A novel protein, amyloid precursor-like protein 2, is present in human brain, cerebrospinal fluid and conditioned media

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A monoclonal antibody, 3B11, was raised to a novel protein, amyloid precursor-like protein 2, which did not recognize amyloid precursor protein. Multiple bands were detected in human brain fractions and cell lysate by Western blotting, indicating the presence of isoforms. 3B11 immunoreactivity was also detected in cerebrospinal fluid and conditioned medium, indicating that the protein is secreted. Immunocytochemistry revealed 3B11 immunoreactivity in sections of human brain.

INTRODUCTION

The identification and cloning of human and mouse cDNAs with high degrees of similarity to amyloid precursor protein (APP), the protein from which the β-amyloid polypeptide found in senile plaques in Alzheimer’s disease brain is derived, indicated that this protein is a member of a larger gene family [1–3]. The 653-amino acid amyloid precursor protein-like protein (APLP1) is 42% identical with 64% similar to that of the 695-amino acid APP (APP-695) [1], and APLP2 (also termed APPH1) is predicted to be a 763-amino acid protein with an amino acid sequence 52% identical with 71% similar to APP-751 [2]. APLP2 has an amino acid sequence 43% identical with 63% similar to APLP1. Virtually all of the identified domains and motifs that characterize APP are present in APLP1 and APLP2 including a hydrophobic membrane-spanning domain, N-glycosylation sites and a Kunitz protease inhibitory (KPI) domain. It has also been reported that APLP2 enters a secretory pathway and that soluble APLP2 derivatives are secreted by transfected cells of neuronal and non-neuronal origin [4]. These similarities between APLP2 and APP imply that APLP2 may have many of the as yet not fully characterized functions of APP. However, the lack of the β-amyloid domain in APLP1 and APLP2 indicates that these proteins cannot act as substrates for the deposition of β-amyloid in Alzheimer’s disease.

High levels of APLP2 mRNA expression have been reported in mouse brain and peripheral tissue [4] as well as human brain, with highest expression in the temporal association cortex [2]. The relative expression of APLP2 mRNA to that of APP has been estimated as 0.55 [4]. The similarity of these proteins raises the problem of distinguishing between them with the use of antibodies; indeed, it was recently demonstrated that widely used immunological reagents generated against APP immunogens fail to discriminate APP from APLP2 [4]. For this reason we raised a monoclonal antibody to a peptide sequence in APLP2 not present in APP and used this to verify the presence of this protein in human brain and cerebrospinal fluid. As the anatomical localization of APLP2 in brain is not known, we investigated this using immunocytochemistry. We have also examined a rat clonal cell line and rat primary neuronal cultures under control and experimental conditions for factors that may affect the metabolism of APLP2.

MATERIALS AND METHODS

Human samples, cell culture and transfection

High-density membrane and supernatant fractions of human frontal cortex were prepared from 12 brains (as described by Arai et al. [5] and modified by Webster et al. [6]) especially collected to minimize post-mortem autolysis (eight cases of Alzheimer’s disease, two of inconclusive diagnosis, one of vascular dementia and one of multisystem degeneration [6]). Resulting preparations were resuspended or diluted (supernatant fraction) with sample buffer [50 mM Tris/HCl, pH 6.8, 100 mM dithiothreitol, 10 mM EGTA, 2 mM PMSF, 4% (w/v) SDS, 0.1% (w/v) Bromophenol Blue, 10% (v/v) glycerol, 1 μg/ml pepstatin and 1 μg/ml leupeptin] and equal volumes pooled.

Lumbar cerebrospinal fluid (LCSF) was obtained from two patients, one with Alzheimer’s disease and one with frontal lobe dementia [7], and ventricular CSF (VCSF) from two patients with bipolar depression undergoing psychosurgery. All were prepared for electrophoresis as described [8].

PC12 cells (ATCC, CRL 1721; Porton Down, Wilts., U.K.) were grown [9] on collagen-coated plates (35 mm diameter) in 3 ml of serum-free Dulbecco’s modified Eagle’s medium (DMEM)/nutrient mix F12 (1:1) without L-glutamine, containing 5 ng/ml sodium selenite, 5 μg/ml insulin and 5 μg/ml transferrin, at 35°C under an atmosphere of 5% (v/v) CO2/95% O2 at an initial seeding density of 7 x 10⁴ cells. After 24 h, 400 ng/ml nerve growth factor (NGF; murine 7S; Promega, Southampton, Hants., U.K.) was added with daily changes of medium (except where stated). Cells were separated from medium by centrifugation (3500 g for 7 min), resuspended in sample
buffer, sonicated and centrifuged (12000 g for 15 min) before electrophoresis. Concentrated medium (6 x; Centricron 10 tubes; Amicon, Stonehouse, Gloucestershire, U.K.) was mixed with concentrated sample buffer to allow for dilution. Oxidative stress was produced as previously described [9] by treatment of PC12 cells for 3 h with 5 mM deoxyglucose and 0.2 µg/ml oligomycin in glucose- and pyruvate-free medium.

Cultures of neurons from the cerebellum and cerebral cortex were prepared from 7-day-old and embryonic (E17) rats respectively and grown by the method of Dutton and Tear [10]. After 7 or 12 days respectively in vitro, cells were exposed to 500 µM glutamate for 30 min and the cells placed in fresh medium (1.5 ml) for a further 24 h. Medium from both culture types was concentrated (2.5 x; Centricron 10 tubes) and cell supernatant, obtained by freezing and thawing cells in distilled water followed by centrifugation (11000 g; 10 min), was mixed with concentrated sample buffer to allow for dilution. Neuroblastoma cells (SH-SY5Y) were grown as previously described [11] and cell lysate prepared for electrophoresis as above.

H4 human neuroglioma cells were stably transfected with mouse APLP1 [1] or human APLP2-763 [2] cloned into a mammalian expression vector containing the CM promoter sequence (pRcCMV; Invitrogen). Cells were maintained in DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, 1% penicillin/streptomycin and, where appropriate, G418 selection (150 µg/ml). To harvest protein, cells were washed in Hanks buffered saline (Gibco/BRL) and processed for electrophoresis (as above). Cells transfected with either APLP1 or APLP2 overexpress the expected mRNA (analysed by Northern blot [12]). In addition, using the appropriate polyclonal antibody (D2-1 [13]), APLP2 was overexpressed in cell lysate (W. Wasco, K. M. Felsenstein and R. E. Tanzi, unpublished work).

Sequences that encode residues 562–681 of mouse APLP2-751 [4] were generated by PCR using a sense primer, 5′-CCGGGAT-CCGGATGAGCTCCCTTACGAAAC-3′ and an antisense primer 5′-CCGGGATTCTTTGTCGCTCAACTGAAAATCC-3′, and mouse APLP2 cDNA [4] as the template. The PCR product was digested with BamHI and EcoRI, gel-purified and subsequently ligated to plasmid pGEX-2T [14] previously digested with BamHI and EcoRI. The resulting plasmid, pGEX-ΔAPLP2, encodes 120 amino acids that are immediately N-terminus to the transmembrane domain of APLP2-751, as a C-terminal fusion protein with glutathione S-transferase (GST). The GST fusion protein was expressed and purified as described [14]. COS-1 cells were transiently transfected with mouse APLP2-751 cDNA, and lysates were prepared as described [13].

Purified secreted APP-695 from transfected 293 cells [15] and baculovirus-expressed APP-751 [16] were gifts from Dr. D. Schenk (Athena Neurosciences, South San Francisco, CA, U.S.A.). Cell lysate from Chinese hamster ovary cells transfected with mouse APLP2 was as previously described [4] and was prepared for electrophoresis (as above). Monoclonal antibody 22C11 [17] was obtained commercially (Boehringer, Lewis, Sussex, U.K.).

Production of a monoclonal antibody to APLP2

From a 98-amino acid sequence of the ectodomain of APLP2 (amino acids 566–664; Figure 1), overlapping tetradecapeptide sequences were synthesized using a previously published method with the addition of a terminal cysteine residue to facilitate coupling to a heterobifunctional agent [18]. Peptides were evaluated for purity by reverse-phase HPLC and plasma-desorption MS (Bio-Ion 20) and subsequently coupled to tuberculin [18]. Female Balb/c mice were primed with a subcutaneous injection of human BCG vaccine [19], followed by three booster immunizations (subcutaneously) of peptide–tuberculin conjugate in Freund’s incomplete adjuvant at 3-week intervals. Groups of three mice each received injections of two overlapping peptide conjugates, e.g., B4 and C4 (Figure 1). After 10 days the mice were bled and serum was screened by electrophoresis and Western blotting to identify high responders. The selected mice [previously injected with peptides B4/C4 (Figure 1)] were given intraperitoneal and intravenous boosts of peptide–tuberculin conjugate (50 µl) on each of the 2 days before spleen removal. The spleens were removed aseptically, and the spleenocytes fused with Sp2 myeloma cells as previously described [20]. The resulting hybridomas were suspended and diluted, then plated out in 96-well tissue culture plates. After 10–14 days, supernatant was taken from each well and tested for specificity by ELISA using plates coated with 1 µg/ml either B4 or C4 peptide. Those hybridomas giving strong positive results were expanded and restetted by ELISA and on Western blots of human brain. The hybridoma clone designated 3B11 was subcloned to confirm monoclonality. The immunoglobulin class of the selected hybridoma was determined to be IgG1, (Immuno Type Kit; Sigma, Poole, Dorset, U.K.).

Electrophoresis and Western blotting

Samples (100 µg of protein) of pooled human frontal cortex, supernatant and high-density membrane fractions [6], LCSF as well as VCSF (equivalent to 14 and 35 µl original volume respectively), baculovirus-expressed APP-751 and purified secreted APP-695 (each 1 µg of protein), PC12 cell lysate (100 µg of protein) and PC12 conditioned medium (corresponding to 0.26 ml original volume) and cortical cell supernatant (corresponding to approx. 2 x 10⁶ cells) and buffer from incubation of primary cultures (corresponding to 77 µl original volume) were separated on SDS/7.5% polyacrylamide gels [6,21]. After electrophoretic transfer to nitrocellulose membranes (Hybond-ECL; Amersham International, Amersham, Bucks., U.K.), residual protein-binding sites were blocked by incubation with 4% semi-fat dried milk and 0.05% Tween 20 in PBS for 1 h at 37 °C. Membranes were subsequently incubated for 1 h at 37 °C with 2.5 ml of undiluted mouse serum or hybridoma culture supernatant diluted 1:20 with blocking solution. After being washed with frequent changes of 0.05% Tween 20 in PBS for 25 min, the nitrocellulose membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG at a dilution of 1:1000 in blocking solution for 1 h at room temperature. After being extensively washed, immunoreactive proteins were
visualized by autoradiography using a chemiluminescence detection system (ECL, Amersham International).

**Immunocytochemistry**

Immunostaining was carried out on human post-mortem brain material from four patients with Alzheimer’s disease that had been briefly fixed (1 h) in 10% formalin and embedded in paraffin wax. Microwave antigen retrieval was performed using the method of Sherriff et al. [22]. The immunocytochemical procedure was also as described in the above study, except that fetal calf serum (1:20) was used as the blocking serum, the diaminobenzidine reaction product was not intensified, and sections were counterstained with haematoxylin. The 3B11 antibody was used at a dilution of 1:5. Serial sections were stained for APP (LN27; Zymed, South San Francisco, CA, U.S.A.; 1:200) and β-amyloid (10D5; Athena; 1:200). For β-amyloid detection, the sections were treated with 90% formic acid for 5 min instead of microwaving. The primary antibody was omitted in control sections.

**RESULTS AND DISCUSSION**

**Monoclonal antibody 3B11 detects a human brain protein with the characteristics of APLP2**

Initially, mice injected with peptide B4/C4 (Figure 1) gave the best response made with those injected with any of the other peptides and therefore these mice were chosen for subsequent antibody production. Serum from mice injected with peptide B4/C4 reacted strongly with two high-molecular-mass protein bands on Western blots of human brain supernatant and pellet fractions (Figure 2a, lane 1). Serum from unjected mice failed to react with any protein bands (Figure 2a, lane 2). Resulting hybridoma supernatants from B4/C4 mice identified the similar bands with less non-specific binding (Figure 2a, lane 3). The expanded clone, 3B11, identified two major protein bands in human brain pellet (Figure 2a, lane 4) and supernatant fractions (Figure 2b, lane 1) with approximate molecular masses between 100 and 140 kDa. The same two bands were identified with polyclonal antibody D2-1 (Figure 2e, lane 1). An example of a non-responding clone is also shown (Figure 2a, lane 5). Specific 3B11 immunoreactivity was abolished if the antibody was preincubated with peptide B4 (10 μg/ml; Figure 2b, compare lanes 1 and 2) but not peptide C4 (not shown). The GST fusion protein containing the ectodomain of APLP2 was also detected by 3B11 (Figure 2b, lane 3). The antibody did not react with purified secreted APP-695, baculovirus-expressed APP-751 or COS-1 cells transfected with APP-770 compared with those transfected with the empty vector (Figure 2b, lanes 4, 5, 9 and 10) in contrast with the monoclonal antibody 22C11 (Figure 2b, lane 6). The monoclonal antibody 3B11 also identified the increased expression of APLP2 in H4 cells transfected with human APLP2 cDNA (Figure 2b, compare lanes 7 and 8), and in COS-1 cells transfected with mouse APLP2-751 cDNA (Figure 2b, compare lanes 9 and 11). The polyclonal antibody D2-1 detected the same band in COS-1-transfected cells (Figure 2e, compare lanes 2 and 4). Increased expression of APLP1 in H4 cells transfected with the mouse APLP1 gene was not detected with 3B11 (not shown). The presence of high-molecular-mass protein bands identified by 3B11 is likely to reflect the existence of APLP2 isoforms, possibly derived by alternative splicing, analogous to APP-KPI and APP-695. Indeed, mRNA [4] and cDNA [2] encoding APLP2

![Figure 2](image-url)

*Figure 2* Western blotting of human brain, cerebrospinal fluid and cultured cells with various antibodies including monoclonal antibody 3B11

(a) High-density membrane fractions from human brain (lanes 1–5) blotted with serum (lanes 1 and 2) or hybridoma supernatant (lanes 3–5) from mice inoculated with B4/C4 peptide (lanes 1, 3 and 4), uninoculated mice (lane 2), selected positive clone (lane 3), expanded positive clone (3B11) lane 4) and negative clone (lane 5). (b) Western blots using monoclonal antibody 3B11 (except lane 6) of human brain supernatant (lanes 1 and 2). GST fusion protein of the 113-amino acid ectodomain of APLP2 (lane 3), baculovirus-expressed APP-751 (lane 4) and purified secreted APP-695 (lane 5). COS-1 cells transfected with APP-770 (lane 10), H4 (lane 7), COS-1 cells (lane 11) transfected with APLP2 cDNA and H4 (lane 8) and COS-1 cells (lane 9) transfected with vector. Baculovirus-expressed APP-751 was also blotted with monoclonal antibody 22C11 (lane 6). (e) Western blots using monoclonal antibody 3B11 of high-density-membrane fractions from human (lane 1) and rat (lane 3) brain, human brain supernatant fraction (lanes 2) and VCSF (lanes 4 and 5) and LFS (lanes 6 and 7). (d) Western blots using monoclonal antibody 3B11 of PC12 cell lysate (control, lane 1; subjected to oxidative stress, lane 2), conditioned medium (lanes 3–12) from 3-day incubation without NGF (lane 3), with NGF (lanes 4–12) and in addition with bradykinin (lanes 10 and 12) for 3 h (lanes 4, 9 and 10), 6 h (lanes 5, 11 and 12), 12 h (lane 6), 16 h (lane 7) and 24 h (lane 8), medium from cerebellar granule (lanes 13 and 14) and cortical (lanes 15 and 17) cell cultures without glutamate (lanes 13 and 15), with glutamate (lanes 14 and 17) and cortical cell supernatant without glutamate (lane 16) and with glutamate (lane 18). (e) Western blots using polyclonal antibody D2-1 of human brain supernatant fraction (lane 1), COS-1 cells transfected with vector (lane 2), with APP-770 (lane 3) and APLP2 (lane 4).
with and without the KPI domain have been detected. These multiple bands may also reflect post-translational modification, as APLP2 has been shown to be a chondroitin sulphate proteoglycan [13]. It is not possible at present to confirm that the highest-molecular-mass band contains APLP2 with the KPI domain, as this sequence is very similar to that in APP-KPI and existing antibodies are likely to cross-react. It may be possible in the future to make specific antibodies to test this.

Detection of APLP2 in subfractions of brain and cerebrospinal fluid

Two high-molecular-mass bands with different mobilities were detected in Immunoblots with 3B11 in pellet and supernatant fractions of human brain and rat brain supernatant, for comparison (Figure 2c, lanes 1, 2 and 3). The pellet fraction also contained lower-molecular-mass species which may be post-mortem degradation products, although it is emphasized that the tissue was specially collected to minimize such effects. APLP2 was also present in both LCSF and VCSF (Figure 2c, lanes 4–7) as two major bands (about 116 and 130 kDa) with an additional lower-molecular-mass band. In one example of LCSF, the highest-molecular-mass band resolved into a doublet (Figure 2c, lane 6).

Immunocytochemical localization of APLP2 in brain

Strong labelling by 3B11 was found in dystrophic neurites surrounding neuritic plaques in the cortex of patients with Alzheimer's disease (Figures 3a and 3b). Serial sections showed that these same neurites were labelled with APP (Figures 3e and 3f) and that they surrounded β-amyloid deposits (Figures 3c and 3d). Pale staining of pyramidal neurons was also seen and, in some sections, astrocytes and possibly microglia also showed some labelling (not shown). The pattern of APLP2 labelling therefore resembles that of APP. However, more detailed studies using 3B11 in normal brain and brain from patient's with Alzheimer's disease are needed to verify these findings.

Detection of APLP2 in a rat clonal cell line and primary cultures

APLP2 was detected in PC12 cell lysate (Figure 2d, lane 1) as three major high-molecular-mass bands but with additional lower-molecular-mass bands, as well as in the cell supernatant of primary cultures of cerebellar granule cells (not shown) and cortical neurons (Figure 2d, lane 16). Trace amounts of APLP2 immunoreactivity were also present in SH-SY5Y neuroblastoma cell lysate (not shown). Oxidative stress reduced cell lysate APLP2 compared with control (Figure 2d, compare lanes 1 and 2).
2). This was also a feature of 22C11 immunoreactivity (Figure 4) (see also ref. [9]).

A single high-molecular-mass immunoreactive band (about 120 kDa) was observed in conditioned medium from PC12 cells (Figure 2d, lane 3) and the primary cultures (Figure 2d, lanes 13 and 15). The release of APLP2 into conditioned medium of PC12 cells was stimulated by NGF (400 ng/ml for 3 days; Figure 2d, compare lanes 3 and 8) in a time-dependent manner (Figure 2d, lanes 4–8) and bradykinin (500 nM for 3 or 6 h; Figure 2d, compare lanes 9 and 10, and 11 and 12). However, brief exposure of cerebellar granule and cortical cultures to glutamate (500 μM for 30 min) led to a reduction in APLP2 immunoreactivity in conditioned medium (24 h after treatment; Figure 2d, compare lanes 13 and 14, and 15 and 17). This was accompanied by reductions in APLP2 immunoreactivity in the cell supernatant of cortical neurons (Figure 2d, compare lanes 16 and 18) and cerebellar granule cells (not shown).

The identification of APLP2 in conditioned medium and cerebrospinal fluid suggests that APLP2 may be processed both in vitro and in vivo to produce a soluble secreted truncated form of the protein. The control of this secretory pathway appears to be qualitatively similar to that for APP (Figure 2; see also refs. [9,23]) as both NGF and bradykinin increase the appearance of APLP2 in conditioned medium. However, whereas 22C11 immunoreactivity was easily detectable in medium after 1 h, the same was not true for APLP2. Thus 3 days incubation of PC12 cells (absence of NGF; Figure 2d, lane 3) or 3 h (presence of NGF, Figure 2d, lane 4) was required for detection of APLP2 in conditioned medium. Similarly, 3 h treatment of PC12 cells with bradykinin was required to see clearly an effect on APLP2 secretion whereas only 1 h was required for 22C11 immunoreactivity (Figure 4, see also ref. [9]). This interpretation is based on the assumption that the antibody 22C11 is detecting mainly APP in conditioned medium; an alternative interpretation is that 3B11 is not as sensitive as 22C11. Resolution of this issue awaits separate quantitative data for the two proteins.

Mismetabolism of APP has been implicated in the pathogenesis of Alzheimer’s disease either as a result of deposition of β-amyloid or loss of function of secreted APP. Cholinomimetics, currently in clinical trials as antidepressant agents, and increased neuronal activity favour non-amyloidogenic APP release in vitro [24] and in vivo [25]. The present study suggests that control of APLP2 release may be similar to that of APP and implies that cholinomimetic treatment of patients with Alzheimer’s disease may have a serendipitous effect on the metabolism of both proteins by restoring the secretion of APP and APLP2 and hence their function.

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