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Production of the Alzheimer's β-amyloid peptide by C6 glioma cells

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The β-amyloid peptide (Aβ) is a 4 kDa proteolytic fragment derived from the β-amyloid precursor protein (βAPP) which is deposited as amyloid fibrils in the brains of patients with Alzheimer's disease. βAPP processing was investigated in C6 glioma cells using several affinity-purified anti-peptide antibodies raised against different domains of the protein. Both direct immunoblot analysis of C6 glioma conditioned medium and metabolic labeling of cells followed by immunoprecipitation of extracellular medium with specific antibodies revealed that these glial cells normally produce and release a soluble 4 kDa peptide which co-migrates with synthetic Aβ (1–40) and is specifically recognized by antibodies raised against N- or C-terminal domains of the β-amyloid peptide. Our results further suggest that glial cells may prove a major source of β-amyloid production in the nervous tissue.

Alzheimer's disease; β-Amyloid peptide; Glial cell

1. INTRODUCTION

The major neuropathological characteristic of Alzheimer's disease is the progressive deposition in the brain and its microvasculature of insoluble aggregates of β-amyloid peptide (AB) [1–3]. This hydrophobic peptide of 39–43 residues (~ 4 kDa) is a small fragment contained within a larger membrane-spanning glycoprotein, the β-amyloid precursor protein (βAPP), which is expressed in most mammalian cells as a combination of different isoforms (mainly βAPP 695, 751 and 770) derived by alternative splicing [2,4,5]. The β-amyloid peptide includes 28 residues just outside the membrane plus the first 11–15 residues of the putative transmembrane domain of βAPP. The understanding of the intracellular processing of βAPP and of the pathways leading to Aβ generation are essential issues in research on Alzheimer disease.

The βAPP can be processed through a constitutive secretory, non-amyloidogenic pathway, which involves its cleavage by an endoproteinase (α-secretase) at position 16 within Aβ, releasing a large soluble βAPP ectodomain and retaining in the plasma membrane a C-terminal fragment of ~ 10 kDa [6,7]. Alternatively, βAPP molecules can be targetted to the endosomal/lysosomal system (probably by re-internalization from the cell surface), where they give rise to a variety of potentially amyloidogenic C-terminal fragments, which are further degraded in an acidic intracellular compartment [8–12].

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Abbreviations: Aβ, β-amyloid peptide; βAPP, β-amyloid precursor protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Moreover, very recent reports have put forward the unexpected finding that a soluble form of the 4 kDa Aβ peptide is produced normally by cultured cells and is present in cerebrospinal fluid [2,13–16]. Initial studies have suggested that the characteristics and extent of Aβ production are dependent on cell type [16]. In this regard, very little is known about βAPP processing in glial cells, despite the fact that substantial expression of the protein has been reported in astrocytes, microglia and C6 glioma cells [17–20]. In this report, we show, using several affinity-purified anti-peptide antibodies raised in our laboratory against different domains of Aβ and βAPP, that active generation and secretion of the 4 kDa Aβ peptide takes place in C6 rat glioma cells.

2. MATERIALS AND METHODS

2.1. *Antibodies*

Antibodies AB74, AB597a, AB597b and Ab597c were generated against synthetic peptides (obtained from Biosynthesis Inc. and Dr. M. Patarroyo) corresponding to residues 74–87, 597–612, 610–625 and 625–638, respectively, of human βAPP (numbering is according to βAPP 695, see [4]). The localization of the antigenic peptides in βAPP is depicted in Fig. 1. For affinity purification, the corresponding peptides were covalently coupled to Affigel-10 or Affigel 102 columns (Bio-Rad) according to the protocol of the manufacturer, and antibodies purified as described [21]. When tested by immunoblotting, these purified antibodies recognized mature βAPP in C6 glioma conditioned medium and cell lysates, and such reactivity was blocked by preabsorption with the corresponding peptide antigens (Morato and Mayor, to be described elsewhere).

2.2. *Cell culture*

Rat C6 glioma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5% fetal calf serum, 10% horse serum and 1% glutamine. When required, 10 ml aliquots of medium were conditioned in the presence of 20×10^6 cells per P100 dish for 12 h, either in the presence or absence of serum. After this time

the medium was removed, cleared of debris by centrifugation at 1,000 × g for 10 min, and concentrated five-fold by lyophylization, prior to analysis by gel electrophoresis and immunoblotting. Total cell lysates (in 50 mM Tris-HCl, pH 7.5, 2% SDS, 150 mM NaCl, 2 mM phenylmethylsulfonylfluoride, 1 mM benzamidine, 1 μg/ml leupeptin, 0.1 μg/ml pepstatin) were also investigated in the same way. In some experiments, conditioned medium was centrifuged at 100,000 × g for 90 min (Beckman TL-100) and the supernatant processed as above, or immunoprecipitated with antibody AB597a, as described below.

2.3. *Metabolic labeling and immunoprecipitation*

C6 glioma cells were grown to about 90% confluence in 10-cm dishes. After removal of culture medium, cells were incubated for 2 h in methionine-free medium, and then labeled for 12 h in the same volume with 1 mCi of ^{35}S Protein labeling Mix (New England Nuclear, Boston), either in the absence or presence of serum. For immunoprecipitation, the medium was made 1× in RIPA-LPT buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8, 1% Nonidet P-40, 0.5% cholic acid, 0.1% SDS, 5 mM EDTA, 1 μg/ml leupeptin, 0.1 μg/ml pepstatin, 2 mM phenylmethylsulfonylfluoride). The affinity-purified antibody AB597a (1:250) and 50 μl of protein A–agarose slurry washed in RIPA-LPT were then added and the mixture incubated at 4°C for 12 h [6]. After washing the agarose beads 3–4 times with RIPA-LPT, the immunoprecipitated proteins were resuspended in reducing SDS sample buffer and analyzed by Tris-Tricine SDS-PAGE on a 16.5% gel [13]. The gel was fixed in acetic acid:ethanol (10:50), incubated in Amplify (Amersham) and labeled proteins visualized by autoradiography.

2.4. *Gel electrophoresis and immunoblotting*

Conditioned medium, total cell lysates or immunoprecipitates were resolved by 16.5% Tris-Tricine SDS-PAGE for improved detection of low molecular weight fragments [22] and electroblotted to Immobilon filters (Millipore). Membranes were blocked in TBS-BSA (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% bovine serum albumin) followed by incubation with the desired purified antibody (1:3,000 in TBS-BSA) for 12 h at 4°C. Filters were washed (3 × 15 min) in TBS containing 0.1% Tween 20 and developed using a chemiluminiscence method (ECL, Amersham Corp.) after incubation with a goat anti-rabbit antibody conjugated to peroxidase. For peptide pre-absorption controls, the antibody was pre-incubated with 100 μg/ml of the peptide immunogen for 12 h at 4°C.

3. RESULTS AND DISCUSSION

The possible production and secretion of low molecular weight βAPP derivatives by C6 glioma cells was initially investigated by resolving the conditioned medium by 16.5% Tris-Tricine SDS-PAGE followed by immunoblot analysis using antibodies raised against different domains of Aβ and βAPP (see scheme in Fig. 1). Fig. 2A shows that a secreted peptide of ~ 4 kDa is recognized by antibodies raised against the N-terminus (AB597a, lane 2), the middle region (AB597b, lane 3), and the C-terminus (Ab597c, lane 4) of the β-amyloid peptide, whereas this band is not immunoreactive with an antibody to the N-terminal domain of βAPP (AB74, lane 1). When the conditioned medium is immunoprecipitated with the antibody raised against the N-terminus of Aβ, a 4 kDa protein can be immunolabeled by both AB597a and AB597c (Fig. 2B, lanes 1 and 3, respectively). Pre-absorption of these antibodies with their corresponding synthetic peptide immunogens abolished reactivity with the 4 kDa peptide (Fig. 2B, lanes 2 and 4). Thus, two different experimental approaches indicate that the 4 kDa protein detected in C6 glioma cells conditioned medium contains both the N- and C-terminal domains of the β-amyloid peptide.

Fig. 3A shows that non-conditioned medium does not display the 4 kDa protein recognized by the AB597a antibody (lane 1), thus ruling out the possibility of a non-cellular origin of this peptide (see also the experiment depicted in Fig. 3B). Fig. 3A also indicates that the 4 kDa protein present in the C6 glioma extracellular medium (lane 3) co-migrates with synthetic Aβ (1–40) peptide resolved in the same gel and labeled with the same AB597a antibody (lane 2), thus further suggesting that the 4 kDa peptide corresponds to the β-amyloid peptide fragment of βAPP. This fragment appears to be released from C6 glioma cells in a soluble form, since when cell-conditioned medium was centrifuged at 100,000 × g for 90 min and analyzed by immunoblotting with the AB597a antibody, the 4 kDa protein was found in the supernatant (Fig. 3A, lane 4). To further demonstrate that the Aβ peptide was produced and secreted by these glial cells, we metabolically labeled C6 glioma cultures with [^{35}S]methionine for 12 h. Immunoprecipitation of the conditioned medium with AB597a followed by electrophoresis and autoradiography revealed a 4 kDa band (Fig. 3B) which was absent in immunoprecipitations with pre-absorbed serum (data not

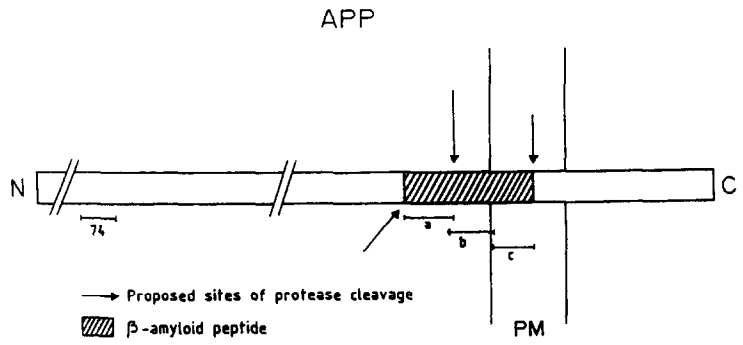

Fig. 1. Schematic representation of the localization of the synthetic peptide antigens within the structure of the β-amyloid precursor protein. AB74 was generated against the N-terminus of βAPP, whereas AB597a, AB597b and AB597c were raised against domains a, b and c of the β-amyloid peptide, respectively. PM, plasma membrane.

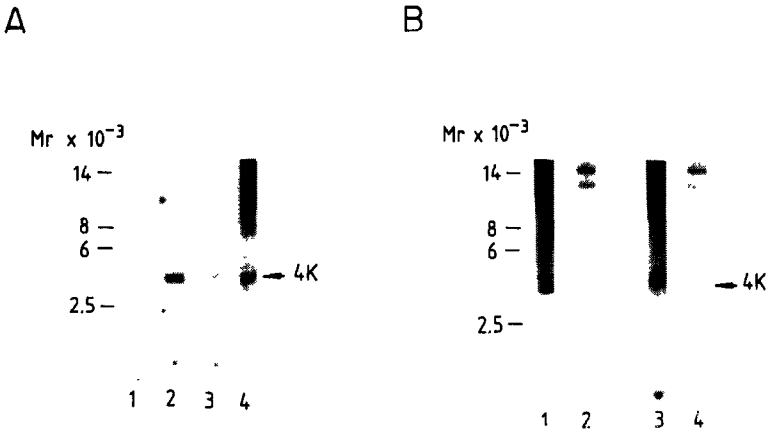

Fig. 2. C6 glioma cells release to the extracellular medium a 4 kDa peptide which is specifically detected by antibodies raised against different domains of the β-amyloid peptide. (A) 25 μl aliquots of concentrated C6 glioma cells conditioned medium were resolved by 16.5% Tris-Tricine SDS-PAGE, transferred to Immobilon membranes and immunoblotted as detailed in section 2 with the affinity-purified antibodies AB597a (lane 2), AB597b (lane 3) and AB597c (lane 4) raised against different domains of Aβ (see Fig. 1), or AB74 (lane 1) raised against an N-terminal domain of βAPP. (B) The 4 kDa peptide is immunoprecipitated from C6 glioma cells conditioned medium. Extracellular medium proteins were immunoprecipitated with the affinity-purified antibody, AB597a, as detailed in section 2 resolved by Tris-Tricine SDS-PAGE and immunoblotted as in A with AB597a (lanes 1 and 2) or AB597c (lanes 3 and 4) without (lanes 1 and 3) or with (lanes 2 and 4) previous pre-absorption with the specific immunogens. The migration of low M_r standards (Pharmacia-LKB) is shown on the left in both panels and the position of the 4 kDa peptide is marked by an arrow.

shown). Similar amounts of the labeled peptide were detected when the labeling was performed in the presence (lane 1) or absence (lane 2) of serum.

In conclusion, several lines of evidence indicate that C6 glioma cells secrete to the medium a soluble 4 kDa peptide which co-migates with synthetic Aβ (1–40) and contains its N- and C-terminal domains, as indicated by immunoreactivity with antibodies raised against these regions of Aβ. Our results are consistent with recent reports showing that Aβ is a normal secretory product of different cell types [13–16]. The production of β-amyloid peptide by glial cells is of particular physiological relevance, given their abundant presence in the nervous tissue. During the preparation of this work, Busciglio et al. [16] have also found that primary cultures of human and rat astrocytes generate high levels of the 4 kDa β-amyloid peptide relative to other cell types in their study, including neurons. In line with this report, our data further suggest that glial cells may be a major source of β-amyloid in the brain. The exact site of production of Aβ within the cells has not been identified to date. Although very recent reports suggest the involvement of endosomes/lysosomes or other acidic components in the initial production and further degradation of potentially amyloidogenic βAPP C-terminal fragments [12], the presence of the 4 kDa peptide has not been reported in lysates from βAPP-transfected cells secreting β-amyloid [2,13,14,23]. The higher levels of production of Aβ in glial cells may explain preliminary results in our laboratory which indicate the presence of the β-amyloid peptide in cell lysates (Morato and Mayor, in preparation), although more detailed subcellular localization studies need to be performed.

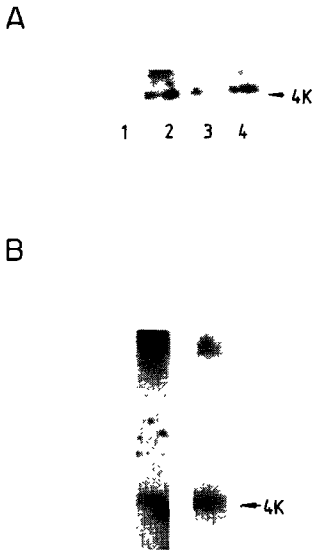

Fig. 3. Characterization of the 4 kDa peptide produced and secreted by C6 glioma cells. (A) The 4 kDa peptide is released in a soluble form and co-migrates with synthetic β-amyloid peptide. Non-conditioned cell culture medium (lane 1), synthetic Aβ (1–40) (lane 2), or C6 glioma cells conditioned medium before (lane 3) and after (lane 4) centrifugation at 100,000 × g for 90 min were resolved by SDS-PAGE, transferred to membranes and immunoblotted with AB597a, as in Fig. 2A. (B) Immunoprecipitation of labeled 4 kDa peptide from the extracellular medium. C6 glioma cells were metabolically labeled with [^{35}S]methionine for 12 h in the presence (lane 1) or absence (lane 2) of serum, and 2 ml aliquots of the conditioned medium immunoprecipitated with AB597a, as detailed in section 2, followed by 16.5% Tris-Tricine SDS-PAGE and autoradiography. The migration of low M_r standards (Pharmacia-LKB) is shown to the left and the position of the 4 kDa peptide is marked by an arrow.

It is worth noting that, under our experimental conditions, C6 glioma cells do not release a truncated 3 kDa β-amyloid peptide that has been detected in the extracellular medium of βAPP-transfected cells, cultured rat neurons and neuroblastoma or pheochromocytoma cell lines [13,15,16,23]. This truncated form lacks the N-terminal portion of Aβ and seems to be derived from the ~ 10 kDa C-terminal fragment of βAPP which remains in the plasma membrane after α-secretase cleavage, thus acting as a marker for the activity of the secretory, non-amyloidogenic pathway [2,23]. Our experimental finding is consistent with previous reports indicating low levels of such constitutive βAPP cleavage and secretion in microglia and astrocytes [19], and with the very low levels of the 3 kDa peptide compared to the full-length Aβ recently reported in astrocytes-conditioned medium [16]. Thus, there may be significant variations in the cellular processing of βAPPP in different cell types, such as neurons and glia. Given the important trophic interactions between neurons and glia, and the reported association of activated astrocytes and microglia with senile plaques found in Alzheimer's disease [2,24,25], the investigation of βAPP processing in glial cells may prove essential for understanding the formation and development of amyloid deposits. The availability of a well-known cell system model, such as C6 rat glioma, that exhibits Aβ generation should allow a detailed study of the regulation of the cellular pathways leading to the formation of β-amyloid peptide in glial cells.

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