Recombinant human amyloid precursor-like protein 2 (APLP2) expressed in the yeast *Pichia pastoris* can stimulate neurite outgrowth

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Abstract The human amyloid precursor-like protein 2 (APLP2) is a member of the Alzheimer's disease amyloid precursor protein (APP) gene family. The human APLP2 ectodomain (sAPLP2) was expressed in the yeast *Pichia pastoris* and the recombinant sAPLP2 was purified from the culture medium in a single step by metal-chelating Sepharose chromatography. The neuritotrophic activity of APLP2 was compared to the APP isoforms sAPP695 and sAPP751 on chick sympathetic neurones. APLP2 had neurite outgrowth-promoting activity similar to that of the APP isoforms. This suggests that APP and APLP2 have a similar or related role and supports the idea of a redundancy in function between the APP-gene family proteins.

Key words: Amyloid precursor-like protein 2; Neuritogenesis

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. The pathology of AD is characterised by the presence of cortical and cerebrovascular amyloid deposits and intracellular neurofibrillary tangles [1]. Extracellular deposits of amyloid have been shown to consist predominantly of aggregates of a 39–43-residue polypeptide Aβ [2,3]. Aβ is derived by proteolytic cleavage from the larger amyloid precursor protein (APP) [4]. APP is part of a gene family, which contains the APP-like proteins (APLP) 1 and 2 [5–10]. At the amino acid level APLP2 has the greatest homology to APP (52% identical and 71% similar) compared with APLP1 which is 42% identical and 64% similar to APP. APLP2 shows conservation of all 12 cysteine residues in the amino-terminus and the putative zinc-, copper- and heparin-binding domains, the putative mitogenic RERMS domain, the Kunitz-protease inhibitor domain (KPI), and the cytoplasmic clathrin/talin-binding domain [7,8,10]. The region of weakest homology between APP and both APLP1 and APLP2 occurs near the transmembrane domain. In APP, this region includes the Aβ domain, but APLP1 and APLP2 both lack a region homologous to Aβ and are therefore considered to be non-amyloidogenic proteins.

APLP2 and APP share a similar pattern of expression in both human and mouse [7,10,11]. APLP2 is present in human brain and cerebrospinal fluid as well as in the supernatant fractions of primary and clonal cultures [11]. This suggests that APLP2 and APP may be processed via a similar mechanism that is capable of yielding secreted species that are C-terminally truncated. In the mouse, APLP2 is detected in all tissues at comparable levels [7], while APLP1 expression is restricted to the brain where it has been found to be expressed in neuronal post-synaptic densities [12]. Neural-specific localisation of APLP1 may imply a specialised function for this protein and thus explain its lower homology to APP and APLP2.

The function of APP remains essentially unknown. APP knockout (APP−/−) mice have been shown to carry a discrete, non-lethal phenotype [13]. In vitro studies have shown that primary hippocampal cultures from APP−/− mice have reduced viability and retarded neurite outgrowth [14] suggesting a role for APP in cell viability and neuritogenesis. In contrast, there was no difference in susceptibility to Aβ toxicity or oxidative stress between primary cortical and cerebellar neurones derived from APP−/− and normal mice [15]. An APLP2 knockout (APLP2−/−) mouse with a deleted exon 1 also appeared phenotypically normal [16]. However, when APLP2−/− and APP−/− mice were crossed to generate APP/APLP2 double knockout mice they showed a reduction in viability with only a 26% survival rate [16]. This indicates APP and APLP2 are required for post-natal survival. The high degree of homology between APP and APLP2 and the lack of a clear phenotype in the APP−/− and APLP2−/− mice or the APP−/− neurones under oxidative stress predicts a shared or overlapping function. However, there are currently few data to show that APLP and APP have similar activities.

In this study we investigated the ability of recombinant APLP2 to stimulate neurite outgrowth from chick sympathetic neurones. A number of studies have shown that APP has neuritogenic activity on a range of neuronal types [17–21]. We show that APLP2 has similar neurite outgrowth-promoting activity to that of APP.

2. Materials and methods

2.1. Antibodies

Mouse monoclonal antibody 22C11 reacts against an epitope present at the N-terminus of both APP and APLP2 [7,22] and was obtained from Boehringer Mannheim.

2.2. Pichia pastoris expression construct

Polymerase chain reaction (PCR) was used to amplify a 1998-bp DNA fragment corresponding to residues 28–693 of human APLP2 (with the KPI domain). Human APLP2 cDNA [10] was used as the PCR template (gift of Drs. Wilma Wasco and Rudolph Tanzi (MIT, Cambridge, MA, USA)). The following oligonucleotide primers were used at a final concentration of 1 pmol: CCG AAT TCT TGG CGC

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TGG CCG GCT ACA and CCC CTC TAG ATC AAC TGC TAC TCA GAC TGA AGT C. The pPIC9-sAPLP2 construct was confirmed by sequence analysis. The pPIC9-sAPLP2 plasmid DNA was linearised with *Sal*I and electroporated into electrocompetent *P. pastoris* GS115 or the protease mutant strains SMD1163, 1165, 1168 [23]. The sAPPα constructs have been previously described [23].

2.3. Expression and purification of sAPLP2 and sAPPα by immobilised copper-chelating chromatography

APLP2-expressing clones were identified by Western blotting culture supernatants with 22C11 as previously described [23]. The highest expressing clones for GS115 and the SMD strains were used for subsequent expression.

Large-scale expression and purification was performed in 200-ml cultures in a 1-l baffled flask. Cultures were induced for 24 h. The yeast supernatant fraction was harvested by centrifugation and phenylmethylsulphonyl fluoride (0.1 mg/ml) was added to inhibit proteolysis. The supernatant fraction was filtered through a 0.2-μm filter (Millipore). A 5-ml HiTrap Chelating Sepharose column (Amersham Pharmacia Biotech) was washed with 5 ml of double-distilled water and then loaded with 0.5 ml of 0.1 M $CuSO_4$. The column was washed again with 5 ml of double-distilled water to remove unbound salts and equilibrated with 5 ml of start buffer (0.02 M sodium phosphate, 0.5 M NaCl, pH 7.2). The filtered yeast supernatant was loaded onto the column using a peristaltic pump (Amersham Pharmacia Biotech) at a flow rate of 3 ml/min. The column was washed with 5 ml of start buffer to remove unbound material. Bound protein was eluted from the column by stripping the copper from the Sepharose matrix with 5 ml of start buffer containing 0.05 M EDTA. The eluted protein was desalted using a PD-10 Sephadex G-25 M Column (Amersham Pharmacia Biotech) into 50 mM Tris-HCl buffer, pH 8 or phosphate-buffered saline.

2.4. SDS-polyacrylamide gel electrophoresis, immunodetection and silver staining

Sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) and Western blotting were performed as described previously [23]. SDS-polyacrylamide gels were silver-stained using a silver staining kit (Bio-Rad).

2.5. Neurite outgrowth assay

Neurite outgrowth assays were performed as previously described [24]. Briefly, 24-well cell culture plates were coated with 0.1 mg/ml polylysine for 1 h, wells were washed with sterile distilled water and then coated with proteins of interest. Proteins were used at a concentration of 10 μg/ml. Each well was rinsed with phosphate-buffered saline following a 2 h incubation, and 1×10^4 cells isolated from 12-day-old chick sympathetic ganglia were added. Cultures were grown for 24 h, then fixed with 2.5% (v/v) glutaraldehyde in phosphate-buffered saline. Computer-assisted image analysis was used to determine neurite length in nine separate fields within the centre of each well. Four wells per treatment group were examined and at least 120 cells were counted for each treatment group. Differences between groups were analysed by a two-tailed Student's *t*-test.

3. Results

3.1. Expression of sAPLP2 in P. pastoris

The APLP2 ectodomain (sAPLP2) from amino acids 23–693 [10] was expressed in *P. pastoris* using the vector pIC9 in frame with the *Saccharomyces cerevisiae* prepro α-factor signal peptide. The pPIC9-sAPLP2 plasmid was transfected into *P. pastoris* strain GS115 and the protease mutant strains SMD1163 (pep4), SMD1165 (pep4, prb1) and SMD1168 (prb1). The pep4 and prb1 genes correspond to the vacuolar proteases proteinase A and proteinase B respectively. These protease mutant strains may reduce proteolysis of recombinant proteins and hence improve protein expression [25–27].

The culture medium from the four different sAPLP2-*P. pastoris* strains were screened by SDS-PAGE and Western blotting with the anti-APP/APLP2 monoclonal antibody 22C11. A major immunoreactive band migrating at 100–110 kDa corresponding to the expected size of sAPLP2 was detected, as well as a number of minor breakdown products (Fig. 1). No immunoreactive bands were seen in supernatant fractions from cells expressing the pIC9 vector alone. There were no significant differences in the breakdown pattern between the different strains. Differences in expression levels were observed between clones and are consistent with our previous work expressing sAPP in *P. pastoris* [23]. The clones yielding the highest immunoreactivity were selected for subsequent analysis.

3.2. Purification of sAPLP2 by copper-chelating chromatography

The ectodomains of both APP and APLP2 have been shown to bind to copper [28]. This copper-binding property was utilised in a single-step purification protocol using immobilised copper-chelating chromatography [29]. The supernatant fractions from the GS115 and the SMD clones were loaded directly on to a copper-charged chelating Sepharose column and non-specific proteins washed away. Bound sAPLP2 was eluted from the column with buffer containing 0.05 M EDTA. A Western blot of a typical purification profile of sAPLP2 from GS115 is shown (Fig. 1). There was a large enrichment of the recombinant sAPLP2 as seen by the increase in immunoreactivity in the eluted fractions (Fig. 1, lane 9). The final purified sAPLP2 from the four different *P. pastoris* strains following buffer exchange and concentration was analysed by silver staining and Western blotting (Fig. 1b). The sAPLP2 doublet was the predominant protein seen by silver staining (Fig. 1b) and all silver-staining bands were recognised by the 22C11 antibody (Fig. 1b). The breakdown products were a minor component of the purified protein. The

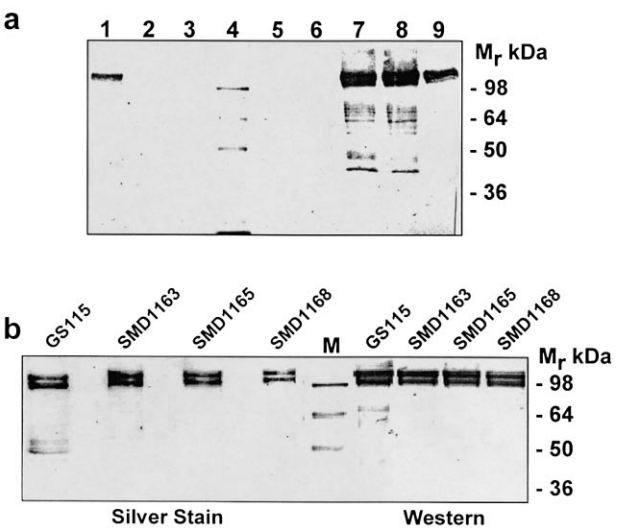

Fig. 1. Purification of sAPLP2 expressed in GS115 on copper-chelating Sepharose chromatography. a: 5-μl aliquots of the starting material (lane 1), flow-through (lane 2), wash (lane 3), 1-ml eluate fractions (lanes 4–9) and molecular weight standards (lane 4). The gel was Western-blotted and probed with 22C11. b: SDS-PAGE analysis of the purified sAPLP2 following buffer exchange and concentration. Silver stain (left-hand panel) and Western blot with 22C11 (right-hand panel) of sAPLP2 expressed in GS115, SMD1163, SMD1165 and SMD1168. Positions of the molecular weight standards (M) are indicated.

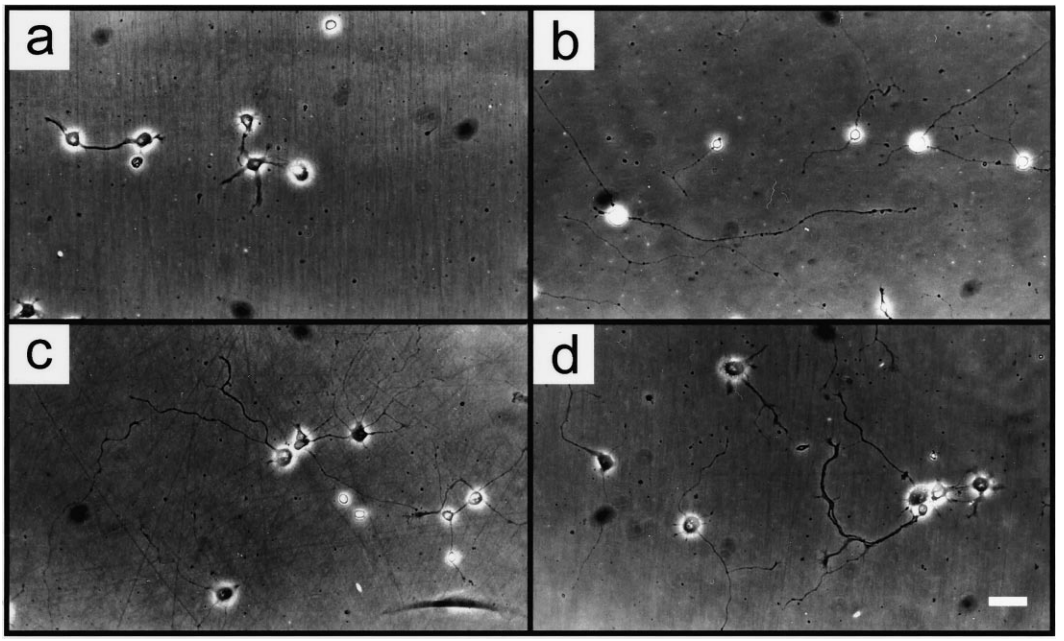

Fig. 2. Phase-contrast micrographs of 12-day embryonic chick sympathetic neurones cultured on substrate-bound (a) polylysine, (b) polylysine and laminin, (c) polylysine and sAPLP2, (d) polylysine and sAPP751α. Bar 20 μm.

nature of the doublet is not clear, but it may represent different glycosylation isoforms. The yield of purified sAPLP2 was approximately 40 mg/l for all the strains. No proteins were purified from the supernatant of a pIC9 vector-alone control culture as determined by silver staining and Western blotting (data not shown). The sAPP695α and sAPP751α proteins (the ectodomains of the APP isoforms 695 and 751 ending at the α-secretase site [23]) were also purified by copper-chelating Sepharose chromatography.

3.3. Neurite outgrowth assay

A number of activities have been proposed for APP including modulation of neurite outgrowth [30]. To determine if this activity is conserved in APLP2, we tested the neuritogenic activity of the recombinant sAPLP2 and compared this to recombinant sAPPα. Substrate-bound sAPLP2, sAPP695α and sAPP751α all stimulated neurite outgrowth from chick sympathetic neurones compared to cells cultured on polylysine alone (Fig. 2). All three proteins demonstrated an increase in the number of neurites per neurone compared with polylysine alone, but less than the laminin-stimulated cultures (Fig. 3a). There was no difference in activity between sAPLP2 and sAPPα. Analysis of neurite length showed that the outgrowth stimulated by sAPLP2 was significantly different from sAPPα. Neurites which were exposed to sAPLP2 generated neurites which were 70% and 47% longer than sAPP695α and sAPP751α respectively (Fig. 3b).

4. Discussion

In this study we used the *P. pastoris* yeast expression system to overexpress the ectodomain of human APLP2. The single-step purification protocol which utilised the endogenous amino-terminal metal-binding sites [28,31] simplified the previous multi-step purification schemes [23,32,33] with no obvious reduction in purity. Expression of APLP2 in the SMD vacuolar protease mutant strains did not improve the expression profile. A similar result was obtained for sAPPα expressed in these strains [23] and indicates the proteolysis of APP and APLP2 in *P. pastoris* was not due to these vacuolar proteases.

This study shows that like sAPP, sAPLP2 is able to stimulate neurite outgrowth, both in number of neurones with neurites and in neurite length. We conclude that the neurite-promoting activity is shared by other members of the APP family. The neuritotrophic activity of APP can be modulated by heparan sulphate proteoglycans with different proteoglycans having contrasting effects [24,34]. While APLP2 can bind to heparin in a similar manner to APP [35], it remains to be determined if it is also modulated by heparan sulphate proteoglycans and how this relates to APP. The retarded neurite development of APP−/− hippocampal neurones [14] and the finding that sAPLP2 has greater neurite length-promoting activity than sAPPα suggests APP and APLP2 have distinct roles in controlling neurite development. Therefore, studying the activities of the APLP molecules and how they relate to

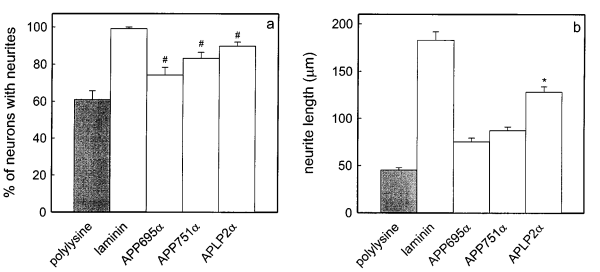

Fig. 3. Effect of substrate-bound sAPLP2, sAPP695α and sAPP751α on chick sympathetic neurones. a: Number of neurones with neurites. Values are means ± S.E.M. Significantly different from polylysine ($P < 0.05$) as assessed by a Student's *t*-test. b: Neurite length. Values are means ± S.E.M. *Significantly different from sAPP695α and sAPP751α ($P < 0.05$) as assessed by a Student's *t*-test.

APP is important in understanding the function of the APP family in AD.

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