Lack of β-amyloid production in M19 cells deficient in site 2 processing of the sterol regulatory element binding proteins

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Abstract The processing of the amyloid precursor protein (APP) and the sterol regulatory element binding protein show remarkable analogies. Following a first lumenal cleavage, both proteins undergo a cleavage within the transmembrane domain by enzymatic activities named γ-secretase and S2P, respectively. We analyzed the processing of APP in the mutant Chinese hamster ovary (CHO) cell line M19 which lacks the S2P gene encoding for a putative metalloprotease. In these cells, we were not able to detect any β-amyloid production from endogenous or transiently overexpressed APP, although the transport of APP along the secretory pathway, its processing by α- and β-secretase, as well as its secretion were normal. This strongly suggests that the γ-secretase cleavage in M19 cells is severely impaired.

Key words: Protein processing; Proteolysis; Amyloid precursor protein; γ-Secretase; Chinese hamster ovary cell

1. Introduction

β-Amyloid (Aβ), a 39- to 43-amino acid peptide thought to play a crucial role in the pathogenesis of Alzheimer's disease, is generated from the amyloid precursor protein (APP) [1,2]. In a process defined as protein ectodomain shedding [3], two enzymatic activities, β- and α-secretase, release APP from membranes [4,5]. Although ubiquitous, these two activities may vary their relative contribution to the secretion of APP in different cells [6]. The β-secretase cleaves at the amino-terminus of the Aβ sequence, yielding the soluble amyloid precursor protein sAPPβ and the membrane-bound C99. The α-secretase cleaves within the Aβ sequence generating sAPPα and C83. The two carboxy-terminal fragments C99 and C83 are either degraded or processed by the γ-secretase to generate Aβ or the shorter peptide P3 [7]. The correct sequence of proteolytic cuts is necessary for the production of Aβ [8,9]. β- and α-secretase require a membrane-bound substrate and thus cleave only APP with an intact transmembrane domain. On the other hand, γ-secretase does not cleave full-length APP. Aβ peptides differ in length at their carboxy-termini [10]. Longer Aβ forms are more prone to form fibrils and are preferentially deposited in plaques [11]. In hereditary Alzheimer's disease, mutations of codon 717 of the APP gene or mutations along most of the sequence of the presenilin genes, significantly increase the proportion of the longer Aβ peptides [12,13]. Drugs inhibiting γ-secretase block Aβ generation [14]. When these studies were extended to include minor Aβ species, it was found that with some inhibitors the reduction of Aβ40 was accompanied by an increase in Aβ42 [15,16]. In addition, Aβ40 and Aβ42 may be formed in different cellular compartments [17]. It was therefore suggested that distinct γ-secretase activities generate Aβ40 and Aβ42.

It is remarkable that γ-secretase(s) cut(s) within the membrane-spanning domain of APP. So far, only one other example of a proteolytic cleavage in a transmembrane region was reported, the processing of sterol regulatory element binding proteins (SREBPs). SREBPs are membrane-bound transcription factors regulating the expression of enzymes involved in the cholesterol and fatty acid biosynthesis and the transcription of the low density lipoprotein (LDL) receptor [18]. Two sequential cleavages release the active transcription factor domain from the membrane of the endoplasmic reticulum. In cholesterol-depleted cells, SREBP-2 is cleaved lumenally at site 1 between the two transmembrane domains [19]. This event is tightly regulated by a cholesterol-sensing protein named SCAP [20]. The cleavage at site 2 occurs thereafter in the first membrane-spanning domain of SREBP-2 by a constitutively active protease [19]. A candidate site 2 protease has been recently identified and named S2P [21]. S2P is a putative metalloprotease with several predicted transmembrane domains, which would define a new class of proteases. Despite the analogies in SREBPs and APP processing, some notable differences exist. First, site 2 is in a domain spanning the membrane in the opposite orientation to that cleaved by γ-secretase. Second, no metalloprotease inhibitors are known to inhibit γ-secretase. Finally, S2P seems to be active in the endoplasmic reticulum [19], whereas the cleavage of APP by γ-secretase is thought to occur in a late compartment of the secretory pathway or in an endocytic/lysosomal compartment [1,2].

To investigate if APP and SREBPs are cleaved by the same or similar enzymes, we analyzed APP processing in M19 cells [22]. These mutant CHO cells lack the S2P gene and thus are deficient in site 2 processing of SREBPs [20]. Our data suggest that M19 cells lack γ-secretase activity, whereas the transport of APP along the secretory pathway as monitored by post-translational modifications such as glycosylation, tyrosine sulfation, processing by α- and β-secretase, as well as its secretion, are not altered compared to parental CHO cells.

2. Materials and methods

2.1. Materials

The cDNA constructs encoding APP_{695}, APP carrying the Swedish $K_{595}M_{596}$-NL mutation (APPswe) and APP truncated at position 40

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Abbreviations: Aβ, β-amyloid; APP, amyloid precursor protein; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SREBP, sterol regulatory element binding protein

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of the Aβ sequence (APP.40) were described previously [9,23]. SPENKC99 was obtained by cloning the C99 cDNA sequence downstream of the human preproenkephalin signal peptide after amplification by polymerase chain reaction. Recombinant protein expression was driven by the cytomegalovirus promoter and by the SV40 polyadenylation signal contained in the vector [24].

The rabbit polyclonal antisera AS NT11, AS APPC, AS 40, AS 16 and the mouse monoclonal antibody β1 were raised as described [9,23], the monoclonal antibody 6E10 was obtained from Senetek. AS NT11 and AS 40 recognise the carboxy-termini of Aβ40 and P3. β1 and 6E10 react with a human specific epitope of Aβ40, Aβ42, full-length APP and sAPPα. AS APPC recognises full-length APP and the carboxy-terminal fragments C99 and C83. AS 16 recognises specifically the free carboxy-terminus of sAPPα.

2.2. Transient cell transfections

Chinese hamster ovary cells CHO-K1 (ATCC CCL61) and M19 cells [22] were grown in Dulbecco's modified Eagle medium (Gibco-BRL), 10% foetal bovine serum (Gibco-BRL), 40 mg/l proline (Sigma) and 1% penicillin/streptomycin (Gibco-BRL) at 37°C/5% CO_2.

For transfection by electroporation, $5–10\times10^6$ CHO or M19 cells were resuspended in 400 μl culture medium. The cells were incubated at room temperature for 5–10 min with 20 μg relevant plasmid in a 0.4-cm gap cuvette (Bio-Rad). The electroporation was performed at room temperature using a Bio-Rad Gene Pulser II set at 350 V and 450 μF. Transfected cells were then seeded in a 10-cm culture dish and incubated for the indicated time.

For lipid-mediated transfection, 1.9×10^6 cells were seeded a day earlier in a 10-cm culture dish. Transfection was performed with FuGene 6 (Boehringer Mannheim) following the instructions of the manufacturer. In brief, 19 μl FuGene 6 diluted in 600 μl PBS were carefully mixed with 6 μg relevant plasmid. Following a 15-min incubation, the transfection mixture was added to 80% confluent cells in 12 ml culture medium.

2.3. Immunoprecipitations and immunoblots

Cell media and cell extracts were prepared and used for immunoprecipitations as previously described [23]. Precipitated proteins were resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to Immobilon P membranes (Millipore) [23,25]. Membranes were blocked with 5% (w/v) low-fat milk powder in phosphate buffered saline, 0.05% Tween-20 (PBST), and incubated overnight at 4°C with first antibodies diluted in PBST. Bound antibodies were detected with goat anti-rabbit IgG (Jackson Immuno-Research Laboratories, Inc.) or goat anti-mouse IgG (Chemicon) conjugated to horseradish peroxidase diluted in PBST using the ECL detection system (Amersham).

2.4. Metabolic labeling and sulfate labeling

For labeling with [^{35}S]methionine/cysteine, CHO and M19 cells were grown to subconfluency in 10-cm culture dishes. Cells were then pulse-labeled overnight and cell media immunoprecipitated as described previously [23].

Labeling with [^{35}S]sulfate was performed as described [26]. Briefly, CHO and M19 cells grown to subconfluency in 10-cm culture dishes were washed once and subsequently starved for 30 min at 37°C/5% CO_2 with sulfate-free medium. Cells were pulse labeled with 1 mCi/ml [^{35}S]sulfate (NEN DuPont, Sulfur-35 as sodium sulfate) for 3.5 h at 37°C/5% CO_2. sAPPα was immunoprecipitated from cell media as described above.

Radiolabeled proteins were analyzed as described previously [23].

3. Results

The processing of APP was analysed in M19 cells, a mutant CHO cell line deficient in S2P processing of the SREBPs [22]. M19 cells grown in regular Dulbecco's modified Eagle medium containing 10% foetal bovine serum were transfected by electroporation with a cDNA encoding human APP_{695}. Two days post-transfection, the cell medium was collected and analysed by immunoprecipitation followed by immunoblotting using the two monoclonal antibodies against Aβ β1 and 6E10. Under these experimental conditions, neither Aβ40 nor Aβ42 were detected in the conditioned medium of transfected M19 cells (Fig. 1A). However, when APP was transfected into the parental CHO cells, a large amount of both Aβ peptides was generated. Analysis of the cell lysates by immunoblotting with the antiserum AS APPC revealed that comparable levels of transgenic APP were expressed in both transfected cell types (Fig. 1B). While a small amount of Aβ was found in cell extracts of transfected CHO cells, no Aβ could be detected in M19 cell extracts (Fig. 1C), indicating that lack of Aβ peptides in the medium of these cells was not due to a defect in protein secretion. This was further confirmed by analysis of sAPPα, the soluble form of APP generated by α-secretase, which was secreted as efficiently by M19 as by CHO cells (Fig. 1D). To monitor the formation of P3, a metabolite of APP generated by α- and γ-secretase cleavage, the cell media were also analysed using the two polyclonal antibodies AS NT11 and AS 40 against the carboxy-terminal region of Aβ. As found for Aβ peptides, M19 cells did not

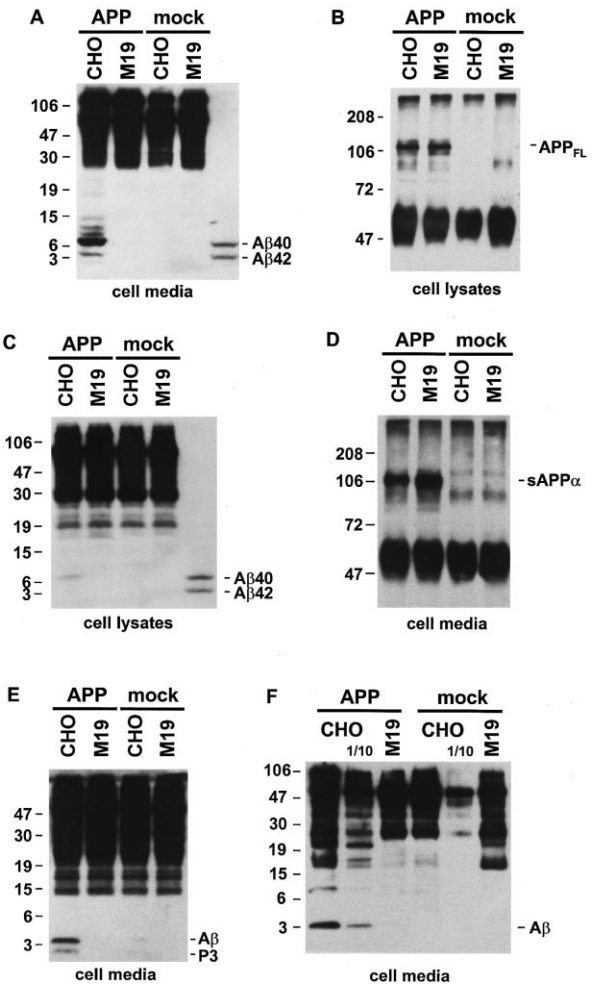

Fig. 1. Lack of Aβ- and P3-peptide production in M19 cells transiently expressing human APP. Cell media and lysates from CHO or M19 cells were collected 2 days post-transfection by electroporation (A–E) or FuGene (F). Mock transfected cells were used as control. Samples were immunoprecipitated with β1 (A,C,F), AS APPC (B), AS 16 (D) or NT11 (E). Precipitated protein was separated by 10% 8 M urea (A,C) or 13.2% 17.4% glycerol (E,F) Tris/bicine SDS-PAGE, or by 10% Tris/glycine SDS-PAGE (B,D). Immunoblots using 6E10 (A–D,F) or AS 40 (E) were developed with the ECL detection system. Synthetic Aβ1-40 and Aβ1-42 were loaded onto the gels as reference (A,B).

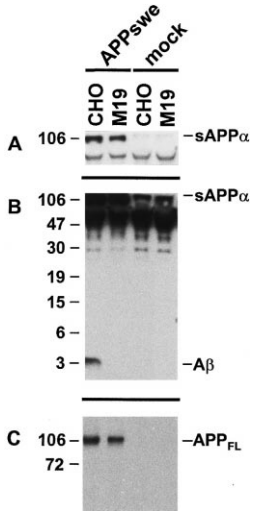

Fig. 2. Transiently expressed APPswe is not processed to Aβ in M19 cells. Cell media from CHO or M19 cells were collected 2 days after transfection, immunoprecipitated with β1, separated on a 13.2% Tris/bicine SDS-PAGE and immunoblotted with 6E10 (A). To visualize Aβ, the same immunoblot was exposed for a longer time (B). Total cell lysates were obtained in sample loading buffer. The samples were then separated on a 8% Tris/bicine SDS-PAGE and immunoblotted with 6E10 (C).

secrete any detectable P3 like-peptides, while these were generated in CHO cells (Fig. 1E). Altogether, these data indicate that M19 cells are deficient in APP processing to Aβ and P3, whereas the expression, the transport along the secretory pathway, the processing by α-secretase and the secretion of APP are similar to the parental CHO cells. Alternatively to electroporation, high levels of APP expression were also achieved by lipid-mediated transfection of the APP cDNA as found by immunoblot using AS APPC or β1 (not shown). Again, we did not find evidence for Aβ generation in M19 cells, whereas Aβ secreted from CHO cells was detectable already in one tenth of the cell medium (Fig. 1F). To further challenge the detection sensitivity of our method, cells were transfected with the APPswe cDNA. As expected, this APP mutant led to a five- to ten-fold increase in Aβ generation in CHO cells (not shown). However, M19 cells expressing APPswe did not secrete any detectable Aβ peptides (Fig. 2). These findings, strongly supporting the notion that the production of Aβ in M19 cells is severely impaired, were consistently reproduced several times.

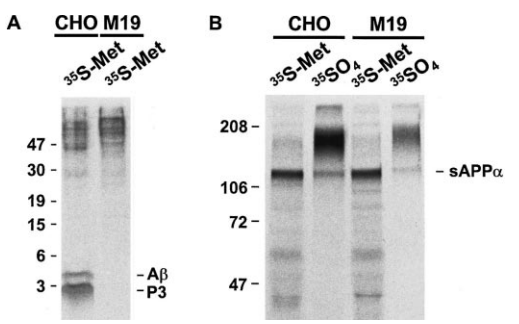

Fig. 3. Lack of Aβ production from endogenous hamster APP in M19 cells. Cells were pulsed overnight with [^{35}S]methionine or for 3.5 h with [^{35}S]sulfate. Cell media were immunoprecipitated with AS NT11 (A) to detect Aβ and P3, or with AS 16 (B) to detect sAPPα. Precipitated protein was resolved by 13.2% Tris/bicine (A) or 10% Tris/glycine SDS-PAGE (B). Molecular weight markers are given on the right of the autoradiogramms.

The generation of Aβ from transfected human APP was analysed in hamster cells. To exclude the possibility of an effect both mediated by the transient transfection or specific for the human sequence in M19 cells, we also investigated the processing of endogenous APP in metabolically labeled cells. Following an overnight pulse, cell media were analysed by immunoprecipitation and electrophoresis. Consistent to the above conclusion, M19 cells completely lacked the capability to generate Aβ- or P3-peptides (Fig. 3A), while soluble sAPPα was formed by both M19 and CHO cells (Fig. 3B). When APP was radiolabeled by sulfation, a post-translational modification occurring in the trans-Golgi/TGN compartment, again sAPPα was released by both mutant and parental CHO cells (Fig. 3B). These data further support the view that in M19 cells APP is efficiently transported along the secretory pathway and correctly post-translationally modified. To monitor the generation of C99, the carboxy-terminal metabolite of APP generated by β-secretase, cells were transfected with APPswe. Cell extracts were then analysed by immunoprecipitation with a polyclonal antibody specific for the carboxy-terminus of APP. We observed that C99 was generated in M19 cells as efficiently as in CHO cells (Fig. 4A). Since β- and α-secretase processing of APP is normal, whereas further processing to Aβ and P3 is deficient, we conclude that M19 cells lack any detectable γ-secretase activity.

Finally, M19 cells were transfected with cDNA constructs encoding artificially truncated APP molecules. APP.40 is truncated at the γ-secretase cleavage site and does not insert into membranes, it is efficiently secreted without any proteolytic processing and, therefore, serves as a marker for secretion [9]. The SPENKC99 construct drives the expression of the β-secretase product C99 which only requires γ-secretase cleavage to generate Aβ [9]. Analysis of the cell media by immunoblot showed that soluble APP-like material corresponding to APP.40 was secreted by both transfected M19 and CHO cells (Fig. 4B). However, M19 cells did not release Aβ from C99 (Fig. 4C). In CHO cells, C99 is processed to Aβ as efficiently

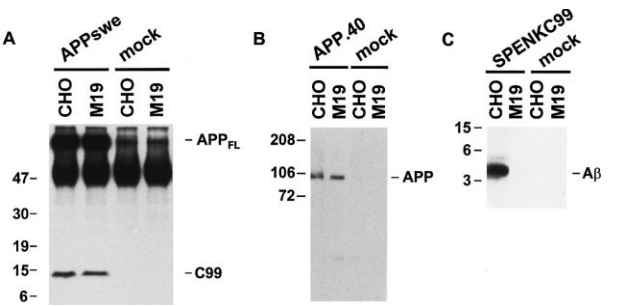

Fig. 4. The γ-secretase activity, but not APP secretion, is severely impaired in M19 cells. CHO or M19 cells were transfected with APPswe (A), APP.40 (B) and SPENKC99 (C). Cell lysates were immunoprecipitated with AS APPC, run on a 10% Tris/bicine SDS-PAGE and immunoblotted with 6E10. No difference was observed between CHO and M19 cells in the steady-state expression of full-length APP (APP$_{FL}$) and C99 (A). To better visualize APP$_{FL}$, a shorter exposure of the same immunoblot is shown at the top. Cell media were immunoprecipitated with β1, run on a 8% or 13.2% Tris/bicine SDS-PAGE and immunoblotted with 6E10 (B,C). Secretion of APP.40 occurs to the same extent in CHO and M19 cells (B), where the production of Aβ from C99 is severely impaired in M19 cells (C).

as APPswe. These data further support the hypothesis that the processing of APP in CHO and M19 cells is generally similar, but that M19 cells lack any γ-secretase activity.

4. Discussion

The processing of endogenous hamster APP and transiently expressed human APP was analyzed in M19 cells. In these cells, we were not able to detect any γ-secretase activity, i.e. the cleavage of the carboxy-terminal fragments C99 and C83 resulting in the generation of Aβ and P3, respectively. In contrast, APP expression, the transport of APP along the secretory pathway, post-translational modifications such as glycosylation (not shown) and tyrosine sulfation, APP processing by α- and β-secretase as well as its secretion, occurred as efficiently as in parental CHO cells.

M19 cells lack the gene for S2P, a putative metalloprotease that is responsible for the cleavage at site 2 within the transmembrane domain of SREBPs [21]. The data presented here suggest that in CHO cells S2P may also have γ-secretase activity. If S2P is indeed the γ-secretase, the enzyme would have the ability to cleave transmembrane domains of both type I and type II orientations. Furthermore, this would mean that γ-secretase belongs to the family of metalloproteases, although no inhibitors of this class of enzymes have been reported to inhibit Aβ generation. On the other hand, S2P could have an indirect effect on Aβ generation by activating the γ-secretase, as known for other proteolytic cascades. However, this appears to be unlikely, since metalloprotease inhibitors should again be active. An alternative possibility is that S2P may be involved in the intracellular transport of APP or γ-secretase, thus in S2P deficient cells the two partners would not come in contact and APP processing would be impaired. Our data suggest that dramatic alterations in the vectorial transport of APP can be excluded (see above). Finally, due to the S2P deletion, M19 cells are not able to upregulate the expression of several enzymes involved in the synthesis of cholesterol and fatty acids as well as the synthesis of the LDL receptor [22]. This defect could lead to a dramatic change in the physicochemical properties of membranes and therefore indirectly affect γ-secretase activity and thus Aβ generation. In preliminary experiments, transient overexpression of S2P in M19 cells did not restore Aβ generation (not shown), supporting the idea of an indirect effect of S2P on γ-secretase processing. This possibility appears attractive in view of the fact that ApoE4, a cholesterol transporting protein, is a risk factor of AD. Interestingly, APP overexpressing transgenic mice lacking the ApoE gene do not form plaques [26].

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