

Generation of β A4 from the amyloid protein precursor and fragments thereof

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The cellular mechanisms underlying the generation of β A4 in Alzheimer's disease and its relationship to the normal metabolism of the amyloid protein precursor (APP) are unknown. In this report, we show that expression of the C-terminal 100 residues of APP, with (SPA4CT) or without (A4CT) a signal sequence in the N-terminal position, in human neuroblastoma cells results in secretion of a 4 kDa β A4-like peptide. In A4CT and SPA4CT expressing SY5Y cells, β A4 generation could not be inhibited by the lysosomotropic amines chloroquine and ammonium chloride but was inhibited by brefeldin A, monensin and methylamine. The last also selectively inhibits APP secretion in neuroblastoma cells [1]. The finding that chloroquine and ammonium chloride inhibit β A4 generation from full length APP but not from A4CT and SPA4CT are consistent with the assumption that the two cleavages necessary to generate β A4 operate in two different compartments. Our data suggest the cleavage which generates the C-terminus of β A4 takes place in the same compartment (late Golgi or endosomal vesicles) in which the APP-secretase operates.

APP; A4CT; β A4; Processing; SY5Y; Alzheimer's disease

1. INTRODUCTION

Alzheimer's disease (AD) is an irreversible neurodegenerative disorder of the human central nervous system. The neuronal dysfunction is correlated with massive deposition of fibrillar aggregates in brain parenchyma and blood vessel walls. The major protein component isolated from these deposits is a small polypeptide of 4–4.5 kDa, called the amyloid β A4 protein due to its relative molecular mass and its partial β -pleated sheet structure [2–5]. The β A4 subunit of the parenchymal deposits consists of 42–43 residues and is synthesized as part of a larger protein precursor (amyloid protein precursor, APP). At least eight different splice-forms of this amyloid protein precursor are encoded by the widely expressed APP gene of chromosome 21 [6–10]. Proteolytic processing of these transmembrane proteins, leading to secretion of the extracellular part (secretory APP, APPsec) [11], occurs within the amyloid β A4 sequence [12,13]. This suggests that the N-terminus of the amyloid β A4 protein is not produced by the cleavage which leads to secretory APP's.

Recent reports have shown that β A4-related peptides are released by cultured cells and can be detected in human cerebrospinal fluid [14–17]. These and the finding that the mutation in the APP gene found in two Swedish kindreds with familial AD, where onset of symptoms and pathology occur much earlier than in

sporadic disease, lead to increased β A4 generation [18], provide further support for the hypothesis that generation and deposition of β A4 contributes both to the onset and the progression of the disorder. The mechanism of the underlying pathological processes is unknown.

In order to study the steps in the processing of APP which lead to β A4 generation, we have expressed, in addition to full length APP695, also the C-terminal 100 residues of APP, with and without a signal sequence, termed by us SPA4CT and A4CT, respectively, in the human neuroblastoma cell line SY5Y. Since β A4 release from A4CT-type APP fragments which carry the β A4 sequence at the N-terminus depends only on the protease generating the C-terminus, we were able to analyse the two cleavages generating the C and N-terminus of β A4 independently from each other.

2. EXPERIMENTAL PROCEDURES

2.1. Cloning procedures

Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation and bacterial transformation were carried out as described by Sambrook et al. [19].

2.2. Plasmid construction

The eucaryotic expression vector CEP/A4CT was obtained by cloning the 1 kb *Sma*I/*Hind*III fragment of SP65/A4CT [20] into pCEP4 (Invitrogen), digested with *Pvu*II/*Hind*III. The resulting plasmid includes methionine codon 596 of APP695 as initiation codon, the amyloid β A4 sequence (codons 597–639/640 of APP695) and the entire C-terminal domain of APP. For construction of CEP/SPA4CT the *Sma*I/*Hind*III fragment from SP65/SPA4CT [20] was cloned into pCEP4, digested with *Pvu*II/*Hind*III. The resulting plasmid CEP/

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SPA4CT encodes the APP signal sequence and two additional residues from APP695 (Leu, Glu) in-frame with the amyloid β A4 sequence and the entire C-terminal domain of the APP. CEP/APP695 was obtained by cloning the *Sma*I/*Hind*III fragment into pCEP, digested with *Pvu*II/*Hind*III.

2.3. Tissue culture and transfection

The subclone neuroblastoma cell line SH-SY5Y was grown in equal parts of Minimum Essential Medium (MEM, with Earle's Salts and L-glutamine) and Ham's F-12 supplemented with nonessential amino acids (Eagle's formulation), penicillin (50 U/ml), streptomycin (40 μ g/ml) and 10% (v/v) fetal calf serum (FCS) (all Gibco/BRL). For stable transfection 5×10^5 cells were seeded onto 60-mm plates, and incubated overnight at 37°C. Cells were about 80% confluent prior to transfection. Cells were then washed three times with Opti-MEM I (GIBCO) prior to addition of the DNA/Lipofectin mixture. The DNA/Lipofectin mixture was produced by combining the DNA solution (10–20 μ g DNA in 1.5 ml Opti-MEM) with the Lipofectin solution (30–50 μ g Lipofectin (BRL, Eggenstein, Germany) in 1.5 ml Opti-MEM). After incubation for 8 h at 37°C, cells were grown for a further 24 to 48 h in cell-culture medium and stable lines were selected by exposure to hygromycin B (300 μ g/ml).

2.4. Metabolic labelling of tissue culture cells and preparation of cell lysates

After removal of the culture medium, the cells were pretreated with 2 ml of MEM (for 6 cm cell culture dishes) lacking methionine for 40 min. Cells were labelled by incubation in 1.5 ml MEM lacking methionine, supplemented with 300 μ Ci of [35 S]methionine for 2–3 h. The medium was then harvested, and the cells were washed once with MEM and scraped from the surface of the dish. For lysis, the cells were resuspended in 0.6 ml of SOL (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF) and placed on ice. After 20 min, samples were centrifuged at $10,000 \times g$ for 5 min, and the supernatants (cell lysates) were stored at -20°C for subsequent analysis.

2.5. Drug treatment

Ammonium chloride (10 mM), chloroquine (20 μ M), brefeldin A (10 μ g/ml), monensin (10 μ M) and methylamine (30 mM) were added to the methionine free medium 30 min prior addition of [35 S]methionine.

2.6. Pulse-chase labelling of tissue culture cells

After removal of the culture medium, the cells were treated with 1.5 ml of MEM lacking methionine for 40 min. Then 500 μ Ci of [35 S]methionine were added, and cells were labelled for 20 min at 37°C. Cells were then washed once with MEM (37°C) and chased in methionine-enriched medium (MEM, 0.3 mg/ml L-methionine, 37°C) for the times indicated in results. After the chase period, the cells were washed twice with PBS before preparation of cell lysates as described.

2.7. Immunoprecipitation [21]

For immunoprecipitation the conditioned medium was first adjusted to 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 2 mM PMSF and then medium and cell lysate were incubated for 30 min with 5 μ l pre-immune serum and 30 μ l (3 mg) protein A-Sepharose (Pharmacia). The samples were centrifuged briefly and the supernatants were incubated with antiserum (5 μ l anti A4CT or 10 μ l anti 2-43) for 60 min at room temperature. Following the incubation, 30 μ l (3 mg) protein A-Sepharose was added for an additional 60 min at room temperature. The insoluble complexes were washed three times with wash A (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Triton X-100, 2 mM EDTA), two times with wash B (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% Triton X-100, 2 mM EDTA) and once with wash C (10 mM Tris-HCl, pH 7.5) before pellets were resuspended in $2 \times$ Laemmli sample buffer. After boiling for 5 min at 100°C , labelled proteins were analyzed by SDS-PAGE and fluorography with EN 3 HANCE (Du Pont).

3. RESULTS

3.1. Expression of A4CT and SPA4CT in SY5Y cells

Stable expression of the C-terminal 100 residues of APP as a cytoplasmic protein (A4CT) or as a transmembrane protein (SPA4CT) was achieved by using the episomal replication of pCEP4 vectors (Invitrogen). Stable cell lines expressing SPA4CT and A4CT, were metabolically labelled with [35 S]methionine, and the immunoprecipitates of cell lysates and conditioned medium obtained with anti A4CT or an antibody raised against synthetic β A4 peptide 2-43 (anti 2-43) were subsequently separated on Tris-tricine gels.

Cell lysates from SY5Y cells expressing SPA4CT and A4CT revealed additional protein bands at 12 kDa (Fig. 1, lane 2) and 11.5 kDa (Fig. 1, lane 3) respectively, which were not present in the control (Fig. 1, lane 1). The bands at higher molecular weights present also in untransfected SY5Y cells correspond to endogenously-expressed APP and fragments thereof. Although the signal peptide of SPA4CT was probably cleaved off [19], the A4CT-band at 11.5 kDa migrated slightly below the SPA4CT-band at 12 kDa. This difference in molecular mass on SDS-PAGE is due to the two additional amino acids present in mature SPA4CT, which were introduced together with the signal peptide sequence (see Section 2.2).

The band at about 10 kDa visible in both untrans-

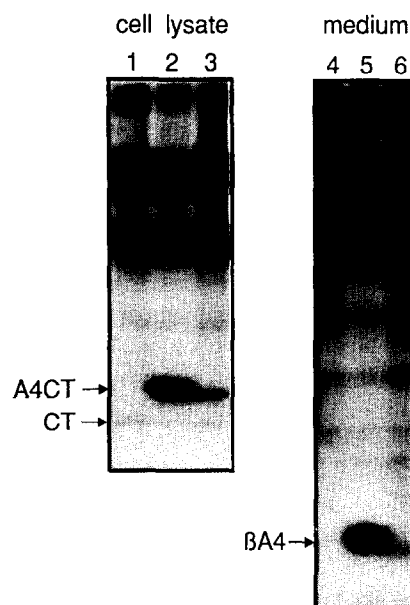


Fig. 1. Expression of A4CT and SPA4CT in SY5Y cells. Cells were transfected with the corresponding constructs, metabolically labelled with [35 S]methionine, and the immunoprecipitates of cell lysates and media were analysed by 16% Tris-tricine-PAGE and fluorography. Lanes 1 to 3: cell-lysates; antibody anti A4CT. Lanes 4 to 6: conditioned medium; antibody: anti 2-43: Lane 1 and 4, nontransfected SY5Y cells; lane 2 and 5, SY5Y cells transfected with CEP/SPA4CT and lane 3 and 6, cells transfected with CEP/A4CT. CT, C-terminal membrane associated APP-fragment after APP-secretase cleavage.

fects and transfected SY5Y cells (Fig. 1 lanes 1–3, CT) corresponds to the C-terminal fragment of the endogenously expressed APP, generated by cleavage by APP secretase within the β A4 sequence.

Analysis of conditioned medium from transfected cells with anti A4CT did not reveal SPA4CT or A4CT signals (data not shown). Identical results were obtained with transiently transfected COS-cells, HeLa cells and PC12 cells (data not shown).

Analysis of conditioned medium from the transfected cells with anti 2-43 revealed a β A4-like peptide at 4 kDa (Fig. 1, lanes 5 and 6), which was not detectable in untransfected SY5Y cells under the conditions used (Fig. 1, lane 4). The β A4 peptide derived from cells expressing SPA4CT migrated slightly above the β A4 peptide secreted from A4CT transfected cells. This is due to the two additional amino acids present in mature SPA4CT (see above) and demonstrates the precursor-endproduct relationship between the 4 kDa band and SPA4CT and A4CT, respectively. The β A4-like peptides could not be detected in cell lysates.

3.2. Time course of β A4 generation in SY5Y cells

To investigate the biogenesis pathway of A4CT and SPA4CT and its metabolism leading to β A4-like peptides, a pulse-chase labelling experiment was performed. The transfected SY5Y cells were labelled for 20 min with [35 S]methionine and chased in complete, methionine-enriched medium for 0–240 min. Immunoprecipitates of cell lysates at 0 min (zero time) revealed the 12 kDa band of SPA4CT and the 11.5 kDa band of A4CT described above (Fig. 2). In this cellular system A4CT had a half-life time of less than 20 min. In contrast, SPA4CT seemed to be more stable and showed a half-life time of about 40 min. The different half-life times

of SPA4CT and A4CT could reflect different degradation pathways of these APP fragments.

Analysis of conditioned medium from the transfected cells with anti 2-43 revealed the presence of β A4-like peptides after 20 min. Thus both, the transmembrane protein SPA4CT and A4CT, which lacks the signal peptide, were processed to β A4-like peptides with similar time courses. Densitometric measuring of SPA4CT and β A4 signals in Fig. 2 showed that about 60% of the precursor SPA4CT was transformed into β A4.

3.3. Compartmentalization of the APP- and A4CT-processing

To identify the compartment in which β A4 is generated, A4CT- and SPA4CT-transfected cells were labeled in the presence of compounds which interfere with distinct processing pathways. Immunoprecipitation of conditioned medium of the stable cell lines with anti 2-43 revealed that for both APP fragments β A4 generation was inhibited by monensin and brefeldin A, but not by the lysosomal protease inhibitors chloroquine and ammonium chloride (Fig. 3). Interestingly, β A4 generation from SPA4CT could be inhibited by methylamine, another acidotropic amine (Fig. 4, medium, lane 'Me'). The generation of β A4 from full length APP695 was partially inhibited by ammonium chloride and fully inhibited by methylamine and brefeldin A (Fig. 4) which is in agreement with other publications [21].

That the different inhibitors are functionally active under the conditions used is shown in Fig. 4. Cell lysates from transfected SY5Y cells incubated with the different compounds used in Fig. 3 and Fig. 4, were analysed by immunoprecipitation with anti A4CT. Ammonium chloride and methylamine strongly stabilized the C-terminal APP-fragment (CT) generated by APP-secretase

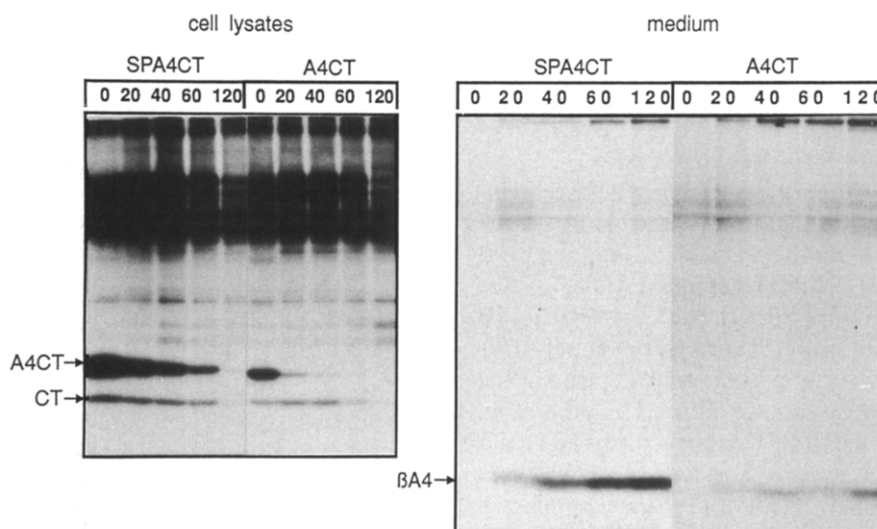


Fig. 2. Half-life time of SPA4CT and A4CT. SY5Y cells transfected with CEP/A4CT and CEP/SPA4CT were labelled for 20 min with [35 S]methionine and chased in methionine-enriched medium for the times indicated. The proteins were analysed by 16% Tris-tricine-PAGE after immunoprecipitation. Cell lysates: analysis of cell-lysates; antibody anti A4CT. Medium: analysis of conditioned medium; antibody anti 2-43. CT, C-terminal membrane associated APP-fragment after APP-secretase cleavage.

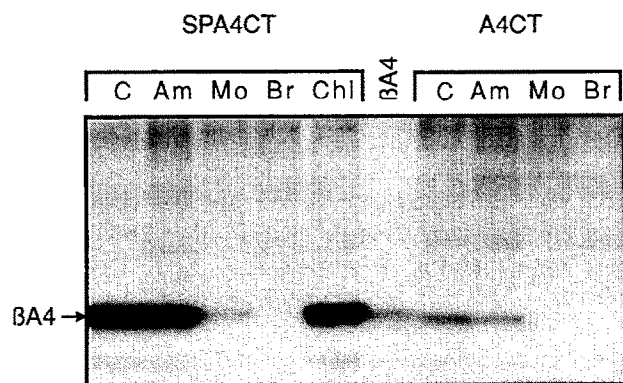


Fig. 3. Compartmentalization of β A4 generation. SY5Y cells transfected with CEP/A4CT and CEP/SPA4CT were labelled with [35 S]methionine for 2 h in the presence or absence of the compounds indicated. The conditioned medium was analysed by immunoprecipitation with anti 2-43 and subsequent Tris-tricine PAGE (16%). Co, control; Am, 10 mM ammonium chloride; Mo, 10 μ M monensin; Br, 10 μ g/ml brefeldin A; Chl, 20 μ M chloroquine; β A4, 1-42 translated in the wheat germ translation system.

and normally degraded in the lysosomal compartment (Fig. 4, cell lysate). Brefeldin A prevented the generation of CT because it blocked APP translocation from the golgi stack to the plasma-membrane.

4. DISCUSSION

In this study we expressed the C-terminal 100 residues of APP with and without a signal sequence at N-terminal position, termed by us SPA4CT and A4CT, respectively, in SY5Y cells. Expression of SPA4CT should allow membrane insertion of A4CT [20] and thus mimic the natural situation of this C-terminal fragment of APP. It is important to note that both APP-fragments start with the β A4 sequence at the N-terminus and need only to be cleaved at the C-terminus of β A4 for its release. By using these stable cell lines, in combination with full length APP expressing cell lines, it became possible to separate functionally the two cleavages necessary to generate β A4 from full length APP.

Expression of SPA4CT and A4CT in SY5Y cells resulted in a 12 and 11.5 kDa protein associated with the cell lysate. The same results were also obtained with HeLa-, COS- and PC-12 cells, transfected with the same APP fragments. Both molecules showed no tendency to aggregate and are not secreted into the medium. This would be expected for cytoplasmic or transmembrane proteins but is in contrast to the findings of Wolf et al. [23] who suggested that A4CT expressed in CV-1 cells aggregates and forms deposits, and to those of Yankner et al. [24] who suggested that A4CT is secreted into the medium and aggregates there.

Analysis of cell lysates and conditioned medium of the stable cell lines with anti 2-43 showed that both A4CT and SPA4CT are processed into β A4-like pep-

tides which are immediately secreted into the medium. We could not detect any intracellular β A4 in the cell lysates of the transfected cells. The pulse-chase experiments showed that the processing of these APP-fragments into β A4 is very fast and efficient, because 60% of SPA4CT is transformed into β A4 in less than 20 min. In this context it is important to mention that SPA4CT seems to be not a good ligand for APP secretase because under the conditions used p3, a peptide generated from A4CT by cleavage of APP secretase [14], could not be detected. Thus our stable transfected cells are a good cellular model for studying β A4 generation and the consequence of the release of the amyloidogenic peptide.

The inhibition of β A4 generation by acidotrophic amines in APP695-transfected cells but not in A4CT- and SPA4CT-transfected cells suggests that the proteolytic cleavage generating the N-terminus of β A4, but not the C-terminal cleavage, occurs in an acidic compartment. Thus we have been able to separate the cleavage which generates the C-terminus from that for the N-terminus of β A4. The C-terminal cleavage is not affected by ammonium chloride and chloroquine, whereas that necessary to generate the N-terminus of β A4 is affected by the lysosotropic amines.

This is in contrast to the findings of Shoji et al. [16], who showed an inhibition of β A4 generation from SPA4CT by chloroquine and ammonium chloride. However, they used concentrations up to 5 times higher

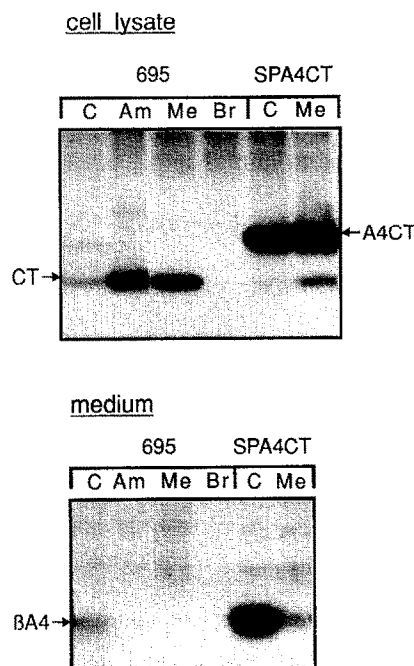


Fig. 4. Compartmentalization of the β A4 generation. SY5Y cells transfected with CEP/695 and CEP/SPA4CT were labelled and analysed as in Fig. 3. Top: cell-lysates; antibody anti A4CT. Bottom: conditioned medium; antibody: anti 2-43; C, control; Am, 10 mM ammonium chloride; Me, 30 mM methylamine; Br, 10 μ g/ml brefeldin A; Chl, 20 μ M chloroquine; CT, C-terminal membrane associated APP-fragment after APP-secretase cleavage.

than used in the current experiments and in this context it is important to mention, that at high concentration ($\geq 100 \mu\text{M}$), chloroquine has been shown to inhibit endocytosis and to be cytotoxic [25]. In our cellular model the concentrations used (chloroquine $20 \mu\text{M}$ and ammonium chloride 10 mM) are slightly below the concentrations which inhibit the general incorporation of radioactivity, indicating that the biosynthetic activity of the cells is not compromised.

Inhibition of βA4 production from APP695, SPA4CT and A4CT elicited by monensin, which is known to inhibit late Golgi and lysosomal function, and by brefeldin A, which causes a redistribution of Golgi into ER without affecting ER or early Golgi function, exclude the possibility that the proteolytic processing which generates the C-terminus and the N-terminus of βA4 takes place within the ER or early Golgi.

Our results also resolve the paradox that A4CT, a protein without a signal sequence, leads to a processing product (βA4) released into the medium. The inhibitory effects of monensin and brefeldin A on βA4 generation from A4CT suggests that the transmembrane domain of A4CT could function as an internal signal peptide and lead to insertion into ER. This is in agreement with the finding that A4CT and SPA4CT also sediment with the cell membrane if expressed in SY5Y cells (data not shown) or with microsomal membranes if expressed in a reticulocyte translation system [20].

Because it has been shown that βA4 generation from SPA4CT is like secretion of APP [1] only inhibited by methylamine but not by the other acidotrophic agents, chloroquine and ammonium chloride, we postulate that the process generating the C-terminus of βA4 occurs in the same or related compartment or maybe elicited by the same mechanism as APP secretion. This proposal is supported by the finding that βA4 was never detected intracellularly. Thus it seems very likely that the cleavage generating the C-terminus of βA4 occurs at or near the plasma membrane in competition with APP-secretase, and that the βA4 generated is immediately lost from the membrane to the extracellular compartment. This suggests that the corresponding compartment carries several distinct proteases releasing APPsec and βA4 , or that APPsecretase and the protease generating the C-terminus of βA4 (βA4aseC) are identical. Finally, the inhibition of the C-terminal cleavage by methylamine but not by the other acidotrophic amines may suggest the involvement in APP processing of transglutaminases, which are specifically inhibited by methylamine.

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