

The amyloid precursor protein of Alzheimer disease is expressed as a 130 kDa polypeptide in various cultured cell types

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The vascular and parenchymal amyloid deposits in Alzheimer disease (AD), normal aging and Down syndrome are mainly composed of a 4 kDa polypeptide (A β), which derives from a larger precursor protein (APP). There is evidence that APP is a transmembrane glycoprotein present in most tissues, but the characteristics of APP in intact cells are not yet known. In order to investigate this issue, we examined the immunoreactivity of fibroblasts of human and nonhuman cell lines with antisera raised to synthetic peptides corresponding to A β and to two other domains of the APP. All three antisera recognized a 130 kDa polypeptide (APP-130) in immunoblots from all cell lines. In fibroblasts, an additional polypeptide of 228 kDa (APP-228) was recognized by the antiserum to A β . In immunoblots of two dimensional gels, APP-130 showed a *pI* of 6.2, while APP-228 failed to focus in the pH range of 4.7–7.0. Sequential extractions of cells with buffer and with Triton X-100 indicate that APP-130 is extractable with nonionic detergents at high ionic strength, whereas 228 kDa APP is a cytosolic component. Immunofluorescence staining is consistent with an intracellular perinuclear and plasma membrane localization. It is concluded that APP-130 and APP-228 are two forms of the APP which result from extensive posttranslational modifications of a smaller original gene product. It is likely that APP undergoes similar posttranslational modifications in different cell types.

Amyloid; Amyloid precursor protein; Alzheimer's disease; (Cell culture)

1. INTRODUCTION

Deposition of amyloid in brain, associated with the formation of senile plaques and amyloid angiopathy, is a major pathological event in Alzheimer disease (AD), Down syndrome (DS) and, to a lesser extent, in normal aging [1]. This amyloid is distinct from that of other amyloidoses [2–6], and derives from a larger precursor protein for which at least three species of mRNA are expressed as a result of alternative splicing [7–13]. Based on the cDNA-derived primary sequence, a putative structure has been proposed which includes an N-terminal signal sequence, three ex-

tracellular domains, a transmembrane region and a cytoplasmic C-terminal domain of 47 residues [7,14]. On the basis of this structure, it has been suggested that APP is a transmembrane glycosylated protein with features typical of a cell surface receptor or an adhesion molecule [7,14,15]. The major component of AD amyloid is a 42 residue polypeptide corresponding to amino acids 597–639 of the deduced APP sequence; its putative location comprises both transmembrane and extracellular domains [7]. Evidence has also been obtained for a precursor-product relationship between APP and amyloid, as APP domains analogous to A β , along with the transmembrane and cytoplasmic domains, have been shown to aggregate and form amyloid fibrils under appropriate conditions [14].

Northern blot analysis indicates that APP is present in a variety of tissues [8,9,11,13]. However,

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scarce and controversial information is available concerning size and subcellular location of APP in intact tissues or cells [16–18]. Some of the controversy may be due to attempts to identify APP in postmortem tissue in which proteins may have undergone autolysis. Thus we have tried to characterize APP in cultured cells. A 130 kDa polypeptide (APP-130) is consistently recognized in human cell lines by three antisera raised to synthetic polypeptides homologous to sequences spanning residues 95 to 658 of the putative APP. In addition to APP-130, one antiserum also recognizes a 228 kDa polypeptide (APP-228) in human fibroblasts. Sequential extractions and immunofluorescence experiments indicate that these two polypeptides reside in different cell compartments including nuclear, perinuclear and plasma membranes and the cytosol. This study has been presented in part [19].

2. MATERIALS AND METHODS

2.1. Cell cultures

Primary cultures of human fibroblasts were obtained from NIGMS Human Genetic Cell Repository. Cultures were kept in a 7% CO₂ humidified incubator and fed with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. K-562 cells, a continuous chronic myelogenous leukemia cell line, and U937 cells, a histiocytic lymphocytoma cell line, were obtained from ATCC and cultured as above in RPMI 1640 medium containing 10% fetal bovine serum. Cos I cells, a monkey kidney cell line was kindly provided by Dr F.M. Rottman.

2.2. Extraction of cells

All operations were done at 0–4°C. Confluent cultures of fibroblasts and Cos I cells were washed free of medium with PBS. Cells were scraped and homogenized in a ground glass homogenizer in 10 mM phosphate buffer, pH 7.0, 2.5 mM PMSF (buffer A). K-562 cells were pelleted at 1000 × g, washed in PBS and homogenized in buffer A. After centrifugation for 60 min at 100000 × g, the cytosolic fraction was obtained and the pellet was homogenized in buffer A containing 1% Triton X-100. After centrifugation at 20000 × g for 20 min, the Triton soluble fraction was removed and the pellet homogenized in buffer A containing 1% Triton X-100 and 0.6 M KCl. After centrifugation as above, the high ionic strength fraction was removed and the pellet dissolved in electrophoresis sample buffer.

2.3. Antibodies

Antisera were raised in rabbits to synthetic peptides corresponding to amino acid sequences 638–658 (C5), 95–110 (N77) and 597–638 (A4) deduced from the APP cDNA [7]. Peptide C5 was coupled to KLH and N77 to thyroglobulin, while A4 was injected uncoupled. Absorptions of the antisera

(diluted 1:20) with the corresponding peptides (100 µg peptide/ml) were done by gentle mixing overnight at 4°C followed by a brief centrifugation.

2.4. Gel electrophoresis and immunoblotting

Polypeptides were resolved in 8% polyacrylamide mini gels. Two-dimensional gel electrophoresis was carried out as described [20], using a pH range of 4.5–7.0 for the first dimension and a 8% polyacrylamide slab gel in the second. Polypeptides were electrotransferred to nitrocellulose [21] and the membranes blocked with 10% non-fat milk at 40°C for 1 h. Incubations with primary antisera were done overnight at 4°C and with peroxidase-labeled goat anti-rabbit for 1 h at room temperature. Diaminobenzidine was used as chromogen.

2.5. Immunofluorescence

Fibroblasts grown on coverslips and cytopins of K-562 cells were fixed in 3% formaldehyde for 20 min, followed by 7 min in acetone at –20°C, or treated only with acetone. Incubations with primary and with fluorescein-labeled antibodies were for 1 h at 37°C. Cells were examined and photographed in a Leitz Dialux microscope equipped with epifluorescence illumination. Photographic exposures of preparations stained with absorbed antisera were of similar length to the automatic exposure required for the nonabsorbed antisera.

3. RESULTS AND DISCUSSION

In immunoblots of total homogenates of human fibroblasts, human myelogenous leukemia (K562), human histiocytic lymphocytoma cell line (U937) and monkey kidney cells (Cos I), all three antisera to the 597–638 (A4), 95–110 (N77) and 638–658 (C5) APP amino acid regions, recognize a polypeptide showing a mobility of 130 kDa in SDS-PAGE (fig.1). The antiserum to the A4 region recognized also a polypeptide migrating at 228 kDa in fibroblasts and a set of less distinct bands of comparable size in K562 cells (fig.1). Absorption of the antisera with the synthetic peptides used as antigen, but not with synthetic peptides to other regions of APP, abolished the reaction (fig.1).

In immunoblots of fibroblasts extracts separated by 2D-PAGE, APP-130 showed a *pI* of 6.2, while the APP-228 failed to focus in the pH range of 4.7–7.0 (fig.2). Thus, these data show that three antisera to different domains of APP specifically recognize a polypeptide of 130 kDa, indicating that this polypeptide is a form of APP which is present in various cell types. The apparent size of this polypeptide is larger than the 78.6 kDa predicted from the derived sequence and the 91.5 kDa polypeptide obtained in a cell-free

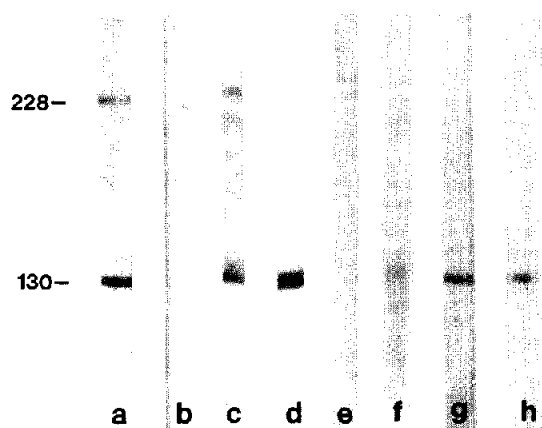


Fig. 1. Immunoblots of total cell extracts with antibodies to APP. Lanes: a, b, human fibroblasts; c–f, K562 cells; g, Cos 1 cells; h, U937 cells. Antibodies used were: A4 (597–638 APP aa sequence) for lanes a, c and h; A4 (597–638 APP aa sequence) absorbed with corresponding peptide, lane b; N77 (95–110 APP aa sequence), lane d; N77 (95–110 APP aa sequence) absorbed, lane e; C5 (638–658 APP aa sequence), lane f. A band of 130 kDa is present in all cell lines. A band of 228 kDa or a series of bands of comparable size are evident in fibroblast and K562 cell preparations immunostained with the A4 antibody. Molecular masses are shown on the left (kDa).

system [7,14]. This difference is consistent with APP being glycosylated [7,14], and possibly being the protein core of a heparan sulfate proteoglycan [15], since glycosylation is likely to result in a slower electrophoretic mobility.

The identical size of APP-130 in the cells examined indicates that APP undergoes the same posttranslational processing, and possibly has the same location and function in various cell types. APP-228, which is consistently detected only in fibroblasts, might represent a highly modified form of APP.

In order to gain insight into the subcellular localization of these polypeptides, fibroblasts were fractionated by sequential extractions with buffer and Triton X-100 at low and high ionic strength (fig.3). APP-228 was extracted in hypotonic buffer, while APP-130 was recovered in the Triton extract at high ionic strength. This analysis indicates that APP-228 is a cytosolic component while APP-130 is associated with another component, possibly membranes. Thus in fibroblasts, there seem to be two forms of APP localized in different cell compartments.

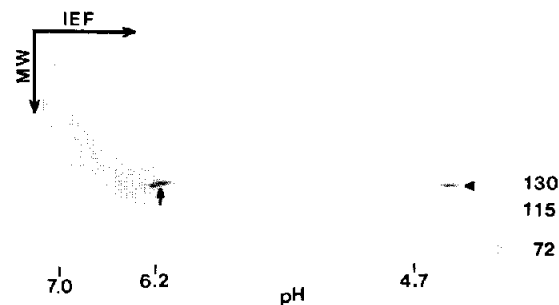


Fig. 2. Immunoblots of human fibroblast extract after separation by two-dimensional gel electrophoresis. APP-130 focus at pH 6.2 (arrow). On the right, a sample of the same extract run only in the second dimension (arrowhead). APP-228 was not detected in the pH range 4.7–7.0. Only the area of interest is shown. Molecular masses are shown on the right (kDa).

Immunofluorescence in cells fixed in formaldehyde showed intracellular staining, that was more intense over the nucleus (fig.4), while in cells that were only permeabilized with acetone, staining was mostly perinuclear, both in fibroblasts and K562 cells (fig.5). In unfixed cytospin preparations, K562 cells showed a light diffuse and strong patchy distribution of the immunofluorescence

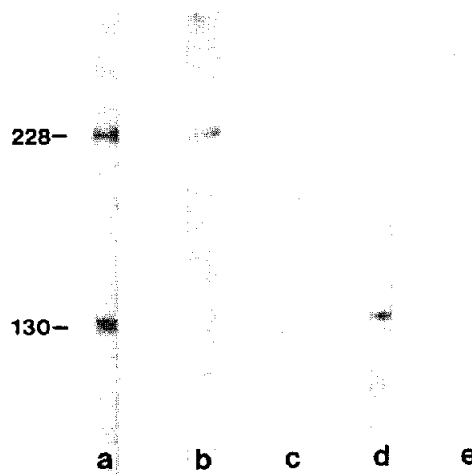


Fig. 3. Immunoblots of fibroblast fractions immunostained with A4. Lanes: a, total homogenate; b, buffer extract; c, Triton extract; d, Triton/KCl extract; e, insoluble. APP-228 is recovered with hypotonic buffer, while APP-130 is extracted with Triton at high ionic strength. These extractions are consistent with a cytosolic location of APP-228 and a possible association with membranes of APP-130.

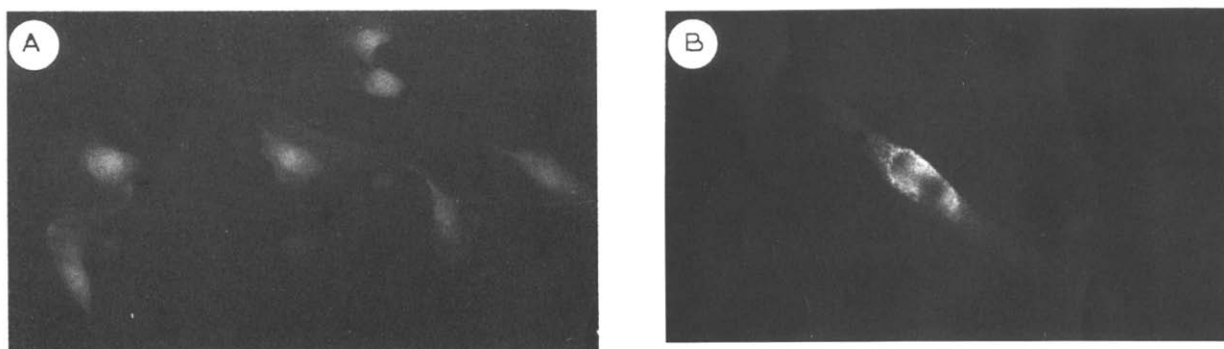


Fig.4. Human fibroblasts fixed in paraformaldehyde. A, diffuse fluorescence is seen around the nucleus; B, at higher magnification the fluorescence is especially noticeable over and around the nucleus, suggesting that APP is located in the nuclear membrane and other perinuclear structures. A, N77 antibody, $\times 250$; B, A4 antibody, $\times 400$.

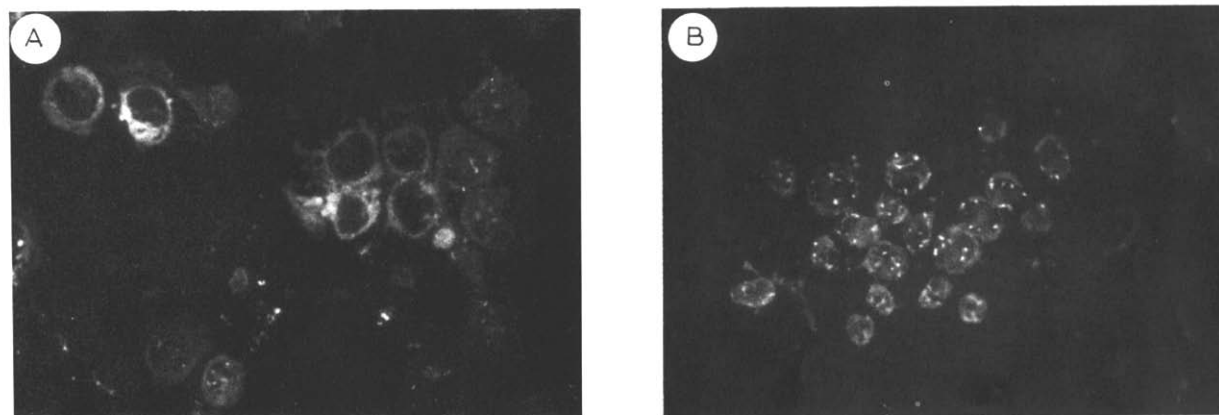


Fig.5. K562 cells. In cells permeabilized with acetone (A) the immunofluorescence is especially prominent in structures immediately around or adjacent to the nucleus, while in unfixed cytopinned cells (B) there is a diffuse, weak immunofluorescence with patches of more intense immunostaining. A, A4 antibody, $\times 200$; B, A4 antibody, $\times 200$.

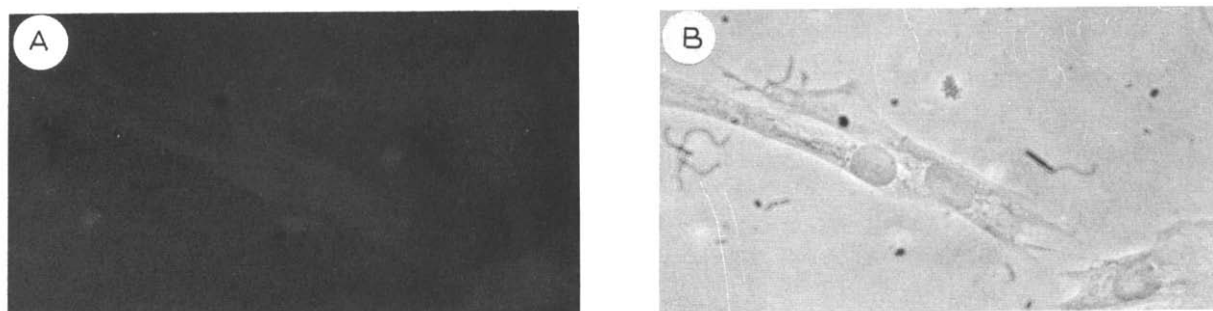


Fig.6. Fibroblasts fixed with paraformaldehyde. No immunofluorescence is seen with the N77 antiserum absorbed with the appropriate peptide. A, Fluorescence, $\times 360$; B, phase, $\times 360$.

(fig.5). Staining was blocked by absorption with the relevant peptide (fig.6). Thus, the pattern of immunostaining we have observed, is consistent with the localization of the APP-130 in the nuclear and possibly other intracellular membranes including, at least in K562 cells, the plasma membrane.

Our findings of a perinuclear immunolocalization of APP in fibroblasts and of a patchy distribution at the surface of K562 cells are similar to the localization in the nuclear envelope and plasma membrane reported in human muscle cells and rat neurons [16,22]. However, a definite localization should be provided by immunostaining at the electron microscope.

In a previous study, we demonstrated that the two antisera to synthetic peptides homologous to APP sequences other than A4 used in this study immunostain deposits located around the core of the senile plaque, which is itself unstained [23]. Our findings showing that the same antibodies recognize the APP, support the hypothesis that the processing of APP leading to the deposition of A4 occurs within the plaque, in the immediate vicinity of the core.

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