Amyloid Precursor Protein Processing in Sterol Regulatory Element-binding Protein Site 2 Protease-deficient Chinese Hamster Ovary Cells*

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Amyloid peptides of 39–43 amino acids (Aβ) are the major constituents of amyloid plaques present in the brains of Alzheimer’s (AD) patients. Proteolytic processing of the amyloid precursor protein (APP) by the yet unidentified β- and γ-secretases leads to the generation of the amyloidogenic Aβ peptides. Recent data suggest that all of the known mutations leading to early onset familial AD alter the processing of APP such that increased amounts of the 42-amino acid form of Aβ are generated by a γ-secretase activity. Identification of the β- and/or γ-secretases is a major goal of current AD research, as they are prime targets for therapeutic intervention in AD. It has been suggested that the sterol regulatory element-binding protein site 2 protease (S2P) may be identical to the long sought γ-secretase. We have directly tested this hypothesis using over-expression of the S2P cDNA in cells expressing APP and by characterizing APP processing in mutant Chinese hamster ovary cells that are deficient in S2P activity and expression. The data demonstrate that S2P does not play an essential role in the generation or secretion of Aβ peptides from cells, thus it is unlikely to be a γ-secretase.

A characteristic neuropathological feature of Alzheimer’s disease (AD)1 are amyloid plaques, containing Aβ, a 39–43-amino acid protein proteolytically derived from a large type 1 transmembrane protein, the amyloid precursor protein (APP)

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‡ The abbreviations used are: AD, Alzheimer’s disease; FAD, familial AD; APP, amyloid precursor protein; APPsw, Swedish mutation of APP695; CTF, C-terminal fragment; SRE, sterol regulatory element; SREBP, SRE-binding protein; S2P, SREBP site 2 protease; SCAP, SREBP cleavage activating protein; CHO, Chinese hamster ovary cells; PS1/PS2, presenilins 1 and 2, respectively; hu, human; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); Bicine, N,N-bis(2-hydroxyethyl)glycine.

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A characteristic neuropathological feature of Alzheimer’s disease (AD)1 are amyloid plaques, containing Aβ, a 39–43-amino acid protein proteolytically derived from a large type 1 transmembrane protein, the amyloid precursor protein (APP)
gamma-Secretase Activity in Site 2 Protease-deficient CHO Cells

Fig. 1. Schematic drawing of APP processing. A typical N-terminal (NH2) signal peptide (open box) is removed before APP is processed by one of two mutually exclusive pathways. a-Secretase cleaves at residue 17 of Aβ (gray box) preventing the generation of the amyloidogenic Aβ peptides. If, however, b-secretase cleaves APP (at position 1 of Aβ), subsequent cleavage by one or more b-secretases generates Aβ40–Aβ42, TMD, transmembrane domain; aAPP, and bAPP, secreted fragments generated by a- and b-secretase cleavage, respectively.

gamma-secretase and the S2P are identical enzymes. Sakai et al. (9) demonstrated that a mutant Chinese hamster ovary (CHO) cell line, CHO M19 (19, 20), is deficient in the S2P activity. Second, the S2P cDNA was cloned by complementation of the M19 defect (21, 22); furthermore, Rawson et al. (22) show that CHO M19 cells do not express the S2P mRNA due to a large deletion encompassing most, it not all, of the S2P gene (22).

In this communication we show that over-expression of human (hu) S2P does not enhance secretion of Aβ from 293 cells, suggesting that either the S2P is not the b-secretase or that the activity is not rate-limiting for processing APP to Aβ. Furthermore, we show that CHO M19 clones process transfected huAPPsw to Aβ40 and Aβ42; both Aβ40 and Aβ42 are generated in proportions indistinguishable from those produced by the wild-type CHO clones. Finally, we confirm that our CHO M19 clones are auxotrophs and that they do not express detectable S2P mRNA. These results definitively show that the S2P is not required for gamma-secretase processing of APP to Aβ40 and Aβ42.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The sense primer (5’-CCG AGA AGA TCT CTG AAG TGA ATG CAG AAT TCC GAC GAT ACT C-3’) containing the Swedish mutation and BglII restriction site and the antisense primer (5’-TAT AGC AGA AGC ACC ATC TGC TAC AG-3’) were used with wild-type APP695 cDNA as template. The PCR-amplified, BglII-digested DNA fragment was subcloned into the wild-type APP695 cDNA to generate pGEM3/APPsw695 (Promega). After confirming the DNA sequence, the APPsw fragment (HindIII-XmnI) was subcloned to generate pCMV/APPsw695. pC/Neo was derived from pC/CMV (Invitrogen). It contains the pC/CMV 1524-bp neomycin resistance expression cassette (KpnI-SaII) fused to the pC/CMV 2178-bp vector backbone (SacI-Xhol fragment). PCR primers for the amplification of codons 1–519 of the human S2P (22) (GenBank™ accession number AF0 19612) were synthesized: 5’-GTC TCT AAG GCT GCT ACT ATG ATT CCG GTC TCG-3’ and 5’-CAT TAC CGT GCT GTC ACG ATC TAC G3’. PCR amplification was performed with random primed human fetal brain cDNA as template. The product was digested with XhoI and cloned into pcDNA3.1 (-) (Invitrogen) between the XhoI and BamHI (blunt) sites to generate S2P/pcDNA3.1.

Stable Cell Lines—A derivative of 293 cells (Cell & Molecular Technologies Inc.) was co-transfected with pCMV-APPSw695 and pHSV-Puro by the calcium phosphate method (24). Individual puromycin-resistant clones were expanded and analyzed for high APP production by Western dot-blot with monoclonal antibody 22C11 (Boehringer). APPsw 293 clone 101 was used in all experiments reported here; similar results were obtained with clone 117. Wild-type CHO K1 (CHO WT) and CHO M19 cells (19, 20) were co-transfected with pC/CMV/APPsw695 and pC/Neo or with pC/Neo alone using the polyethylene glycol-LT1 (Mirus). Individual G418 resistant colonies were expanded and assayed for high APP production by Western blot with antibody 22C11.

Transient Transfections and Cell Culture—293 cells over-expressing APPsw (clone 101) were transiently transfected with S2P/pcDNA3.1, pcDNA3.1, or a b-galactosidase-containing vector using DMEHIE-C (Life Technologies, Inc.). 293 clone 101 cells were grown in Dulbecco’s modified Eagle’s medium with 10% FBS and 1% pen/strep/gm containing 5 μg/ml puromycin. CHO clones were grown in F12 with 10% fetal bovine serum, 10 μg/ml gentamycin, and 0.5 mg/ml G418.

Electrophoretic Separation of Aβ40 and Aβ42, Immunoprecipitations, and Westerns—Tris-Bicine urea gels were used to resolve Aβ40 and Aβ42 as described (25) using synthetic peptides from Quality Controlled Biochemicals, Inc. as standards. Proteins were transferred to polyvinylidene difluoride membranes as described (26).

For 293 cells over-expressing APPsw and transiently transfected with human S2P or vector control, 20 μl of 24-h conditioned medium was analyzed on Tris-Bicine urea gels. Following transfer, the blot was boiled in phosphate-buffered saline for 5 min and blocked for 2 h in 5% milk. The blot was probed overnight with 10 μg/ml E110 (Senetek), washed, and then treated with biotinylated-secondary antibody (Amersham Pharmacia Biotech) for 30 min. The blot was washed, incubated with streptavidin linked to horseradish peroxidase (Amersham Pharmacia Biotech), washed, and exposed to ECL film. For CHO clones, 48-h conditioned medium samples were collected from nearly confluent plates. One-ml samples were immunoprecipitated overnight with 10 μg of G6 (Senetek) and analyzed on gels/Westerns as described above.

Quantitative Analysis of S2P mRNA—S2P mRNA expressed during transient transfection experiments was quantified using solution hybridization to [35S]UTP riboprobes (27). Riboprobes corresponding to huS2P amino acids 437–519 and 250–321 (22) in both sense and antisense orientations were used to quantify the S2P mRNA.

RT-PCR—cdNA template generated with random primers and total cellular RNA from CHO M19 and WT clones was used to amplify S2P or α-tubulin by PCR. Codons 254–519 of S2P (22) were amplified with sense, 5’-GTT GGG GTG CTC ATC ACT GAA-3’ and antisense, 5’-CAT CGT GCT GTC ACC ACC CAG-3’, primers yielding a product of 790 bases. α-Tubulin was amplified with sense, 5’-AAG AAG TCC AAG CTG GAG TTC TC-3’ and antisense, 5’-GTC GTG TGT CTG ATC AGT CAC AC-3’, primers yielding a product of 700 base pairs.

RESULTS

Over-expression of the SREBP S2P Does Not Enhance Production of Aβ40 or Aβ42 from APPsw—To test the hypothesis that the S2P may be identical to the APP γ-secretase, we generated 293 cells stably expressing human APP695 with the Swedish mutation (APPsw) (28). If S2P is the same enzyme as the γ-secretase, transient over-expression in cells containing substrate (e.g. APP-over-expressing cell lines) could lead to an increase in the production of either Aβ40, Aβ42, or both.

Analysis of Aβ40 and/or Aβ42 secretion from 293 cells transiently transfected with S2P by quantitative urea-gel/Western blot was carried out on conditioned media samples (Fig. 2). Synthetic Aβ40 and Aβ42 peptides are well separated by this gel system with Aβ42 migrating faster than Aβ40 (Fig. 2, lanes 4 and 5), and our linear range of detection is from about 20 pg to 10 mg. Because this gel system does not separate peptides strictly on the basis of size, it is possible to identify the bands only by co-migration with known peptide standards (and by antibodyspecificity). (Some of the slower migrating bands in the conditioned media samples are dimers of the 40 and 42 peptides, but others, both slower and faster migrating in Figs. 2 and 3B, have not been identified by us, but see Ref. 26.)

APPsw-expressing 293 cells, transiently transfected with either vector control (Fig. 2, lane 1) or a b-galactosidase-containing vector (lane 3), secrete Aβ40 and Aβ42 in a ratio of about 10:1 as expected (29). Transient expression of human S2P in these cells (lane 2) did not lead to any detectable increase in
Aβ_{40} or Aβ_{42} production nor to a change in the ratio of the two Aβ species (Fig. 2, compare lane 2 to control lanes 1 and 3). These results suggest that over-expression of S2P does not lead to increased γ-secretase activity in these cells.

To show that the S2P cDNA was being expressed during this transient assay, we first determined that the transient transfection efficiency in this experiment was about 80% (based on β-galactosidase expression; data not shown). Next we performed a quantitative solution hybridization assay to detect S2P mRNA (see “Experimental Procedures” and Ref. 27) in the same cells from which the conditioned media were collected. This assay revealed that S2P transfected cells contained on average about 800 copies of S2P mRNA, whereas untransfected cells contained less than 20 copies per cell; nonetheless, no change in Aβ_{40} or Aβ_{42} production could be detected (Fig. 2).

**CHO Cells Lacking S2P Expression Process Hu APPsw to Aβ_{40} and Aβ_{42}**—Recent data indicate that the cholesterol auxotroph CHO M19 contains a deletion spanning most, if not all, of the S2P gene and thus represents a cellular S2P gene knock-out (22). If the activity of S2P is responsible for the γ-secretase activity of the S2P gene and thus represents a cellular S2P gene knock-out CHO M19 contains a deletion spanning most, if not all, of the S2P gene; nonetheless, no change in Aβ_{40} or Aβ_{42} production could be detected (Fig. 2).

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Because endogenous production of Aβ is very low in CHO cells, and it is not recognized by the 6E10 antibody used in our gel assay, both CHO WT and CHO M19 cells (19, 20) were stably transfected with pBCB/APPsw695 which encodes hu APPsw. Individual geneticin-resistant clones, which secreted roughly equivalent amounts of hu APPs and vector control clones for CHO WT and CHO M19, were selected for further analysis (Fig. 3A).

Most non-neuronal cell lines, including these CHO cells, express the 751 or 770 splice variants of APP. Endogenous intracellular APP expressed by these cells is detected as the upper band in extracts from all clones (Fig. 3A). It is well separated from the smaller APPs which is detected in all of the pBCB/APPsw695 transfected clones (lower band in lanes 2, 3, 5, and 6) but not in the vector control clones (lanes 1 and 4).

Conditioned media from these six clones were collected and concentrated by immunoprecipitation and the hu Aβ_{40} and hu Aβ_{42} production was analyzed on the urea-gel/Western described above (Fig. 3B). As expected, CHO WT clones expressing the APPsw processed and secreted hu Aβ_{40} and hu Aβ_{42} at a ratio of about 10:1 (Fig. 3B, lanes 2 and 3). The CHO M19 clones also processed and secreted hu Aβ_{40} and hu Aβ_{42} in a ratio of about 10:1 and at a level reflecting total APPsw expression (Fig. 3B, lanes 5 and 6). These data demonstrate directly that the S2P-deficient CHO M19 cells are not deficient in γ-secretase activity.

Following the collection of the conditioned media used above, we incubated these cultures in serum-free media to confirm that the CHO M19 clones lack S2P expression. We found that all of the CHO M19 clones died in serum-free media (within 24–48 h) as would be expected for cholesterol auxotrophs, whereas the CHO WT clones remained attached and appeared healthy for many days (data not shown). Furthermore, we used RT-PCR to demonstrate that only the CHO WT clones express mRNA for the S2P (Fig. 4). α-Tubulin-specific primers were used as a positive control, and RT-PCR generated a product of the expected 700 base pairs in CHO WT (lanes 1-3) and CHO M19 clones (lanes 4-6). In contrast, the S2P specific primers led to the generation of a 790-base pair fragment in the CHO WT clones (lanes 7-9), but no product was generated from cDNA derived from any of the CHO M19 clones (lanes 10-12). Similarly, Northern blot analysis for S2P expression showed that CHO WT cells expressed the ~4-kilobase S2P transcript, CHO M19 cells were negative (data not shown).

**DISCUSSION**

S2P stands out among the many proteases suggested to be γ-secretase because it has several properties that make it an ideal candidate. Only S2P and γ-secretase have been postulated to cleave substrates within the membrane in vivo (6, 9, 10). S2P is widely expressed as is γ-secretase. S2P activity is (indirectly) enhanced by a transmembrane protein, SCAP, which is located in the endoplasmic reticulum (12), and γ-secretase activity appears to require another endoplasmic reticulum transmembrane protein, PS1 (17). We therefore directly tested the hypothesis that the γ-secretase is the same enzyme as the SREBP S2P (7). First we show that over-expression of the recently cloned S2P in cells expressing APPsw does not lead to any enhancement of the production of Aβ_{40} or Aβ_{42} (Fig. 2) suggesting that either the S2P is not the γ-secretase or that γ-secretase activity is not limiting. The immediate substrates...
of γ-secretase are the ~10- and ~12-kDa C-terminal fragments (CTFs) generated by α- and β-secretase cleavage of APP, respectively (see Fig. 1 and Ref. 30). Using antibodies that recognize these CTFs, we detect both of these substrates in the 293 APPswe-expressing cells (data not shown), thus it would appear that the generation of additional Aβ (and p3) by over-expression of the authentic γ-secretase could occur in these cells. Although we did not directly show that the S2P protein was enzymatically active in our transfected 293 cells, using a similar protocol, Rawson et al. (22) documented that S2P cleavage was restored and cholesterol auxotrophy of M19 cells was corrected in CHO cells expressing S2P. Because over-expression of the S2P mRNA in 293 cells did not lead to generation of additional Aβ, we conclude that it is unlikely to be the same enzyme as the γ-secretase.

Second, using S2P-deficient CHO M19 (9, 19, 20, 22) we show here that these cells process transfected hu APPswe to both Aβ40 and Aβ42. Neither the amount nor the ratio of Aβ40 or Aβ42 produced by the mutant CHO M19 clones is different from that produced by the CHO WT clones. Based on these experimental results we conclude that the SREBP S2P is not identical to the γ-secretase enzyme(s) responsible for the generation of the C termini of Aβ and Aβ42.

Although the similarities between γ-secretase and the S2P are intriguing, there are a few differences. First, the substrates have different membrane insertion topology; APP is a type I, single transmembrane protein (C terminus is cytosolic, N terminus is luminal) whereas SREBP has two transmembrane domains (both N and C termini are cytosolic). Second, although it is not known how PS1 and/or PS2 regulate the γ-secretase activity (direct versus indirect), it is clear that SCAP directly interacts with and regulates the SREBP site 1 protease (18); thus the S2P cleavage is only indirectly regulated by SCAP.

Perhaps, as originally suggested by Brown and Goldstein (7), the S2P and γ-secretase(s) are members of the same protease family. Rawson et al. (22) recently cloned the S2P, and although they found numerous related genes from different organisms (human, hamster, Sulfolobus sp., Drosophila sp., Caenorhabditis elegans, and Schistosoma mansoni), the S2P appears to be a novel member of a new family of metalloproteases. The cDNA encoding the S2P contains the signature sequence (HEXXH) of zinc metalloproteases; however it does not belong to any of the well-recognized protease families (31, 32). Analysis of the sequences suggests that the S2P is a polytopic membrane protein with four or five transmembrane domains, even the hydrophilic active site, HEXXH, appears to be buried in a very hydrophobic region of the protein. These features, along with a serine repeat of variable length (in different species) and a cysteine-rich domain, may be hallmarks of this new family. Finding S2P related proteases based on these motifs or by direct homology screening may yet provide a fruitful end to the search for the elusive γ-secretase(s).

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