**Involvement of Cell Surface Glycosyl-phosphatidylinositol-linked Aspartyl Proteases in α-Secretase-type Cleavage and Ectodomain Solubilization of Human Alzheimer β-Amyloid Precursor Protein in Yeast**

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Human β-amyloid precursor protein (APP) introduced into yeast undergoes α-secretase-type cleavage, suggesting that yeast have α-secretase-like protease(s). Here we report that two structurally and functionally related glycosyl-phosphatidylinositol-linked yeast aspartyl proteases, Mkc7p and Yap3p (collectively termed yapsin), are responsible for α-secretase-type cleavage of APP expressed in yeast, resulting in release of soluble APP into the extracellular space. Disruption of MKC7 and YAP3 in a vacuolar protease-deficient strain abolished this APP cleavage/release, and APP cleavage/release could be restored by introduction of MKC7 or YAP3 on a single copy plasmid. Purified Mkc7p cleaved an internally quenched fluorogenic APP peptide substrate at the α-secretase cleavage site. Measurement of proteolytic activity either in yeast homogenates or on the yeast cell surface revealed that most Mkc7p and Yap3p activities were localized at the cell surface. These results establish a molecular basis for α-secretase-type cleavage in yeast and support the generally held concept that α-secretase cleavage of APP occurs at the cell surface.

The β-amyloid precursor protein (APP),1 a type-1 transmembrane protein, can undergo at least two alternative pathways of proteolytic processing. β-Amyloid peptide (Aβ), the component of cerebral plaques associated with Alzheimer’s disease, is produced by proteolytic processing of APP and includes 28 residues of the N-terminal luminal domain and the first 12–14 residues of the transmembrane domain (1). Alternatively, cleavage of a Lys-Leu bond in the luminal portion of the Aβ region of APP by a membrane protein-solubilizing proteolytic activity, termed “α-secretase,” releases the luminal portion of APP as a soluble protein and precludes the formation of Aβ (1). There has been intensive interest in identifying the enzymes that cleave APP to determine their relationship to the etiology of Alzheimer’s disease. However, the responsible molecules remain unknown. In particular, molecular identification of α-secretase could provide insight into the general mechanism of integral membrane protein cleavage (2) and clarify the role of this enzyme in the pathogenesis of Alzheimer’s disease.

We have reported that the yeast *Saccharomyces cerevisiae* has α-secretase-like activities: human APP expressed in *S. cerevisiae* undergoes proteolytic cleavage at the α-secretase site (3, 4). Existence of an α-secretase-like activity was also observed in insect cells (5), suggesting that the enzymes are widely conserved among metazoans. Therefore, identification of yeast secretases could lead to the discovery of counterparts among other phyla. Here we demonstrate that homologous glycosyl-phosphatidylinositol (GPI)-linked aspartyl proteases, Mkc7p (6) and Yap3p (7, 10–12), termed “yapsin” (33), are responsible for α-secretase cleavage of human APP in yeast.

**EXPERIMENTAL PROCEDURES**

**Yeast Media and Strains—**Liquid synthetic media were as described (13). All strains were derived from the W303 background as described (14). The strains were as follows: CRY2, *MATa, can1-100, ade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3-1 (14); HKY36–6B, CRY2 pep4::HIS3, prb1Δ::LEU2 (6); HKY26, CRY2 yap3Δ::LEU2, mkc7Δ::HIS3 (6); HKY27, CRY2 yap3Δ::LEU2, mhc7Δ::HIS3, pep4Δ::HIS3, prb1Δ::LEU2 (6); HKY20, CRY2 yap3Δ::LEU2 (6); HKY21, CRY2 mhc7Δ::HIS3 (6).

**Plasmids—**DNA manipulations followed standard methods (15). Plasmids pMP-APP751 and pMP were as described (3). pMP-APP751 consists of the prepro-segment of prepro-α-factor that includes processi ng signals provided by residues 1–151 of human APP751, under control of the GAL1 promoter on the yeast plasmid pBMY258 (16). pMP consists of the prepro-α-factor structural gene MFA1, deleted of three of the four α-factor repeats, under control of the GAL1 promoter on the yeast plasmid pBMY258. pRS314 is a CEN plasmid harboring TRP1 (17). pRS4MKC7 is a SpeI-Smal fragment containing the MKC7 gene inserted into yeast pBMY258. pRS4YAP3 is a SacI-XbaI fragment containing the YAP3 gene inserted into yeast plasmid pRS314. pRS316 is a CEN plasmid harboring URA3 (17). pRS6MKC7 is a SpeI-Smal fragment containing the MKC7 gene inserted into yeast plasmid pRS316. pY0324 is a SacI-Smal fragment containing the MKC7 gene inserted into yeast plasmid pRS316. pY0324 is a 2-μm replication origin (18) and was a gift from Dr. Ohysh (University of Tokyo, Tokyo, Japan). pY04MKC7 is a SpeI-Smal fragment containing the MKC7 gene inserted into yeast plasmid pRS316. pRS4YAP3 is a SacI-XbaI fragment containing the MKC7 gene inserted into yeast plasmid pRS316. pY0324 is a 2-μm replication origin (18) and was a gift from Dr. Ohysh (University of Tokyo, Tokyo, Japan).
null mutations in MKC7 and YAP3 block generation of the C-terminal fragment of human APP in yeast (A), and production of the C-terminal fragment is restored by introducing a plasmid encoding MKC7 or YAP3 (B). Rabbit polyclonal affinity-purified antibody against the cytoplasmic domain of APP was used to immunoprecipitate APP-related polypeptides from [35S]methionine-labeled yeast cell lysates. Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (discontinuous gel 7.5%/15%) and autoradiography. For both panels, arrowheads indicate the positions of the pro-α-factor-APP fusion protein (upper band) and near full-length APP (lower band) generated by removal of the pro-α-factor region by Kex2 cleavage (lower band) (3). The arrows indicate the position of the nonamyloidogenic C-terminal APP fragment, the product of α-secretase-type cleavage. Open arrowheads indicate the positions of potentially amyloidogenic C-terminal APP fragment, possibly the product of β-secretase-type cleavage. A, each strain was transformed with pMF-APP751 (odd lanes) or pMF (even lanes). Lanes 1 and 2, wild type (CRY2); lanes 3 and 4, mck7Δyap3Δ (HKY24); lanes 5 and 6, pep4Δprb1Δ (KRY36–5B); lanes 7 and 8, mck7Δyap3Δ pep4Δprb1Δ (HKY26). + indicates that the endogenous wild-type gene is present at normal levels, −, pMKY76 (mck7Δyap3Δ pep4Δprb1Δ) was transformed with the following plasmids: lane 1, pRS 314 and pMF-APP751; lane 2, pRS4MKC7 and pMF-APP751; lane 3, pRS4MKC7 and pMF; lane 4, pRS4ΔYAP3; lane 5, pRS4ΔYAP3 and pMF; lane 6, pYOMKCY7 and pMF-APP751; lane 7, pYOMKCY7 and pMF. + indicates that the HKY26 background has been transformed with a single copy of a wild-type gene. ++ indicates transformation with multiple copies of a wild-type gene. − indicates that the endogenous genetic deficiencies characteristic of HKY26 have not been genetically corrected.

To construct pG5YAP3, the BamHI and SalI sites were introduced upstream of NdeI site and downstream of the BstEII site of YAP3, respectively, by PCR. Then the NdeI-BstEII fragment from the PCR product was replaced with the original fragment from YAP3. This was digested with BamHI and SalI and ligated to pG5, which had been cut with BglII and SalI.

Radio labeling and Immunoprecipitation—Radio labeling was performed as described (14, 20). Yeast strains containing pMF-APP751 or pMF were grown at 30 °C in low sulfate medium containing 100 mM ammonium sulfate and 2% galactose (14). Cultures were harvested at a density of ∼10⁷ cells/ml and subjected to sulfate depletion for 30 min by resuspension in low sulfate medium containing 20 μM ammonium sulfate and 2% galactose, following which cells were labeled for 30 min with 150 μCi of [35S]methionine/ml (NEN Life Science Products). Labeled cells (1 ml) were chilled on ice after addition of 10 mM sodium azide, harvested by centrifugation, and washed once with 50 mM Tris–HCl, pH 7.0, containing protease inhibitors (10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 100 μM N-tosyl-L-phenylalanine chloromethyl ketone, 100 μM N-tosyl-L-lysine chloromethyl ketone, 1 mM benzamidine–HCl, 25 μM pepstatin A). Cells were stored at −80 °C prior to lysis. Cell lysis was performed as described (14). To immunoprecipitate the C-terminal fragment of human APP, 2 μl of affinity-purified rabbit antibody 369 against the C-terminal 645–694 residues of APP369 (21) and 30 μl of 50% Pansorbin slurry (Calbiochem) were added to the lysate made from 1 ml of labeled cells. Immunoprecipitates were washed and then solubilized in SDS sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 30%, v/v glycerol, 5% 2-mercaptoethanol, 5 mM EDTA) at 97–98 °C for 3 min, as described previously (14) and subjected to SDS-polyacrylamide gel electrophoresis and fluorography.

Immunoprecipitation of released soluble APP (sAPP) was performed as described (4), because most sAPP is bound to the surface of yeast cells (4). A 1-ml aliquot of labeled cells was washed once with 50 mM Tris–HCl, pH 10.5, containing 5 mM dithiothreitol, resuspended in 1 ml of the same buffer, and then incubated for 30 min at 30 °C. After centrifugation at 40,000 × g for 15 min to pellet the cells, the clear supernatant was mixed with 10 μl of mouse monoclonal antibody 22C11 (22) against the N-terminal intralumenal domain of APP, 5 μl of rabbit anti-mouse IgG, and 25 μl of 50% protein A-Sepharose (Amersham Pharmacia Biotech) slurry, to immunoprecipitate the sAPP. The immunoprecipitates were solubilized in SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis and fluorography.

Assay of Cell Surface Proteolytic Activity—Assay of cell surface proteolytic activity was performed essentially as described (25). Cells were grown to a density of 10⁷/ml (Klett value 70), harvested, washed in 0.15 M NaCl, and then resuspended in 1/4× volume of reaction buffer (100 mM sodium citrate, pH 4.0, 5 mM CaCl₂) containing protease inhibitors 0.5 mM phenylmethylsulfonyl fluoride/0.1 mM N-tosyl-L-phenylalanine chloromethyl ketone/0.1 mM N-tosyl-L-lysine chloromethyl ketone/1 mM benzamidine hydrochloride). To assay cell surface proteolytic activity, 25 μl of cell suspension and 25 μl of reaction buffer containing either 40 μM APP IQ substrate or 40 μM pro-α-factor IQ substrate were mixed and incubated at 37 °C for 30 min. Following termination of reaction by addition of 650 μl of 1× Tris HCl, pH 10, yeast cells were removed by centrifugation at 10,000 × g for 15 min. Fluorescence of the supernatant was determined with a Perkin-Elmer L8-5B fluorimeter (λex = 358 nm; λem = 490 nm). 1 unit of activity was defined as an increase in fluorescence equivalent to 1 pmol GluEDANS/min. To assay
total activity, cells were lysed by three cycles of freezing on dry ice and thawing at 0°C in reaction buffer containing protease inhibitors and 1% Triton X-100.

RESULTS AND DISCUSSION

α-Secretase Cleavage of Human APP Expressed in Yeast: Blockade by Double Disruption of MKC7 and YAP3—Double disruption of MKC7 and YAP3 greatly diminished α-secretase site cleavage of APP, as evidenced by the levels of cell-associated APP C-terminal fragment (Fig. 1A, lane 1 versus lane 3), and α-secretase-type APP cleavage was completely abolished when the double disruption was created in a vacuolar protease-deficient strain (Fig. 1A, lane 5 versus lane 7). Expression of MKC7 (Fig. 1B, lane 2 versus lane 1) or YAP3 (Fig. 1B, lane 4 versus lane 1) under a wild-type promoter on a single copy yeast centromeric plasmid restored this cleavage. The YAP3-transformed cells showed greater APP α-secretase activity than did the MKC7-transformed cells (Fig. 1B, lanes 2 and 4). Overexpression of MKC7 greatly enhanced α-secretase-type APP cleavage (Fig. 1B, lane 6). These results indicate that both Mkc7p and Yap3p can catalyze α-secretase site APP cleavage in intact cells, although some of this cleavage can involve vacuolar proteases. Of note, it was frequently possible to detect a slowly migrating APP C-terminal fragment (Fig. 1B) potentially consistent with a "β-C-terminal-fragment" or "C100-fragment" bearing Aβ at its N terminus (26) and thus constituting the immediate precursor of Aβ. Immunochemical and radiochemical characterization of this APP C-terminal fragment is underway.

In yeast, most of the α-secretase-cleaved N-terminal APP fragment (i.e. sAPP) associates with the exterior of the cells, and only a small proportion of this sAPP appears in the medium (4). Treatment of the cells with 5 mM dithiothreitol at pH 10.5 for 30 min releases much of this cell surface-associated sAPP (4). Disruption of MKC7 in a vacuolar protease-deficient strain abolished generation of this cell surface-associated sAPP (Fig. 2). Introduction of either MKC7 or YAP3 on a single copy

| Genotype | Plasmid | Activity | Total<sup>a</sup> | units/10<sup>6</sup> cells |
|----------|---------|----------|-------------------|--------------------------|
| Experiment A (a) | | | | |
| wt | wt | | 12.2 ± 2.9 | 11.8 ± 0.4 |
| Δ | Δ | | 14.2 ± 2.3 | 12.2 ± 0.5 |
| Δ | Δ | | 3.1 ± 0.7 | 2.0 ± 0.3 |
| Δ | Δ | | 3.1 ± 0.4 | 1.9 ± 0.3 |
| Experiment B (a) | | | | |
| Δ | Δ | pRS6MKC7 | 12.1 ± 2.4 | 10.4 ± 1.1 |
| Δ | Δ | pRS4YAP3 | 4.4 ± 1.2 | 4.8 ± 0.3 |
| Δ | Δ | pRS6MKC7 & pRS4YAP3 | 14.0 ± 2.0 | 11.3 ± 1.3 |
| Δ | Δ | pRS341 & pRS316 | 2.5 ± 0.9 | 1.8 ± 0.4 |
| Experiment C (a) | | | | |
| wt | wt | | 11.3 ± 1.4 | 7.1 ± 2.5 |
| Δ | Δ | | 3.4 ± 0.1 | 4.8 ± 0.1 |
| Experiment D | | | | |
| Δ | Δ | pG5-MKC7 (a) | 760 ± 50 | 1,840 ± 50 |
| Δ | Δ | pG5-MKC7 (APP) | 271 ± 50 | 53.5 |
| Δ | Δ | pG5-YAP3 (a) | 68 ± 13 | 148 ± 1 |
| Δ | Δ | pG5-YAP3 (APP) | 376 ± 13 | 84.6 ± 1 |
| Δ | Δ | pG5 (a) | 3.1 ± 50 | 1.6 ± 0.6 |
| Δ | Δ | pG5 (APP) | 1.5 ± 50 | ND |
plasmid restored generation of this fragment, and introduction of multicycop MCK7 increased sAPP production. (Fig. 2). Thus, some APP undergoes Mck7p- or Yap3p-mediated cleavage to produce an intracellular C-terminal fragment and a cell surface-associated, sAPP-like N-terminal APP fragment.

Cleavage of APP Peptide at the α-Secretase Site by Purified Mck7p—V_{max}/K_{m} for this IQ substrate by purified Mck7p was determined,2 and the cleavage site identified by Edman degradation and MALDI-TOF-MS indicated that >95% of the cleavage occurred at the Lys-Leu bond (the α-secretase site).3 Mck7p cleaved this substrate at pH 4.0 with a V_{max}/K_{m} of 0.03 min^{-1}, normalized to 1 μg/ml Mck7p, equivalent to a k_{cat}/K_{m} of 3.3 × 10^{4} M^{-1} s^{-1} assuming a fully active enzyme preparation. Although this reaction was approximately 2 orders of magnitude less than those reactions observed for cleavage of substrates based on pro-α-factor cleavage sites at pH 4.0, the V_{max}/K_{m} for the α-secretase site in APP was increased ~20-fold when tested at pH 6.0 (k_{cat}/K_{m} ~ 6.6 × 10^{5} M^{-1} s^{-1}).2 Localization of Yapsin at the Cell Surface—GPI-anchored proteins appear to be delivered to the cell surface (27). Yap3p in particular was reported to be localized to the plasma membrane by subcellular fractionation (12). Therefore, we directly measured the proteolytic activity of Mck7p and Yap3p on the cell surface using both an APP-based IQ substrate as well as an IQ substrate based on pro-α-mating factor (Table I) that is a better substrate for these enzymes.2 Evidence that Mck7p and Yap3p are at the cell surface came from experiments using the highly efficient pro-α-factor IQ substrate. Significant proportions of proteolytic activity were held concept that 3 H. Komano and R. S. Fuller, unpublished results.

Because the latter two activities may be identical at the molecular level, it will now be possible to investigate the roles of aspartyl- and metalloproteinases as ectodomain solubilizing enzymes in various cases. Beyond α-secretase-type APP cleavage and arguably more relevant to Alzheimer’s disease pathobiology, highly sensitive methods now enable the investigation of whether β-, γ-, and/or θ-secretase activities exist in yeast, enabling a complete reconstitution of Aβ generation in S. cerevisiae. Such studies are now in progress (34, 35).

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