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Atypical Processing of Amyloid Precursor Fusion Protein by Proteolytic Activity in *Pichia pastoris*

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Secretases catalyze the production of important proteolytic products of the amyloid precursor protein. We expressed a fusion protein that contained horseradish peroxidase, fragment 590-695 of amyloid precursor protein, and c-myc and polyhistidine tags in *Pichia pastoris.* It secreted a 50-kDa N-terminal fragment; a 15-kDa C-terminal fragment accumulated in cells. The N-terminal fragment exhibited peroxidase activity and reacted with antibodies specific for peptides within the sequences −2 to 15 and 21-37 of β-amyloid peptide. The C-terminal fragment reacted with antibodies that recognize the sequences 649-664 and 676-695 of amyloid precursor protein and the C-terminal c-myc tag. To locate the cut site, the C-terminal fragment was metabolically labeled with either [^{35}S]Met or [^{3}H]Lys and radiosequenced. A major component, derived from a cleavage at Gly^{25}-Ser^{26} of β-amyloid, was detected. Results suggest a predominant atypical cleavage, like that observed in Down Syndrome fibroblasts, occurs between the α- and γ-sites.

The amyloid precursor protein (APP) plays a key role in Alzheimer's Disease (1). APP is the precursor of the 4 kDa βA4 amyloid peptide, that accumulates in extracellular plaques in the brains of AD patients (2). APP 695, one of 4 known isoforms, contains the βA4 sequence, a single membrane spanning domain and a short cytoplasmic region (3). APP 695 contains these domains at C-terminal amino acids 625-695. As reviewed by Evin *et al.* (4) proteolytic processing of mammalian APP 695 is catalyzed by three types of secretases. Each produces a mixture of CTFs, with predominant cleavage at the following sites: the β-site (Met^{595}-Asp^{596}); the α-site (Lys^{612}-Leu^{613}) and two γ-sites (Val^{636}-Ile^{637}) or (Ala^{638}-Thr^{639}). The responsible proteases, usually referred to as secretases, have not been characterized and their exact specificity and numbers remain uncertain.

In *Saccaromyces cerevisiae,* a combination of immunological and sequence analysis showed that cleavage of APP occurred at the same α-site as in mammalian cells (5, 6). Recently, it was reported that Yap3p and Mkc7p, GPI-linked aspartyl proteases associated with the cell surface are α-secretases in *S. cerevisiae* (7) Le Broque *et al.* (8) expressed APP in the methylotrophic yeast, *Pichia pastoris.* They showed that APP was cleaved at or near the α-site and to a minor extent at the α-site. Furthermore processing of APP by the organism led to the secretion of a 4 kDa fragment with the immunological properties of βA4. This suggested the presence of a γ-secretase in *P. pastoris.*

De Strooper *et al.* (9) reported the expression of a construct which codes for horseradish peroxidase (HRP) fused with amino acids 590-695 (HRP-APP$_{590\text{-}695}$) of murine APP in mammalian cells. This included the βA4, trans-membrane and cytoplasmic domains of APP. As with full length APP, the cells appeared to cleave that construct predominantly at α-secretase sites. However, unlike APP secretion products, the fusion protein products were not secreted from MDCK cells in a polarized manner.

We have expressed HRP-APP$_{590\text{-}695}$ in *P. pastoris.* A combination of immunological and radiosequencing analyses indicated that the fusion protein was predominantly cleaved at an atypical site, Gly^{25}-Ser^{26} of βA4 in APP.

EXPERIMENTAL PROCEDURES

Strains, plasmids, and enzymes. A *P. pastoris* expression kit, vector pPICZ B, and Zeocin were purchased from Invitrogen. *E. coli*

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Abbreviations used: (HRP-APP$_{590\text{-}695}$), a fusion protein whose exact structure is described in Fig. 1; APP, amyloid protein precursor; AD, Alzheimer's disease; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; NTF, N-terminal fragments of HRP-APP$_{590\text{-}695}$; CTF, C-terminal fragments of HRP-APP$_{590\text{-}695}$; HRP, horseradish peroxidase; PCR, polymerase chain reaction.

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DH5α and all restriction and modification enzymes were from Gibco-BRL. Pml I and Pme I were from Biolabs. Dr. De Strooper provided us with a gene in pSG5 plasmid that encodes HRP fused in tandem with the βA4, trans-membrane and cytoplasmic domains of murine APP 695 (9).

DNA construct preparation, transformation, and expression in Pichia pastoris. DNA manipulations were performed as described by Sambrook *et al.* (10). DNA encoding the sequence from the N-terminal methionine of HRP to the C-terminal Asn^{695} of APP was amplified by PCR. The following primers were used: 5′ ATG CAG TTA ACC CCT ACA 3′ (sense) and 5′ AGT TCT GCA TTT GCT CAA A 3′ (antisense). The DNA fragment was gel-purified using the GeneClean II (Bio 101) kit and cloned into the Pml site of the pPICZaB expression vector. To confirm that the gene was in frame with the α-factor secretion signal and the C-terminal tag, sequencing was performed. Ten μg of DNA were linearized with Pme I, transformed into *P. pastoris* strain GS115 by electroporation and induced for 24 hours as described in Invitrogen pPICZα A,B,C, instruction manual.

Antibodies. Rabbit anti-HRP polyclonal antibodies were from Dako Corp. The peroxidase conjugated anti-c-myc monoclonal antibodies (clone 9E10) were from Boehringer. Dr. D. Selkoe provided us with a panel of antibodies used in this study and immunogen B for C8. These include rabbit polyclonal antisera against the indicated sequences in APP: C8 (amino acids 676-695) (11), R 1155 (amino acids 649-664) (11), R1963 (amino acids 617-633) (12), R1736 (amino acids 595-611) (13). Immunogenic peptide A was synthesized at the Protein Research Facility, UIC.

SDS-PAGE, Western blotting, and immuno-precipitation. SDS-PAGE was performed by the Laemmli method (14) or by the Tris-Tricine system modification (15). Gels were blotted onto PVDF membrane followed by treatment with primary and HRP labeled secondary antibodies. Blotted proteins were visualized with an enhanced chemiluminescence detection system (ECL) from Amersham, as described by the manufacturer.

Metabolic labeling and radiosequencing. Transformed *P. pastoris* were induced in 1 ml of amino acid free YNB medium containing 200 μCi/ml [^{35}S]Met or 1mCi/ml [^{3}H]Lys. After 24 h, horseradish peroxidase activity was measured in the culture medium and cells were pelleted and stored at −80°C. Thawed cells were extracted in TSA buffer (10 mM Tris-HCl, pH 8.0, 0.15M NaCl, 0.01% sodium azide) containing 0.2% Triton X-100 and a protease inhibitor cocktail (Boehringer) by vortexing in the presence of glass beads. The extracts were incubated with Protein G-agarose absorbed with monoclonal anti-c-myc antibodies (Calbiochem) overnight at 4°C. Immunoprecipitates were washed, solubilized in SDS sample buffer and subjected to 12% Tris-Tricine SDS-PAGE. After electrophoresis labeled proteins were blotted onto ProBlott membranes (Applied Biosystems). Membranes were subjected to autoradiography. Strips that corresponded to the visualized 15 kDa band were excised and sequenced as described (16).

Enzyme activity and protein assay. Peroxidase activity was assayed by measuring the rate of conversion of 1,2-phenylenediamine at 540 nm (9). Activity is reported as OD_{540}/min/ml. Protein concentrations were determined according to the manufacturer's instructions, using a Bio-Rad protein assay kit, with bovine serum albumin as a standard.

Purification of secreted recombinant peroxidase. After induction, the culture supernatant (800 ml) was filtered to remove any residual particles and lyophilized in the presence of PMSF. Purification of the recombinant HRP was carried out with an Immunopure Protein A IgG Orientation Kit (Pierce). The immuno-affinity column was prepared by binding 5 mg of rabbit anti-HRP antibody to the Protein A column according to manufacturer's instructions. The concentrated sample was diluted 2 times in 10 mM Tris-HCl, pH 7.5 and applied to the column. The column was washed with Binding Buffer and eluted with Elution Buffer supplied in the Kit. Aliquots of 0.5 ml fractions were assayed for HRP activity, and fractions containing the enzyme were pooled and stored at −20°C.

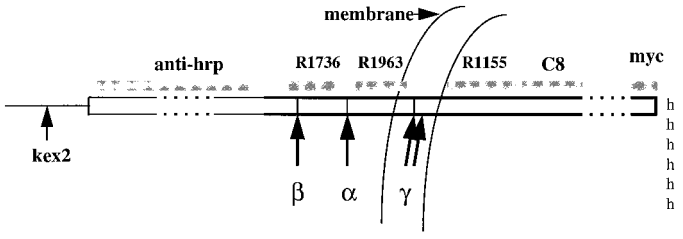

FIG. 1. Diagram of the structure and antigenicity of the expressed fusion protein, HRP-$APP_{590-695}$. The line indicates a portion of the α-mating factor signal sequence which is cleaved at the KEX 2 site. This is followed by the HRP-APP fusion rectangle. The dotted regions indicate that the drawing is not to scale. The APP 695 cut sites are marked (β = 595-596, α = 612-613, γ = 636-637, or 638-639). The gray bars represent regions that are recognized by antibodies. R1736 recognizes APP 695 sequence 595-611, R1963 recognizes 617-633, R1155 recognizes 649-664, and C8 recognizes 676-695.

Isolation of the cellular CTFs from APP. The cells were harvested from 800 ml of induced culture and the cell lysate was prepared as described in *Pichia pastoris* Expression Kit manual. The supernatant containing 139 mg of total protein was loaded on a His-Bind column (15 ml) from Novogen prepared according to manufacturer's instructions. The column was washed with Breaking Buffer:50 mM sodium phosphate, pH 7.4 containing 5 mM PMSF, 1 mM EDTA, mixture of protease inhibitors (pepstatin, leupeptin, antipain, chymostatin) and the bound protein was eluted with Elution Buffer 1 (20 mM Tris-HCl, pH 7.9, 60 mM imidazole, 0.5M NaCl) followed by Elution Buffer 2 (10 mM Tris-HCl, pH 7.9-500 mM imidazole, 250 mM NaCl). All buffers contained 0.1% SDS. Fractions were assayed by Western blot using peroxidase conjugated anti-c-myc monoclonal antibodies and combined antibody binding fractions eluted with Elution Buffer 1 were used for the second chromatography on the same column. The reactive samples were lyophilized, resuspended in water and separated by 15% Tris-Tricine SDS-PAGE. The fragments were visualized with Coomassie Blue, excised from the gel and electro-eluted in 50 mM ammonium bicarbonate-0.1% SDS buffer on the Model 422 ElectroEluter (BioRad). Eluted protein was lyophilized, dissolved in water and desalted on Micro Bio-Spin P-6 column (BioRad).

RESULTS

Kinetics of induction and processing of HRP-$APP_{590-695}$. Figure 1 depicts the structure of the gene product expressed in *P. pastoris.* The protein includes an N-terminal peptide that is a portion of the secretion signal of yeast α-mating factor from *S. cerevisiae.* It should be cleaved at the indicated KEX 2 site. An additional C-terminal peptide containing a c-myc epitope and a polyhistidine tag, is attached to the C-terminus of the APP portion. Figure 2A and 2B show the time course of appearance of HRP and the 15 kDa CTF. HRP accumulated continuously and reached a steady state 24 hours after induction. Anti-c-myc positive bands appeared at approximately 15 kDa and 30 kDa, 2 hours after induction. Elution and re-electrophoresis of the 15 kDa band resulted in the

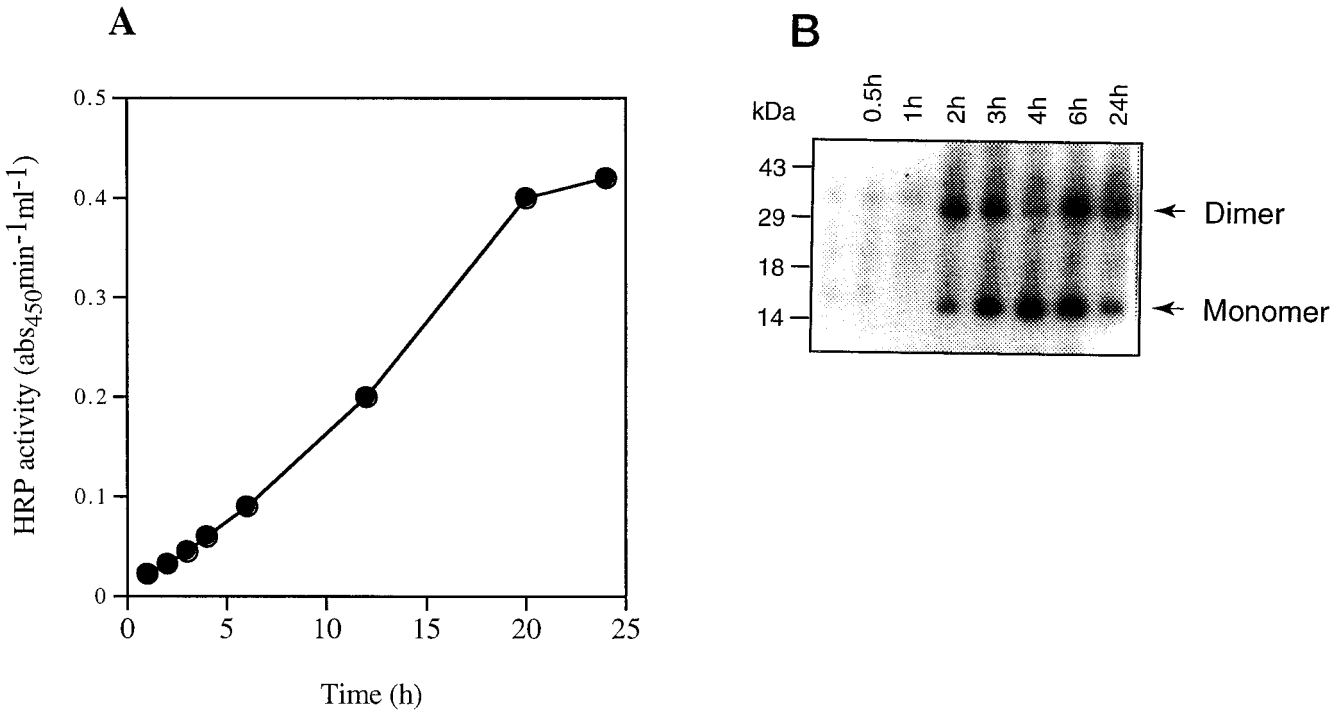

FIG. 2. Kinetics of expression of HRP-$APP_{590-695}$ fragments in *Pichia pastoris*. (A) NTF: After induction, aliquots of the yeast culture were removed at the indicated times and centrifuged, and supernatants were analyzed by measuring the HRP. (B) CTF: The cells were lysed in SDS sample buffer by boiling for 5 min, and the extract was applied to SDS-PAGE. CTF was estimated by Western blotting with anti-myc antibody.

re-appearance of the 30 kDa band, which did not dissociate after boiling in SDS and β-mercaptoethanol. Therefore the 30 kDa band is a dimer of the 15 kDa band.

Physico-chemical characterization of NTF and CTF. To identify fusion protein cleavage sites, the secreted and cell associated fragments were purified. CTF was purified by chromatography on a His-Bind column and gel electrophoresis. As shown in Fig. 3A, greater than 90% of the Coomassie staining material in the final preparation was associated with a diffuse 15 kDa protein. Attempts at standard N-terminal sequencing of purified CTF led to the detection of multiple amino acids after each cycle. When the top and bottom halves of the 15 kDa CTF bands were excised and subjected to re-electrophoresis, protein in the top half retained a lower relative mobility than protein in the bottom half. These results suggested the pool of CTF was heterogeneous.

NTF was purified by immuno-affinity chromatography using anti-HRP columns. The final preparation was greater than 90% on SDS-PAGE (Fig. 3A). Unlike in mammalian cells (9) HRP activity was measured without added hemin. Sodium azide (0.01%) completely inhibited the secreted HRP activity. The 50 kDa NTF is larger than authentic glycosylated horseradish peroxidase. NTF is also glycosylated since it reacted positively with a carbohydrate detection kit (Sigma).

Immunological characterization of NTF and CTF. The purified fragments were subjected to immunological analysis. Approximately equal amounts of native HRP, purified NTF, and purified CTF were run on SDS-PAGE and immunoblotted with the indicated antibodies (Fig. 3B-E). Antibody binding was performed in the presence of 200 μg/ml of HRP to minimize reaction of endogenous anti-HRP in rabbit serum with the NTF. R1736, which detects sequence 595-611 of APP (−2 to 15 of βA4) (see Fig. 1) reacted strongly with NTF under these conditions (Fig. 3B). R1963 (which detects sequence 21 to 37 of βA4) also reacted with NTF (Fig. 3C). In the presence of Peptide A, the immunogen of R1963, no reaction could be detected (data not shown). R1155 recognized only CTF (Fig. 3D). C8 appeared to bind to both NTF and CTF (Fig. 3E). However, the C8 immunogen, Peptide B, inhibited the reaction of CTF with C8, but had no effect on its binding to NTF. Conversely, Pre-immune serum C8 reacted nonspecifically with NTF, but did not react with CTF (data not shown). Thus the specific binding of C8 was with CTF. The antibody analysis indicated that the cut site was included in the region which is defined by epitopes recognized by R1963 and R1155.

Radiosequencing of CTF. To determine the cleavage site more exactly, the CTF was radiosequenced. Metabolically radiolabeled CTF was obtained by inducing equal amounts of the same batch of cells in the

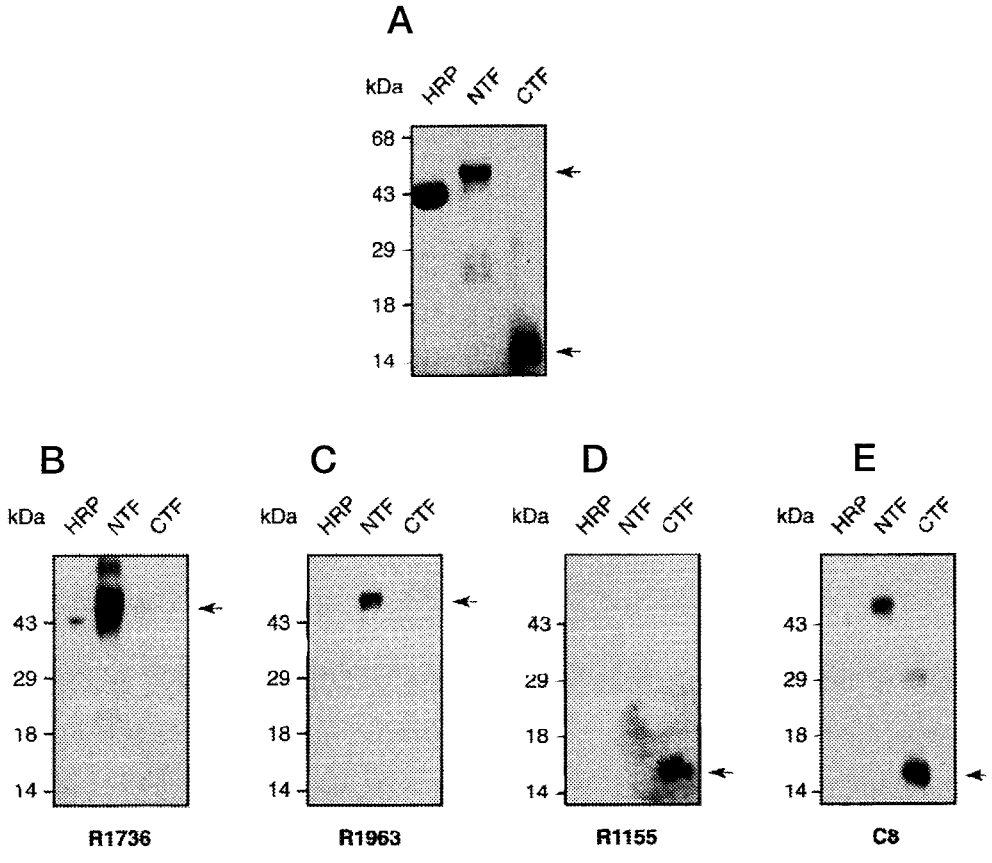

FIG. 3. Characterization of purified NTF, and CTF fragments with a panel of antibodies. (A) Authentic HRP and purified NTF and CTF samples were run on 12% SDS/Tris-Tricine gel and stained with Gelcode blue staining reagent. After electrophoresis of similar samples, the gel was blotted and probed in the presence of 200 μg/ml of HRP with the following APP site-specific antibodies: (B), R1736; (C), R1963; (D), R1155; (E), C8. Arrows indicate 50-kDa NTF and 15-kDa CTF fragments.

presence of either [^{35}S]Met or [^{3}H]Lys. The CTF was purified from the cell lysates by immunoprecipitation with monoclonal anti-c-myc antibody and SDS-PAGE. When the CTF was isolated and subjected to amino-terminal radiosequence analysis (Fig. 4A) the [^{35}S]Met signal reached a major peak at cycle 10. A minor peak was detected at cycle 6. The [^{3}H]Lys signal attained major peaks at cycles 3 and 9 and a minor peak at cycle 6 (Fig. 4B). The results indicated that CTF could have been produced by a cut at Gly^{25}-Ser^{26} of βA4 in the fusion protein (see Fig. 4C).

DISCUSSION

In this work we employed HRP-APP$_{590\text{-}695}$, to study APP processing in *P. pastoris* because we expected that replacement of the APP ectodomain with the reporter, HRP, would be more advantageous for analysis of processing products and secretases. Recent studies have indicated that *P. pastoris* might be a potential source of secretases (8). We found that the fusion protein is cleaved at an atypical secretase site.

P. pastoris NTF contained HRP activity and antigenicity. Because of additional peptide sequences at the N- and C-termini of NTF (see Fig. 1) its mobility on SDS-PAGE (50 kDa) was slower than that of native HRP (44 kDa). The apparent molecular weight of CTF (15 kDa) was greater than that calculated for a fragment generated by a predominant α-cut (13.5 kDa). However because of the heterogeneity of the band and the abnormally slow migrations of CTFs (5, 17) additional procedures were required to identify the cut sites.

NTF specifically bound to R1963 antibodies that recognize sequences between the α- and γ-site of βA4. CTF specifically bound to R1155 and C8 antibodies, which recognize most of the sequence C-terminal to the γ-site of βA4. These results suggested the CTF was derived from an enzyme activity that cleaves amino acids in the sequence between 617 and 664 of APP (C-terminal to the predominant α-site and close to the transmembrane region, as shown in Fig. 1). Le Brocque *et al.* (8) suggested that APP in *P. pastoris* was cleaved primarily at or near the α-site. This conclusion was based on

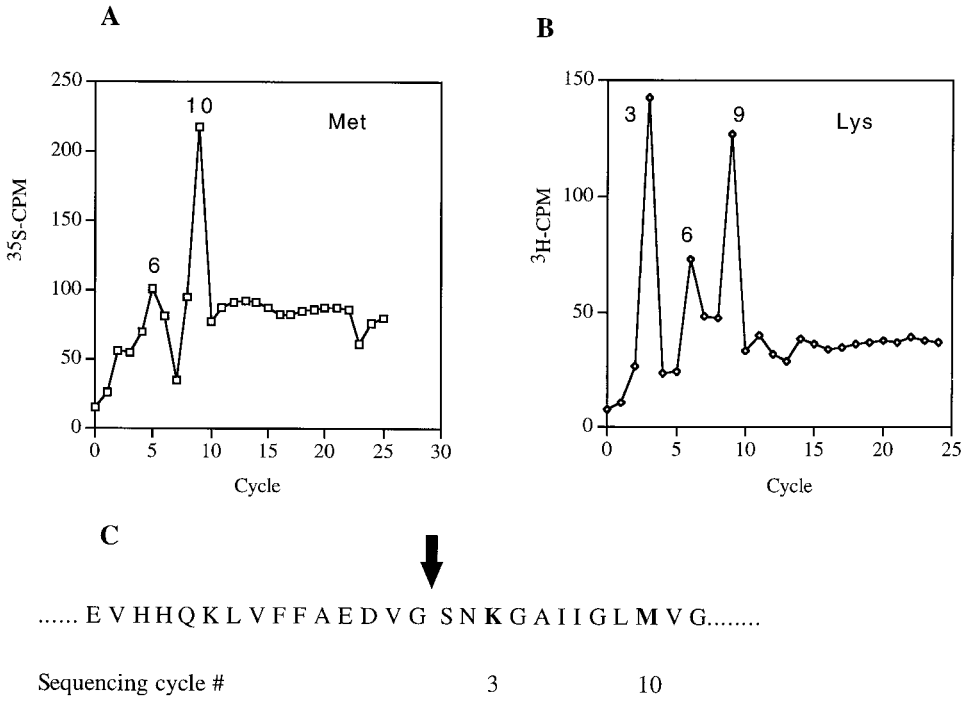

FIG. 4. Radiosequence analysis of 15 kDa CTF isolated from *P. pastoris* cell lysates. Transformed *P. pastoris* were induced for 24 h in medium containing either (A) 200 μCi/ml [^{35}S]Met or (B) 1 mCi/ml [^{3}H]Lys. Cells were lysed in TSA buffer and extracts immunoprecipitated with monoclonal anti-c-myc antibodies. After extensive washing immunoprecipitates were solubilized in SDS sample buffer, subjected to 12% Tris-Tricine SDS-PAGE, and blotted onto ProBlott membranes. Membranes were subjected to autoradiography, and strips that corresponded to the visualized 15-kDa band were excized and sequenced as described (22). (C) Partial sequence of βA4 peptide illustrating secretase cleavage site at Gly^{26}-Ser^{27} (denoted by arrow).

the reactivity of secreted NTF with monoclonal antibodies that recognize residues 1-17 of βA4 and the non-reactivity of the concomitant CTF with monoclonal antibodies directed to residues 1-16. Our results are in agreement. APP is probably processed in *P. pastoris* by the same secretase as the fusion protein. Determination of the N-terminal sequence of the APP and fusion derived CTF products could provide further evidence for this idea. A similar issue relates to the processing of HRP-APP in some mammalian cells. De Strooper *et al.* (9) reported that a monoclonal antibody to amino acids 17-24 of βA4 did not bind with NTF, but we showed that a polyclonal antibody to amino acids 21-37 does bind to the NTF generated in *P. pastoris.* Sequence analysis of the respective CTF sequences could also resolve this difference.

Possible differences between the processing of APP and HRP-APP$_{590\text{-}695}$ in *P. pastoris* can be due to the exchange of HRP with the APP ectodomain (9). Also, the C-terminal extension in our fusion protein might affect its processing. An example of an amino acid change at a distance from a secretase site affecting its cleavage has been reported (19).

To find the exact cleavage site in HRP-APP$_{590\text{-}695}$ in *P. pastoris* we performed sequence analysis of the radiolabeled CTF fragments (Fig. 4). These data suggest that an atypical cleavage of this substrate occurs between the α- and γ-sites. The predominant [^{35}S]Met radiosignal occurred at cycle 10 indicating that two cut sites within the immunologically defined sequence were possible (Gly^{25}-Ser^{26} and Iso^{41}-Ala^{42}). The [^{3}H]Lys labeled CTF sequencing left only the Gly^{25}-Ser^{26} as an option. Only that cut could produce a peptide, . . .VG<-> SNKGGAIIGLKM. . ., which would release [^{3}H]Lys on the third cycle and methionine at the 10th cycle. The release of [^{3}H]Lys on the sixth and ninth cycle represents the sequence of other as yet unidentified peptides. The finding of multiple peptides is in agreement with our other observations regarding the heterogeneity of CTF.

The CTF found in this study is identical to an APP derived component that is produced by fibroblasts from Down's syndrome patients (18). Moreover the sequence around the cut site in *P. pastoris* is almost the same, except for the first amino acid, as the fragment (FGS-NKG. . .) that was detected as a minor component in *S. cerevisiae* by Zhang *et al.* (5). They found a major fragment that was derived from a typical α-cleavage. Because of the limitations of the sequencing of a minor component in the presence of a major component, the **F** could have been an error. This suggests that a similar atypical cut might take place in fibroblasts of Down Syndrome patients and in *S. cerevisiae.*

The HRP-APP$_{590-695}$ expressed in *P. pastoris* may offer a convenient tool for future investigations directed toward identification of yeast and related human brain enzymes and their inhibitors. The characterization of these enzymes will require their purification and genetic analysis. These, in turn, require highly sensitive and simple detection methods such as the HRP secretion assay described here.

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