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EXPRESSION, PURIFICATION, AND NEUROTROPHIC ACTIVITY OF AMYLOID PRECURSOR PROTEIN-SECRETED FORMS PRODUCED BY YEAST

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Summary: The secreted form of amyloid precursor protein (APP^S) including most of the extracellular domain of APP is released from the cell surface, suggesting physiological significance of APP^S *in vivo*. We used the methylotrophic yeast *Pichia pastoris* as a host system for the production of recombinant APP^S ($rAPP^S$). Two $rAPP^S$s derived from isoforms of APP (APP695 and APP770) were secreted into the culture medium from the yeast, which carried cDNA encoding the N-terminal portion of APP under the control of a *P. pastoris* alcohol oxidase promoter. Like APP^Ss produced by the transfected COS-1 cells, the purified $rAPP^S$s from yeast were shown to be biologically active in terms of neurite outgrowth of embryonic rat neocortical explants. These $rAPP^S$s could be valuable tools for investigating the biological functions of APP^Ss.

Alzheimer's disease is characterized by the deposition of amyloid fibrils in the intracellular neurofibrillary tangles, extracellular plaques, and congophilic angiopathy. The major component of the extracellular amyloid is amyloid β protein (Aβ) derived from its larger precursor, amyloid precursor protein (APP) (1-3). APP is translated predominantly from three alternatively spliced mRNAs to yield polypeptides of 695, 751, and 770 amino acids (aa), which consist of a 17-residue signal peptide at the amino terminus, a large extracellular domain, a single membrane-spanning region, and a short intracellular carboxyl terminus. Each peptide is refered as APP695, APP751, and APP770, respectively. The latter two peptides contain a Kunitz-type serine protease inhibitor (KPI) domain (4-6). The major pathway of APP metabolism involves an enzymatic cleavage within the Aβ sequence (7, 8), release of APP-secreted forms (APP^Ss) including most of the extracellular domain from the cell surface, and their accumulation in plasma and cerebrospinal fluid (9-11), suggesting physiological significance of APP^Ss *in vivo*. On the other hand, membrane-bound APPs have a receptor-like architecture (1) with a region in the cytoplasmic domain capable of complexing with GTP-binding protein (11).

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Recently, several biological functions of APP^{s}s have been reported. Purified APP^{s}s were shown to have trophic activity on cultured fibroblasts (12), cortical neuronal cells (13) and PC12 cells (14). It was also reported that APP^{s}s protect culture neurons from hypoglycemia-induced cytotoxicity (15), influence blood coagulation (16, 17), and play a role in cell-substrate adhesion (18, 19). However, the molecular mechanisms of the biological activities of APP^{s}s remaine unclear. Conventionally, APP^{s}s have been purified from brain (20, 21) or prepared from the medium of cells overexpressing APPs (6, 13), but their yield is not very high. For elucidating the biological function of APP^{s}s, a sufficient amount of purified APP^{s}s is required.

We report here the expression and purification of APP^{s}s produced by the methylotrophic yeast, *Pichia pastoris*. The obtained recombinant APP^{s}s (rAPP^{s}s) was shown to be biologically active in terms of neurite outgrowth of neocortical explants.

MATERIALS AND METHODS

Construction of the rAPP^{s} expression vectors: For the construction of each plasmid, *Escherichia coli* JM109 was used as a host. A 1.85-kilo base (kb) *Bam*HI-*Eco*RI fragment derived from APP695 cDNA (1) was ligated to pUC118 digested with *Bam*HI and *Eco*RI. The oligonucleotides shown in Fig. 1A were inserted into the *Eco*RI site of the obtained plasmid to create a stop codon next to the Lys^{612} codon of APP695. After insertion of a synthetic linker (5'-GGAATTCC-3') into the *Sma*I site of the plasmid to create a new *Eco*RI site, a 1.9-kb *Eco*RI fragment was prepared from it and inserted into the *Eco*RI site of pHIL-D2 (Invitrogen, CA), resulting in plasmid pAPE1 (Fig. 1A). Furthermore, a 0.9-kb *Kpn*I-*Xho*I fragment of the 1.9-kb *Eco*RI fragment was replaced with a 1.1-kb *Kpn*I-*Xho*I fragment derived from APP770 cDNA (6) and the obtained 2.1-kb *Eco*RI fragment was inserted into the *Eco*RI site of pHIL-D2 (Fig. 1A), resulting in plasmid pAPE2. *P. pastoris* GS115 (*his4*) was transformed according to the method described previously (22). Briefly, after digestion of each plasmid with a restriction enzyme, *Not*I, *P. pastoris* was transformed with the obtained *Not*I fragments containing a gene cassette for expression of rAPP^{s} and a *his*4 gene. Colonies of His^{+} Mut^{-} phenotype, expected to be recombined at the *AOX1* sequence on the yeast chromosome, were then selected. Expression of rAPP^{s}s by the transformants was induced by the method described previously (23).

Purification of the expressed rAPP^{s}s: The culture medium was separated from the transfected yeast by centrifugation and 200 to 800 ml of the medium was concentrated to 15 ml by pressure filtration using a YM-30 Amicon ultrafilter. The obtained sample was passed through a heparin Sepharose column (0.5 x 1 cm, Bio-Rad). The column was washed with 60 ml of phosphate-buffered saline. Bound protein was eluted with a linear gradient of 0.15-1.65 M NaCl at a flow rate of 1 ml/min over 40 min. Pooled fractions containing rAPP^{s} from the heparin Sepharose column were added to an equal volume of 4.0 M $(NH_4)_2SO_4$ in 50 mM Na_2HPO_4, pH 7.0, buffer and loaded onto an alkyl-Superose column (0.5 x 5 cm, Pharmacia) at a flow rate of 0.5 ml/min. The column was washed with 10 ml of 2.0 M $(NH_4)_2SO_4$ in 50 mM Na_2HPO_4, pH 7.0, buffer. Bound protein was eluted with a linear gradient of 2.0-0 M $(NH_4)_2SO_4$ at a flow rate of 0.5 ml/min over 60 min. Fractions corresponding to the first peak containing pure rAPPs were pooled and dialyzed against phosphate-buffered saline. The purification of each rAPP^{s} was monitored by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by silver staining or Western blotting with antibody 22C11 (Boehringer Mannheim).

Explants of rat neocortical tissue: The effect of rAPP^{s}s on neurite outgrowth from rat neocortical explants was examined by the method described previously (24). In brief, neocortical tissues from embryonic 17-day rats were cleaned of meninges and chopped into pieces of various sizes (50-200 μm in diameter). The explants were then suspended in serum-free Dulbecco's modified Eagles's medium and plated in a polyethylenimine-coated 24-well plate. Purified rAPP^{s}s were added to the culture medium 2 h after the seeding. After 2 days of incubation, the explants were fixed with 2% glutaraldehyde and stained with Coomassie Brilliant Blue R-250 to visualize the neurite outgrowth. The extent of neurite outgrowth was determined under phase-contrast microscopy as described elsewhere (25).

RESULTS AND DISCUSSION

Two isoforms of rAPP^S were produced in a yeast expression system using the methylotrophic yeast *P. pastoris* as a host. In brain, APP^{S}s are known to be mainly released from the cell surface by the cleavage of APP between Lys and Leu (residues 612 and 613 of APP695) (7, 8). For expression of the recombinant APP695-secreted form (rAPP695^S) and the recombinant APP770-secreted form (rAPP770^S), DNA fragments encoding 612 aa (residues 1-612 of APP695) and 687 aa (residues 1-687 of APP770) were inserted downstream of the *P. pastoris* alcohol oxidase (*AOX1*) promoter, resulting in plasmid pAPE1 and pAPE2 (Fig. 1A), respectively. The transformants of *P. pastoris* obtained with pAPE1 and pAPE2 were named APE1 and APE2, respectively. Each transformant was incubated in the culture medium containing glycerol as a carbon source to accumulate cells and then the medium was changed to one containing methanol to induce expression of rAPP^{S}s. After incubation, the cells were harvested by centrifugation. The pelleted cells and the supernatant were examined for their produced proteins by Western blotting using antibody 22C11 (Fig. 1B). In the supernatants of

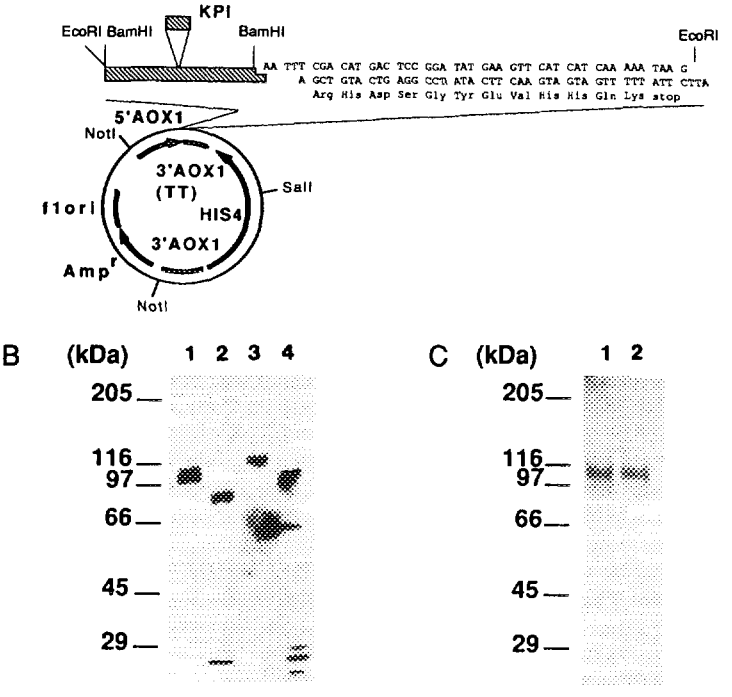

Fig. 1. Expression and purification of rAPP^{S}s in yeast. **A**. Schematic map of pAPE1 and pAPE2. Hatched box indicates a fragment derived from APP cDNA without a KPI domain in pAPE1 or with a KPI domain in pAPE2 (see Materials and Methods). The nucleotide sequence of the synthetic oligonucleotides encoding the C-terminal amino acid sequence is shown. **B**. Identification of rAPP^{S}s produced by recombinant yeasts APE1 (lanes 1 and 2) and APE2 (lanes 3 and 4) by Western blotting. Proteins from 15 μl of the culture medium (lanes 1 and 3) and proteins (20 μg) from cell lysates (lanes 2 and 4) were subjected by SDS-PAGE, blotted on nitrocellulose paper, and immunologically detected with antibody 22C11. **C**. Eluates (200 ng protein) from a heparin column (lane 1) and an alkyl-Superose column (lane 2) were subjected to SDS-PAGE and the bands were detected by silver staining.

APE1 and APE2, a 100-kilodalton (kDa) band of rAPP695^S and a 120-kDa band of rAPP770^S were observed, respectively. Their molecular weights are quite similar to that of purified APP^{S}s from cerebrospinal fluid (9). The positive band in each cell lysate was 15 kDa shorter than that in the supernatant, indicating that most of the product anchored in the cells might not be glycosilated.

The expressed rAPP^{S}s were purified from the culture media by the procedure described in "Materials and Methods". After centrifugation, the supernatant was concentrated by ultrafiltration, and loaded onto a heparin column. Previous work (21) showed that APPs from the brain was eluted from a heparin column between 0.3 and 0.4 M NaCl. In our case, bound rAPP^{S}s were also eluted at about 0.4 M NaCl (Fig. 1C, lane 1). The crude rAPP^S was then applied to an alkyl-Superose column and pure rAPP^S was fractionated by elution with a 2.0-0 M linear gradient of $(NH_4)_2SO_4$ (Fig. 1C, lane 2). The purification of each rAPP^S was monitored by Western blotting with antibody 22C11 (data not shown). By densitometric analysis of the immunoblots, about 4.5 mg and 1 mg/l of the secreted rAPP695^S and rAPP770^S, respectively, were estimated to be accumulated in the supernatant of culture medium. The amino terminal amino acid sequence of the purified material completely matched the sequence predicted for the cDNA of APP between residues 18 and 27, indicating that the signal peptide of APP was properly processed on secretion from yeast cells in the same manner as that from mammalian cells.

To investigate the biological activity of rAPP^{S}s induced by the yeast expression system, we examined the effects of APP^{S}s on neurite outgrowth of cultured neocortical explants (see Materials and Methods). First, the APP695^S and APP770^S purified from COS-1 cells transfected with cDNAs (26), were added to cultured rat neocortical explants. As shown in Fig. 2A, APP^{S}s promoted neurite outgrowth. The effect was further analyzed semi-quantitatively by determining the percentage of explants with neurites longer than the diameter (25) (Fig. 2B). When APP^{S}s produced by COS-1 cells were added, the percentage of explants with longer neurites increased in a dose-dependent manner. The increase was significant at concentrations higher than 30 ng of each APP^S per ml and the saturation dose was 100 ng/ml, indicating that the KPI domain is irrelevant to this neurotrophic activity. Similar results were obtained by the addition of rAPP^S produced by yeast cells (Fig. 3A) and its ability to promote the neurite outgrowth was suppressed by the addition of antibody 22C11 (Fig. 3B), indicating that the neurite outgrowth is specifically enhanced by rAPP^S.

Araki et al. (13) also demonstrated that APP^{S}s produced by COS-1 cells had a neurite-promoting effect on rat primary cultured neocortical neurons at a concentration of 40 nM and Jin et al. (27) reported that APP695s produced by *E. coli* promoted neurite extension of a clonal CNS neuronal line at concentrations from 10 to 100 nM. These effective concentrations were about 20- to 100-fold higher than that found in our study. It is likely that the assay system using neocortical explants is more sensitive for detecting the biological activity of APP^S.

We developed a system for the efficient production of pure and biologically active rAPP^{S}s using a yeast expression system. Their molecular weights calculated from SDS-PAGE and elution profiles of a heparin column were quite similar to those of APP^{S}s purified from the human brain. In recombinant yeasts, the leader sequence was accurately cleaved showing that the

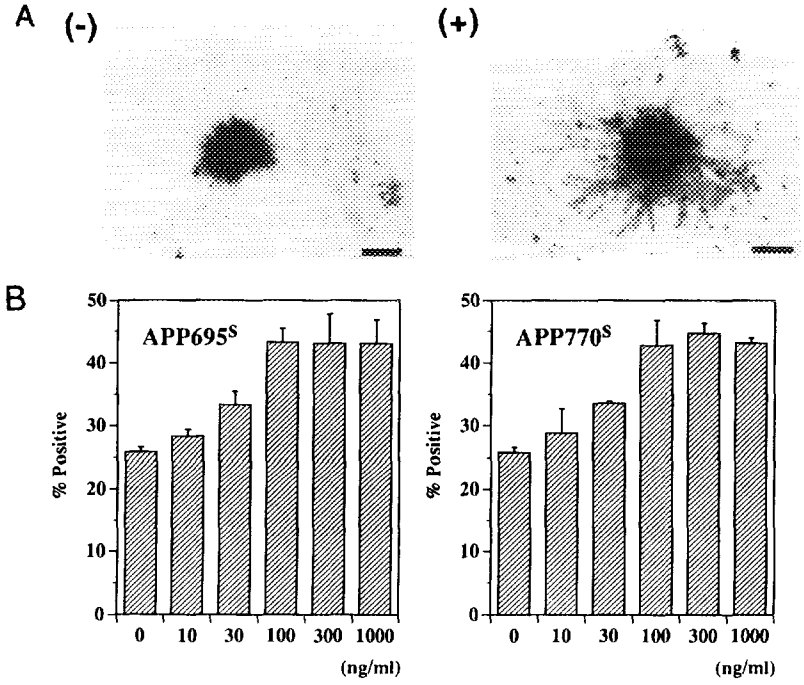

Fig. 2. Effect of APP^{S}s produced by COS-1 cells on neurite outgrowth in neocortical explants. **A**. Neurite outgrowth in explants with (+) and without (-) APP695^{S} (100 ng/ml). Bars indicate 100 μm. **B**. Semi-quantitative analysis of neurite promoting activity of APP^{S}s. Each column represents mean ± SD (bars).

signal peptide of APP is correctly recognized and processed in *P. pastoris*. Furthermore, rAPP^{S} produced by yeast had the same neurotrophic activity in neocortical explants as APP^{S} produced by COS-1 cells. These results indicated that rAPP^{S} has properties equivalent to those of native

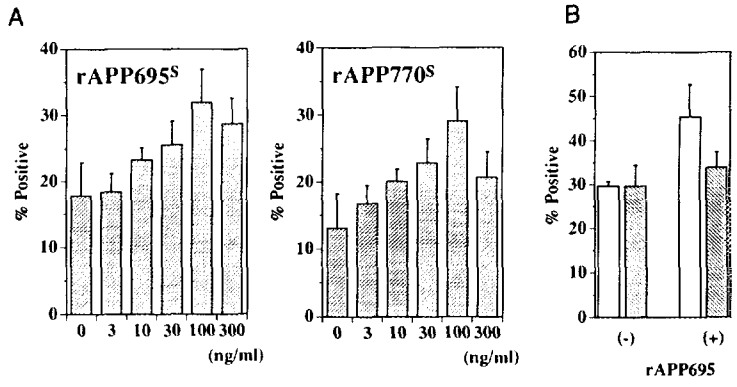

Fig. 3. Effect of rAPP^{S}s produced by yeast on neurite outgrowth in neocortical explants. **A**. Neurite-promoting activity of rAPP^{S}s in neocortical explants. Data are mean ± SD (bars) values. **B**. Inhibitory effect of antibody 22C11 on neurite outgrowth promoted by rAPP695^{S}. Antibody 22C11 (hatched bars) or normal mouse IgG (open bars) was added (500 ng/ml) at the time of rAPP695^{S} addition (100 ng/ml) and neurite outgrowth was determined after 2 days of culture.

APP^S. The analysis of APP and APP homolog function using yeast system has been reported (28-30). Wagner et al. (28) also expressed native and active KPI domains of APP in *P. pastoris*. Therefore, the expression system using *P. pastoris* should be useful in studying the biological functions of APP^S.

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