Biochem. J. (1995) 311, 299–305 (Printed in Great Britain)

Lysosomal processing of amyloid precursor protein to Aβ peptides: a distinct role for cathepsin S

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To investigate the potential contribution of the lysosomal compartment in the processing of amyloid precursor protein (APP) to amyloid β-peptides (Aβs), we stably overexpressed a series of lysosomal proteases (the cysteine proteases, cathepsins B, L and S, and the aspartic protease, cathepsin D) in a human kidney epithelial cell line (293) transfected to express high levels of βAPP. Preliminary experiments indicated that 293 cells endogenously synthesize cathepsins B, L and D, but not cathepsin S. Aβ secretion was assessed by immunoprecipitation and ELISA and found to be increased ~2-fold following cathepsin S expression, but to be unchanged (cathepsins B, L) or decreased (cathepsin D) in the other double transfectants. E-64d, an inhibitor of lysosomal cysteine proteases, significantly reduced Aβ secretion by the cathepsin S transfectants, but had no effect on cells expressing the other proteases. Radiosequencing of Aβ secreted by cathepsin S-expressing cells revealed that a previously unreported variant beginning at Met −1 (relative to the most common Aβ N-terminus, Asp −1) accounted for most of the increase in Aβ secretion. Immunostaining of human brain sections revealed cathepsin S in cortical neurons and glia in samples of brain from patients with Alzheimer's disease. These results provide evidence in living cells for a pathway in which cathepsin S generates Aβ from amyloidogenic fragments of βAPP in the endosomal/lysosomal compartment. This pathway appears to be inducible, distinct from a constitutive pathway used by 293 and other cells to generate Aβ, and may be relevant to the pathogenesis of Alzheimer's disease.

INTRODUCTION

Alzheimer's disease (AD) is characterized by the presence of the 4 kDa amyloid β-peptide (Aβ) within plaques and vascular deposits of affected brains. Aβ is derived by proteolytic processing of an integral membrane protein, β-amyloid precursor protein (βAPP) [1], which is encoded by a gene on chromosome 21 and can exist in four major isoforms determined by alternative mRNA splicing (βAPP 563, 695, 751 and 770) [1–5]. After post-translational processing in the endoplasmic reticulum and Golgi, βAPP can undergo proteolysis within the Aβ domain by an enzyme termed α-secretase to generate a soluble form of βAPP (APP_s) [6,7] and an ~10 kDa membrane-associated C-terminal fragment [8]. Alternatively, βAPP, which contains a potential internalization sequence in its C-terminal cytoplasmic tail [9], can be re-internalized from the cell surface and degraded in the endosomal–lysosomal system, where multiple C-terminal fragments, some of which contain the entire Aβ region, can be detected in cultured cells and human cerebral cortex [10–12]. Although controversy exists as to whether Aβ is a direct cause of AD, there is ample circumstantial evidence implicating βAPP and Aβ in its pathogenesis, including the finding of early AD neuropathology in trisomy 21, in which the gene dosage and expression of βAPP are increased, and several mutations of the βAPP gene that result in early-onset, familial forms of AD [13–16].

Soluble Aβ is constitutively secreted by a number of cell types [17–19], and recent evidence implicates the endosomal compartment as a site of Aβ generation [20]. A related protein, p3, is also secreted; it is a C-terminal fragment of Aβ with an N-terminus at or adjacent to the α-secretase site. Numerous enzymes present in mammalian brain have been shown in experiments *in vitro* to have potential Aβ N-terminal cleaving ability, including multicatalytic protease [21], clipsin [22], a calcium-activated cathepsin G-like serine protease [23], metalloendopeptidase 24.15 [24] and cathepsin D [25]. Cathepsin G also has this activity [26] but is not detectable in human brain [27]. None of these proteases has actually been linked to Aβ secretion *in vivo*.

Most previously reported work examining specific candidate proteases in APP processing has employed either denatured APP or synthetic oligopeptide derivatives as model substrates. Because cleavage of these model substrates may not reflect the Aβ-generating potential of proteases *in vivo*, we have investigated the effects of a series of acidic lysosomal proteases (the cysteine proteases cathepsins B, L and S, and the aspartic protease cathepsin D) on Aβ production in an intact cell system. This was done by co-transfecting 293 cells to stably overexpress βAPP and a protease. Our results indicate that among the proteases tested, only cathepsin S is capable of generating Aβ variants under these *in vivo* conditions.

EXPERIMENTAL

Materials

E-64d was a gift from Dr. Masaharu Tamai, Taisho Pharmaceutical Co. Ltd., Ohmiya-shi, Japan. Bovine cathepsin S was from Dr. Robert Mason, Virginia Polytechnic Institute, Blacksburg, VA, U.S.A. Cathepsin B cDNA in the pBluescript

Abbreviations used: Aβ, amyloid β-peptide; AD, Alzheimer's disease; βAPP, β-amyloid precursor protein; DMEM, Dulbecco's modified Eagle's medium; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; E-64d, ethyl-(+)-(2*S*,3*S*)-3-[(*S*)-3-methyl-1-(3-methylbutylcarbamoyl)-butylcarbamoyl]-2-oxiranecarboxylate; MBP, maltose-binding protein; p3, the C-terminus of Aβ; RT, room temperature; TBS, Tris-buffered saline.

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SK⁻ vector (Stratagene) was obtained from Dr. Bonnie Sloane, Wayne State University, Detroit, MI, U.S.A. JPM-565 was from Dr. Daniel Rich, and was synthesized and iodinated as described [28].

Co-expression of cathepsins and βAPP in K293 cells

Kidney 293 cells stably expressing βAPP 695 or the V642I mutant form of βAPP 695 were as described and were maintained under G418 selection [8,29]. Cathepsin S cDNA in pcDNA-I (Invitrogen) [28] was excised by *Kpn*I and *Xho*I digestion and inserted into the same restriction sites of pCEP4 (Invitrogen); the construct was linearized by *Stu*I digestion. A cathepsin B cDNA in pBluescript was removed with *Bam*HI digestion and inserted into the *Bam*HI site of pCEP4 and linearized by digestion with the restriction enzyme Asp 700 (Boehringer-Mannheim). Cathepsin D and cathepsin L cDNAs were obtained by PCR using reverse-transcribed human alveolar macrophage RNA [28] and primers that encoded regions including the start methionines and stop codons. The sequences were confirmed by sequencing. Cathepsin D and L PCR products were subcloned into the pCR II vector (TA cloning system, Invitrogen) according to the manufacturer's instructions. Cathepsin L and D cDNAs in the appropriate orientations were excised by *Xho*I and *Hin*dIII digestion and ligated into the same restriction sites of the pCEP4 vector. Cathepsin D was cloned into pCEP4 in both sense and antisense orientations. 293 cells expressing a βAPP cDNA were grown to ~50% confluence, suspended at 2×10^6/ml in Dulbecco's modified Eagle's medium (DMEM) on ice, electroporated in 0.4 cm cuvettes with 0.5 ml of cell suspension and 5 μg of DNA at 250 V and 250 μF, placed on ice for 10 min, then added to complete medium (DMEM containing 10% fetal-calf serum, penicillin, streptomycin and 800 μg/ml G418). After 48 h, the medium was changed and hygromycin B added at 100 μg/ml. Cysteine protease transfectants were analysed ~2 weeks after transfection by active-site radiolabelling with ^{125}I-JPM-565 (an iodinated analogue of E-64), followed by SDS/PAGE and autoradiography, as described previously [28], using aliquots of 10^5cells. To assess cathepsin D expression, transfectants were analysed ~2 weeks after transfection by Western blotting and pepstatin-inhibitable degradation of ^{125}I-fibrin, as follows. Aliquots of 10^6 cells were washed once with PBS, lysed directly in reduced SDS/PAGE sample buffer, passed six times through a 25-gauge needle to shear DNA, and electrophoresed on a 12% acrylamide gel [28]. Protein in gels was transferred to nitrocellulose using a Pharmacia 2117 Multiphor II semi-dry electrophoresis unit according to the manufacturer's recommendations. Blots were blocked in 5% non-fat dry milk in PBS for 1 h, incubated with polyclonal rabbit IgG against human cathepsin D (Vital Products, Inc.) at 1:500 dilution in PBS/milk for 1 h, washed three times with PBS/milk, incubated with ^{125}I-labelled goat polyclonal antibody to rabbit IgG (DuPont–New England Nuclear) at 1:1000 dilution in PBS/milk for 1 h, then washed three times in PBS/milk and three times in PBS, followed by autoradiography. Cathepsin D proteolytic activity was assessed by degradation of ^{125}I-fibrin as described [30] with the following modifications. Cells were washed in PBS, then lysed at 6.67×10^6 cells/ml in 1% Triton X-100, 1 mM EDTA, 100 mM sodium acetate, pH 3.5, 50 mM NaCl, containing 100 μM E-64 and 20 mM PMSF, on ice for 20 min. Lysates were clarified by centrifugation, and aliquots of 50 μl were treated with pepstatin (1 μM) or an equivalent volume of dimethyl sulphoxide vehicle, followed by incubation with 39.5 μg of ^{125}I-fibrin (specific radioactivity 7600 c.p.m./μg). Trichloroacetic acid-soluble c.p.m. after 60 min incubation were measured with a gamma counter. Cathepsin D activity is defined as difference in c.p.m. for samples without and with pepstatin, after correction for c.p.m. at time zero.

Characterization of Aβ production by immunoprecipitation, ELISA and radiosequencing

Cell monolayers in 10-cm-diam. tissue-culture dishes were metabolically labelled overnight with 300 μCi of [^{35}S]methionine (~1000 Ci/mmol) or 2.5 mCi [^{3}H]phenylalanine (100 Ci/mmol) in 5 ml of medium specifically deficient in the respective amino acids and in the presence of 10 μM E-64d or diluent. The polyclonal antibody R1280 [31], generated against synthetic $A\beta_{1-40}$, was used for immunoprecipitation from [^{35}S]methionine- or [^{3}H]phenylalanine-labelled lysates or conditioned media as described [17]. Immunoblotting of cell lysates using the antibody designated αC7 [8,32], which recognizes amino acids 676–695 of βAPP [1], was as described [33], after overnight incubation with E-64d or diluent. ELISA of Aβ in conditioned medium was as described [18]; transfected 293 cells were placed in 35-mm-diam. wells, cultured to confluence, then washed with DMEM followed by addition of fresh medium containing E-64d (10 μM final concentration, from 10 mM stock in dimethyl sulphoxide) or diluent. Media were collected after 24 h and assayed in blind fashion. Cells were then detached by treatment with trypsin and EDTA and counted. Statistical significance was determined by Wilcoxon signed rank test (for paired samples, ±E-64d treatment) or Wilcoxon rank sum test. Immunoprecipitated, [^{3}H]phenylalanine- or [^{35}S]methionine-labelled Aβ was blotted on to poly(vinylidene difluoride) membrane and sequenced essentially as described in [17]. ^{3}H or ^{35}S activity for each cycle of sequencing was determined by liquid scintillation counting. For a given experiment, the proportion of Aβ existing as the Met −1 species was calculated by dividing the c.p.m. in cycle 5 (Met −1) by the total c.p.m. in cycles 4 and 5.

Anti-cathepsin S antibody

A cathepsin S–maltose binding protein (MBP) fusion protein was used to immunize rabbits as described previously [34]. The resulting antiserum recognizes a single ~28 kDa band in our gel system that co-migrates with cathepsin S (as detected by active-site radiolabelling) in immunoblots of lysates of human alveolar macrophages and cathepsin S-transfected 293 cells, but not in immunoblots of lysates of untransfected 293 cells. Antiserum was purified by adsorption to cathepsin S–MBP blotted to nitrocellulose after separation by SDS/PAGE, followed by elution at low pH, as described in [35]. Specificity of the resulting antibody preparation was determined by adsorbing the purified antibody with cathepsin S–MBP or cathepsin L–MBP [34] blotted on to nitrocellulose, followed by immunoblotting of cell lysates containing cathepsin S as above; adsorption to cathepsin S, but not cathepsin L, abrogated the ability of the purified antibody to recognize cathepsin S.

Localization of cathepsin S by immunofluorescence and immunohistochemistry

Immunofluorescence studies were performed on βAPP 695 and βAPP 695/cathepsin S transfectants that were plated on poly(L-lysine)-coated 12 mm² coverslips, cultured for 24 h, washed with Tris-buffered saline (TBS; 20 mM Tris, 150 mM NaCl, pH 7.4) and fixed with methanol at −20 °C for 5 min. Cells were washed three times with TBS and incubated with blocking solution (10% goat serum in TBS) for 1 h at room temperature. Blocking

solution was removed and primary antibody (1:100 affinity-purified rabbit antiserum to cathepsin S in blocking solution) applied for 1 h at room temperature (RT). Cells were washed five times with TBS, incubated with 1:800 fluorescein isothiocyanate-conjugated polyclonal goat IgG raised against rabbit IgG (Boehringer-Mannheim) for 1 h at RT, and washed five times with TBS. Coverslips were mounted with Fluorosave reagent (Calbiochem) on to glass slides and stored in the dark at 4 °C.

Immunohistochemical analysis of human brain autopsy tissue was carried out using briefly fixed (1 h in 10 % buffered formalin), paraffin-embedded 8-μm-thick sections of hippocampus. Sections were baked at 56 °C for 1 h before being deparaffinized in Histoclear (National Diagnostics, Atlanta, GA, U.S.A.) and rehydrated. Endogenous peroxidase activity was quenched with 0.3 % hydrogen peroxide in methanol for 20 min. Sections were washed in water and treated with 80 % formic acid for 10 min at RT. After rinsing in water, the sections were incubated in a blocking solution of 10 % goat serum in TBS for 20 min. Affinity-purified rabbit anti-cathepsin S antibody (1:25–1:100) was applied for either 1 h at RT or overnight at 4 °C. The horseradish peroxidase avidin–biotin complex system (rabbit Elite ABC kit, Vector Laboratories, Burlingame, CA, U.S.A.) and diaminobenzidine were used to visualize bound antibody. Sections were counterstained with haematoxylin, dehydrated, cleared in Histoclear and coverslipped with Permount.

RESULTS

Expression of lysosomal proteases

293 cells expressing βAPP 695 were transfected with cDNAs encoding the cysteine proteases cathepsins B, L and S, then lysed and subjected to active-site radiolabelling with an iodinated E-64 analogue [28]. Cathepsins L and S were expressed at high levels in cells (Figure 1a, lanes 2,4 and 5), but polyclonal cathepsin B transfectants did not demonstrate expression above control levels (results not shown). Transfection of 293/βAPP 695 cells with cathepsin B cDNA was repeated, followed by screening of individual clones by active-site radiolabelling. Three of 20 clones expressed cathepsin B at levels moderately above control, and one of these was selected for study (Figure 1a, lane 3). Active-site radiolabelling of an equivalent number of human alveolar macrophages is shown for comparison (Figure 1a, lane 6).

Labelling of 293/βAPP 695 cell lysates yields four cysteine protease bands, at 42–43 kDa (doublet), 39 kDa, 36 kDa and 33 kDa (Figure 1a, lane 1). Overexpression of cathepsins B and L (Figure 1a, lanes 3 and 5) allows identification of the 39 and 33 kDa bands as cathepsin B (procathepsin and single-chain mature form, respectively), and the 42–43 kDa and 36 kDa bands as cathepsin L (procathepsin and intermediate form, respectively), in agreement with prior reports [36–38]; note that cathepsin S is not detectable except with cathepsin S transfection. The fully processed single-chain (30 kDa) and two-chain (25 and 5 kDa) forms of cathepsin L do not incorporate label, as previously reported [37]. Overexpression of cathepsins B and S results in a loss of procathepsin L (Figure 1a, lanes 1–3), suggesting that cathepsins B and S, in addition to cathepsin L itself [39], process cathepsin L precursors. Labelled cathepsin S migrates at 28 kDa, as described previously [28], rather than at 24 kDa as reported by others [40]; its identity was confirmed by immunoblotting and co-migration of active-site-labelled human cathepsin S and purified bovine cathepsin S in our gels (results not shown).

Cathepsin D transfection was confirmed by immunoblotting of cell lysates, shown in Figure 1(b). Immature forms of 51, 44 and 34 kDa, and the mature 31 kDa form [41], are increased in

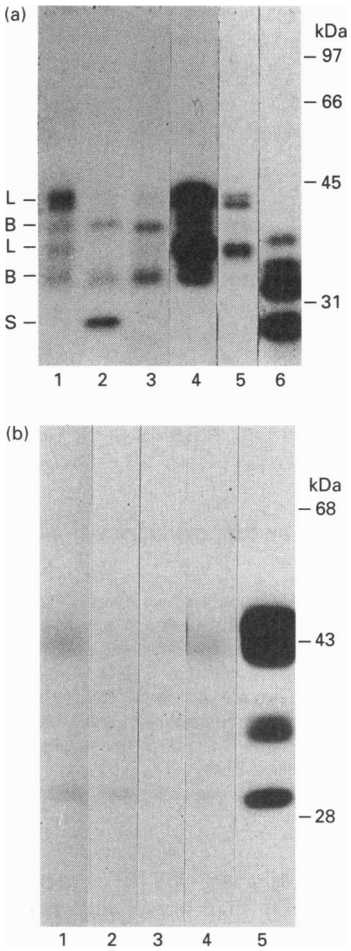

Figure 1 Transfection of βAPP 695-overexpressing 293 cells with lysosomal proteases

(**a**) Active-site labelling of cysteine proteases. Aliquots of 10^5 cells were permeabilized with Triton X-100 at pH 5 and incubated with an iodinated analogue of E-64, followed by SDS/PAGE on 12% gels and autoradiography. Lane 1, pCEP4 vector alone; lane 2, transfection with cathepsin S cDNA; lane 3, cathepsin B cDNA; lane 4, cathepsin L cDNA; lane 5, cathepsin L cDNA, short exposure; lane 6, human alveolar macrophage lysate. Exposure times: lanes 1–4 and lane 6, 5 days; lane 5, 1 day. Positions of cathepsins B, L and S are indicated. (**b**) Cathepsin D immunoblotting. Lysate of 10^6 cells was run on SDS/12%-PAGE, transferred to nitrocellulose, and incubated with polyclonal rabbit antiserum to human cathepsin D, then with iodinated secondary antibody. Exposure time: 3 days. Lane 1, no protease transfection; lane 2, cathepsin L transfection; lane 3, cathepsin S transfection; lane 4, transfection with cathepsin D in antisense orientation; lane 5, cathepsin D, sense orientation.

the cathepsin D transfectants (Figure 1b, lanes 1 and 5). Expression of cathepsins S and L, and transfection with an antisense cathepsin D cDNA, reduced levels of the two major forms minimally or not at all (Figure 1b, lanes 2–4). Cathepsin D activity was measured by the ability of cell lysates to solubilize iodinated fibrin at pH 3.5 in a pepstatin-inhibitable manner [30]. Cathepsin D lysates degraded 560 ng of fibrin/h per 10^6 cells, which was a 16-fold increase over 293/βAPP 695 cells.

Influence of lysosomal protease expression on Aβ secretion and APP processing

The effect of protease expression on Aβ secretion was assessed by immunoprecipitation of conditioned media (Figure 2). For cells overexpressing a cysteine protease, parallel experiments were

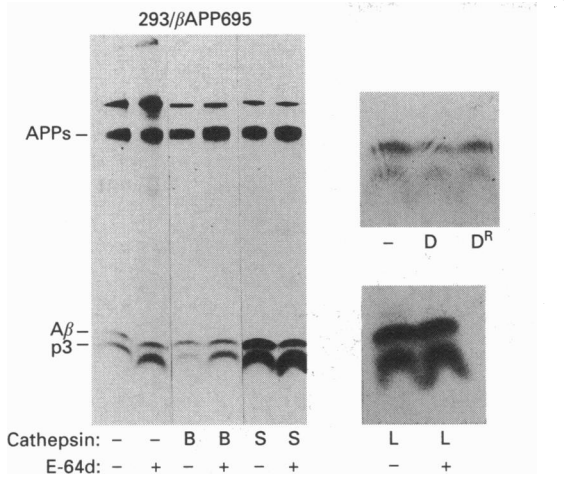

Figure 2 Aβ secretion by cells overexpressing βAPP 695 and specific lysosomal proteases

Conditioned media were immunoprecipitated with an antibody (R1280) raised against $A\beta_{1-40}$. Confluent 10 cm dishes of cells were labelled overnight with [^{35}S]methionine, with or without E-64d, followed by immunoprecipitation of conditioned media, SDS/PAGE and autoradiography. Main panel, cells transfected with cathepsin B or S cDNA as indicated. Insets on right-hand side: Aβ and p3 bands only from additional immunoprecipitations. Lane 1, cells without protease transfection; lane 2, cathepsin D transfection; lane 3, transfection with cathepsin D cDNA in antisense orientation; lanes 4 and 5, parallel samples of cells transfected with cathepsin L, with (lane 5) or without E-64d.

done with or without 10 μM E-64d [42], a membrane-permeable analogue of E-64 [43] that irreversibly inactivates cysteine proteases. Transfection with cathepsin B did not change the level of Aβ secretion seen in 293/βAPP 695 cells, and in both the untransfected and transfected lines, Aβ secretion was not significantly influenced by cysteine protease inhibition. In contrast, 293/βAPP 695 cells expressing cathepsin S produced an increased amount of Aβ, and this Aβ secretion was reduced by cysteine protease inhibition. These findings were confirmed in at least three separate experiments for each set of cells. Aβ secretion by cells overexpressing cathepsin L was not inhibited by E-64d, and cells overexpressing cathepsin D produced Aβ at levels equivalent to those of 293/βAPP 695 cells with or without control transfection (antisense cathepsin D cDNA) (Figure 2). Overexpression, or inhibition, of the various cysteine proteases had no significant effect on the level of p3 release (Figure 2).

Conditioned media were collected from the same cells and assayed for Aβ by ELISA [18]. Only cathepsin S expression resulted in an increase in Aβ secretion above control levels, and this increase was largely inhibited by E-64d (Table 1). In a separate set of experiments, Aβ secretion was significantly reduced in 293/βAPP 695 cells overexpressing cathepsin D, compared with 293/βAPP 695 cells, or cells transfected with control or cathepsin L cDNA (Table 1). Note that baseline Aβ secretion varied between sets of experiments, which were done in different batches over several months; thus, results are grouped to combine experiments performed at similar time periods. The ~ 2-fold increase in Aβ secretion caused by cathepsin S expression was seen in four sets of experiments, two of which are shown in Table 1, with baseline Aβ secretion by 293/βAPP 695 cells ranging from 0.6 to 2.5 ng/10^6 cells per 24 h.

To assess further the effect of cathepsin S expression on processing of APP, immunoblots of cell lysates probed with an antibody against the C-terminus of APP [32] were done to

Table 1 Results of ELISA determination of Aβ secretion in APP-transfected 293 cells co-transfected with specific cathepsins

APP-expressing cells were co-transfected with various cathepsins as indicated and cultured overnight in the absence or presence of E64d. The results represent experiments done in three different time periods. Each data point represents the mean ± S.D. of at least three sets of cells.

Enzyme co-transfected	E64d (10 μM)	Aβ secretion (ng/10^6cells per 24 h)
Experiment 1		
None	–	0.78 ± 0.26
	+	0.77 ± 0.30
Cathepsin B	–	0.59 ± 0.07
	+	0.56 ± 0.08
Cathepsin L	–	0.56 ± 0.03
	+	0.41 ± 0.04
Cathepsin S	–	1.50 ± 0.37*
	+	1.06 ± 0.31**
Experiment 2		
None	–	2.25 ± 0.66
	+	2.11 ± 0.76
Cathepsin S	–	3.93 ± 0.86*
	+	2.39 ± 0.79**
Experiment 3		
None	–	1.57 ± 0.5
Cathepsin L	–	1.57 ± 1.2
Antisense cathepsin D		1.60 ± 1.1
Cathepsin D		0.79 ± 0.05*

* Significantly different ($P < 0.05$) from control cells.
** Significantly different ($P < 0.05$) between without and with E64d.

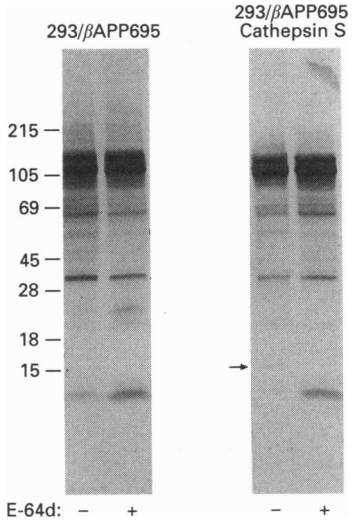

Figure 3 Immunoblotting of cell lysates with antibody C7, which recognizes C-terminal fragments of APP

Cells were incubated overnight with or without E-64d inhibition, then lysed. Equal amounts of protein were added per lane. Protease and APP transfections, and E-64d treatments, were as indicated. Development of blots was with an alkaline phosphatase system. Similar results were seen in at least three experiments.

examine the pattern of cell-associated APP and C-terminal fragments thereof. Figure 3 displays results for cells expressing βAPP 695 with and without cathepsin S co-transfection. In both

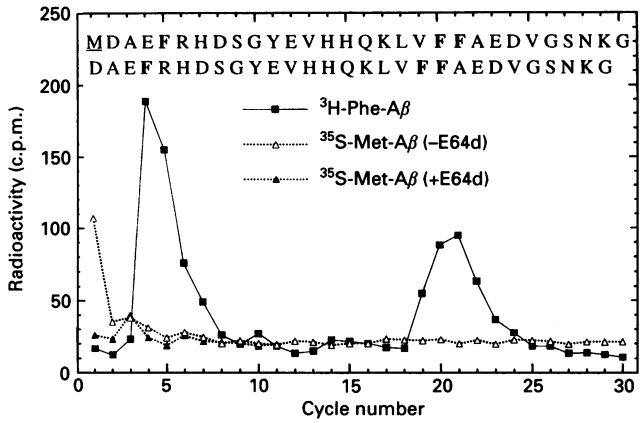

Figure 4 Radiosequencing of immunoprecipitated Aβ peptides, as described in the Experimental section

Plots show c.p.m. obtained from radiosequencing of [^{3}H]phenylalanine- and [^{35}S]methionine-labelled, immunoprecipitated Aβ bands from cells expressing cathepsin S and wild-type APP. [^{35}S]Methionine c.p.m. are shown for Aβ obtained from cells incubated with or without E-64d. The c.p.m. in cycle 5 ([^{3}H]phenylalanine-labelled Aβ) and in cycle 1 ([^{35}S]methionine-labelled Aβ, without E-64d) are as expected for an Aβ peptide starting at Met −1. Sequences of the Met −1 and Asp 1 Aβ species are shown above, aligned to illustrate that the sequencing cycles at which [^{3}H]phenylalanine and [^{35}S]methionine activity were observed correspond to the positions in the two peptides at which the respective residues are known to occur.

cell lines, E-64d causes an increase in the ~ 10 kDa fragment resulting from α-secretase action, analogous to previous results using leupeptin [17] and suggesting that lysosomal cysteine proteases are involved in degradation of this fragment. Similar results were seen in cathepsin B- and L-expressing cells (results not shown). In cells expressing cathepsin S, levels of the ~ 10 kDa fragment are reduced and return to control values with E-64d inhibition. In addition, a faint C-terminal band at 15 kDa (which should contain the entire Aβ region) is seen in the cathepsin S-expressing cells and is abolished by treatment with E-64d.

Radiosequencing of Aβ peptides

To determine the N-termini of Aβ peptides released by cells expressing βAPP 695 and cathepsin S, radiosequencing of immunoprecipitated Aβ was done. Radiosequencing of the [^{3}H]phenylalanine-labelled, immunoprecipitated Aβ (Figure 4) revealed a ^{3}H peak at cycle 4, consistent with the previously described predominant Aβ N-terminus Asp 1 [17], and also a peak at cycle 5 consistent with an Aβ species starting at Met −1. In the experiment shown in Figure 4, the Met −1 species is calculated to be 45% of the total Aβ. In two additional experiments, the Met −1 peptide represented 27% and 37% of the total Aβ (results not shown), resulting in a mean value of 36% (S.D., 9%). It should be noted that these calculations assume no spillover of ^{3}H from cycle 4 into cycle 5. To the extent that some spillover does occur, then the calculated fractions of Met −1 peptide to the total Aβ could be overestimated. To confirm the existence of a Met −1 N-terminus, we radiosequenced [^{35}S]methionine-labelled Aβ from the same cells incubated in the presence or absence of E-64d. The peak obtained at cycle 1 (Figure 4) indicates a Met −1 N-terminus, and disappears after treatment with E-64d as expected.

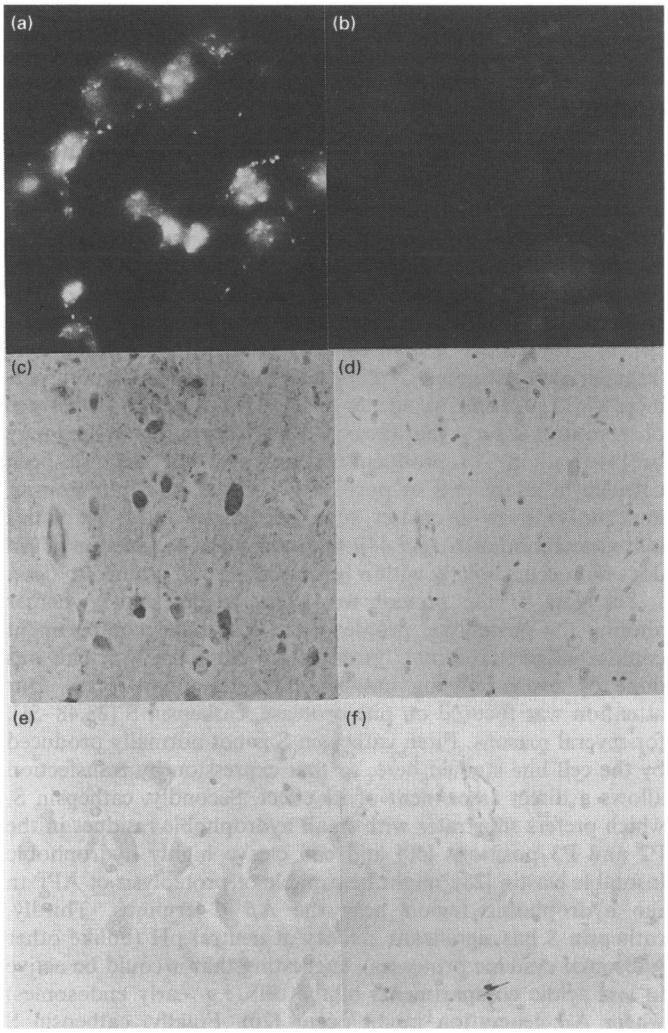

Figure 5 Immunostaining with polyclonal anti-cathepsin S antibodies

(**a**) and (**b**) Immunofluorescent staining of 293 cells expressing βAPP 695, with and without cathepsin S co-transfection respectively, using polyclonal, affinity-purified cathepsin S antibodies. Only cathepsin S-transfected cells show immunoreactivity. (**c**) and (**d**) Immunohistochemistry of hippocampus from AD brain. Reaction is seen in some AD neurons and glia (**c**); Specificity was confirmed by blocking the staining by adsorption of the antibody to nitrocellulose-bound recombinant cathepsin S prior to use (**d**), as described in the Experimental section. (**e**) and (**f**) Immunohistochemistry of hippocampus from brain of a 60-year-old female without AD pathology.

Immunohistochemical detection of cathepsin S in human brain

Immunofluorescent staining of 293 cells before and after cathepsin S transfection revealed prominent cathepsin S antigen expression only in cathepsin S transfectants, in a granular, perinuclear distribution suggestive of a lysosomal localization (Figures 5a and 5b). Immunostaining of multiple cases of AD brain revealed staining of variable numbers of cortical neurons and glial cells in all cases. A representative example of hippocampal neuronal staining is shown in Figure 5(c). Staining was markedly attenuated by adsorption of affinity-purified antibody to cathepsin S fusion protein (Figure 5d) but not by adsorption to cathepsin L fusion protein (results not shown). In contrast, little or no cortical cellular staining was seen in samples of normal aged brain. Representative cathepsin S immunostaining

of sections of a normal 60-year-old brain are shown in Figures 5(e) and 5(f).

DISCUSSION

Accumulating evidence suggests that secretion and deposition of Aβ peptides, which are derived by normal constitutive proteolytic processing of APP, are directly linked to the pathogenesis of AD (reviewed in [46,47]). A major gap in current knowledge of this process is an understanding of the specific proteases that generate Aβ and the cellular compartment(s) in which this occurs. APP is reinternalized from the cell surface to the endosomal–lysosomal system [10] where a number of potentially amyloidogenic fragments are generated [11,12], and inhibition of acidic compartments with alkalinizing agents reduces Aβ secretion [19]; these observations suggest that the endosomal–lysosomal system may be involved in Aβ production. However, Aβ has not been detected in intact cells or purified lysosomes, and inhibitors of multiple lysosomal proteases, such as leupeptin and E-64, do not inhibit secretion of Aβ [19,44], suggesting that production of Aβ does not occur directly within lysosomes in cells studied to date.

The goal of the present work was to determine whether altering the proteolytic profile of the lysosomal compartment could result in endosomal/lysosomal production of Aβ. This was done by overexpressing individual lysosomal proteases. Our attention was focused on one protease, cathepsin S [28,48–51], for several reasons. First, cathepsin S is not normally produced by the cell line studied here, so that expression by transfection allows a direct assessment of its effect. Secondly, cathepsin S, which prefers substrates with small hydrophobic residues in the P2 and P3 positions [40] and can cleave highly hydrophobic insoluble elastin [28], might be capable of proteolysis of APP in the hydrophobic region near the Aβ C-terminus. Thirdly, cathepsin S has significant activity at neutral pH (unlike other lysosomal cysteine proteases), suggesting that it could be active in less acidic compartments of the cell (e.g. early endosomes) where Aβ generation might occur [20]. Finally, cathepsin S appears to be a regulated and inducible gene product (in contrast to ubiquitous lysosomal proteases such as cathepsins B, L and D) [34] that has been detected in adult rat brain [48,52]. Thus, if cathepsin S is capable of generating Aβ, its potential dysregulation or induction in the brain could plausibly be linked to the pathogenesis of β-amyloid deposits in AD.

Our results indicate that cathepsin S is capable of augmenting Aβ secretion by 293 cells above basal, constitutive levels (Figure 2a). Quantification of Aβ secretion by ELISA of conditioned media reveals that this increase is $\sim$2-fold over cells not expressing cathepsin S (Table 1), and that this augmentation is largely blocked by the cysteine protease inhibitor E-64d (in contrast, Aβ secretion is not affected by E-64d in cells not expressing cathepsin S). Our results are consistent with the generation of Aβ in endosomes/lysosomes by cathepsin S in a manner that is distinct from the constitutive pathway in these cells, but cannot exclude an indirect mechanism, such as proteolytic activation of another protease in the cell. Furthermore, this enhancement of Aβ generation is specific for cathepsin S among the cathepsins we examined. Overexpression of cathepsins B, L and D does not increase Aβ secretion, and in the case of cathepsin D results in decreased Aβ secretion (Figure 2, Table 1).

Radiosequencing of Aβ secreted by cathepsin S-expressing cells reveals, in addition to the most common Asp 1 peptide, a novel species beginning at Met -1 (relative to Asp 1 of the predominant Aβ species). Met -1 Aβ has not been described previously [53], although other Aβ variants have been noted (e.g., Val -3, Ile -6, Arg 6 and Glu 11) [17,18,54,55]. There is recent evidence that there are cell-specific differences in Aβ cleavage: in Madin–Darby canine kidney cells, Aβ is found to be 80% Arg 5, 10% Val -3 and 10% Asp 1 [54]. Because the ELISA data show that cathepsin S augments total Aβ production $\sim$2-fold and the radiosequencing indicates on average that the Met -1 peptide accounts for about one-third of the total Aβ, the fraction of Met -1 species can only account for about two-thirds of the increase in Aβ ascribed to cathepsin S. This suggests that there is either some production of Asp 1 peptide by cathepsin S or some conversion of Met -1 Aβ to the Asp 1 species by an endogenous methionine aminopeptidase activity. Cathepsin S is not predicted to hydrolyse the Met -1/Asp 1 amide bond, as this cleavage would require lysine in the S2 position; cathepsin S preferentially cleaves substrates with small, hydrophobic residues at P2 and P3 [40]. Cathepsin L, which most closely resembles cathepsin S in sequence and substrate specificity [28,52], has been reported to cleave a synthetic analogue of the Aβ N-terminus at the Lys -2/Met -1 bond [56].

We also investigated the effect of cathepsin S transfection on 293 cells overexpressing a βAPP mutant (Val 642 $\rightarrow$ Ile) [29] associated with early-onset, familial AD. We reasoned that an alteration in amino acid sequence at the Aβ N-terminus might enhance its degradation by cathepsin S. However, the increase in Aβ secretion that we observed in such doubly transfected cells was also $\sim$2-fold (results not shown).

This is the first description of a means to increase Aβ secretion in cells expressing equal amounts of βAPP. Although the observed increase is modest, the facts that a βAPP mutation associated with early-onset, familial AD results in a 5–8-fold increase in Aβ secretion in transfected cells [57,58], and that trisomy 21 (in which there is a 50% increase in βAPP gene dosage) is associated with early AD pathology, suggest that changes in Aβ secretion of the magnitude produced by cathepsin S could be relevant to increased Aβ accumulation in the brain. The Met -1 form of Aβ has not been detected in clinical samples of AD brain; thus, if the cathepsin S pathway described here is operative *in vivo*, there must be additional proteolytic processing of the Met -1 form.

The extent of expression of cathepsin S in normal and diseased human brain has not previously been investigated. Cathepsin S mRNA is expressed in rat brain, probably predominantly in microglia [45,52], consistent with its expression in human monocytes and lung macrophages [28,34]. Northern blotting of normal human brain RNA, however, does not reveal detectable cathepsin S [34]. We used an affinity-purified polyclonal antibody to cathepsin S to examine cathepsin S expression in human brain. This antibody preparation recognizes cathepsin S, but not cathepsins B, L or H, on immunoblots of lysates of human alveolar macrophages, and demonstrates cathepsin S expression in lung macrophages by immunostaining techniques essentially identical to those used here [34]. Also, the antibody confirms expression of cathepsin S in transfected 293 cells in a pattern consistent with a lysosomal distribution (Figure 5). As expected, there was little or no detectable cathepsin S in normal brain. In contrast, focal cellular immunostaining of cortical neurons and glial cells was found in AD brain (Figure 5). Although glial expression is not unexpected, the prominent neuronal immunostaining is unanticipated. These data do not establish sites of cathepsin S synthesis, and it is possible that neurons have internalized cathepsin S produced by other cells. An extensive immunohistochemical analysis of cathepsin S expression in AD and other disease states will be published separately [59]. In summary, our data indicate that an alternative, lysosomal pathway of Aβ production can exist in cells expressing cathepsin S. Because cathepsin S has characteristics of an inducible gene

(e.g. multiple predicted AP-1 binding sites and a consensus heat shock element sequence at -168 to -145 bp, [34]) and appears to be up-regulated in AD brain (Figure 5), we propose that this is an inducible lysosomal pathway that may play a role in the course of β-amyloidosis in AD.

We thank Dr. Carmen Vigo-Pelfrey and Dr. Dale Schenk of Athena Neurosciences, for performing the ELISA assays; Dr. Bonnie Sloane for her generous gift of the cathepsin B cDNA; and Dr. Robert Mason for the kind gift of purified bovine cathepsin S as well as helpful discussions. We are also grateful to Lisa Natkin for excellent technical assistance. This work was supported by National Institutes of Health Grants HL48261, HL44712 and AG 06173, and a Parker B. Francis Fellowship (J. S. M.).

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Received 17 October 1994/11 May 1995; accepted 26 May 1995