Generation of the Amyloid-β Peptide N Terminus in Saccharomyces cerevisiae Expressing Human Alzheimer’s Amyloid-β Precursor Protein*

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The Alzheimer’s amyloid-β precursor protein (βAPP) is a type 1 membrane-spanning protein from which the Alzheimer’s disease amyloid-β peptide (Aβ) is proteolytically derived. To date, attempts to identify the enzymes responsible for Aβ generation have failed. Here we report the accumulation of Aβ-immunoreactive peptides in yeast expressing human βAPP. Characterization of these peptides by metabolic labeling, immunoprecipitation with Aβ-specific antibodies, and N-terminal radiosequencing indicates that these peptides include the Aβ peptide at their N termini. The Aβ-like peptides generated in yeast were recovered predominantly as 8- and 12-14-kDa species. A 4-kDa species was recovered either when a protease-deficient strain was used to prevent breakdown or when the 8- and 12-14-kDa species were treated with disaggregating agents. The likely existence in yeast of enzymes generating the Aβ N terminus indicates that the molecular identification of yeast β-secretase-like enzymes may be accomplished using genetic screens or empirical approaches based upon the sequenced genome of Saccharomyces cerevisiae.

Alzheimer’s disease (AD) is characterized by an intracranial amyloidosis that develops in an aging-dependent manner. This amyloidosis appears to be dependent on the production of the amyloid-β peptide (Aβ) from the amyloid-β precursor protein (βAPP) (1, 2). βAPP is a type 1 transmembrane protein, which can be proteolytically processed through two mutually exclusive pathways. Cleavage of a lysine-leucine bond within the luminal portion of the Aβ region of βAPP, a reaction catalyzed by a membrane-associated proteolytic activity termed α-secretase, releases the luminal portion of βAPP as a soluble protein (βAPPs). Alternatively, Aβ is produced by the successive proteolytic processing of βAPP by at least two activities termed β-secretase and γ-secretase. Aβ includes the final 28 residues of the N-terminal luminal domain and the first 12–14 residues of the transmembrane domain of βAPP. All known genetic alterations underlying familial AD increase the accumulation of Aβ in the brain, suggesting that an important early step in the pathogenesis of AD involves production or deposition of Aβ. This invariant pathological phenotype has made Aβ metabolism a potential target for therapeutic intervention.

Isolation of the enzymes responsible for Aβ generation has proven extremely challenging. We have sought to overcome many of the barriers inherent in mammalian enzyme discovery by searching in a simpler organism for a gene homologous to one of the mammalian secretases. For these studies, we chose yeast because of the ease of genetic manipulation. More specifically, we selected the budding yeast Saccharomyces cerevisiae because it is the only yeast for which the genome has been fully sequenced. Combined genetic and biochemical approaches have identified several important proteolytic processing enzymes in yeast, which have proven homologous to enzymes that catalyze the same reaction in higher eukaryotes (4, 5).

It was previously demonstrated that yeast could execute an α-secretase-like cleavage of human βAPP (6, 7), a reaction recently discovered to be catalyzed by yapsins (yeast glycosylphosphatidylinositol-linked aspartyl proteases, Mkc7 and Yap3) (8, 9). Here we report that yeast also possess one or more β-secretase-like activities, which metabolize human βAPP751 into peptides bearing the Aβ sequence at their N termini.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal antibody 4G8 (Senetek, Maryland Heights, MO) recognizes an epitope between residues 18–39 of Aβ. Monoclonal antibodies 26D6 (a gift from Sibia) and 6E10 (Senetek) recognize an epitope between residues 1–17 of Aβ. Rabbit polyclonal antibody 6A3 was raised against a 50-mer peptide derived from the cytoplasmic domain of βAPP (10). The yeast strains used were: CRY1 (MATa can1–100, ade2–101, his3–11, -15, leu2–3, -112 trp1–1, ura3–1), CRY2 (MATa, CB018 (CRY1 pep4::HIS3, prb1::hisG, pcr1::hisG), BFY106-4D (CRY2 hess2::HIS3) CB017 (CRY2 pep4::HIS3, prb1::hisG, pcr1::hisG, hess2::HIS3) (6). Yeast Expression Constructs—Plasmid construction was as described previously (6). pBM-MFA1-100 (pMFA) contains the prepro-α-factor structural gene MFA1, under the control of the GAL1 promoter, with three of the four α-factor repeats deleted on the CEN4 ARS1 URA3 plasmid, pBM258. Human βAPP751 sequences from Glu19 to the stop codon were subcloned into pMFA resulting in an in-frame fusion of prepro-α-factor to Glu19 of βAPP751 (pMFA-βAPP751; Fig. 1). To express the Swedish mutation of βAPP (11), a tandem mutation (K651N,M652L) was introduced into pMFA-βAPP751 using a QuikChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA). Plasmids were introduced into yeast strains by lithium acetate transformation (12).

Yeast Media, Growth, and Radiolabeling—Yeast strains were grown in synthetic complete medium (13) lacking uracil and containing either 2% (w/v) glucose (SGalC-ura) or galactose (SGalC-gal). Cells were grown at 30 °C in SGalC-ura to a density of ~1 × 10⁷ cells/ml. Cells were washed twice with SGalC-ura and equal amounts of cells were resuspended in 5 ml of SGalC-ura and incubated at 30 °C for 2–4 h in the presence of EXPRESS-[35S]methionine/cysteine labeling mix (250
Preparation of Yeast Cell Lysates—Labeled cells were harvested by centrifugation and washed twice in sterile H$_2$O. Acid washed glass beads (Sigma) (~250 µl) were added to the cells with 100 µl of extraction buffer (0.5% deoxycholate, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 50 µg/ml aprotinin, 20 µg/ml leupeptin, 2 µg/ml pepstatin, 50 mM Tris, pH 7.4). The mixture was vortexed at full speed for 2 min and then centrifuged at 14,000 rpm in a microcentrifuge for 5 min at 4 °C. An equal volume of a solution (Buffer A) containing 20 mM sodium phosphate, pH 7.4, 200 mM sodium chloride, 2% Triton X-100, 2 mg/ml sodium azide, 1 mM phenylmethylsulfonyl fluoride, and 50 µg/ml aprotinin was added, and the supernatant fractions were subjected to immunoprecipitation.

Immunoprecipitation—A 400-µl aliquot of each supernatant was incubated with 5 µl of 369 and immunoprecipitated with protein A-Sepharose (Amersham Pharmacia Biotech) to preclear full-length βAPP and C-terminal fragments of βAPP. The resulting supernatants were then incubated with either 1 µl of 4G8, 1 µl of 26D6, or 3 µl of 6E10 and proteins immunoprecipitated with 3 µl of rabbit affinity-purified antibody to mouse IgG (Cappel) followed by protein A-Sepharose. Sepharose pellets were washed twice in a solution containing 150 mM sodium chloride, 10 mM Tris-HCl, 5 mM EDTA, 0.1% Triton X-100, and 100 mg/ml bovine serum albumin, once in phosphate-buffered saline, and resuspended and heated for 3 min at 100 °C in 25 µl of tricine loading buffer (Novex, San Diego, CA). Samples were subjected to SDS-polyacrylamide gel electrophoresis on 10–20% tricine gels (Novex) and autoradiography (Kodak). For quantitative analyses, autoradiographic densities were quantified using a Bio-Rad PhosphorImager and software version 2.0.

Sequence Analysis—Cells (~5 × 10⁷) were doubly labeled with 0.5 mCi/ml [3H]phenylalanine (NEN Life Science Products) and 250 µCi/ml [35S]methionine (NEN Life Science Products) for 4 h at 30 °C in SGalC-ura. Lysates were prepared, and proteins were immunoprecipitated with 4G8 as described above. Samples were subjected to SDS-polyacrylamide gel electrophoresis on 10–20% tricine gels. Labeled peptides were transferred to polyvinylidene difluoride sequencing membranes (Millipore) at 35 V for 90 min. After autoradiography, bands were excised from the membrane and eluted with acetonitrile, and the peptides subjected to 12–22 cycles of Edman degradation in an automated sequencer (Rockefeller University Protein Facility). Fractions were collected and radioactivity was measured by liquid scintillation spectrometry (Beckman LS 5801).

Disaggregation Assays—Cells were harvested and lysed as described above except that the extraction buffer was replaced by 100 µl of either 70% formic acid or 100% hexafluoroisopropanol (HFIP). Lysates were sonicated for 4 × 30 s followed by centrifugation at 14,000 rpm at 4 °C for 1 min to remove unbroken cells. Formic acid-treated lysates were re-equilibrated to pH 7 with 2 mM Tris-HCl, pH 8.8. HFIP-treated lysates were lyophilized and redissolved in 6 µl urea. Samples were adjusted to 0.5% SDS and heated at 100 °C for 3 min. An equal volume of Buffer A was added to samples, and proteins were immunoprecipitated overnight using antibody 4G8.

**Fig. 1.** Yeast expression construct and structure of the amyloid-β precursor protein. pBM-MFα1-100 was fused to a region of βAPP encoding amino acids 19–751, which can be cleaved by Kex2 to release βAPPp1-751. The plasmid was maintained in Ura(-) medium, and expression of the βAPP encoding region was under galactose induction.

**Fig. 2.** Recovery of intracellular Aβ-immunoreactive fragments from a yeast strain expressing pBM-MFα1-100 without or with wild type βAPP or Swedish βAPP. Cells were harvested after 2 h of metabolic labeling (see “Experimental Procedures”). Cell lysates were sequentially immunoprecipitated, first with an antibody to remove C-terminal fragments (369) and then with an antibody for Aβ (4G8, 6E10, or 26D6), and proteins were subjected to SDS-PAGE. Arrows indicate positions of two amyloidogenic fragments with apparent molecular mass of 8 and 12–14 kDa. WT, wild type.

**Fig. 3.** Radioactivity profile of [3H] incorporation into 8- and 12–14-kDa Aβ-immunoreactive species. Cells expressing wild type βAPP were harvested after 4 h of metabolic labeling in SGal-ura containing [35S]methionine and [3H]phenylalanine. Lysates were sequentially immunoprecipitated with 369 (preclearing) followed by 4G8. The proteins recovered by 4G8 were subjected to SDS-PAGE and transferred to nitrocellulose. The 8- and 12–14-kDa bands were excised and eluted from the nitrocellulose after which Edman degradation was performed. Fractions were diluted in liquid scintillation fluid, and the tritium incorporation was measured by liquid scintillation spectrometry. Radioactivity was present in fractions 4, 19, and 20 of both species, as would be expected for a peptide bearing Aβ at its N terminus. CPM, counts/minute.
RESULTS AND DISCUSSION

Previous work (6, 7) demonstrated that human βAPP can be expressed in S. cerevisiae and successfully trafficked through the secretory pathway. In the current study, S. cerevisiae expressing human βAPP<sub>19–753</sub> fused to a yeast α-mating pheromone precursor, prepro-α-factor (Fig. 1), were metabolically labeled with [35S]methionine in galactose-containing growth medium. After this labeling period, the yeast were harvested, and the media were collected. Cell lysates were precleared with 369, 14-kDa bands, their N termini were radiosequenced by labeling with [3H]phenylalanine. Lysates from these cells were immunoprecipitated with 4G8, separated on 10–20% tricine SDS-PAGE, and subjected to Edman degradation, and analyzed as described under “Experimental Procedures." The 4-kDa Aβ-like species is indicated by an arrowhead. B, Kex2<sup>−</sup> cells were metabolically labeled, and lysates were prepared and analyzed as described in the legend to Fig. 3. Radioactivity was present in fractions 4, 19, and 20 of the 4-kDa species, as would be expected for a peptide bearing Aβ at its N terminus. CPM, counts/minute.

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Fig. 4. Analysis of a 4-kDa Aβ-like species from a Kex2-deficient yeast strain. A, wild type (Kex2<sup>+</sup>) and Kex2-deficient (Kex2<sup>−</sup>) strains were transformed with wild type human βAPP and analyzed as described under “Experimental Procedures." The 4-kDa Aβ-like species is indicated by an arrowhead. B, Kex2<sup>−</sup> cells were metabolically labeled, and lysates were prepared and analyzed as described in the legend to Fig. 3. Radioactivity was present in fractions 4, 19, and 20 of the 4-kDa species, as would be expected for a peptide bearing Aβ at its N terminus. CPM, counts/minute.

Fig. 5. Effect of disaggregating agents on electrophoretic mobilities of Aβ-like species in yeast A. Cells were harvested after 2 h of metabolic labeling in SGal-ura. The cells were lysed, and the proteins were extracted with 0.5% deoxycholate/0.5% Nonidet P-40 (lanes 1 and 3), 70% formic acid (lane 2), or 100% HFIP (lane 4) as described under “Experimental Procedures." Proteins were sequentially immunoprecipitated with antibody 369 (preclearing) and antibody 4G8. 4G8-immunoprecipitated proteins were subjected to SDS-PAGE and autoradiography. B, relative intensities of the three Aβ-like bands were determined using NIH Imagequant software, version 2.0. ND refers to a signal below the limit of detection.

It seemed possible that the absence of a typical 4-kDa Aβ-like species might be attributable to protease activity, to aggregation, or to both. Evidence in support of the first possibility was obtained by screening a series of βAPP-expressing yeast strains, deficient in one or another protease, for their ability to generate an Aβ-immunoreactive species. In a strain deficient for Kex2 (BFY-106-4D), an Aβ-immunoreactive species with apparent molecular mass of 4 kDa was detected (Fig. 4A). N-terminal radiosequencing of this 4-kDa species was consistent with the N terminus of Aβ (Fig. 4B). One explanation for the appearance of the 4-kDa band only in a Kex2-deficient strain could be that the presence of Kex2 results in degradation of Aβ. Alternatively, the absence of Kex2 could affect the activity of an as yet unidentified enzyme, which promotes the formation of the 4-kDa species, or lead to a missorting of βAPP to an inappropriate intracellular compartment, such as the vacuole, where proteases are highly active. Any of these possibilities could account for the generation of the 4-kDa Aβ-like species in yeast strains deficient for Kex2. Evidence in support of the possibility of peptide aggregation was obtained by sonication of yeast lysates in the presence or absence of formic acid or HFIP. Following either treatment, the recovery of the 8- and 12–14-kDa species was reduced (but not abolished), and a 4-kDa band became apparent (Fig. 5), suggesting that the larger species represent, at least in part, dimers and trimers of Aβ. The determination of the exact C terminus of this 4-kDa species will be required to determine whether this peptide corresponds to...
Aβ 1–40 or 1–42, both of which occur in mammalian cells. Preliminary studies, using an high pressure liquid chromatography system, which is capable of resolving these two variants, suggest that the 4-kDa species is predominantly similar to Aβ42.²

The discovery that yeast strains can process full-length human βAPP into an Aβ-like species, together with the ease of genetic manipulation in yeast, should make it possible to conduct genetic screens designed to identify Aβ N terminus-generating activities and to study βAPP trafficking.

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