Ca^{2+}-Dependent 68-Kilodalton Protease in Familial Alzheimer's Disease Cells Cleaves the N-Terminus of β-Amyloid†

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ABSTRACT: Lymphoblastoid cells derived from patients with early- and late-onset familial Alzheimer's disease express a Ca^{2+}-dependent, 68-kDa protease which forms an SDS-stable and heat-labile complex with the β-amyloid precursor protein. Utilizing this property, we prepared the protein by heat-dissociation of its immunoprecipitate with an antibody raised against the extracellular part of the β-amyloid precursor protein. Disuccinimidyl suberate cross-linking analysis showed that in the presence of Ca^{2+} this protein binds to a synthetic oligopeptide corresponding to the first 12 amino acids of β/A4-amyloid and its N-terminal flanks. Thin-layer chromatography of a reaction mixture of the 68-kDa protein and the oligopeptide demonstrated its proteolytic activity in the presence of Ca^{2+}. Subsequent N-terminal amino acid sequencing of the cleaved fragment showed the cleavage site of the oligopeptide to be the Lys^{-2}–Met^{-1}–$Asp^{\beta A4-1}$ bonds. This protease also cleaves a natural substrate of 110-kDa β-amyloid precursor protein, thereby generating the 16-kDa preamyloid peptide that accumulates abnormally in familial Alzheimer's disease lymphoblastoid cells. It does not, however, cleave the $Gln^{\beta A4-15}$–Lys^{16}–Leu^{17} bond that is regarded to be the normal proteolytic site for the secretion of the β-amyloid precursor protein. Analysis of the effects of protease inhibitors suggests that this 68-kDa protease is a Ca^{2+}-dependent serine protease.

Abnormal and progressive deposition of β-amyloid (β/A4),[1] as in senile plaque and cerebrovascular amyloid, is a major neuropathological hallmark in Alzheimer's disease (AD) (Müuller-Hill & Beyreuther, 1989; Selkoe, 1989). The earliest pathological finding in the brain is the extracellular nonfibrous deposition of β/A4 (diffuse plaque) (Selkoe, 1989; Mann & Esiri, 1989; Yamaguchi et al., 1990); 4-kDa β/A4 is generated by cleavage of the β-amyloid precursor protein (APP) (Kitaguchi et al., 1988; Tanzi et al., 1987; Ponte et al., 1988). In addition to APP695, APP has alternately spliced isoforms of APP751 and APP770 that carry a Kunitz-type protease inhibitor (KPI) domain that has antitryptic activity (Ponte et al., 1988; Kitaguchi et al.; Tanzi et al., 1988). β/A4 is deposited abnormally not only in the brain but also in such extraneural cells as epidermal and intestinal interstitial cells, and lymphocytes (Neve et al., 1988; Münnig et al., 1992). Moreover, the APP^{717} point mutation within the transmembrane domain is linked to a group of familial AD families (Goate et al., 1991), and the $\beta/A4^{22}$ point mutation in hereditary cerebral hemorrhage with amyloidosis (Dutch type) causes β/A4 deposition, predominantly in cerebral vessels (Levy et al., 1990). A clear understanding of the mechanism of β/A4 deposition therefore is essential for understanding the pathogenesis of AD. We used familial AD and control lymphoblastoid cells to make a detailed comparative analysis of APP and β/A4 processing, and verified that familial AD cells abnormally accumulate 16 kDa, the β/A4 cytoplasmic domain preamyloid, because of deficient intra-β/A4 proteolysis (Matsumoto & Fujiwara, 1991, 1993). Abraham et al. (1990, 1991) reported that a Ca^{2+}-dependent serine protease is increased in AD and aged normal brains and that it cleaves both bonds of the Lys^{-2}–Met^{-1}–Asp^{1}–Ala^{2} sequence of the β/A4 N-terminus. Protease activity also is inhibited by protease nexin 2 and α_1-antichymotrypsin (Abraham et al., 1991), indicative that an imbalance between protease and protease inhibitors may cause the aberrant processing of APP. In AD brain cells, in which the abnormal APP processing pathway that accumulates amyloidogenic fragment(s) with β/A4 predominates, a β-pleated sheet conformation as amyloid is easily induced under physiological conditions (Selkoe et al., 1988; Beyreuther et al., 1992; Estus et al., 1992). In normal brain cells, intra-β/A4 proteolysis by a postulated APP secretase precludes β/A4-amyloid formation (Esch et al., 1990; Allsop et al., 1991; McDermott & Gibson, 1991).

We found independently that familial AD lymphoblastoid cells uniquely express a Ca^{2+}-dependent 68-kDa protein which forms an SDS-stable and heat-labile complex with an extracellular APP fragment possibly through the KPI domain (Matsumoto & Fujiwara, 1991, 1993). We here present substantial evidence that the 68-kDa protein expressed in familial AD cells is a protease and give the results of the analysis of its biochemical characteristics.

MATERIALS AND METHODS

Cell Culture. Epstein–Barr virus-transformed lymphoblastoid cells that we had established (Matsumoto & Fujiwara, 1991) were used as the normal controls (NL27KO, NL7KO), and late-onset (BEH16212, QDH18601, MI7810) and early-onset (LH11401, KG4012, AM12618, L801) familial AD cells (Bird et al., 1989) were supplied by D. G. Schellenberg,

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[1] Abbreviations: AD, Alzheimer's disease; APP, β-amyloid precursor protein; β/A4, β/A4-amyloid; KPI, Kunitz-type protease inhibitor; cAPP, central APP; A4C; A4C, βA4-cytoplasmic domains; $NaCl/P_i$, phosphate-buffered saline; FBS, fetal bovine serum; TLC, thin-layer chromatography.

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T. D. Bird, and G. M. Martin of the Alzheimer's Disease Research Center, University of Washington, Seattle. These lymphoblastoid cells were routinely cultured in RPMI1640 medium with 15–20% fetal bovine serum, unless otherwise specified.

Preparation of APP in the Media, Cell Lysate, and Membrane by Immunoprecipitation. For extracellular protein preparation, at least 50 mL of conditioned media of serum-free 24-h cultures of 1×10^8 cells was collected by centrifugation and dialyzed for 8 h at 4 °C against two changes of buffer (2 L) consisting of ice-cold 5 mM Tris-HCl, pH 7.5, and 14 mM NaCl, after which the sample was condensed to one-tenth its original volume. For cell lysate preparation, 3×10^7 cells were lysed in ice-cold buffer (1.5 mL) consisting of 50 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 1% Nonidet P40, 20 mM NaCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride, 0.1 mM leupeptin, and 0.2 unit/mL trypsin inhibitor. After sonication in a Branson sonifier, the crude extract was obtained by centrifugation at 15000*g* for 10 min. For the membrane-enriched fraction, cells suspended in homogenization buffer consisting of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 0.2 mM $MgCl_2$, protease inhibitors (see above), and 0.3 mg/mL DNase I were disrupted in a glass homogenizer on ice. After the removal of nuclei, the homogenate was loaded on a cushion of 25% Ficoll 400 (Pharmacia) and centrifuged at 100000*g* at 4 °C for 14 h. The membrane-enriched fraction at the interface between the upper aqueous and lower Ficoll phases was collected and washed with the homogenization buffer and then pelleted by centrifugation at 50000*g* for 30 min. All the samples were kept frozen at −80 °C until use.

Immunoprecipitation was done with affinity-purified polyclonal rabbit anti-cAPP antibody raised against a fusion protein of protein A and the 144–654th residue peptide of APP751 (Matsumoto & Fujiwara, 1991, 1993). APP and its fragments in the cell lysate and media were immunoprecipitated with anti-cAPP antibody using protein A–Sepharose beads, as described elsewhere (Matsumoto & Fujiwara, 1991, 1993). After intensive washes, the immune complex that bound to the protein A–Sepharose beads was suspended in Laemmli sample buffer (Laemmli, 1970) and then separated by 10% SDS–PAGE. Full-length cytosolic APP, isoalted as the 120-kDa natural APP substrate, was prepared either by SDS–PAGE or by nondenaturing gel electrophoresis without SDS (Goldenberg, 1989) after cAPP immunoprecipitation; then the gel was stained with Coomassie brilliant blue R-250. The gel strip containing the 120-kDa APP was recovered by electroelution (Hunkapiller & Lujan, 1986). Characteristics of substrates for both disuccinimidyl suberate cross-linking analysis and proteolytic analysis described below were indistinguishable, and these substrates were positive for the sodium periodate oxidation reaction (Kondoh et al., 1991), suggesting that glycoconjugates of these APP molecules are intact. Two-dimensional polyacrylamide gel electrophoresis of APP polypeptides was done by the method of O'Farrell (1975). The molecular mass markers used in SDS–PAGE were 110-kDa β-galactosidase 84-kDa transferrin, 47-kDa glutamic dehydrogenase, 33-kDa carbonic anhydrase, 24-kDa soybean trypsin inhibitor, and 16-kDa lysozyme (Bio-Rad and Pharmacia).

APP Substrates and Radioiodination. Three APP substrates were used for disuccinimidyl suberate cross-linking and proteolysis: (i) a synthetic 18-residue oligopeptide (P18), His-Ser^{592}-Glu-Val-Lys-Met-Asp$^{\beta A4-1}$-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val$^{608(\beta A4-12)}$ (the N-terminal addition of His for radioiodination), for the N-terminal region of β/A4 that was synthesized in a peptide synthesizer (Applied Biosystems) and purified by high-pressure liquid chromatography; (ii) a 10-residue oligopeptide substrate (β/A4–8/17), Ser$^{\beta A4-8}$-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu$^{\beta A4-17}$-Cys (added for linkage to keyhole limpet hemocyanin), within the β/A4 sequence also was synthesized and purified as above; (iii) 120-kDa full-length APP was purified as described above. These substrates were radioiodinated with $Na^{125}I$ (specific activity 629 GBq/mg, NEN) by the modified chloramine-T method (Markwell, 1982).

Heat Release of 68-kDa Protease from 120-kDa Complex. The 120-kDa complex from the serum-free culture media of familial AD cells is composed of a 68-kDa protein and a 53-kDa APP peptide that carries KPI (Matsumoto & Fujiwara, 1991). High molecular mass (>100 kDa) proteins in total extracellular protein in serum-free medium of AM12618 early-onset familial AD cells were enriched by centrifugation through a limit filtration column (Ultrafree C3-THK, Millipore). The trapped protein (approximately 10% of the original amount) was immunoprecipitated with anti-cAPP antibody on protein A–Sepharose and then washed 5 times. The anti-cAPP immune complex with the 120-kDa protein complex was heated at 65 or 55 °C for 15 min to dissociate the 68-kDa protein and the 53-kDa APP, after which the protein A–Sepharose beads were spun down. Dissociated rabbit IgG was removed by passage through a protein A–Sepharose column with an elution buffer composed of 10 mM sodium phosphate and 20 mM NaCl. The eluate was dialyzed against ice-cold 1 mM sodium phosphate buffer, pH 7.5, containing 0.1 mM $CaCl_2$ and then condensed 10-fold by lyophilization. The final solution containing the purified 68-kDa protein was separated by 10% SDS–PAGE. Some samples were purified further by removing the copurified 53-kDa APP fragment: The heat-dissociated complex (at 55 °C) was run through a limit filtration column to remove any undissociated 120-kDa complex. The passed-through fraction then was immunoprecipitated as above to adsorb the 53-kDa APP fragment on the protein A–Sepharose beads. The supernatant obtained was passed through a protein A–Sepharose affinity column to remove anti-cAPP antibody. The final passed-through fraction showed a single 68-kDa band on SDS-PAGE (Figure 3, *lane 4*). Heating the complex at 55 °C for longer than 60 min or at 65 °C completely inactivated the proteolytic activity. The yield of 68-kDa protein was estimated as follows: From 10 mg of total extracellular protein of AM12618 familial AD cells, 1 mg of high molecular mass protein (>100 kDa) was prepared. After first-round immunoprecipitation with anti-cAPP antibody, the amount of 120-kDa complex obtained was 3.2 μg. The complex which is composed of the 68-kDa protein and the 53-kDa APP fragment was then heat-dissociated and subjected to second-round immunoprecipitation. The final yield of 68-kDa protein was 0.7 μg (approximately 10 pmol). The amount of 68-kDa protein prepared differs between familial AD cell strains used; From 10 mg each of extracellular proteins of L801 or KG4012 early-onset familial AD cells, 6 or 14 pmol of the 68-kDa protein was prepared, respectively. Quantitation of protein was performed by using the Protein Assay Kit (Bio-Rad) or by comparing the intensity of the 68-kDa protein and the known amount of bovine serum albumin (69.5 kDa) on a Coomassie blue-stained gel. We also released the 120-kDa complex by acidification at pH 4.0. After neutralization, rabbit IgG was removed by passing the complex through a protein A–Sepharose column as described above.

Analysis of Specific Binding of the 68-kDa Protein to APP Substrates by Disuccinimidyl Suberate Cross-Linking. A 5-μg extracellular (10 μL) or a 10-μg cytosol (10 μL) protein sample (Figure 2), or the purified 68-kDa protein (1 pmole in 4 μL) as described above (Figure 4, left panel), was incubated at 37 °C for 15 min with 5 nM ^{125}I-P18 (6×10^4 cpm), or with ^{125}I-labeled natural 120-kDa APP (approximately 1 pmole in 6 μL) (Figure 4, right panel) in buffer composed of 50 mM sodium phosphate, pH 7.5, and 1 mM $CaCl_2$ with or without 5 mM EDTA at a final volume of 20 μL. To cross-link the protein and ^{125}I-labeled substrate, the mixture was treated with 1 mM (final) disuccinimidyl suberate at 4 °C for 20 min (Tsudo et al., 1987). Cross-linking was terminated by the addition of 50 mM (final) Tris-HCl, pH 7.5, and some samples were immediately passed through a limit filtration column (Ultrafree, Millipore) to remove un-cross-linked molecules that might enhance artifactual binding. Cross-linked complexes were separated by 10% SDS–PAGE, after which the gel was fixed in 30% methanol/10% acetic acid, dried, and exposed to Fuji RX X-ray film at –85 °C for 16 h.

Proteolysis of P18 by the 68-kDa Protease and Amino Acid Sequencing of the Cleavage Site. Fifty picomoles of ^{125}I-P18 (for autoradiography) or unlabeled P18 (5 μg for amino acid sequencing) was incubated for 1 or 3 h at 37 °C with 0.07–2 pmol of the purified 68-kDa protein in a buffer composed of 10 mM sodium phosphate, pH 7.5, and 1 mM $CaCl_2$. A 2-μL sample of each reaction product was spotted on a microcrystalline cellulose TLC plate (Schleicher & Schuell) and then separated by ascending chromatography for 3 h at room temperature with a solvent of *n*-butanol–pyridine–acetic acid–water (15:10:3:12 v/v). The air-dried plate was autoradiographed to verify the proteolysis of ^{125}I-P18. For amino acid sequencing, a scaled-up reaction was run with 5 μg of unlabeled P18 and 13.3 pmol (20 μL) of the protease. The cellulose containing the cleaved FI fragment (see Figure 5A) was scraped off, after which the FI peptide was extracted with 50% acetic acid and then microsequenced in an Applied Biosystems 470A protein sequencer (Tempst & van Beeumen, 1983) to determine its N-terminal amino acids.

Proteolysis of β/A4–8/17 and Full-Length APP by the 68-kDa Protease. After radioiodination, the β/A4–8/17 oligopeptide (10 ng, 10 pmol) and 120-kDa full-length APP (approximately 1.5 pmol) substrates were incubated separately with various amounts of the purified protease under standard reaction conditions with or without 5 mM EDTA. Thereafter, the reaction mixture containing ^{125}I-β/A4–8/17 was separated by TLC, and that containing ^{125}I-labeled natural APP by 10% SDS–PAGE. Separation of the samples was verified by autoradiography.

Specific Binding of a Serine Protease Inhibitor to the 68-kDa Protein and Effects of Class-Specific Protease Inhibitors on Its Proteolytic Activity. Purified 68-kDa protein (2 pmol in 8 μL) and 100 pmol of a specific serine protease inhibitor, [1,3-^{3}H]diisopropyl fluorophosphate (222 GBq/mmol, 0.17 nmol/μL; NEN), were incubated in the reaction buffer as described at 37 °C for 15 min. The reaction mixture was then separated by 10% SDS–PAGE without heat denaturation before loading. The gel was fixed as above, soaked in en^{3}Hance autoradiography enhancer (NEN) for 30 min and then in ice-cold water for 30 min, and dried. The dried gel was exposed to Fuji-RX X-ray film for fluorography (48 h). The authentic protease inhibitors and their final concentrations were as follows: thiol protease inhibitors, 5 mM iodoacetamide and 20 mM E64; serine protease inhibitors, 2 and 10 mM phenylmethanesulfonyl fluoride, 25 μg/mL leupeptin, and 1 mM diisopropyl fluorophosphate; aspartic protease inhibitor, 0.1 ng/mL pepstatin; chymotrypsin-type serine protease inhibitor, 15–30 μg/mL chymostatin; Ca^{2+}-dependent protease inhibitors, 5 mM EGTA and 5 mM EDTA. Their effects on the cleavage of ^{125}I-P18 (10 pmol) by 1 pmol of 68-kDa purified protease (2 μL for this preparation) were examined in separate treatments during a 1-h incubation period at 37 °C under standard cleavage conditions.

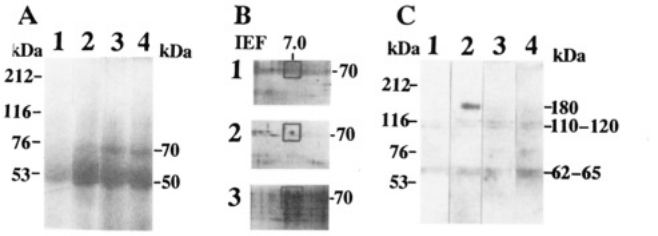

FIGURE 1: 68-kDa KPI binding protein in familial Alzheimer's disease cells. *Panel A*: SDS–PAGE profiles of anti-cAPP immunoprecipitates from 300 μg per lane of extracellular protein after boiling (Coomassie brilliant blue staining), *Lane 1*, NL27KO (normal); *lane 2*, MI7810 (late-onset type); *lane 3*, AM12618 (early-onset type); *lane 4*, L801 (early-onset type). *Panel B*: Silver-stained two-dimensional gel profiles of anti-cAPP immunoprecipitates of secreted proteins (300 μg per panel). *Lane 1*, NL27KO (normal); *lane 2*, M17810 (late-onset type); *lane 3*, AM12618 (early-onset type). *Panel C*: Anti-cAPP immunoblots of 40 μg of protein per lane of membrane-enriched fraction after boiling for 5 min (*lanes 3* and *4*) and not boiled (*lanes 1* and *2*). *Lanes 1* and *3*, NL27KO (normal); *lanes 2* and *4*, AM12618 (early-onset type).

RESULTS

Detection of the 68-kDa Protein. To verify the specific expression of the 68-kDa protein in familial AD cells, each extracellular protein was immunoprecipitated with 1 μL of anti-cAPP antibody. The immune complex formed was dissolved in 15 μL of Laemmli sample buffer (Laemmli, 1979) and then separated by SDS–PAGE after heating the sample at 65 °C for 15 min (Figure 1A). Samples prepared from NL27KO normal cells (Figure 1A, *lane 1*) did not express the 68-kDa protein, but MI7810 late-onset (Figure 1A, *lane 2*) and both AM12618 and L801 early-onset type familial AD cells (Figure 1A, *lanes 3 and 4*) did. As expected, preimmune serum did not coprecipitate the 68-kDa protein from the AM12618 extracellular proteins (data not shown). The broad band at about 50 kDa represents rabbit IgGs and the 53-kDa APP fragment that forms a complex with the 68-kDa protein (Matsumoto & Fujiwara, 1991). Two-dimensional gel electrophoresis of the same anti-cAPP immunoprecipitates of the secreted proteins from the MI7810 and AM12618 familial AD cells (Figure 1B, *lanes 2 and 3*) confirmed the presence of a 68-kDa protein spot with a p*I* of 7.0 that was not present in NL27KO normal cells (Figure 1B, *lane 1*). It should be noted, however, that the 68-kDa protein prepared from early-onset familial AD cells exhibits at least two 68-kDa spots with slightly different p*I*s (7.0 and 6.9). This is possibly due to microheterogeneity in glycosylation of the 68-kDa protein, since we have clarified the nature of the 68-kDa protein as a glycoprotein by sodium metaperiodate reaction (data not shown).

Figure 1C shows the anti-cAPP immunoblot for the membrane-associated APP and the APP binding protein in the isolated membrane fraction. Membrane-associated APP has 110- and 120-kDa full-length and 65-kDa C-terminal polypeptides. The latter, shorter peptides are derived from

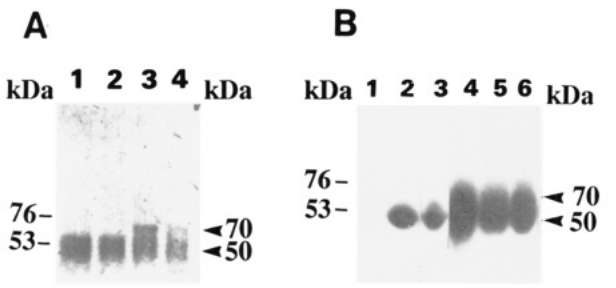

FIGURE 2: Disuccinimidyl suberate cross-linking of the total extracellular and cytosol proteins. ^{125}I-P18 (5 nM) was incubated with 5 μg of extracellular and 10 μg of cytosol proteins in the presence of 1 mM $CaCl_2$. Cross-linking with disuccinimidyl suberate was followed by SDS–PAGE and autoradiography. *Panel A*: Extracellular proteins. *Lane 1*, NL27KO (normal); *lane 2*, NL7KO (normal); *lane 3*, AM12618 (early-onset familial AD); *lane 4*, KG4012 (early-onset familial AD). *Panel B*: Cytosol proteins in the presence of Ca^{2+}. *Lane 1*, NL27KO (normal) with 5 mM (final) EDTA; *lane 2*, NL27KO (normal); *lane 3*, NL7KO (normal); *lane 4*, AM12618 (early-onset familial AD); *lane* 5, M17810 (late-onset familial AD); *lane 6*, KG4012 (early-onset familial AD).

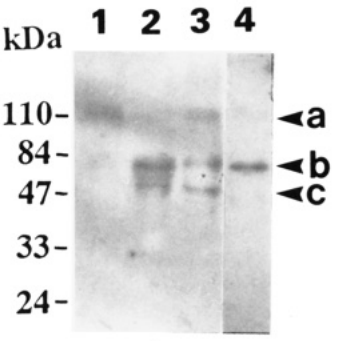

FIGURE 3: Purification of 68-kDa protease by heat dissociation. Extracellular proteins (300 μg) of AM12618 cells were limit-filtered to remove proteins smaller than 100 000 daltons. The 120-kDa complex in the enriched high molecular mass proteins was immunoprecipitated by anti-cAPP antibody. The precipitate was heated at 55 or 65 °C for 15 min or was acidified to pH 4.0 to dissociate the 68-kDa protease from the 53-kDa APP and antibody IgG. After IgG was removed by passage through a protein A–Sepharose affinity column, the eluant was separated by SDS–PAGE and stained with Coomassie brilliant blue. *Lane 1*, dissociation by acidification; *lane 2*, dissociation by heat at 65 °C for 15 min: *lane 3*, dissociation by heat at 55 °C for 15 min; *lane 4*, further purification of the 55 °C dissociated sampled shown in *lane 3* (see Materials and Methods). Arrowheads indicate (*a*) the 120-kDa complex, (*b*) the 68-kDa protease, and (*c*) the 53-kDa APP fragment.

40–50-kDa N-terminal truncation in the PEST regions in the extracellular domain (Matsumoto & Fujiwara, 1991; Siman & Christoph, 1989). The 180-kDa band in the AM12618 membranes represents an SDS-stable, heat-labile complex and its dissociation of 120-kDa full-length APP that has the KPI domain and the 68-kDa protein (Figure 1C, *lanes 2 and 4*, respectively). This 180-kDa complex is not present in NL27KO normal cell membranes (Figure 1C, *lanes 1 and 3*). These results indicate clearly that the 68-kDa protein is expressed in familial AD cells and that it forms a heat-labile complex with the membrane-associated full-length APP that has KPI. Therefore, the 68-kDa protein most probably is a KPI binding serine protease.

Familial AD cell lysates from the peptide sequence analysis showed no distinct fragment cleaved at the N-terminus of β/A4 (Matsumoto & Fujiwara, 1993), but the possibility remains that the 68-kDa protease is inhibited by protease inhibitors, such as the KPI domain of APP (Matsumoto & Fujiwara, 1991). We therefore used a synthetic oligopeptide, P18 (2200 daltons), and the disuccinimidyl suberate cross-linking method (Tsudo et al., 1987) to determine whether a specific protein(s) bind(s) to P18; 5 nM ^{125}I-P18 reacted with both the extracellular (5 μg) and cytosolic (10 μg) proteins. Cross-linking produced only a 50-kDa band with the secreted proteins from NL27KO and NL7KO normal cells (Figure 2A, *lanes 1 and 2*), whereas the two 50- and 70-kDa bands made complexes with the extracellular proteins from AM12618 and KG4012 early-onset familial AD cells (Figure 2A, *lanes 3 and 4*). These complexes are formed in the presence of Ca^{2+} but not in its absence nor by addition of 5 mM EDTA (data not shown). Similar results were obtained for cytosol proteins of normal (NL27KO, NL7KO) cells (Figure 2B, *lanes 2 and 3*) and of both early-onset familial AD (AM12618, L801) (Figure 2B, *lanes 4 and 6*) and late-onset familial AD calls (MI7810) (Figure 2B, *lane 5*). Addition of 5 mM EDTA completely inhibited the cross-linking (Figure 2B, *lane 1*). Expression of the 48-kDa protein is similar in both normal and familial AD cells, but the 68-kDa protein appears to be specific to familial AD cells (Figure 2A,B).

Preparation of the 68-kDa Protein. To isolate the 68-kDa protein, we utilized the property that this protein is coprecipitated by anti-cAPP antibody. An extracellular protein sample (300 μg) from AM12618 early-onset familial AD cells was subjected to limit filtration to remove proteins of less than 100 000 daltons, after which the filtered protein was immunoprecipitated with anti-cAPP antibody. When the immune complex was dissociated by acidification (pH <4.0) and then passed through a protein A–Sepharose affinity column to remove rabbit IgGs, a single 120-kDa band appeared (Figure 3, *lane 1*). Heating the complex for 15 min at 65 °C dissociated it completely, and at 55 °C partially, into the 68-kDa protein and 53-kDa APP fragment (Figure 3, *lanes 2 and 3*, respectively). These results show that the 120-kDa band is a heat-labile complex composed of the 68-kDa protein and secreted 53-kDa APP fragment.

The heat-dissociated (55 °C for 15 min) complex prepared from AM12618 extracellular protein was applied to a limit filtration column to remove any undissociated 120-kDa complex, after which the pass-through fraction was immunoprecipitated with anti-cAPP antibody to remove the 53-kDa APP fragment. Its supernatant was passed through a protein A–Sepharose affinity column to remove the cAPP antibody. The fraction from the final passage contained only the 68-kDa protein (Figure 3, *lane 4*), verification that it is a component of the 120-kD complex. This result also shows that the 68-kDa protein can be prepared by immunoprecipitation of total extracellular protein with anti-cAPP antibody followed by heat dissociation.

Disuccinimidyl suberate cross-linking analysis showed that the 68-kDa protein in total cytosol and extracellular proteins of familial AD cells binds to ^{125}I-P18 (Figure 2). To ascertain the reproducibility of binding of the purified 68-kDa protease, we again performed the cross-linking analysis (Figure 4A, left panel). In the presence of Ca^{2+}, the signal intensity of the 70-kDa radioactive band increased as a function of the amount of 68-kDa protein added (Figure 4A, *lanes 3–5*). The absence of Ca^{2+} completely inhibited formation of the cross-linked complex (Figure 4A, *lane 6*); therefore, the purified 68-kDa protein bound to 2-kDa ^{125}I-P18 containing the N-terminal sequence of β/A4 in the presence of Ca^{2+}. This indicates that the 68-kDa protein present in the mixture of extracellular and cytosol proteins from familial AD cells is identical to the protein prepared as described above. Furthermore, binding of the 68-kDa protein to a natural APP substrate was analyzed using the same method (Figure 4B, right panel). One picomole of 68-kDa protease prepared from

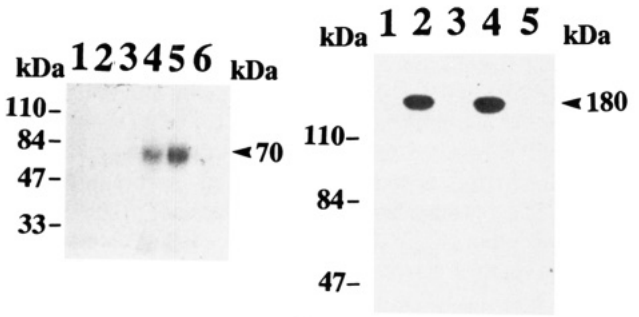

FIGURE 4: Disuccinimidyl suberate cross-linking analysis of the 68-kDa protein. Conditions were the same as in Figure 2, except that purified 68-kDa protein was cross-linked and that samples were subjected to limit-filtration immediately after termination of cross-linking. Left panel: Cross-linking with 5 nmol of ^{125}I-P18 substrate. *Lane 1*, no protease; *lane 2*, 5 pmol of protease without substrate; *lane* $_3$, 0.05 pmol of protease; *lane 4*, 0.5 pmol of protease; *lane 5*, 5 pmol of protease; *lane 6*, 5 pmol of protease with 5 mM (final) EDTA. Right panel: Cross-linking with 120-kDa ^{125}I-native APP (approximately 1 pmol). *Lane 1*, no 68-kDa protein; *lane 2*, 1 pmol of 68-kDa protein prepared from AM12618 cells; *lane 3*, 1 pmol of 68-kDa protein (AM12618) in the absence of Ca^{2+}; *lane 4*, 1 pmol of 68-kDa protein prepared from KG4012 cells; *lane 5*, 1 pmol of 68-kDa protein (AM12618) in the presence of 5 mM (final) EDTA.

media of AM12618 familial AD cells was cross-linked with 1 pmol of ^{125}I-labeled natural APP in the presence of Ca^{2+} (Figure 4B, *lane 2*), but the cross-linking was abolished in the absence of Ca^{2+} (Figure 4B, *lane 3*) or in the presence of 5 mM (final) EDTA (Figure 4B, *lane 5*). The same amount of 68-kDa protease prepared from media of KG4012 early-onset familial AD cells also cross-linked this substrate (Figure 4B, *lane 4*). A unique appearance of the single 180-kDa band indicates that the cross-linking condition (4 °C for 20 min) does not induce proteolysis of the natural APP. This result demonstrates that binding of the 68-kDa protease to oligopeptide P18 is not a nonspecific artifactual result, but a specific event recognizing the amino acid sequence and/or three-dimensional structure around the β/A4 N-terminus.

Characterization of the 68-kDa Protease. We studied the proteolytic action of the 68-kDa, P18 binding protein. A 10 pmol (20 ng) sample of the ^{125}I-P18 oligopeptide substrate was made to react with the 68-kDa protein in 10 mM phosphate buffer, pH 7.5, and 1 mM $CaCl_2$ at 37 °C for 1 h, after which cleavage of ^{125}I-P18 was analyzed by thin-layer chromatography (Figure 5). A 5 pmol (20 μL) sample of protease was prepared from 400 μL of AM12618 extracellular proteins by heat-dissociation of 55 °C; 0.5 pmol (2 μL) of 68-kDa protein cleaved ^{125}I-P18 completely (Figure 5A, *lane 1*), whereas lesser amounts of the 68-kDa protease cleaved it only partially (0.125 pmol) (Figure 5A, *lane 2*) or not at all (0.025 pmol) (Figure 5A, *lane 3*). The protease prepared by heat treatment at 55 °C for longer than 30 min had markedly decreased proteolytic activity, and at 55 °C for 60 min or at 65 °C its proteolytic activity was completely lost (data not shown). In contrast, 0.5 pmol (2 μL) of protease prepared from AM12618 cell (Figure 5B, *lanes 1–3*) or QDH18601 cell (Figure 5B, *lane 4*) extracellular proteins reacted with increasing amounts of P18 oligopeptide substrate. It cleaved 10 pM P18 completely (Figure 5B, *lanes 1 and 4*) and the 100 pM substrates partially (Figure 5B, *lane 2*), but had no effect on the 1 nM substrates (Figure 5B, *lane 3*). These results indicate that the 68-kDa protease derived from the extracellular proteins of AM12618 and QDH 18601 familial AD cells cleaves the P18 oligopeptide in the presence of Ca^{2+}.

Furthermore, we determined the site on the P18 oligopeptide cleaved by the 68-kDa protease. After running a scaled-up

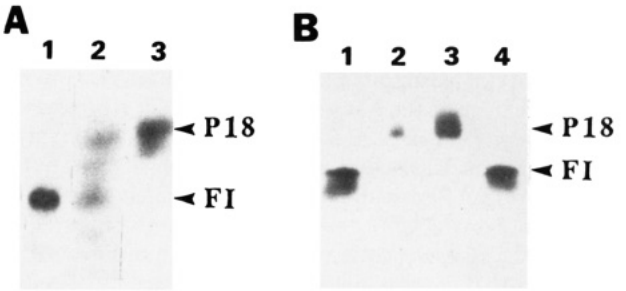

FIGURE 5: Proteolysis of ^{125}I-P18 by the 68-kDa protease. Autoradiogram of thin-layer chromatography. *Panel A*: Proteolysis of 10 pM ^{125}I-P18 by various amounts of protease prepared from AM12618 extracellular proteins. *Lane 1*, 0.5 pmol of protease; *lane 2*, 0.125 pmol of protease; *lane 3*, 0.025 pmol of protease. *Panel B*: Proteolysis by a fixed amount of purified 68-kDa protease. Substrates were mixtures of P18 and ^{125}I-P18: 0.5 pmol of protease heat-dissociated at 55 °C (*lanes 1–3*) or at 65 °C (*lane 4*) reacted with 10 pM ^{125}I-P18 only (*lane 1*), with 10 pM ^{125}I-P18 and 90 pM P18 (*lane 2*), or with 10 pM ^{125}I-P18 and 990 pM P18 (*lane 3*). Arrowheads indicate the migration positions in the thin-layer chromatogram. *P18*, substrate P18; *FI*, the large fragment of cleaved P18. The small fragment was run off.

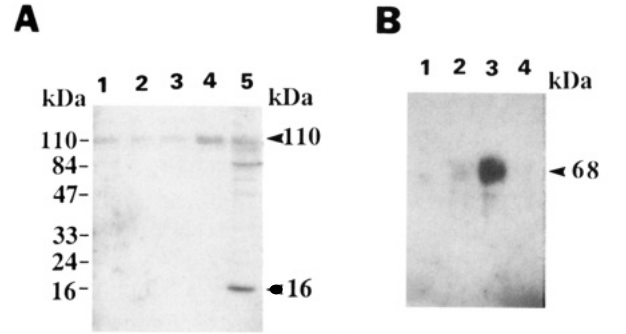

FIGURE 6: Proteolysis of natural APP by the 68-kDa protease and its specific binding to a serine protease inhibitor. *Panel A*: autoradiogram of SDS–PAGE of the 120-kDa ^{125}I-labeled natural APP cleaved by the 68-kDa protease. Natural ^{125}I-APP substrate (approximately 1.5 pmol) was incubated with 0.03–0.9 pmol of protease at 37 °C for 1 h and then separated by SDS–PAGE after boiling. *Lane 1*, no protease; *lane 2*, 0.9 pmol of protease with 5 mM EDTA; *lane 3*, 0.03 pmol of protease; *lane 4*, 0.3 pmol of protease; *lane 5*, 0.9 pmol of protease. The arrowhead indicates the migration position of ^{125}I-β/A4–8/17. *Panel B*: Fluorogram of SDS–PAGE to detect binding of 100 pmol of [1,3-^{3}H]diisopropyl fluorophosphate to the protease. *Lane 1*, no protease; *lane 2*, with 0.2 pmol of protease; *lane 3*, with 2 pmol of protease; *lane 4*, with 2 pmol of protease and 5 mM (final) EDTA.

reaction for 3 h of 5 μg of unlabeled P18 and the protease from the AM12618 cell lysate, we determined the N-terminal amino acid sequence of the FI fragment which migrated to exactly the same position as shown in Figure 5. The result was a mixture of the two N-terminal sequences of Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr (43 pmol) and Met-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly (20 pmol) (data not shown). This implies that the 68-kDa protease cleaves the β/A4 N-terminal Lys–Met–Asp bond before Met by 32% and after it by 68%.

To analyze the substrate specificities of the 68-kDa protease, we investigated whether this protease cleaves the Esch site, Gln^{15}-Lys^{16}-Leu^{17} (Esch et al., 1990), within the β/A4–8/17 oligopeptide substrate, which corresponds to the 8–17th amino acid residues of β/A4. This protease, however, did not cleave this substrate (data not shown). ^{125}I-Labeled 120-kDa full-length APP also was used as a natural substrate (Figure 6A). Under identical conditions for proteolysis, the 68-kDa protease cleaved the natural substrate dose dependently (Figure 6A, *lanes 3–5*), cleavage being completely inhibited by addition

of 5 mM EDTA (Figure 6A, *lane 2*). This cleavage yields both a main 16-kDa preamyloid fragment that corresponds to the β/A4 cytoplasmic domain of APP and a 90-kDa protein which appears to be the N-terminal fragment of APP cleaved at the same site (Figure 6A, *lane 5*). In addition to this major site, the protease cleaved at a less preferable site within the middle part of APP because two minor fragments of 47 and 65 kDa were produced in almost equal amounts (Figure 6A, *lane 5*). The 16-kDa peptide appears at a migration position identical to that of the abnormally accumulated protein seen in both early- and late-onset familial AD cells (Matsumoto & Fujiwara, 1991, 1993).

The characteristics of this protease were analyzed further to clarify its reaction with class-specific protease inhibitors. Specific binding of the purified 68-kDa protease to a serine protease inhibitor, [1,3–^{3}H]diisopropyl fluorophosphate (100 pmol), was analyzed (Figure 6B). A 68-kDa band was detected in the fluorogram in a dose-dependent fashion (0.1 or 2 pmol) of the 68-kDa protease added (Figure 6B, *lanes 2 and 3*, respectively). This band, however, was not detected in the absence of the protease (Figure 6B, *lane 1*) or in the presence of 5 mM (final) EDTA (Figure 6B, *lane 4*). This result clearly demonstrates specific binding of this serine protease inhibitor to the 68-kDa protease. Densitometry of the ^{125}I-labeled bands indicates that, under the condition of protease inhibitor excess, the binding occurs in linear dose-dependent fashion of the purified 68-kDa protease added. The following class-specific protease inhibitors were used to characterize the activity of the 68-kDa protease by TLC (data not shown): The inhibitors of thiol proteases, 5 mM iodoacetamide and 20 mM E64, had no effect on 68-kDa protease activity toward P18. The serine protease inhibitors, 2 mM phenylmethanesulfonyl fluoride and 25 μg/mL leupeptin, did not inhibit its activity; However, 10 mM phenylmethanesulfonyl fluoride and 1 mM diisopropyl fluorophosphate inhibited cleavage. An aspartic protease inhibitor, 0.1 ng/mL pepstatin, also did not inhibit its activity, whereas 15 and 30 μg/mL chymostatin, a chymotrypsin-type serine protease inhibitor, inhibited the cleavage of P18. Addition of the Ca^{2+}-dependent protease inhibitor 5 mM EGTA completely inhibited cleavage. The 68-kDa protease seems to be a Ca^{2+}-dependent serine protease.

DISCUSSION

We separated the 48- and 68-kDa proteins that respectively form the 50- and 70-kDa disuccinimidyl suberate cross-linked complexes with ^{125}I-P18 in the presence of Ca^{2+} (Figure 2). These two proteins were present in both the extracellular and cytosol fractions. The 48-kDa protein is expressed in both normal cells and familial AD cells, whereas the 68-kDa protein appears to be specific to familial AD cells (Figure 2). Our recent result of Western analysis using anti-68-kDa protease antibody indicates that the 48-kDa protein detected in the disuccinimidyl suberate cross-linking analysis is not detected by this antibody, suggesting these two proteins do not share antigenic epitope(s) in common (unpublished observation). Also, the 68-kDa protein prepared from early-onset familial AD cells seems to be a glycoprotein with heterogeneous glycoconjugates. Results shown in Figure 1 suggest that this 68-kDa protein is a serine protease because it forms an SDS-insoluble, heat-labile complex with the secreted APP fragment and that of the membrane-associated APP, and because its proteolytic activity was blocked by diisopropyl fluorophosphate, a specific active-site-directed inhibitor of serine protease. The 68-kDa protease cleaves oligopeptide P18 at either bond of Lys^{-2}–Met^{-1}–$Asp^{\beta A4-1}$ and the 120-kDa natural APP substrate in the presence of Ca^{2+}, but does not cleave the Esch's site at $Gln^{\beta A4-15}$-Lys-$Leu^{\beta A4-17}$ within β/A4. The characteristics of binding and cleavage of the 68-kDa protease toward APP substrates and a protease inhibitor suggest that the activity of this protease is performed in an equimolar fashion. The reason why a fragment cleaved at the Lys–Met–Asp bond of the β/A4 N-terminus was not detected in cytosol has yet to be clarified (Matsumoto & Fujiwara, 1993). Plausible reasons for this would be the following: (i) the abundant APPs with the KPI domain present in familial AD lymphoblastoid cells may inactivate the 68-kDa protease by forming an insoluble complex (Figures 1 and 3), and this accounts for the failure to split β/A4; (ii) enhanced cleavage at the N-terminal bond of β/A4 produces the 16-kDa self-aggregated preamyloid which may resist cleavage by the protease.

The significance of 68-kDa protease expression in the abnormal accumulation of the 16-kDa preamyloid peptide in familial AD cells is of interest. The 16-kDa peptide seems to be neurotoxic because in mouse embryonal carcinoma cells (M19, differentiating into neuronal tissue) transfected with β/A4 cytoplasmic sequences of APP cDNA its intracellular accumulation and that of 14-kDa C-terminally truncated fragments result in neuronal degeneration (Fukuchi et al., 1992). The mechanism by which these peptides accumulate in lymphoblastoid cells therefore may be related to β/A4 deposition in the brain. One possible explanation is that the enhanced activity of this protease in familial AD cells generates more amyloidogenic APP fragments, eventually leading to the enhanced accumulation of preamyloid peptide. In normal cells, however, increased activity of the postulated APP secretase that splits the Gln^{15}–Lys^{16}–Leu^{17} bonds within β/A4 would preclude preamyloid accumulation (Estus et al., 1992; Esch et al., 1990; Allsop et al., 1991; Maruyama et al., 1991). In the human brain, in which APP695 devoid of the KPI domain predominates, serine proteases that split the β/A4 N-terminus in AD brain should be more active than in other tissues, which may lead to massive β/A4 deposition. In contrast, Citron *et al.* (1993) have reported that a missense mutation at the Lys and Met residues immediately N-terminal to β/A4 present in Swedish familial AD patients is responsible for the increased deposition of β/A4 in human kidney 293 cells transfected with mutated APP cDNA. This means that proteolysis at these two N-terminal bonds, both of which are target cleavage sites for the 68-kDa protease (Matsumoto & Fujiwara, 1993), is responsible for the accumulation of amyloidogenic β/A4 C-terminal fragments. Cleavage at the N-terminus of β/A4 in the endosomal–lysosomal system (Haass et al., 1992; Golde et al., 1992) therefore is not necessarily an undesirable event that leads to β-amyloid accumulation in the cytoplasm. It is uncertain whether the specific expression of this protease is a primary abnormality of familial AD cells or whether it is a secondary response of these cells that allows processing of amyloidogenic fragments.

By formic acid extraction of cytosol proteins, we reported that the 16-kDa fragment appearing in SDS–PAGE of the cytosol of familial AD cells is a mixture of at least 4 peptides and that the N-terminus of the major peptide is 30 amino acids N-terminal to the first residue of β/A4, whose peptide is the only discrete fragment separated in normal cells (Matsumoto & Fujiwara, 1993). Although the total amount of the 16-kDa protein as well as mRNA expression is larger in familial AD cells than in normal cells, its deficient removal

in familial AD cells cannot be simply attributed to the saturation state of the putative secretase pathway for normal APP processing, since peptide components that constitute the 16-kDa protein seem to be different between familial AD and normal cells (unpublished observation). As we could not find a fragment with a confirmed amino acid sequence at the N-terminus of β/A4, the amino acid sequences N-terminal to β/A4 are considered to be related to the abnormal accumulation of the preamyloid. Cleavage analysis of the natural APP substrate *in vitro* (Fgiure 6A) indicates that the 68-kDa protease contributes, at least in part, to the generation of the amyloidogenic 16-kDa fragments present in familial AD lymphoblastoid cells. Cleavage of APP at the N-terminus of β/A4 therefore does not appear to be a prerequisite for β-amyloid generation as understood thus far (Selkoe et al., 1988; Estus et al., 1992); rather, it may be a separate secretory step that prevents β/A4 accumulation in the cytosol that, in addition, may provide a pathway for the extracellular accumulation of β/A4 (Seubert et al., 1993). Conceivably, the expression of the 68-kDa protease in familial AD cells is a secondary response that prevents the intracellular accumulation of β/A4 through activation of an alternative secretory pathway for APP processing.

The 68-kDa protease differs from the multicatalytic proteinases (>500 000 daltons) and calpains (≈80 000 daltons) in being expressed in both the extracellular and cytosol fractions. Abraham et al. (1991) reported that a 68-kDa, Ca^{2+}-dependent serine protease that is increased in AD and aged normal brain cells cleaves each bond in the Lys^{-2}-Met^{-1}-$Asp^{\beta A4-1}$-Ala^{2} sequence of the β/A4 N-terminal and is inhibited by the serine protease inhibitors protease nexin-2 and α_1-antichymotrypsin. They now have reported that this proteolytic activity is the action of three different proteases: a 28-kDa Ca^{2+}-dependent serine protease which preferentially cleaves the Lys–Met bond of the oligopeptide substrate at the N-terminus of β/A4; a cysteine-dependent metalloprotease that requires dithiothreitol for its proteolysis at the Met–Asp bond of the same oligopeptide; and another cysteine-dependent protease that cleaves the Lys–Met bond of the same substrate (Abraham et al., 1993). This 28-kDa protease appears to have cathepsin G-like epitopes, and the first cysteine-dependent protease to be homologous to rat endopeptidase (EC 3.4.24.15) (Abraham et al., 1993). The 68-kDa protease from lymphoblastoid cells does not show cross-reactivity to the anti-cathepsin G antibody (unpublished observation) and does not have a subunit structure because its size in SDS–PAGE is unaltered in the presence of reducing agents. The proteolysis results shown in Figures 5 and 6 indicate that our 68-kDa protease does not require dithiothreitol for its activity. These proteases isolated independently from different sources have a common property: specific proteolysis at the N-terminus of β/A4. One difference is that our 68-kDa protease does not cleave the Asp^{1}–Ala^{2} bond of the β/A4 oligopeptide, the major cleavage site for the 68-kDa protease from AD brain (Abraham et al., 1990). This cleavage site has not, however, been documented in the further purified stage (Abraham et al., 1993). It seems unlikely that the 68-kDa protease found in human lymphoblastoid cells is identical to any of the three β/A4 N-terminus-splitting proteases found in the brain, but its identity must be clarified by cDNA cloning.

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