the processing of APP, we studied the C-terminal fragments (CTFs) associated with the membrane of the yeast transfected with APP_{695}. Immunoprecipitation of a Triton X-100 extract from the yeast membrane fraction, followed by electrophoresis on 15% Tris-glycine gels and Western blotting with Ab54, yielded a predominant product of 15 kDa, plus minor products ranging from 18 to 21 kDa (Figure 4A). The 120 kDa band corresponds to membrane-associated full-length APP. Immunoprecipitation with mAb 6E10 followed by Western blotting with Ab54 revealed two bands of 16 and 17 kDa (Figure 4B, lane 2). The 16 kDa band had an electrophoretic mobility identical to that of the recombinant C-97 protein and could correspond to CTFβ (Figure 4B, lane 1). The 23 kDa broad band corresponds to the immunoprecipitating antibody light chains. To confirm that some cleavage occurred at the N-terminus of βA4, we used mAb NTβ4 to immunoprecipitate the CTFs. mAb NTβ4 has been shown to recognize specifically the free N-terminus of βA4 *(48)* and thus constitutes a suitable tool to detect the products of β-secretase cleavage. NTβ4 immunoprecipitates were

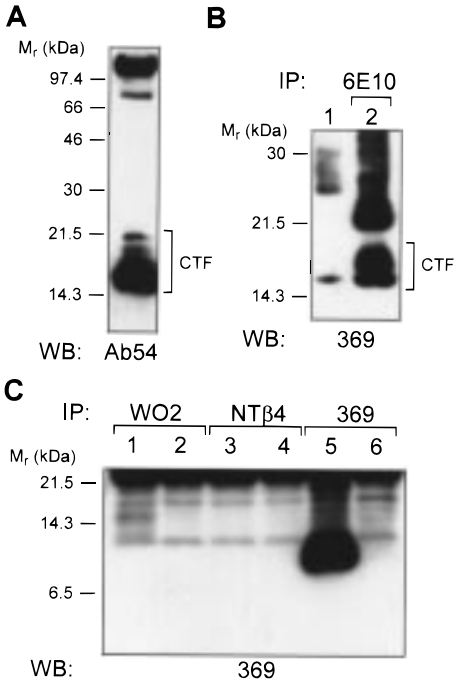

FIGURE 4: Characterization of APP membrane-associated carboxyl-terminal fragments. (A) Immunoblot of 1% Triton-X 100 extract from membrane pellet of APP_{695} transfected yeast, using C-terminal antiserum Ab54. The yeast sample was resolved on a 15% Tris-Glycine gel. C-terminal fragments of 15−21 kDa are detected (indicated as CTF). (B) Detection with Ab54 of APP C-terminal amyloidogenic fragments immunoprecipitated by mAb 6E10 from the 1% Triton-X 100 extract from the yeast membrane pellet (lane 2). C-97 protein (APP residues 598−695) expressed in *P. pastoris* in shown in lane 1 for comparison. The proteins were resolved on a 15% Tris-Glycine gel. (C) Immunoprecipitation of APP C-terminal fragments with the three anti-βA4 antibodies: mAb WO2, which is directed to βA4 residues [1−16], mAb NTβ4, specific for βA4 free amino-terminus, and polyclonal 369, raised to APP cytoplasmic domain. The immunoprecipitates were resolved on a discontinuous 10%−15% Tris-Tricine gel, and the blot was probed with 369. Lanes 1, 3, and 5 correspond to the yeast membrane extract, and lanes 2, 4, and 6 correspond to a lysosomal fraction from human brain, prepared as previously described (*37*).

analyzed in comparison with WO2 and 369 immunoprecipitates, using Tis-Tricine gels. A unique CTF band of approximately 10.5 kDa was immunoprecipitated by NTβ4 from the APP_{695} yeast membrane extract, as detected by APP C-terminal antiserum 369 (Figure 4C, lane 3). This apparent molecular weight is consistent with the size of CTFβ. A similar band was observed when immunoprecipitating with NTβ4 a lysosome-enriched fraction from human brain (Figure 4C, lane 4). mAb WO2 immunoprecipitated from the yeast membrane extract the 10.5 kDa species, plus a 15 kDa fragment (Figure 4C, lane 1). A fragment slightly larger than CTFβ was also immunoprecipitated by mAb 6E10 (Figure 4B, lane 2), and this is likely to derive from alternative cleavage upstream from the β-secretase cleavage site. In contrast, only the 10.5 kDa species was immunoprecipitated from the brain lysosomal fraction with mAb WO2 (Figure 4C, lane 2), suggesting a predominant cleavage at the β-secretase site. Antiserum 369 immunoprecipitated a very abundant 9 kDa band from the yeast membrane extract (Figure 4C, lane 5). Longer exposure of the blot also detected the 9 kDa in the lysosome-enriched fraction from human brain (data not shown). This 9 kDa band would correspond to the α-secretase cleavage product.

Secretion of βA4 Peptide by Pichia pastoris. Immunoprecipitation of 5 mL of culture medium from APP_{695}-transfected *P. pastoris* with mAb 1E8 followed by immunoblotting with mAb WO2 gave a 4 kDa product (Figure 5A, lane 7) with an electrophoretic mobility similar to that of the product secreted from human neuroblastoma cells SH-SY5Y (Figure 5A, lane 4). There was no similar product precipitated from the corresponding total yeast lysate (Figure 5A, lane 6) or from a Triton-X 100 extract from the membrane-associated fraction (Figure 5A, lane 5). The medium from the pHIL-S1-transfected control (Figure 5A, lane 3) and the corresponding lysate (Figure 5A, lane 2) and membrane-associated extract were also negative (Figure 5A, lane 1). Immunoprecipitation of the APP_{695} transfected yeast medium with mAb WO2 and NTβ4 (Figure 5B, lanes 2 and 4, respectively) revealed a similar 4 kDa species with an apparent electrophoretic mobility identical to that of synthetic βA4 peptide (Figure 5B, lane 1). Immunoprecipitation of the medium from control yeast with these two antibodies was negative (Figure 5B, lanes 3 and 5). mAb 1E8 and WO2 also immunoprecipitated a 7 kDa species which could not be immunoprecipitated by mAb NTβ4, and this is likely to derive from a N-terminal cleavage upstream from the β-secretase cleavage site. mAb 1E8 is expected to immunoprecipitate the p3 peptide (α-secretase product) as well as βA4, but because the blots were developed with mAb WO2, p3 could not be detected in these experiments.

DISCUSSION

Characterization of the proteolytic mechanisms which release βA4 from its precursor, including identification of the responsible proteases, is of paramount importance to understanding AD pathogenesis and will offer new therapeutic approaches. In this report, we studied APP processing in the methylotrophic yeast, *P. pastoris*, transfected with APP cDNA. APP was secreted in the culture medium by cleavage within the βA4 domain, as has been described for mammalian and insect cells (*10*, *50*, *51)*. The sAPP ectodomain secreted by *P. pastoris* contained the N-terminal portion of βA4, suggesting that it is derived from cleavage at, or near, the α-secretase site. The concomitant production of a major membrane-associated C-terminal fragment, with the correct size to be a product of cleavage at, or near, the α-secretase site, and which is not detected by antibodies directed to βA4 [1−16] provides indirect evidence for a major α-secretase-like secretory processing occurring in *P. pastoris*. APP cleavage−secretion appears to be well-conserved in all species as it has also been demonstrated not only in mammalian cells but also in baculovirus (*42*) and in the yeast *S. cerevisiae* (*43*,*44*). Recent studies with *S. cerevisiae* have shown involvement of two GPI-linked aspartyl proteases, Mkc7 and Yap3, in the processing of APP through the secretory pathway (*52*) and that these may constitute the majority of *Saccharomyces* α-secretase activity (Komano, Seeger, Gandy, and Fuller, personal communication).

Of relevance to AD, we found that *P. pastoris* was also able to process APP by cleavage at the β-secretase site and to secrete the βA4 peptide. To demonstrate cleavage at the β-secretase site, sAPPβ species were immunoprecipitated from the yeast medium with a monoclonal antibody specific for the sAPPβ C-terminus and which does not cross-react

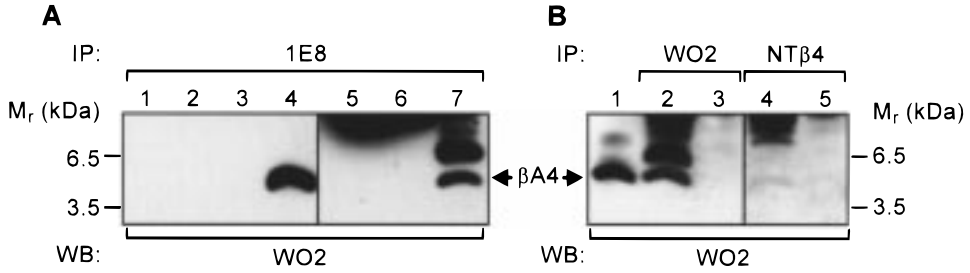

FIGURE 5: Immunodetection of βA4 peptide from *P. pastoris* culture supernatant by immunoprecipitation with three different antibodies. (A) Western blot analysis with mAb WO2 (anti-βA4 [1−16]) of culture media and cell lysates immunoprecipitated with mAb 1E8 (anti-βA4 [13−28]). Lanes 1−3 correspond to *P. pastoris* transfected with the pHIL-S1 vector: lane 1 is the immunoprecipitate of 1% Triton extract from the yeast membrane pellet; lane 2 is the immunoprecipitate from the yeast lysate supernatant; and lane 3 is the immunoprecipitate of the yeast culture medium. Lane 4 corresponds to the culture medium of human neuroblastoma cells SH-SY5Y (positive control) *(72)*. Lanes 5−7 correspond to *P. pastoris* transfected with APP_{695}: lane 5 is the immunoprecipitate from 1% Triton extract of the yeast membrane pellet; lane 6 is the immunoprecipitate of the yeast lysate supernatant; and lane 7 is the immunoprecipitate from the culture medium. (B) Immunoblot analysis with mAb WO2 of immunoprecipitates from *P. pastoris* culture media. Lane 1 corresponds to a standard synthetic peptide βA4 [1−40]; lanes 2 and 4 represent immunoprecipitates (with WO2 and NTβ4, respectively) of the culture medium from *P. pastoris* transfected with APP_{695}; and lanes 3 and 5 represent immunoprecipitates (with WO2 and NTβ4, respectively) of the culture medium from *P. pastoris* transfected with the pHIL-S1 vector (negative control).

with sAPPα. sAPPβ major species were detected as a doublet of 100−110 kDa, similar to sAPPβ from human brain (data not shown). Immunocharacterization of membrane-associated C-terminal fragments also confirmed cleavage at the β-secretase site. A CTF species which contains the free N-terminus of βA4 as well as the APP cytoplasmic domain was found, with an eletrophoretic mobility similar to that of a recombinant protein corresponding to the last 97 C-terminal residues of APP. Besides cleavage at the N-terminus of βA4, a significant amount of APP was processed by *P. pastoris* through a cleavage upstream from βA4 N-terminus (corresponding to a shift of molecular weight of 3−4 kDa). Such alternative cleavages have also been described, particularly in hippocampal neurons (*53*), in platelets (*54*) (Li and Masters, unpublished results), in fibroblasts of Down Syndrome subjects (*55*), and in lymphoblastoid cells from AD patients (*56*).

We also show that *P. pastoris* can secrete a 4 kDa peptide with electrophoretic mobility and immunoreactive properties identical to those of βA4, either of synthetic origin, or that produced by human neuronal SH-SY5Y cells. This species was characterized as βA4 with three monoclonal antibodies directed to distinct epitopes of βA4. However, these antibodies could not discriminate between the (X-40) and (X-42) forms of βA4, thus the precise site of γ-secretase cleavage in *P. pastoris* remains to be determined. The fact that βA4 can be produced by lower eukaryotic cells has not been described previously. Studies by Essalmani et al. (*42*) suggest that the baculoviridae are unable to produce βA4. Besides this 4 kDa species, we found that *P. pastoris* also generates a 7 kDa species which could correspond to the product of an alternative proteolytic N-terminal cleavage. A similar product is also found in platelets (Li and Masters, unpublished observations). Thus, it appears that *P. pastoris* is able to process APP through proteolytic cleavages similar to those used by mammalian cells.

It was not unexpected that *P. pastoris* would contain the cellular machinery to secrete APP since it has also been shown to secrete angiotensin converting enzyme (ACE), another membrane-inserted protein (*57*). APP α-secretion resembles the secretion of other membrane-anchored proteins (*58*, *59*) (reviewed by Hooper et al., ref *60*); it can be stimulated by phorbol ester treatment (*61*), and is inhibited by metalloprotease inhibitors in the same way as is the secretion of interleukin-6, transforming growth factor α (TGFα), tumor-necrosis factor α (TNFα), or ACE (*62*). This α-cleavage would be predicted to occur in the secretory pathway common to all cells, and the finding that APP is secreted by *P. pastoris* is a further piece of evidence that the relevant cellular machinery is conserved from yeast to neurons. TNFα secretase has recently been cloned and identified as a disintegrin metalloprotease (*63*, *64*). We may speculate that the secretases for other growth factors, the ACE convertase and the APP α-secretase itself, are similar proteases, with possibly individual differences to confer specificity toward their particular substrates. *P. pastoris* may be able to secrete all of these proteins by means of the α-secretase ancestor of broad specificity.

Concerning the release of the βA4 peptide, the β- and γ-secretases of APP have properties and subcellular compartmentalization that are quite different from those of the secretases of the membrane-anchored proteins. There could be at least two distinct mechanisms for βA4 production (*13*). A pool of βA4 is produced in the endocytic/recycling pathway (*13*, *65*) and/or in the late secretory pathway (*66*, *67*) to be rapidly secreted, and it will be important to study with organelle-specific inhibitors whether the secretion of βA4 we identified in *P. pastoris* corresponds to one of these mechanisms. A substantial amount of βA4 is also found intracellularly, particularly in neurons (*13*), and there is now evidence for the generation of βA4 (X-42) in the endoplasmic reticulum (*22*−*24*). βA4 release shares similarities with the proteolytic release of the sterol regulatory element binding protein SBREP which also requires two sequential cleavages, the first one occurring in the lumen of the ER and the second within a transmembrane domain (*68*), to generate a functional protein fragment which regulates cholesterol synthesis and uptake. The protease involved has recently been cloned and identified as a polytopic zinc metalloprotease (*69*).

Whether βA4 release serves a particular function, or if it is only part of a cellular degradation pathway remains unclear. The use of *P. pastoris* mutants deficient in proteasome and in vacuolar proteases should permit further clarification of a possible involvement of the ER proteasome degradation system and of the endosomal/lysosomal system in βA4 generation in yeast. Alternatively, the creation of

new mutants may prove necessary to identify the β- and γ-secretases and to help with the identification of the related proteases in the human brain.

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REFERENCES

1. Glenner, G. G., and Wong, C. W. (1984) *Biochem. Biophys. Res. Commun. 120*, 885−890.
2. Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., McDonald, B. L., and Beyreuther, K. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4245−4249.
3. Kang, J., Lemaire, H., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K., Multhaup, G., Beyreuther, K., and Müller-Hill, B. (1987) *Nature 325*, 733−736.
4. Haass, C., Schlossmacher, M. G., Hung, A. Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B. L., Lieberburg, I., Koo, E. H., Schenk, D., Teplow, D. B., and Selkoe, D. L. (1992) *Nature 359*, 322−325.
5. Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M., Whaley, J., Swindlehurst, C., McCormack, R., Wolfert, R., Selkoe, D., Lieberburg, I., and Schenk, D. (1992) *Nature 359*, 325−327.
6. Jarrett, J. T., and Lansbury, P. T., Jr. (1993) *Cell 73*, 1055−1058.
7. Hardy, J. (1997) *Trends Neurosci. 20*, 154−159.
8. Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T. D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) *Nat. Med. 2*, 864−870.
9. Schubert, D., Jin, L. W., Saitoh, T., and Cole, G. (1989) *Neuron 3*, 689−694.
10. Weidemann, A., König, G., Bunke, D., Fischer, P., Salbaum, J. M., Masters, C. L., and Beyreuther, K. (1989) *Cell 57*, 115−126.
11. Sisodia, S. S., Koo, E. H., Beyreuther, K., Unterbeck, A., and Price, D. L. (1990) *Science 248*, 492−495.
12. Evin, G., Beyreuther, K., and Masters, C. L. (1994) *Amyloid 1*, 263−280.
13. Tienari, P. J., Ida, N., Ikonen, E., Simons, M., Weidemann, A., Multhaup, G., Masters, C. L., Dotti, C. G., and Beyreuther, K. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 4125−4130.
14. Thinakaran, G., Teplow, D. B., Siman, R., Greenberg, B., and Sisodia, S. S. (1996) *J. Biol. Chem. 271*, 9390−9397.
15. Haass, C., Lemere, C. A., Capell, A., Citron, M., Seubert, P., Schenk, D., Lannfelt, L., and Selkoe, D. J. 1995) *Nat. Med. 1*, 1291−1296.
16. Chyung, A. S. C., Greenberg, B. D., Cook, D. G., Doms, R. W., and Lee, V. M. Y. (1997) *J. Cell Biol. 138*, 671−680.
17. Perez, R. G., Squazzo, S. L., and Koo, E. H. (1996) *J. Biol. Chem. 271*, 9100−9107.
18. Haass, C., Hung, A. Y., Schlossmacher, M. G., Teplow, D. B., and Selkoe, D. J. (1993) *J. Biol. Chem. 268*, 3021−3024.
19. Golde, T. E., Estus, S., Younkin, L. H., Selkoe, D. J., and Younkin, S. G. (1992) *Science 255*, 728−730.
20. Klafki, H. W., Abramowski, D., Swoboda, R., Paganetti, P. A., and Staufenbiel, M. (1996) *J. Biol. Chem. 271*, 28655−28659.
21. Citron, M., Diehl, T. S., Gordon, G., Biere, A. L., Seubert, P., and Selkoe, D. J. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 13170−13175.
22. Wild-Bode, C., Yamazaki, T., Capell, A., Leimer, U., Steiner, H., Ihara, Y., and Haass, C. (1997) *J. Biol. Chem. 272*, 16085−16088.
23. Cook, D. G., Forman, M. S., Sung, J. C., Leight, S., Kolson, D. L., Iwatsubo, T., Lee, V. M. Y., and Doms, R. W. (1997) *Nat. Med. 3*, 1021−1023.
24. Hartmann, T., Bieger, S. C., Brühl, B., Tienari, P. J., Ida, N., Allsop, D., Roberts, G. W., Masters, C. L., Dotti, C. G., Unsicker, K., and Beyreuther, K. (1997) *Nat. Med. 3*, 1016−1020.
25. Maruyama, K., Kametani, F., Usami, M., Yamoa-Harigaya, W., and Tanaka, K. (1991) *Biochem. Biophys. Res. Commun. 179*, 1670−1676.
26. Sisodia, S. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6075−6079.
27. Massagué, J., and Pandiella, A. (1994) *Annu. Rev. Biochem. 62*, 515−541.
28. Beldent, V., Michaud, A., Wei, L., Chauvet, M.-T., and Corvol, P. (1993) *J. Biol. Chem. 268*, 26428−26434.
29. Arribas, J., and Massagué, J. (1995) *J. Cell Biol. 128*, 433−441.
30. Citron, M., Teplow, D. B., and Selkoe, D. J. (1995) *Neuron 14*, 661−670.
31. Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A. Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe, D. J. (1992) *Nature 360*, 672−674.
32. Cai, X., Golde, T. E., and Younkin, S. G. (1993) *Science 259*, 514−516.
33. Citron, M., Diehl, T. S., Capell, A., Haass, C., Teplow, D. B., and Selkoe, D. J. (1996) *Neuron 17*, 171−179.
34. Tischer, E., and Cordell, B. (1996) *J. Biol. Chem. 271*, 21914−21919.
35. Tjernberg, L. O., Näslund, J., Thyberg, J., Gandy, S. E., Terenius, L., and Nordstedt, C. (1997) *J. Biol. Chem. 272*, 1870−1875.
36. Ladror, U. S., Snyder, S. W., Wang, G. T., Holzman, T. F., and Krafft, G. A. (1994) *J. Biol. Chem. 269*, 18422−18428.
37. Evin, G., Cappai, R., Li, Q. X., Culvenor, J. G., Small, D. H., Beyreuther, K., and Masters, C. L. (1995) *Biochemistry 34*, 14185−14192.
38. Brown, A. M., Tummolo, D. M., Spruyt, M. A., Jacobsen, J. S., and Sonnenberg-Reines, J. (1996) *J. Neurochem. 66*, 2436−2445.
39. Ishiura, S., Tsukahara, T., Tabira, T., and Sugita, H. 1989) *FEBS Lett. 257*, 388−392.
40. Kojima, S., and Omori, M. (1992) *FEBS Lett. 304*, 57−60.
41. Mundy, D. I. (1994) *Biochem. Biophys. Res. Commun. 204*, 333−341.
42. Essalmani, R., Guillaume, J. M., Mercken, L., and Octave, J. N. (1996) *FEBS Lett. 389*, 157−161.
43. Zhang, H., Komano, H., Fuller, R. S., Gandy, S. E., and Frail, D. E. (1994) *J. Biol. Chem. 269*, 27799−27802.
44. Hines, V., Zhang, W., Ramakhrisna, N., Styles, J., Mehta, P., Kim, K. S., Innis, M., and Miller, D. L. (1994) *Cell. Mol. Biol. Res. 40*, 273−284.
45. Ohsawa, I., Hirose, Y., Ishiguro, M., Imai, Y., Ishiura, S., and Kohsaka, S. (1995) *Biochem. Biophys. Res. Commun. 213*, 52−58.
46. Henry, A., Masters, C. L., Beyreuther, K., and Cappai, R. (1997) *Protein Expression Purif. 10*, 283−291.
47. Nordstedt, C., Caporaso, G. L., Thyberg, J., Gandy, S. E., and Greengard, P. (1993) *J. Biol. Chem. 268*, 608−612.
48. Stephens, D. J., and Austen, B. M. (1996) *J. Neurosci. Res. 46*, 211−225.
49. Ida, N., Hartmann, T., Pantel, J., Schröder, J., Zerfass, R., Förstl, H., Sandbrink, R., Masters, C. L., and Beyreuther, K. (1996) *J. Biol. Chem. 271*, 22908−22914.
50. Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., McClure, D., and Ward, P. J. (1990) *Science 248*, 1122−1124.
51. Lowery, D. E., Pasternack, J. M., Gonzalez-DeWhitt, P., Zürcher-Neely, H., Tomich, C. C., Altman, R. A., Fairbanks, M. B., Heinrikson, R. L., Younkin, S. G., and Greenberg, B. D. (1991) *J. Biol. Chem. 266*, 19842−19850.
52. Zhang, W., Espinoza, D., Hines, V., Innis, M., Mahta P., and Miller, D. L. (1997) *Biochim. Biophys. Acta 1359*, 110−122.

53. Simons, M., De Strooper, B., Multhaup, G., Tienari, P. J., Dotti, C. G., and Beyreuther, K. (1996) *J. Neurosci. 16*, 899−908.
54. Ghiso, J., Gardella, J. E., Liem, L., Gorevic, P. D., and Frangione, B. (1994) *Neurosci. Lett. 171*, 213-216.
55. Zhong, Z., Higaki, J., Murakami, K., Wang, Y., Catalano, R., Quon, D., and Cordell B. (1994) *J. Biol. Chem. 269*, 627−632.
56. Matsumoto, A., and Fujiwara, Y. (1993) *Eur. J. Biochem. 217*, 21−27.
57. Sadhukhan, R., Sen, G. S., and Sen, I. (1996) *J. Biol. Chem. 271*, 18310−18313.
58. Ramchandran, R., and Sen, I. (1995) *Biochemistry 34*, 12645−12652.
59. Williams, T. A., Gouttaya, M., Tougard, C., Michaud, A., Chauvet, M. T., and Corvol, P. (1997) *Mol. Cell. Endocrinol. 128*, 39−45.
60. Hooper, N. M., Karran, E. H., and Turner, A. J. (1997) *Biochem. J. 321*, 265−279.
61. Buxbaum, J. D., Gandy, S. E., Cicchetti, P., Ehrlich, M. E., Czernik, A. J., Fracasso, R. P., Ramabhadran, T. V., Unterbeck, A. J., and Greengard, P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6003−6006.
62. Arribas, J., Coodly, L., Vollmer, P., Kishimoto, T. K., Rose-John, S., and Massagué, J. (1996) *J. Biol. Chem. 271*, 11376−11382.
63. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Bolani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Cerretti, D. P. (1997) *Nature 385*, 729−736.
64. Moss, M. L., Jin, S.-L. C., Milla, M. E., Burkhart, W., Carter, H. L., Chen, W.-J., Clay, W. C., Didsbury, J. R., Hassler, D., Hoffman, C. R., Kost, T. A., Lambert, M. H., Leesnitzer, M. A., McCauley, P., McGeehan, G., Mitchell, J., Moyer, M., Pahel, G., Rocque, W., Overton, L. K., Schoenen, F., Seaton, T., Su, J.-L., Warner, J., Willard, D., and Becherer, J. D. (1997) *Nature 385*, 733−736.
65. Koo, E. H., and Squazzo, S. L. (1994) *J. Biol. Chem. 269*, 17386−17389.
66. Busciglio, J., Gabuzda, D. H., Matsudaira, P., and Yankner, B. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2092−2096.
67. Xu, H. X., Sweeney, D., Wang, R., Thinakaran, G., Lo, A. C. Y., Sisodia, S. S., Greengard, P., and Gandy, S. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3748−3752.
68. Brown, M. S., and Goldstein, J. L. (1997) *Cell 89*, 331−340.
69. Rawson, R. B., Zelenski, N. G., Nijhawan, D., Ye, J., Sakai, J., Hasan, M. T., Chang, T. Y., Brown, M. S., and Goldstein, J. L. (1998) *Mol. Cell 1*, 47−57.
70. Moir, R. D., Martins, R. N., Small, D. H., Bush, A. I., Milward, E. A., Multhaup, G., Beyreuther, K., and Masters, C. L. (1992) *J. Neurochem. 59*, 1490−1498.
71. Walsh, D. M., Williams, C. H., Kennedy, H. E., and Allsop, D. (1994) *Biochem. Soc. Trans. 22*, 14S.
72. Fuller, S. J., Storey, E., Li, Q.-X., Smith, A. I., Beyreuther, K., and Masters, C. L. (1995) *Biochemistry 34*, 8091−8098.

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