A Novel Metalloprotease in Rat Brain Cleaves the Amyloid Precursor Protein of Alzheimer's Disease Generating Amyloidogenic Fragments†

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ABSTRACT: The amyloid protein (Aβ or βA4) is the major constituent of amyloid plaques in the Alzheimer's disease brain. Aβ is cleaved from the amyloid precursor protein (APP) by a mechanism which is poorly understood. Cell culture studies suggest that APP may be cleaved by secretases within the late Golgi compartment. Studies performed so far have mainly used exogenous APP and synthetic peptides as substrates. For this study, a Golgi and plasma membrane-enriched fraction was isolated from rat brain and incubated at 37 °C at pH 7.2 to study the degradation of endogenous APP. The breakdown of APP was accompanied by the concomitant generation of Aβ-containing C-terminal fragments, in a time-dependent fashion. The metal ion chelators EDTA and 1,10-phenanthroline inhibited this degradation. The inhibition by EDTA was reversed by 50 μM Zn^{2+} but not by other metal ions. The protease activity was not inhibited by cysteine, serine or aspartic protease inhibitors nor was it inhibited by compounds which are inhibitors of known metalloendopeptidases and matrix metalloproteinases (cFP, phosphoramidon and TIMP-2). Our data suggest that a novel Zn^{2+}-dependent metalloprotease activity associated with a Golgi and plasma membrane-enriched fraction can degrade endogenous APP to generate Aβ containing C-terminal fragments. This protease may generate amyloidogenic fragments of APP which may serve as precursors for Aβ.

The 4 kDa amyloid protein Aβ[1] (or βA4) is the major constituent of amyloid plaques that are observed in the cortex and hippocampus of the brain of individuals affected with Alzheimer's disease (Price et al., 1991). Aβ arises from proteolytic cleavage of the amyloid precursor protein, APP (Kang et al., 1987), through the activity of proteases termed secretases which remain to be characterized [for reviews see Evin et al. (1994) and Checler (1995)].

α-Secretase, the major enzyme which frees APP from the membrane, cleaves within the Aβ sequence, resulting in an N-terminal secreted fragment (sAPPα) and a membrane-associated C-terminal derivative which contains only a portion of the Aβ sequence (Esch et al., 1990; Oltersdorf et al., 1990; Sisodia et al., 1990). β-Secretase cleavage occurs at the N-terminus of the Aβ sequence and also results in C-terminal fragments (Esch et al., 1990; Estus et al., 1992; Golde et al., 1992; Haass et al., 1992a; Seubert et al., 1993; Cheung et al., 1994; De Strooper et al., 1995). C-terminal fragments accumulating from both of these cleavages (α and β) are potentially amyloidogenic. The fragments are further processed by the action of a γ-secretase which releases the C-terminus of Aβ. This processing results in 4 kDa (Aβ) and 3 kDa (p3) products, generated in association with β- and α-secretase cleavages, respectively (Haass et al., 1992a; Shoji et al., 1992; Busciglio et al., 1993; Koo & Squazzo, 1994; Fuller et al., 1995). Other minor cleavages have been observed near the membrane in the vicinity of the β-secretase cleavage site (Haass et al., 1992b, 1994; Busciglio et al., 1993; Cheung et al., 1994; Simons et al., 1996). It is now clear that Aβ is also produced by normal cellular metabolism (Haass et al., 1992b, 1993; Shoji et al., 1992; Busciglio et al., 1993; Suzuki et al., 1994; Fuller et al., 1995).

APP may be processed through several pathways, and numerous studies with cell culture systems have tried to define the exact compartmentation of the different secretases. α-Secretase activity may be associated with the plasma membrane (Maruyama et al., 1991; Sisodia, 1992; De Strooper et al., 1993; Arribas et al., 1996). However, many other studies have demonstrated that processing of APP can occur intracellularly in the late Golgi compartment or in

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[1] Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; APLP2, amyloid precursor-like protein 2; Aβ, the amyloid protein obtained from APP cleavage; cFP, cFP-AAY-pAB(*N*-[1(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate); MBP, myelin basic protein; PVDF, polyvinylidene difluoride; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PAP, peroxidase anti-peroxidase; ECL, enhanced chemiluminescence; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid, disodium salt; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane; NEM, *N*-ethylmaleimide; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; TPCK, tosylphenylalanyl chloromethyl ketone; TIMP-2, tissue inhibitor of metalloproteinase-2.

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secretory vesicles (Esch et al., 1990; Sambamurti et al., 1992; De Strooper et al., 1993; Kuentzel et al., 1993). Evidence for γ-secretase activity has been observed both in the endocytic pathway (Koo & Squazzo, 1994) and the late secretory pathway (Busciglio et al., 1993). Overall, there is substantial evidence to suggest that the Golgi compartment may be involved in APP processing (Caporaso et al., 1992, 1994; Busciglio et al., 1993; Haass et al., 1993; Kuentzel et al., 1993; Xu et al., 1995).

Preparations of crude brain homogenates are frequently used as starting material to locate candidate proteases which may participate in APP processing. A major obstacle in identifying Golgi-associated proteases in crude homogenates is the presence of abundant and very active lysosomal proteases which may mask more specific secretases. In addition, synthetic peptides and exogenous APP, when used as substrates to study APP processing, may only partially mimic the actual events *in vivo*. To overcome these problems, we isolated a Golgi and plasma membrane-enriched fraction from rat brain homogenates and demonstrated that it contained APP. Incubation of this isolated membrane fraction was accompanied by degradation of the endogenous APP, suggesting co-localization of APP with a protease. The APP fragments generated contained Aβ and were potentially amyloidogenic. The protease was identified as a novel Zn^{2+}-dependent metalloprotease.

MATERIALS AND METHODS

Materials

cFP was synthesized in the Peptide Biology Laboratory (Baker Medical Research Institute, Prahran, Australia). Recombinant human TIMP-2 was purchased from Oncogene Science (Cambridge, MA). All other protease inhibitors used were purchased from Sigma Chemical Co. (St. Louis, MO). The Enhanced Chemiluminescence kit and donkey anti-rabbit immunoglobulins conjugated to horse radish peroxidase were from Amersham Corporation (Buckinghamshire, England). PVDF (Immobilon P) transfer membrane was from Millipore Corporation (Bedford, MA). The bicinchoninic acid (BCA) protein assay kit was from Pierce Chemical Co. (Rockford, IL). Goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase were from Promega Corporation (Madison, WI). A rabbit polyclonal anti-human brain MBP antibody and rabbit PAP were purchased from Dakopatts (Denmark). Anti APP antibodies were 22C11 (Boehringer Mannheim, Germany), a monoclonal antibody which recognizes an epitope near the N-terminus of APP (Weidemann et al., 1989), affinity-purified rabbit polyclonal antibody 369A against residues 645−694 of the cytoplasmic domain of APP_{695} (Buxbaum et al., 1990), a kind gift from Dr. Samuel Gandy (Cornell University Medical Center, New York, NY), and antiserum 1-25 raised against residues 1-25 of the rat Aβ sequence (Walsh et al., 1993), a gift from Dr. David Allsop (SmithKline Beecham Pharmaceuticals, Harlow, U.K.). The anti-APLP2 antiserum raised to mouse APLP2 (Thinakaran et al., 1995) was a gift from Dr. Sangram Sisodia (Johns Hopkins University, Baltimore, MD).

Subcellular Fractionation of Rat Brain Homogenates

Subcellular fractions were prepared from brains of 150−200 g Sprague-Dawley rats using a modification of the method described by Koenig (1974). The majority of white matter was removed and the brains were homogenized with five strokes of a Potter-Elvehjem homogenizer at 1000 rpm in 5.67 vol of 0.3 M buffered sucrose containing 5 mM $MgCl_2$, 10 μM NaCl, 20 mM Tris-HCl, pH 7.2, 1 μg/mL aprotinin, 0.1 μg/mL pepstatin A, and 0.2 mM PMSF. All experiments were performed with samples containing these protease inhibitors, which were added in the first step of the fractionation. The exclusion of these protease inhibitors from the initial fractionation step did not alter the results.

The homogenate was centrifuged in a Beckman GPR centrifuge at 1200*g* for 10 min at 4 °C to remove nuclei and cellular debris. The resultant pellet was resuspended in 20 mL of the 0.3 M buffered sucrose and re-centrifuged at 1200*g*. The post-nuclear supernatant fractions from both centrifugation steps were pooled and further centrifuged at 100000*g* for 20 min at 4 °C in a Beckman L8-M ultracentrifuge with a Beckman 70.1 Ti rotor. The pellet from the 100000*g* fraction was resuspended in the 0.3 M buffered sucrose solution and layered on a discontinuous gradient comprising 0.8, 1.0, 1.2, and 1.4 M sucrose in 20 mM Tris-HCl, pH 7.2. The gradients were centrifuged at 55000*g* for 1.5 h at 4 °C in a Beckman L8-M ultracentrifuge with a Beckman SW40 rotor. Material at the interfaces of the sucrose gradient was collected and centrifuged at 100000*g* for 3 h at 4 °C to pellet particulate material. Pellets were washed once with the 0.3 M buffered sucrose solution. Fractions were designated F1−F5 where F1 was the lightest (sedimented to the 0.3−0.8 M sucrose interface), F2 (0.8−1.0 M), F3 (1.0−1.2 M), F4 (1.2−1.4 M), and F5 was the pellet at the bottom of the gradient. Fractions were frozen and stored in aliquots at −80 °C.

Fractions for electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Samples were then fixed in 1% osmium tetroxide, dehydrated in acetone and embedded in Epon-Araldite. Sections were stained with uranyl acetate and lead citrate before examination using a Siemens 102 electron microscope.

Enzyme Assays

Fractions from the sucrose gradient were assayed for the lysosomal marker enzyme β-glucuronidase (Jacox & Feldmahn, 1955), the Golgi marker α-mannosidase II (Storrie & Madden, 1990) and the plasma membrane marker 5′ nucleotidase (De Duve et al., 1955). The amount of protein in each fraction was determined using the bicinchoninic acid (BCA) protein assay kit from Pierce Biochemicals using bovine serum albumin as a standard.

SDS Polyacrylamide Gel Electrophoresis and Western Blotting

Aliquots of the gradient fractions containing 10 μg of protein were subjected to electrophoresis on 8.5% or 15% mini SDS polyacrylamide gels (Laemmli, 1970). Electrophoresis was at 40 mA per gel, and proteins were then transferred to polyvinylidene difluoride (PVDF) membranes at a constant current of 1 A for 3 h. Membranes were blocked in 0.5% hydrolyzed casein in phosphate-buffered saline, pH 7.4, for 1 h, and proteins were detected using various antibodies. Detection was performed with a secondary antibody conjugated to alkaline phosphatase and developed with Fast Red/naphthol AS-MX substrate. Alterna-

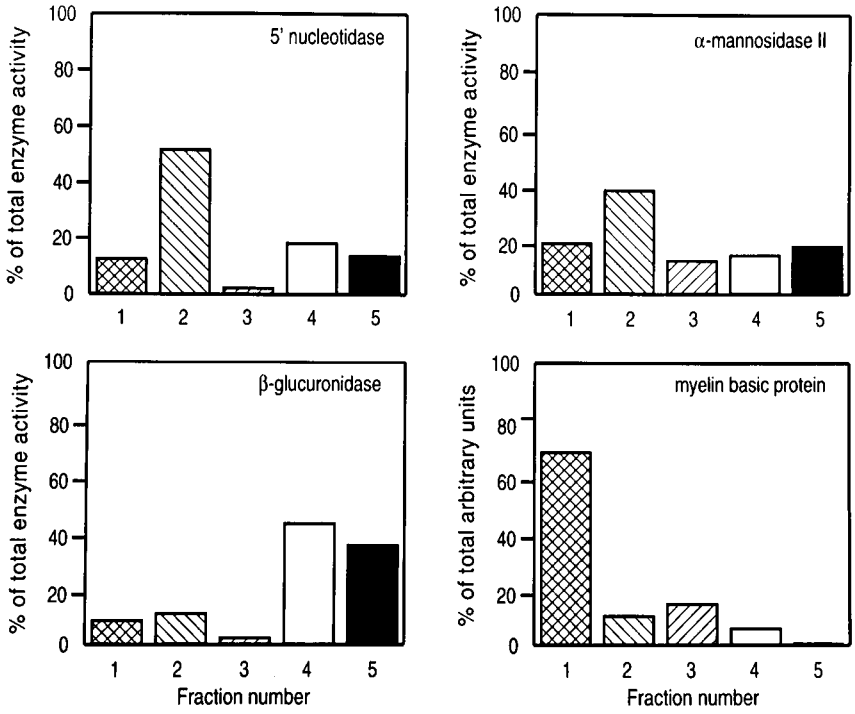

FIGURE 1: Analysis of subcellular fractions from rat brain homogenates. (i) Assay of different enzymes from subcellular fractions. Bands were collected at the interfaces of F1 (0.3−0.8 M sucrose), F2 (0.8−1.0 M), F3 (1.0−1.2 M), F4 (1.2−1.4 M), and F5 (bottom pellet). Fractions were assayed for α-mannosidase II (Golgi), 5′ nucleotidase (plasma membrane), and β-glucuronidase (lysosomes). Results are expressed as a percentage of the total enzyme activity in all five subcellular fractions. Similar results were obtained for $n = 5$ separate fractionations. (ii) Quantification of myelin basic protein (MBP) in the different subcellular fractions. The 18 and 14 kDa isoforms of MBP were quantified using the NIH Image (version 1.57) program. Results are expressed as a percentage of the total arbitrary units in all five fractions.

tively, a secondary antibody conjugated to peroxidase was used, the signal enhanced with an antibody against the peroxidase (PAP) and detection performed using the enhanced chemiluminescence (ECL) method (Rosewell & White, 1978). Bands from western blots were quantified by densitometry using the NIH Image (Version 1.57) program written by W. Rasband (National Institutes of Health, Bethesda, MD). The results represent experiments which were performed at least three times each.

RESULTS

Characterization of Subcellular Fractions

To identify Golgi proteases that may cleave APP, rat brains were homogenized and the crude homogenates subjected to sucrose density gradient centrifugation to separate fractions enriched in different subcellular organelles. Material collected from the sucrose gradient was assayed for specific proteins. The majority of the Golgi enzyme α-mannosidase II and plasma membrane enzyme 5′ nucleotidase was localized in the lighter gradient fractions, relatively well separated from the lysosomal enzyme β-glucuronidase, which was observed in F4 and F5 (Figure 1). The assays for these different enzyme activities were performed routinely with every new fractionation. Western blot analysis using an antibody to myelin basic protein (MBP) followed by quantification of the 18 and 14 kDa MBP isoforms using the NIH Image program indicated that most of the myelin was present in F1 at the top of the gradient (Figure 1). The presence of myelin in F1 and lysosomes in F4 and F5 were confirmed by electron microscopic studies (data not shown). F2 was used for all further studies on endogenous proteolytic processing at pH 7.2 as it was enriched in Golgi and plasma membranes and depleted of myelin and lysosomes.

Proteolytic Degradation of Endogenous APP and APLP2 in a Membrane-Enriched Fraction and Generation of Amyloidogenic APP Fragments

To investigate the degradation of endogenous APP and APLP2 in F2 and the subsequent generation of fragments, aliquots were incubated in 20 mM Tris-HCl pH 7.2 for various times at 37 °C and the generated products were analyzed by SDS−PAGE and western blotting. Several antibodies were used for this characterization. Antiserum 1-25 was raised against residues 1-25 of the rat Aβ sequence (Walsh et al., 1993) and therefore recognizes only APP and not APLP2. Antiserum 369A was raised against residues 645−694 of the cytoplasmic domain of APP. Monoclonal antibody 22C11 recognizes an epitope near the N-terminus of APP (Weidemann et al., 1989). Both 369A and 22C11 were raised to regions of APP which are well conserved with the related molecule APLP2 and have been shown to also detect this protein (Slunt et al., 1994). Antiserum D2-II raised to mouse APLP2 recognizes several forms of this molecule (Thinakaran et al., 1995).

APLP2 was detected in F2 but was hardly degraded upon incubation at 37 °C, and only minor fragments of approximately 24 and 14 kDa were generated (Figure 2). High molecular weight material (110−120 kDa) was also recognized by the polyclonal antibody 1-25 (Figure 3A). Incubation at 37 °C resulted in the degradation of these bands in a time-dependent manner, with the concomitant release of 60 and 17 kDa fragments (Figure 3B). Since antibody 1-25 recognizes an epitope within the Aβ region, it specifically

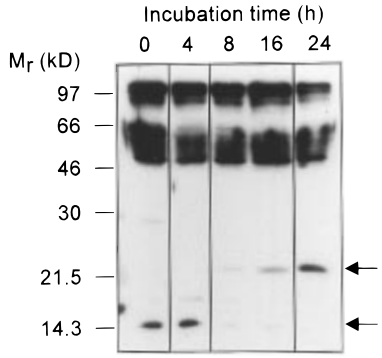

FIGURE 2: Western blot analysis of APLP2 degradation. F2 was incubated at 37 °C, aliquots removed at various times, electrophoresed on a 15% Tris-glycine gel and electroblotted onto PVDF. Blots were analyzed with antiserum D2-II raised to mouse APLP2. APLP2 is detected in all incubations analyzed. Arrows show the APLP2 degradation products observed.

detects APP and fragments of APP containing Aβ. These results indicate that a protease activity capable of degrading APP to release Aβ-containing fragments was present in this fraction. These products are therefore potentially amyloidogenic and may be further processed to generate Aβ.

A major band with an apparent molecular mass of 110−120 kDa was also detected with antiserum 369A and monoclonal antibody 22C11 (Figure 3C,E). These antibodies are directed to the C-terminus and N-terminus of APP respectively. Degradation products containing the C-terminus epitope recognized by 369A showed apparent molecular masses ranging from 80 kDa to less than 14 kDa (Figure 3C,D). Two of these products (60 and 17 kDa) were also detected by antibody 1-25 (Figure 3A,B) indicating that they were specifically derived from APP.

N-terminal processing of the 110−120 kDa material was evident from the generation of fragments recognized by 22C11 (Figure 3E,F). Several fragments were observed, but these were distinct from the C-terminal fragments detected by 369A. As mentioned previously, 369A and 22C11 do not discriminate between APP and APLP2 products, and since both these molecules were present endogenously, fragments resulting from both would be expected. The proteolytic activity in F2 was capable of degrading the 110−120 kDa material as observed with antibodies (1-25), 369A and 22C11 (Figure 3). In contrast, it did not seem to be as active against APLP2 (Figure 2).

Effect of pH on Protease Activity

As lysosomal proteases are known to be active at low pH, we tested the pH-dependence of the protease activity in F2. Aliquots of F2 were incubated for 18h at 37 °C either alone in acetate or Tris buffers of varying pH, or in the presence of leupeptin and pepstatin. Samples were then subjected to SDS−PAGE on 8.5% Tris-glycine gels, immunoblotted onto PVDF, and analyzed with the antiserum 369A, and the amount of material remaining at 110−120 kDa was quantified. Protease activity was observed over a range of pH values tested (Figure 4). However, degradation activity at lower pH was inhibited by the addition of leupeptin and pepstatin, while at pH 7.5, APP degradation was not only preserved but even increased, probably due to the protection of the APP-degrading enzyme itself by the inhibitors.

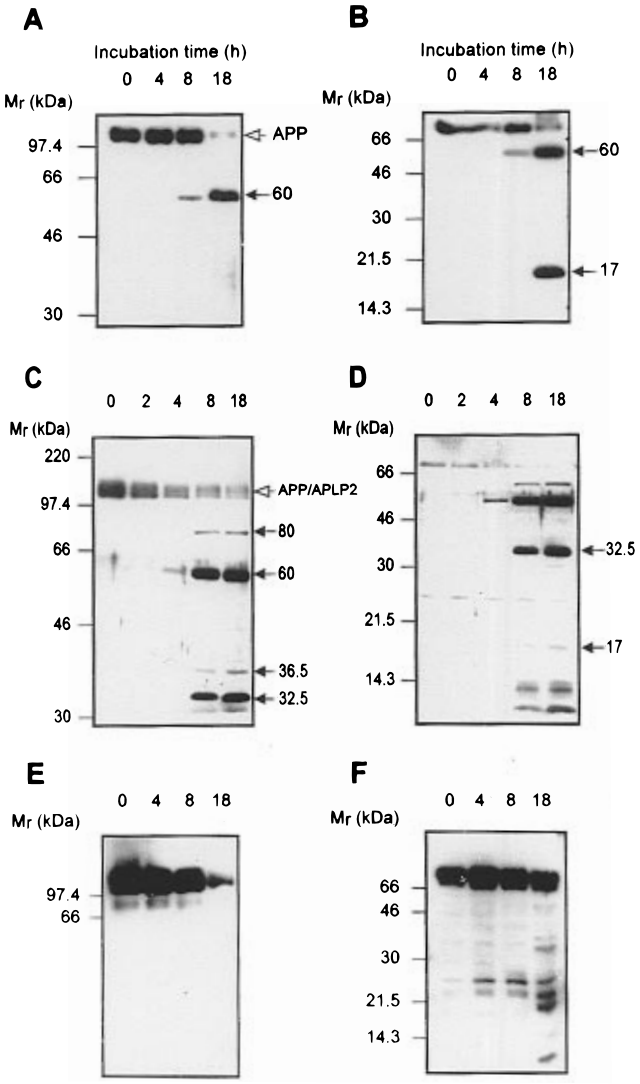

FIGURE 3: Proteolytic degradation of APP and APLP2 in F2 during incubation at 37 °C. Aliquots of F2 containing 10 μg of protein were removed after various times, subjected to electrophoresis on 8.5% (A, C, E) and 15% (B, D, F) Tris-glycine gels, and transferred onto PVDF membranes. Blots were developed with an antibody raised to residues 1-25 of the rat Aβ sequence, Ab (1-25) (A, B); 369A, an antibody to the cytoplasmic domain of APP_{695} (C, D) or 22C11, directed to the N-terminus of APP (E, F). Aβ and C-terminal containing fragments are indicated by the solid arrows. The position of undegraded APP or APLP2 is shown by the open arrow.

Although F2 is probably contaminated with a protease activity derived from lysosomes, it is unlikely therefore that the protease we found to be active at neutral pH is of lysosomal origin.

Effect of Inhibitors on Protease Activity

To determine the class of the protease involved in the degradation of the 110−120 kDa material, F2 was preincubated with different protease inhibitors for 15 min at 25 °C and then further incubated at 37 °C for 18 h. The degradation products were characterized by western blotting. Incubation in the presence of the metal-ion chelator EDTA inhibited degradation, as no products were observed (Figure 5). As we have seen previously, the lysosomal protease inhibitor leupeptin and the aspartyl protease inhibitor pepstatin had no effect on degradation of the 110−120 kDa material (Figures 4 and 5). A new 46 kDa product was

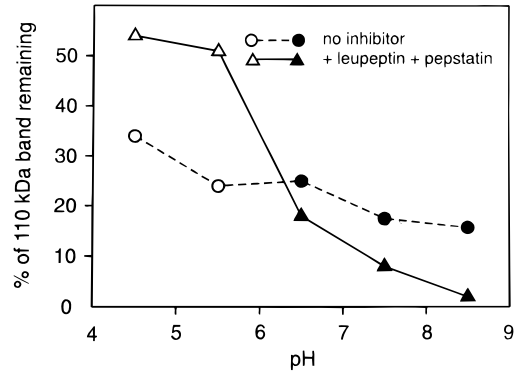

FIGURE 4: pH dependence of protease activity and the effect of the protease inhibitors leupeptin and pepstatin. Aliquots of F2 were incubated for 18 h at 37 °C in acetate (○, △) or Tris (●, ▲) buffers of different pH, in the absence or presence of the protease inhibitors leupeptin (100 μg/mL) and pepstatin (2 μg/mL). Samples were electrophoresed on an 8.5% Tris-glycine gel, blotted onto PVDF, and analyzed with the antiserum 369A. The amount of high molecular weight material remaining after incubation with or without the protease inhibitors was quantified using the NIH Image program. Undigested controls were taken as 100%. The amount of material remaining after incubation was expressed as a percentage of the undigested control. Degradation was observed at all pH values tested. Leupeptin and pepstatin inhibited this degradation at low pH conditions.

observed in the presence of pepstatin (Figure 5). Several other protease inhibitors were also tested for their ability to inhibit degradation (Table 1). 1,10-Phenanthroline, like EDTA, was effective in inhibiting degradation. In addition to leupeptin and pepstatin, inhibitors of serine proteases (AEBSF), cysteine proteases (E-64, NEM, iodoacetamide), chymotrypsin-like activity (TPCK), the 72 kDa gelatinase (TIMP-2), and metallo-endopeptidases (phosphoramidon and cFP) had no effect on the protease activity (Table 1).

Effect of Divalent Metal Ions on Protease Activity

The effect of divalent metal ions on the protease activity was studied by incubation of F2 with various ions (Figure 6). Zn^{2+} (50 μM) reversed the inhibition by EDTA. Addition of 50 μM Zn^{2+} to incubations containing 1 mM EDTA resulted in breakdown to produce bands of 60 and 14 kDa. In addition, a minor fragment of 31 kDa was also observed. Other metal ions tested (Fe^{2+}, Ca^{2+}, and Mg^{2+}) did not reactivate the protease activity, providing evidence for the specificity of the Zn^{2+} effect. These results suggest that a Zn^{2+}-dependent metalloprotease is responsible for APP degradation.

DISCUSSION

Many studies using cells in culture have suggested that proteases in the Golgi compartment may play a major role in APP processing (Caporaso et al., 1992; Busciglio et al., 1993; Haass et al., 1993; Kuentzel et al., 1993). In the present study, we have isolated a subcellular fraction from rat brain that is enriched in Golgi and plasma membranes in order to study the processing of endogenous membrane-bound APP.

APP and APLP2 were both present in the Golgi and plasma membrane-enriched fraction (F2). Bands of 110−120 kDa were detected by several different antibodies. Incubation of this fraction at 37 °C resulted in the degradation of high molecular weight material with the generation of a

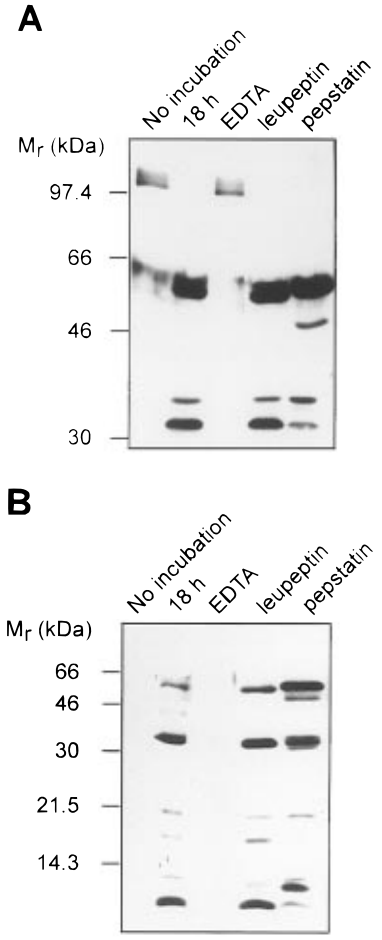

FIGURE 5: Proteolytic degradation of 110−120 kDa material in the presence of protease inhibitors. Aliquots of F2 were pre-incubated with different protease inhibitors for 15 min at 25 °C, then further incubated at 37 °C for 18 h. Samples were then electrophoresed on (A) 8.5% and (B) 15% Tris-glycine gels, transferred onto PVDF membranes, and analyzed with the antiserum 369A. Final concentrations of inhibitors used were EDTA, 1 mM, leupeptin, 100 μg/mL, and pepstatin, 2 μg/mL.

range of C-terminal containing fragments, indicating the presence of a protease activity. Studies have shown that antibodies like 22C11 and 369A which are raised to well-conserved regions will not discriminate between APP and APLP2 (Slunt et al., 1994). However, two of the C-terminal products (the 60 and 17 kDa fragments) were also recognized by an antibody raised to residues 1-25 of rat Aβ. Since these fragments contain Aβ, they cannot be derived from APLP2, which does not contain a homologous sequence to Aβ. The C-terminal fragments recognized by 369A which were not detected by Ab (1-25) may be smaller fragments of APP containing the C-terminus but devoid of the Aβ domain.

Antiserum D2-II detects full-length APLP2 and a range of other APLP2 peptides, which have a different pattern to APP on the western blots (Thinakaran et al., 1995). This antibody only detected minor amounts of APLP2 fragments. APLP2 may be more resistant to the protease and hence may not be degraded as extensively or in the same manner as APP. Alternatively, D2-II may not be capable of recognizing fragments generated within this system and more specific antibodies raised to different regions of APLP2 should be used.

Further studies using inhibitors specific for the different classes of proteases indicated that the APP-degrading enzyme

Table 1: Effect of Inhibitors on APP-Degrading Protease Activity in a Golgi and Plasma Membrane-Enriched Subcellular Fraction from Rat Brain Homogenates

inhibitor	concentration	specificity of inhibitor	inhibition of protease activity[a]
EDTA	1 mM	metallo/metal activated	+
1,10-phenanthroline	2 mM	metallo/metal activated	+
TIMP-2	2 μg/mL	72 kDa gelatinase	−
phosphoramidon	20 μM	endopeptidase 24.11	−
CFP	5 μM	endopeptidase 24.15 and 24.16	−
E-64	10 μM	cysteine	−
NEM	1 mM	cysteine	−
iodoacetate	10 μM	cysteine	−
AEBSF	1 mM	serine	−
leupeptin	100 μg/mL	serine/trypsin-like	−
TPCK	0.1 mM	serine/chymotrypsin-like	−
pepstatin	2 μg/mL	aspartic	−

[a] (−) No inhibition of protease activity. (+) Inhibition of protease activity.

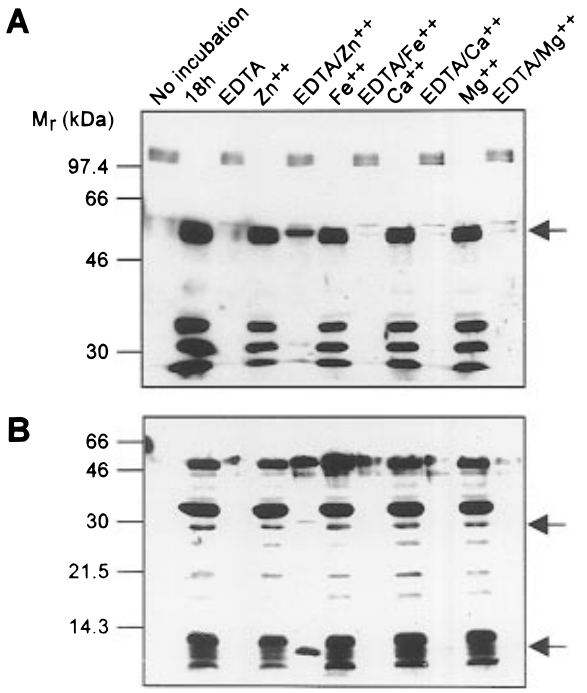

FIGURE 6: Effect of divalent metal ions on protease activity. Aliquots of F2 were pre-incubated with 50 μM of the metal ions Zn^{2+}, Fe^{2+}, Ca^{2+}, and Mg^{2+} in the presence or absence of 1 mM EDTA for 15 min at 25 °C. Samples were then incubated at 37 °C for a further 18 h and electrophoresed on (A) 8.5% and (B) 15% Tris-glycine gels, immunoblotted, and analyzed with antiserum 369A as described previously. The major degradation fragments in the incubation of EDTA with Zn^{2+} (lane 5 from the left) are shown by the arrows.

was a metalloprotease. EDTA inhibited degradation of the 110−120 kDa material as shown using the antiserum 369A. No smaller products were detected with 369A or by Ab (1-25) in the presence of EDTA [data not shown for Ab (1-25)]. Inhibition of the degradation of high molecular weight material by EDTA was reversed by Zn^{2+}, whereas other metal ions did not reverse the inhibitory effect. However, inhibitors of the neutral zinc metallo-endopeptidases 24.15 and 24.16 (cFP), 24.11 (phosphoramidon), and the 72 kDa gelatinase (TIMP-2) did not prevent degradation. This indicates that the APP-degrading protease is distinct from these metallo-endopeptidases. Previous studies have demonstrated that membranes isolated from PC12 cells and bovine chromaffin granules contain full-length APP which can be released into a soluble fraction upon incubation at 37 °C (Ripellino et al., 1994; Vassilacopoulou et al., 1995). An integral membrane metallo-endopeptidase has been shown to cleave membrane-bound APP at the α-secretase site in H4 human neuroglioma cells expressing the N-terminal 493 amino acids of human placental alkaline phosphatase fused to the C-terminal 105 amino acids of APP (Boseman-Roberts et al., 1994). These metalloproteases appear likely to be involved in APP secretion and release from the membrane.

It has been suggested that endopeptidase 24.15 and endopeptidase 24.11 may be involved in APP processing. A metalloprotease identified as 24.15 was isolated from human brain and was seen to cleave a synthetic peptide spanning the β-secretase site (McDermott et al., 1992; Seubert et al., 1992). Studies by Papastoitsis et al. (1994) have shown that a metalloprotease with high sequence homology to 24.15 cleaved both human recombinant APP and a synthetic substrate. Several other metalloproteases have been reported to cleave synthetic peptides (McDermott & Gibson, 1991; Schönlein et al., 1994). The 72 kDa gelatinase has been shown to cleave synthetic peptides at the α-secretase (Miyazaki et al., 1993) and the β-secretase sites (LePage et al., 1995). Other studies using rat cortical membrane fractions incubated with synthetic Aβ peptides have identified a protease sensitive to EDTA, EGTA, and Zn^{2+} (Allsop et al., 1991). This protease was activated by Ca^{2+} and Mn^{2+}, indicating that it is unlikely to be similar to the protease observed in our studies. Recent studies using Chinese hamster ovary cells have demonstrated the release of several membrane-bound cell-surface proteins by a metalloprotease sensitive to 1,10-phenanthroline, and the reversal of this activity by the addition of Zn^{2+} (Arribas et al., 1996). The protease observed in our studies possesses similar characteristics and may therefore play a similar role in the release of APP from the membrane.

C-terminal fragments which have been shown to accumulate in different systems may be amyloidogenic and thereby contribute to the formation of Aβ (Esch et al., 1990; Estus et al., 1992; Golde et al., 1992; Haass et al., 1992a; Seubert et al., 1993; Cheung et al., 1994; De Strooper et al., 1995). Our studies and those of other groups have characterized amyloidogenic C-terminal fragments in lysosomal fractions from human (Evin et al., 1995) and rat (Sun et al., 1994) brain. Aβ-containing C-terminal fragments are also produced by stimulated platelets (Li et al., 1995) and rat hippocampal neurons transfected with APP_{695} (Simons et al.,

1996). In the present study, C-terminal fragments of 60 and 17 kDa were recognized by Ab 1-25 and 369A when the high molecular weight (110−120 kDa) material was cleaved. A similar 60 kDa fragment of APP has been observed by Baskin et al. (1991) in stressed PC-12 cells. The 17 kDa fragment would correspond to that observed in human brain and platelets (Shoji et al., 1992; Evin et al., 1995; Li et al., 1995) and in insect Sf9 cells infected with baculovirus-bearing human APP (Gandy et al., 1992).

Evidence for β-secretase activity in the Golgi compartment has been reported in many different cell culture systems (Caporaso et al., 1992, 1994; Busciglio et al., 1993; Haass et al., 1993; Kuentzel et al., 1993; Thinakaran et al., 1996). Our results do not provide sufficient evidence to indicate that the protease detected in this Golgi and plasma membrane-enriched fraction is a β-secretase. However, one may speculate that the fragments produced here which contain the entire Aβ could be transported to an endosomal/lysosomal compartment for further processing by other proteases including a γ-secretase. The characterization of C-terminal fragments with an antibody raised specifically to Aβ is especially important as it indicates that these fragments are potentially amyloidogenic and are suitable substrates for subsequent processing to release Aβ.

The studies by Ripellino et al. (1994), Boseman-Roberts et al. (1995), and Vassilacopoulou et al. (1995) suggest that APP may have to be membrane-bound to be cleaved by secretases. Since the protease we identified in this study is associated with a membrane-enriched fraction, it may be potentially important for APP processing. Ultimately, the purification and cloning of a cDNA encoding the protease will be a necessary first step toward determining whether this novel metalloprotease is an APP secretase.

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