We have investigated the functional relationship between metalloendopeptidase EC 3.4.24.15 (MP24.15) and the amyloid precursor protein involved in Alzheimer’s disease (AD) and discovered that the enzyme promotes $\beta$-degradation. We show here that conditioned medium (CM) of MP24.15 antisense-transfected SKNMC neuroblastoma has significantly higher levels of $\beta$. Furthermore, synthetic-$\beta$ degradation was increased or decreased following incubation with CM of sense or antisense-transfected cells, respectively. Soluble $\beta$1–42 was degraded more slowly than soluble $\beta$1–40, while aggregated $\beta$1–42 showed almost no degradation. Pre-treatment of CM with serine proteinase inhibitors 4-(2-aminoethyl)benzenesulfonyl fluoride and diisopropyl fluorophosphate completely inhibited $\beta$ degradation. Additionally, $\alpha$-antichymotrypsin (ACT), a serpin family inhibitor tightly associated with plaques and elevated in AD brain, blocked up to 60% of $\beta$ degradation. Interestingly, incubation of CM of MP24.15-overexpressing cells with ACT formed an SDS-resistant ACT complex, suggesting an ACT-serine proteinase interaction. Recombinant MP24.15 alone did not degrade $\beta$. Diisopropyl fluorophosphate-radiolabeled CM from MP24.15-overexpressing cells contained increased levels of several active serine proteinases, suggesting that MP24.15 activates one or more $\beta$-degrading serine proteinases. Thus, ACT may cause $\beta$ accumulation by inhibiting an $\beta$-degrading enzyme or by direct binding to $\beta$, rendering it degradation-resistant. Identification of the $\beta$-degrading enzyme and MP24.15’s role in its activation is underway. Pharmacological modulation of either enzyme may provide a means of regulating $\beta$ in the brain.

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia in the elderly (1). Evidence indicates that accumulation of amyloid-$\beta$ (A$\beta$) deposits in senile plaques and in cerebrovasculature is associated with the pathophysiology of AD (2). The A$\beta$ peptide is composed of 40–42 amino acids (3). The events leading to its formation from the transmembrane amyloid precursor protein (APP) involve proteolytic cleavage by enzymes that have been termed: 1) $\beta$-secretase, which cleaves at the amino terminus of $\beta$, and 2) $\gamma$-secretase, which cleaves at the carboxyl terminus. In the senile plaques $\beta$ is associated with a number of proteins, including $\alpha$-antichymotrypsin (ACT) (4), which is a serine proteinase inhibitor of the serpin family as well as an acute-phase protein (5). Thus, we hypothesized 10 years ago that ACT may play a role in APP processing (4, 6).

Soluble A$\beta$ peptide can be detected in the conditioned media of a variety of cultured mammalian cells in vitro (7–9), as well as in serum and cerebrospinal fluid in vivo (10). The majority of secreted A$\beta$ is 40 amino acids in length ($\beta$1–40), but approximately 10% of all A$\beta$ is 42 amino acids in length ($\beta$1–42) (11). Little is known about how secreted A$\beta$ is degraded and cleared from the extracellular milieu. The excessive cerebral accumulation of A$\beta$ that occurs in AD could be explained in part by a decreased ability of the brain to degrade and clear A$\beta$. If neural cells can be shown to release specific A$\beta$-degrading proteinases, changes in the activity of such proteinases and/or their up-regulation could represent a therapeutic approach to AD.

We have explored the role of the metalloendopeptidase MP24.15 in the degradation of A$\beta$. MP24.15 is a thiol-dependent enzyme that was purified to homogeneity from AD brain as a candidate $\beta$-secretase (12). McDermot et al. (13) also identified a partially purified MP24.15 as a potential $\beta$-secretase using synthetic peptide substrates. In vitro, MP24.15 has been shown to be involved in the inactivation of a number of neuropeptides, including somatostatin, bradykinin, substance P, and neurotensin (14–16). The cDNA coding for MP24.15 was subsequently cloned from a human brain library (17, 18). In an attempt to further examine the $\beta$-secretase properties of MP24.15 under physiologic conditions, we transfected human neuroblastoma cells with MP24.15 cDNA in the sense and antisense directions to test its activity on the endogenous, membrane-bound APP. A$\beta$ amounts produced by cells containing the mock-, sense-, or antisense-transfected cDNA were compared. Contrary to our expectations, we observed higher A$\beta$ levels in the conditioned medium (CM) of antisense-transfected cells than in the CM of sense- or mock-transfected cells, while APP levels remained unchanged. These unexpected results prompted us to further investigate MP24.15. Here we report that MP24.15 is involved in the degradation of A$\beta$ rather than its production.

Previous reports have shown that rat microglia, astrocytes, and human THP-1 monocyte cells are important in the formation (19) or clearance of A$\beta$ and amyloid fibers (20) when A$\beta$ is added to the culture medium, but that rat neurons do not possess this degradative potential (21). Proteinase inhibition studies have also been utilized to determine which class of proteinase is responsible for the degradation of A$\beta$ in the culture media of microglial cells. Mentlein et al. (22) determined...
that lipopolysaccharide-activated microglial cells release a 200-kDa metalloproteinase that preferentially degrades soluble, but not polymerized, Aβ. Naidu et al. (23) found that the levels of soluble Aβ peptide released to the media of Chinese hamster ovary cells are reduced over time due to the activity of multiple types of proteinases, including those from the metallo, aspartyl, and thiol classes. Banks et al. (24) identified an erythrocyte-derived metalloproteinase that shows a loss of Aβ degradation activity in the presence of aluminum. Other proteinases including the lysosomal enzyme, cathepsin D (25), and extracellular proteinases, including matrix metalloproteinase 2/gelatinase-A (26) have been reported to be involved in the processing of Aβ. Insulin degrading enzyme (IDE), a 110-kDa metalloproteinase, has additionally recently been shown to bind to and degrade radioiodinated Aβ peptide (27–29). This degradation by IDE occurs in rat brain and liver, human brain synaptic membrane fractions, and microglial cells in culture.

Additionally, there have been several reports of serine proteinases that degrade the Aβ peptide. One such 68-kDa proteinase isolated from human brain partially degrades Aβ purified from human brain (30). Another 28-kDa serine proteinase was found in conditioned medium of Chinese hamster ovary cells transfected with APP695. Although this serine proteinase originated from the trypsin cell passing solution, it was shown to complex with α2-macroglobulin (α2M) serum component and to degrade both Aβ1–40 and 1–42 (31).

In parallel to the direct proteolysis of Aβ, alternative methods of Aβ trafficking and processing have been studied. The serpin-enzyme complex receptor was identified in cells for its ability to complex with IDE as their major Aβ-degrading activity in the presence of aluminum. Other proteinases have also shown that serine proteinases complex receptor was identified in cells for its ability to degrade both Aβ and Aβ peptide isolated from human brain partially degrades Aβ purified from human brain (30). Another 28-kDa serine proteinase was found in conditioned medium of Chinese hamster ovary cells transfected with APP695. Although this serine proteinase originated from the trypsin cell passing solution, it was shown to complex with α2-macroglobulin (α2M) serum component and to degrade both Aβ1–40 and 1–42 (31).

In parallel to the direct proteolysis of Aβ, alternative methods of Aβ trafficking and processing have been studied. The serpin-enzyme complex receptor was identified in cells for its ability to bind to IDE and other serine proteinase inhibitors, and investigations have also shown that serpin-enzyme complex receptor mediates Aβ internalization and degradation (32). The proteinase inhibitor α2M was shown to bind Aβ directly, possibly mediating Aβ uptake by the α2M uptake receptor, or low-density lipoprotein receptor-related protein (33, 34). The receptor for advanced glycation end products has also been shown to interact with Aβ and it may contribute to neurotoxicity that results in dementia (35). It is likely that different cells utilize different mechanisms for the clearance of Aβ whereas microglial cells may use IDE as their major Aβ-degrading enzyme while neuronal cells may use a serine proteinase.

Identification of the proteinases involved in Aβ catabolism is therefore critically important for the development of therapeutics to prevent or treat AD. Our approach has been to focus on the metalloproteinase MP24.15. Its role in the proteolytic cascade leading to Aβ degradation holds great promise for direct intervention in the pathophysiology of AD.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Transfections**—SKNMC human neuroblastoma cell line (ATCC, MD) were cultured in minimal Eagle’s medium supplemented with various batches of 10% heat-inactivated fetal bovine serum (FBS). Cells were regularly monitored for growth and viability by trypan blue exclusion assay. The MP24.15 cDNA was obtained from Dr. G. Huber (Hoffman la Roche, Basel, Switzerland (18)). A construct containing the full-length MP24.15 cDNA (2551 base pairs) in the pCDNA3.1/Zeo (Invitrogen, CA) was used for transfection of the SKNMC cells. The cells were plated at 105 cells/100-mm dish and allowed to attach for 18–24 h. The medium was then changed, and the cells were transfected 2 to 4 h later with 20 μg of plasmid DNA by the calcium-phosphate method using a Calcium Phosphate Mammalian Cell Transfection Kit (5 Prime, Inc., Boulder, CO). The initial incubation of the plates (4 h, 37 °C, 5% CO2) was followed by a 15% glucose shock (3 min at room temperature). After washing with serum-free Dulbecco’s modified Eagle’s medium, the cells were allowed to recover overnight in minimal Eagle’s medium containing 10% FBS. The following day the cells were split into 5 plates and were incubated with minimal Eagle’s medium containing 10% FBS and 400 μg/ml Zeocin (Invitrogen) for selection. Medium was changed every 3–4 days and stably transfected clones were collected. MP24.15 antisense constructs were cloned into the pCDNA3.1/Zeo plasmid as well. These constructs contain the KpnI/SmaI fragment of the MP24.15 cDNA (1191 base pairs) and were subcloned into the plasmid in the antisense direction. The SKNMC cells were transfected with the antisense construct as above. All transfected cells were grown in Dulbecco’s modified Eagle’s medium containing 10% FBS and Zeocin. Cells were grown to confluence in minimal Eagle’s medium containing 10% FBS in 100-mm dishes. Once confluent, the plates were washed twice with serum-free and phenol-red free Dulbecco’s modified Eagle’s medium and then incubated for 18–24 h with the same medium at 37 °C (in some experiments, N2 supplement (Life Technologies, Inc.) was added to the serum-free medium). The following day, the CM was collected and centrifuged at 1500 rpm to pellet the cell debris. The plates were washed twice with ice-cold phosphate-buffered saline, and the cells were harvested using cell scrapers (Nalge Nunc Intl., Naperville, IL) in the presence of 300 μl of homogenizing/lysis buffer containing 1 mM EGTA, 1 mM EDTA, 1 mM AEBSF, 1 μM leupeptin, and 1% Triton X-100 in phosphate-buffered saline. Cells lysates were sonicated until all visible particle matter disappeared. Protein levels of each of the CM samples were measured using the BCA protein assay reagent kit (Pierce, Rockford, IL). 15–20 μg of protein from each sample was electrophoresed on a 10% Tris glycine SDS-polyacrylamide gel (Bio-Rad) or on 10–20% Tris-Tricine gels (Novex, San-Diego, CA). Gels were then soaked for 20 min in blotting buffer and blotted onto Immobilon-P (Millipore, Bedford, MA) for 2 h at 200 ma for the glycine gels or 2 h at 100 V for the Tris-Tricine gradient gels. After transfer the blots were incubated with 5% milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20). Blots were incubated with the specific primary antibody as indicated in each figure. After 3 washes with TBST (15 min each), secondary antibody (Sigma) conjugated to alkaline-phosphatase was applied, and the blots were washed again three times as above. Reactions were developed in the presence of alkaline-phosphatase premixed substrate reagents kit (Bio-Rad). For the detection of MP24.15, we used monoclonal antibody IVD6 generated in our laboratory (36). For the IDE detection, we used the monoclonal antibody that was kindly provided by Dr. Richard A. Roth, Stanford University. For detection of ACT, we used sheep anti-human ACT antibodies (Atlantic Antibodies, Stillwater, MN).

**Immunoprecipitation**—Conditioned medium supernatants (1–3 ml) were precleared by incubation with 50 μl of goat anti-mouse conjugated affinity gel-Sepharose (Cappel, Organon Teknika Corp. West Chester, PA) for 1 h at 4 °C on a rocker platform. Samples were centrifuged at 6000 rpm for 5 min, at 4 °C, and the precleared supernatants were incubated with the 6E10 (1:300) monoclonal antibody against Aβ1–16 (37) for 5 or 18 h on a rocker at 4 °C. The goat anti-mouse antibody affinity gel (50 μl) was added to the incubation and the roller continued for 4 h at 4 °C. The immunoprecipitated complex was collected by centrifugation (same as above) and washed three times 20 min each with STEN buffer containing 100 mM Tris, pH 7.6, 300 mM NaCl, 4 mM EDTA, 0.4% Nonidet P-40 as follows: the first wash contained STEN buffer + 0.5 mM NaCl; the second wash STEN buffer + 0.1% SDS; and the third wash, STEN buffer alone. All buffers also contained 20 μM phenylmethylsulfonyl fluoride and a protease inhibitor mixture (Sigma). The pellet obtained following the last wash and centrifugation was resuspended in 12 μl of 100 mM Tris-HCl, pH 7.4, 2 μl of Na3VO4 (0.2 mM), and one Iodo-Bead (Pierce). The mixture was incubated for 20 min and the iodinated peptide separated from the unbound free iodine on a Sepharose G-25 spin column (5 Prime→3 Prime, Inc.). CM (10 μl) of MP24.15 sense-, antisense-, or mock-transfected cells were incubated with 10 μl (5 μM) iodinated
Expression of MP24.15 in human neuroblastoma cells. Western blot using monoclonal antibody IVD6 demonstrates MP24.15 levels in cell lysates after transfection with the MP24.15 cDNA in the sense direction (A) and in the antisense direction (B). A: 1, nontransfected cells; 2, mock-transfected cells; 3, MP24.15-transfected cells; 4, M markers. B: 1, mock-transfected cells; 2–6, MP24.15 antisense-transfected cells clones.

MP24.15 and Amyloid-β Degradation

Strategies that overexpression of MP24.15 in these cells results in higher levels of this enzyme in cell extracts (Fig. 1A), as well as in CM (Fig. 4) than the endogenous levels of mock-transfected cells. Fig. 1B shows the expression of MP24.15 in different clones of SKNMC cells transfected with antisense constructs. Among these antisense clones, some exhibit undetectable or significantly lower levels of MP24.15 expression (lanes 2 and 4–6) as compared with mock-transfected clones (lane 1), while others (lane 3) display endogenous levels of MP24.15 expression. APP and APP fragments, including the naturally secreted Aβ, were monitored by immunoprecipitation and Western blot analysis of cell lysates and CM of transfected cells with monoclonal antibody 6E10. Interestingly, when we measured the amounts of APP and Aβ in the lysates and CM of the various cells, we observed that soluble Aβ levels in the CM of the MP24.15 antisense-transfected cells were high, while Aβ was not detectable in CM of mock-transfected cells. The amounts of APP in lysates and CM of all cells were comparable (Fig. 2). These results suggest that the reduced level of MP24.15 caused an increase in Aβ, indicating that MP24.15 may be involved in Aβ degradation.

To determine the role MP24.15 plays in Aβ degradation, CM of MP24.15 sense-, antisense-, and mock-transfected cells were incubated with synthetic [125I]Aβ1–40 and [125I]Aβ1–42 for 4, 8, 16, and 32 h at 37 °C, and Aβ degradation was monitored as described under “Experimental Procedures.” We found that (a) CM of MP24.15 sense-transfected cells exhibit the highest Aβ-degrading activity; (b) CM of mock-transfected cells, which express the endogenous MP24.15, exhibit an intermediate Aβ degrading activity; (c) CM of antisense-transfected cells, which contain the lowest MP24.15 levels, exhibit the lowest Aβ-degrading activity (Fig. 3A). Aβ incorporated with Tris buffer or with medium containing 10% heat-inactivated fetal bovine serum, as a control, is not detectably degraded during the 32-h incubation period (Fig. 3A). The difference in Aβ degradation rates of CM of MP24.15-overexpressing cells and CM of mock-transfected cells is more clearly demonstrated in the shorter time course experiment described in Fig. 3B.

Serum-free CM did not exhibit any Aβ-degrading ability (data not shown), but in the presence of serum the amount of Aβ degradation directly correlates with MP24.15 levels. The effect of the serum was independent of the lot used. Comparison of the degradation rates of Aβ1–40 and 1–42 clearly demonstrates that Aβ1–42 catabolism is slower than that of Aβ1–40 under the same conditions (Fig. 3A). Additionally, Aβ1–42 partially aggregates during the incubation time in 10 mM Tris buffer and appears as a high molecular weight band in the lanes that contain Aβ1–42 alone in Tris buffer, but not in

RESULTS

The Metalloendoproteinase EC 3.4.24.15 Is Involved in Amyloid β Peptide Degradation—In order to establish the role MP24.15 plays in APP processing, we stably transfected the human neuroblastoma cell line SKNMC with the full-length cDNA of MP24.15, and with a fragment that contains most of the cDNA in the antisense orientation. Western blot analysis using our monoclonal antibody IVD6 against MP24.15 demonstrated that overexpression of MP24.15 in these cells results in higher levels of this enzyme in cell extracts (Fig. 1A), as well as in CM (Fig. 4) than the endogenous levels of mock-transfected cells. Fig. 1B shows the expression of MP24.15 in different clones of SKNMC cells transfected with antisense constructs. Among these antisense clones, some exhibit undetectable or significantly lower levels of MP24.15 expression (lanes 2 and 4–6) as compared with mock-transfected clones (lane 1), while others (lane 3) display endogenous levels of MP24.15 expression. APP and APP fragments, including the naturally secreted Aβ, were monitored by immunoprecipitation and Western blot analysis of cell lysates and CM of transfected cells with monoclonal antibody 6E10. Interestingly, when we measured the amounts of APP and Aβ in the lysates and CM of the various cells, we observed that soluble Aβ levels in the CM of the MP24.15 antisense-transfected cells were high, while Aβ was not detectable in CM of mock-transfected cells. The amounts of APP in lysates and CM of all cells were comparable (Fig. 2). These results suggest that the reduced level of MP24.15 caused an increase in Aβ, indicating that MP24.15 may be involved in Aβ degradation.

To determine the role MP24.15 plays in Aβ degradation, CM of MP24.15 sense-, antisense-, and mock-transfected cells were incubated with synthetic [125I]Aβ1–40 and [125I]Aβ1–42 for 4, 8, 16, and 32 h at 37 °C, and Aβ degradation was monitored as described under “Experimental Procedures.” We found that (a) CM of MP24.15 sense-transfected cells exhibit the highest Aβ-degrading activity; (b) CM of mock-transfected cells, which express the endogenous MP24.15, exhibit an intermediate Aβ degrading activity; (c) CM of antisense-transfected cells, which contain the lowest MP24.15 levels, exhibit the lowest Aβ-degrading activity (Fig. 3A). Aβ incorporated with Tris buffer or with medium containing 10% heat-inactivated fetal bovine serum, as a control, is not detectably degraded during the 32-h incubation period (Fig. 3A). The difference in Aβ degradation rates of CM of MP24.15-overexpressing cells and CM of mock-transfected cells is more clearly demonstrated in the shorter time course experiment described in Fig. 3B.

Serum-free CM did not exhibit any Aβ-degrading ability (data not shown), but in the presence of serum the amount of Aβ degradation directly correlates with MP24.15 levels. The effect of the serum was independent of the lot used. Comparison of the degradation rates of Aβ1–40 and 1–42 clearly demonstrates that Aβ1–42 catabolism is slower than that of Aβ1–40 under the same conditions (Fig. 3A). Additionally, Aβ1–42 partially aggregates during the incubation time in 10 mM Tris buffer and appears as a high molecular weight band in the lanes that contain Aβ1–42 alone in Tris buffer, but not in
those lanes containing medium with 10% serum as control (data not shown). Interestingly, in all lanes where Aβ degradation occurs an approximately 30-kDa band is seen, suggesting that iodinated Aβ binds to a CM protein that may represent an Aβ-degrading serine proteinase.

The Inhibitory Profile of the Aβ Degrading Activities—To further characterize the Aβ degrading activity found in the SKNMC CM, we examined the effects of different proteinase inhibitors using the Aβ degradation assay described above. Using concentrations known to inhibit other proteinases, we incubated the various inhibitors with the CM of the sense-, antisense-, or mock-transfected cells for 30 min at 37 °C prior to the addition of the iodinated Aβ peptide for an additional incubation of 3 h. As seen in Table I, the inhibitory profile clearly demonstrates that both a metalloproteinase and a serine proteinase are involved in Aβ degradation. DFP and AEBSF, two serine proteinase inhibitors, completely inhibited the Aβ degradation. ACT, a serine proteinase inhibitor of the serpin family, inhibited 46–60% of the Aβ degrading activity. The inhibitor Cpp-Ala-Ala-Phe-pAB, which is a non-permeable specific inhibitor of MP24.15, had a partial inhibitory effect on the Aβ peptide degradation, while Zincov had only a slight inhibitory effect. 1,10-Phenanthroline (4 mM) and insulin (1–10 mM) which are both known to completely inhibit the IDE (29, 38, 39), had low inhibitory effects on Aβ degradation (Table I).

Serum-free Medium Does Not Degrade Aβ—To test whether recombinant human MP24.15 (active against the specific fluorescent substrate Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp)-OH) can degrade radiolabeled Aβ, we mixed recombinant MP24.15 with 125I-Aβ. No Aβ degradation was observed (data not shown). We observed that CM of sense-transfected cells grown in serum-free medium, with or without the N2 supplement, exhibit no Aβ degrading activity. Our trial to reconstitute the Aβ degrading activity in serum-free CM with recombinant MP24.15 was un-
The inhibitory profile of 125I-β peptide degradation by conditioned media of SKNMC cells

The various inhibitors were incubated with the conditioned media for 20 min at 37 °C prior to the addition of equal volume 125I-β. Tubes were then incubated for an additional 4 h at 37 °C. Autoradiographs were read in a densitometer. Percent inhibition was calculated as 1-

| Inhibitor | Concentration | % inhibition in mock-transfected cells | % inhibition in MP sense-transfected cells |
|-----------|---------------|----------------------------------------|------------------------------------------|
| No inhibitor | mM | 0 | 0 |
| AEBSF | 2 | 100 | 100 |
| DFP | 7 | 100 | 100 |
| ACT | 0.005 | 60.6 | 46.5 |
| EDTA | 0.10 | 42.6 | 35.1 |
| Zinc | 1.6 | 7.8 | 9.8 |
| 1,10-Phenanthroline | 4 | 30 | 25.5 |
| Cpp-Ala-Ala-Phe-pAB | 0.15 | 48.4 | 30 |
| Pepstatin | 0.15 | 21.8 | 15.2 |
| Insulin | 0.001 | 0 | 0 |
| ACT | 0.01 | 11.8 | 100 |
| 0.1 | 100 |

*Note that 1 mM 1,10-phenanthroline is known to completely inhibit IDE, but in our system 4 mM 1,10-phenanthroline inhibits only 25–30% of the β degrading activity.

*Also, 1 μM insulin has been shown to completely inhibit IDE, while in our system 1 μM insulin had no effect at all and 10 μM insulin only partially inhibited β degradation. Insulin at 100 μM is 20 times more concentrated than the 125I-β used in the assay (5 μM) and the inhibition is believed to be nonspecific.

successful, indicating that serum is needed in addition to MP24.15 for β degradation to occur (data not shown).

Dependence on one or more serum factors was also observed and reported earlier by Qiu et al. (31), who described β degradation from Chinese hamster ovary cells by a serine proteinase complexed with α2M. We tested whether the factor missing in the serum-free medium was α2M by adding methylamine-activated α2M to our serum-free CM. In our system no β degrading activity was present in the serum-free CM regardless of the presence of α2M (data not shown). Altogether, these results indicate that a serine proteinase and MP24.15 must be present for β degradation to occur in SKNMC cells, but that MP24.15 does not directly degrade β.

**IDE Is Not a Major β-Degrading Enzyme in SKNMC Cells**—Recently it has been reported that IDE is the enzyme responsible for β degradation by microglial cell cultures. In order to determine whether IDE is present in our SKNMC cells and their CM, and to determine whether it is involved in β degradation, cell lysates and CM of the sense-, antisense-, and mock-transfected cells were analyzed by Western blot using monoclonal antibodies against IDE. As shown in Fig. 4, although there is a significant difference in the amount of MP24.15 between MP24.15 sense-, antisense-, and mock-transfected cells, all samples contained comparable amounts of IDE in both their cell extracts and CM. Thus, MP24.15, but not IDE, levels correlated with β degradation ability. Furthermore, insulin inhibited β degradation completely only at 100–200 μM (Table I). This concentration is 20 times higher than that of 125I-β (5 μM) in the assay, and thus this effect is likely nonspecific. Insulin at 1 or 10 μM, which is known to completely inhibit IDE activity, was only a weak inhibitor of β degradation by the neuroblastoma CM (Table I).

**Levels of MP24.15 and IDE in HEK293 and IMR-32 Cells**—We analyzed by Western blot analysis the levels of MP24.15 and IDE expression in CM and cell lysates of two other non-transfected cell lines, human embryonal kidney (HEK293) and human neuroblastoma (IMR-32), in order to compare their endogenous expression of MP24.15 and their ability to degrade 125I-β peptide with the corresponding properties of SKNMC. We found that HEK293 and IMR-32 cells produce equivalent levels of MP24.15 and IDE when compared with SKNMC cells (data not shown). The cell extracts possess the majority of the MP24.15 and IDE, but each of the CM shows detectable levels of these enzymes as well. Furthermore, the β degrading activity of these cells was similar to that of SKNMC cells (data not shown).

**Degradation of Aggregated β by CM of MP24.15 Transfected Cells**—We wished to test the β degrading activity of the various CM on pre-aggregated β. 125I-β1–40 and 1–42 were aggregated for 7 days at room temperature before incubation with CM of the MP24.15 sense- or mock-transfected cells for 4 or 18 h. Mixtures were then separated on 10–20% Tris-Tricine gels. Samples of the pre-aggregated 125I-β1–40 and 1–42 were spun at maximum speed in an Eppendorf centrifuge, the supernatant separated from the pellet, and both the pellets (Fig. 5, lanes 7) and the supernatants (lanes 8) were run on the gels. β1–40 had smaller soluble aggregates and most of the radioactivity stayed in the supernatant, while most of the β1–42 radioactivity was in the pellet and was seen on the gel as high molecular weight aggregates. Consistent with previous results by others, and as demonstrated in the autoradiograph in Fig. 5, β1–42 had higher aggregation ability than β1–40 during this period of time, and was much more resistant to degradation. β1–40 exhibited almost no aggregation and was consequently degraded by the CM.

**Characterization of the Serine Proteinase That Degrades β Peptide in SKNMC Neuroblastoma Cell Medium**—We performed 14-C-DFP labeling experiments in order to identify active serine proteinases in the CM. We have repeatedly found that 14-C-DFP strongly labels a band of 26–28 kDa and several high molecular mass bands at about 200 to 230 kDa and higher in serum-containing CM of MP24.15 overexpressing cells (Fig. 6). The same bands are labeled in the mock- and antisense-transfected cell CM, but to a much lesser extent. Thus, there is a direct correlation between the amount of MP24.15 that is expressed in the cells and found in CM and the intensity of the 14-C-DFP-labeled serine proteinases (Fig. 6). Serum-free CM of mock-, sense-, and antisense MP24.15-transfected cells did not contain any 14-C-DFP-labeled serine proteinases (data not shown). In addition, we found the same pattern of 14-C-DFP labeling in CM of non-transfected SKNMC, HEK293, and IMR-32 cells (data not shown).

**ACT Inhibits β Degradation and Forms an SDS-resistant Complex with a Proteinase in the CM of MP24.15-transfected Cells**—As mentioned above (Table I) we found that the serine proteinase inhibitors AEBSF and DFP completely inhibit β degradation by neuroblastoma CM. We therefore tested ACT, a serine proteinase inhibitor whose levels we previously found to be elevated in AD brain, for a possible inhibitory role in β degradation. Fig. 7 demonstrates that β degradation activity by CM of both mock- and MP24.15-transfected cells was partially inhibited by 5 μM ACT (Fig. 7, lanes 3 and 4). Since ACT is a serine proteinase inhibitor from the serum family and members of the serpin family form SDS-insoluble complexes with their serine proteinases, we examined the ability of CM to form complexes with ACT. These complexes can be detected by Western blot analysis using antibodies to ACT. We incubated CM of non-transfected, mock-transfected, and MP24.15-transfected cells with 0.75 μM ACT for 30 min at 37 °C. The samples were then boiled in Laemmli sample buffer and separated on a 7.5% gel. ACT and ACT-proteinase complexes are demonstrated on the blot with anti-human ACT antibodies. As a positive control, a sample of ACT was incubated in parallel with cathepsin G, a
known substrate for ACT inhibition, at a 1:1 molar ratio. Fig. 8 demonstrates an ACT complex of approximately 100 kDa formed with a factor from CM of MP24.15 sense-transfected cells (panel A, lane 4 and panel B, lanes 3 and 4) and a complex of 90 kDa formed with cathepsin G (panel B, lane 2). In order to compare the ACT-cathepsin G complex with the ACT-CM protease complex, we added ACT and cathepsin G to the CM of mock- or MP24.15 sense-transfected cells. Two different ACT complexes are observed. An ACT-cathepsin G complex of 90 kDa is seen in panel B, lane 2, which contains medium, serum, ACT, and cathepsin G. In panel B, lanes 3 and 4, which contain ACT, cathepsin G, and CM of mock- and sense-transfected cells, respectively, the amount of the 90-kDa ACT-cathepsin G complex is lower, and a new complex of approximately 100 kDa is seen. In panel B, lane 4, where the level of MP24.15 is higher than that in lane 3, the level of ACT-CM protease is also higher. Note that as the ACT-cathepsin G complex diminishes in panel B from lanes 2 to 4, the ACT-CM protease complex increases, suggesting that the CM proteinase competes with cathepsin G for binding to ACT.
DISCUSSION

Excessive deposition of Aβ, the main constituent of the extracellular amyloid plaques, is an early pathological hallmark of AD. Understanding the normal clearance mechanism for secreted Aβ and finding methods to accelerate this process could be extremely beneficial for the prevention, delay, or treatment of AD. The data presented in this article demonstrate that the metalloendopeptidase EC 3.4.24.15 (MP24.15) is a necessary element in the pathway that leads to the degradation of Aβ by human neuron-like cells. Our results show a direct correlation between the expression levels of MP24.15 of healthy SKNMC cells and the degradation of naturally produced or radiolabeled Aβ by their CM. Media from cells overexpressing MP24.15 degrade Aβ more rapidly than CM of mock-transfected cells, which contain only the endogenous MP24.15. Antisense-transfected cells express only very low levels of MP24.15 and, likewise exhibit the lowest levels of Aβ degradation. This correlation is striking in light of the fact that the concentration of this enzyme declines with age. In addition, MP24.15 levels are decreased further in brain regions with the greatest accumulation of Aβ. In AD this decline is even more pronounced (40).

MP24.15 was previously examined as a candidate β-secretase by several groups after they discovered that this neuropeptidase cleaves test peptides containing the β secretase cleavage site (12, 13, 41). The most rigorous of these studies showed that neither the overexpression of MP24.15 nor the use of specific inhibitors dramatically altered Aβ levels or APP processing in these cells, as expected of a β-secretase (41–43). In contrast, our own studies showed that MP24.15 does generate amylloidogenic fragments from recombinant APP (12); however, as we have shown here, there is no obvious or dramatic increase in Aβ production when cells genetically altered to produce high levels of the MP24.15 were analyzed. On the contrary, we were surprised to find that reducing the levels of MP24.15 using antisense techniques produced an acute accumulation of soluble Aβ in the conditioned medium. Given this new discovery, it seemed likely that MP24.15 was involved in the degradation of Aβ rather than in its generation.

The distribution of neuronal MP24.15 in the brain is widespread and enriched in areas displaying high levels of its known substrates (i.e. substance P, etc.) (44). Neuronal loss in AD may be responsible for the observed decreased activity of MP24.15. A continuum of work on the physiologic role for MP24.15 has generated a wealth of data with regard to its function in the central nervous system and to its level of expression in specific brain regions. Several of these studies have noted that the level of MP24.15 expression in the temporal cortex decreases with age and that neuropeptides, such as substance P or somatostatin, two substrates of MP24.15, decrease as well in aging brain tissues (44–46). Additionally, when AD patients were compared with age-matched controls that lacked any overt neuropathology or psychiatric illness, their MP24.15 levels were found to be drastically reduced. The diminished levels were especially striking in the temporal and parietal cortices where Aβ deposits are heaviest. We hypothesize that these reductions in MP24.15 activity, especially in AD brains, lead to a loss of capacity to remove soluble Aβ, to higher levels of soluble Aβ, Aβ aggregation, and eventually more plaque formation.

There have been a number of proteinases that have been implicated in Aβ degradation, but the IDE has emerged as a strong candidate. Degradation of Aβ by IDE occurs in rat or human membrane preparations from brain homogenates and in a variety of cell types, but not neurons (27–29). We have found in our system that IDE is not the major contributor to Aβ degradation. We observe no correlation between the level of IDE present in cell lysates or CM and the rate of Aβ degradation. We also report that neither insulin (at 1 μM or 10 μM) nor 1,10-phenanthroline (at 1 mM), both effective inhibitors of IDE (38, 47), do not substantially block Aβ degradation. Only insulin at 100 μM completely inhibited Aβ degradation by the SKNMC cells CM (Table I). This concentration of insulin is 20 times higher than 125I-Aβ in the assay (5 μM) and may represent nonspecific inhibition. Similarly, 4 mM 1,10-phenanthroline inhibited only 25–30% of the Aβ degradation by CM of SKNMC neuroblastoma cells. While our serum-free CM contains both MP24.15 and IDE there is neither any Aβ degrading activity nor 14C-DFP labeled (i.e. active) serine proteinases. This contrasts again with Qiu et al. (29), who report that microglial IDE degrades Aβ in serum-free medium. The dependence on serum in SKNMC cells further distinguishes these two activities.

In exploring the inhibitor profile of SKNMC CM we suggest that MP24.15 is required for the activation of an Aβ-degrading serine proteinase(s). The Aβ degradation by CM of MP24.15 sense-transfected or mock-transfected cells is completely inhibited by AEBSF and DFP (Table I), while Zincov, 1,10-phenanthroline, insulin, and the MP24.15 inhibitor CPP-Ala-Ala-Phe-pAB only partially inhibit Aβ degradation. The inhibitory effect of these metalloproteinase inhibitors was low (between 7 and 30%) in comparison to AEBSF or DFP, which demonstrate 90–100% inhibition. Since serine proteinase and Aβ degrading activity correlate with the MP24.15 expression level, these results indicate that the main Aβ degrading activity in these cells involves one or more serine proteinases that are activated or induced by MP24.15. Autoradiographs of the Aβ degradation assay show a distinct band at 28 kDa which may be the serine proteinase responsible for the degradation of Aβ (data not shown). This band suggests an acyl-intermediate complex between the 125I-labeled Aβ and the proteinase that degrades it. This band is clearly distinct from aggregates of Aβ and is only seen in lanes that show Aβ degradation.

We have previously found that ACT mRNA is highly elevated in AD brain and that ACT protein is tightly associated with Aβ in both diffuse and classical plaques (4). Fitting well with our serine proteinase inhibitory profile, we report here that ACT also inhibits Aβ degradation by CM of mock- or MP24.15-transfected cells. This inhibition of Aβ degradation may occur by two possible mechanisms. First, by binding to the active site of the serine proteinase, ACT may block the proteinase from degrading Aβ. We demonstrate the formation of an SDS-stable ACT-serine proteinase complex in the CM of the MP24.15-overexpressing cells (Fig. 8) (which possesses reduced Aβ-degrading activity) (Fig. 7). Although the identity of this ACT-inhibited Aβ-degrading serine proteinase has not yet been determined, preliminary results from the 14C-DFP labeling experiment (Fig. 6) and the ACT complex formation (Fig. 8) support a molecular mass of 26–28 kDa. The formation of an ACT-proteinase complex, that migrates at 100 kDa, suggests that this proteinase does not excise the 8-kDa reactive site loop of ACT under these conditions. In contrast, the ACT-cathepsin G complex migrates at 90 kDa, as expected. The second mechanism for ACT’s inhibition of Aβ degradation would be through direct binding to Aβ peptide. We have shown that ACT binds Aβ to form an SDS-stable complex rendering ACT a weaker inhibitor of chymotrypsin (48). Recently the binding of Aβ to ACT was described in structural detail (49). Binding of ACT to Aβ could render Aβ a poorer substrate for degradation. We have shown that when both ACT and cathepsin G are added to either CM of mock- or MP24.15-transfected cells, two ACT complexes are formed, one of 90 kDa and one of 100 kDa (Fig. 8). Because the amounts of these two ACT complexes are in-
versely correlated it is suggested that the activated serine protease in the CM binds to ACT with a higher affinity than cathespin G does.

It should also be noted that preliminary measurements of $\alpha_\beta$ deposits in double transgenic mice expressing human ACT and human APP demonstrate a higher amyloid load than that of human APP expressing singly transgenic mice of similar age. These in vivo results support the in vitro findings reported here that ACT inhibits $\alpha_\beta$ catabolism.

The data presented in Fig. 6 demonstrate the direct relationship among MP24.15 expression levels, the concentration of $^{14}$C-DFP-inhibitable active serine proteinase(s), and $\alpha_\beta$ degradation. The higher the MP24.15 expression, the more labeling by $^{14}$C-b cells, but the A solution used to pass the cells in culture. When complexed with is caused by a chymotrypsin-like activity from the trypsin ing may lead to the design of pharmaceutical agents that can distribution to A DFP-inhibitable active serine proteinase(s), and A mounting that pharmacological up-regulation of MP24.15 could be A that MP24.15 is necessary as part of a proteolytic cascade for may activate a serine proteinase by: 1) activating the protein- bands above 200 kDa (Fig. 6). Since $\alpha_\beta$ monomeric antibody to IDE. We also thank Dr. Elizabeth Simons, Qui 2M to the conditioned serum-free medium of the SKNMC Annu. Rev. Cell Biol. 2. Selkoe, D. J. (1994) (1992) Nature 374, 322–325. Qui, W. Q., Borth, W., Ye, Z., Haass, C., Teplow, D. B., and Selkoe, D. J. (1996) J. Biol. Chem. 32730–32738. Matsumoto, A., Enomoto, T., Fujisawa, Y., Baba, H., and Matsumoto, R. (1996) Neurosci. Lett. 220, 159–162. Qiu, W. Q., Borth, W., Ye, Z., Haass, C., Teplow, D. B., and Selkoe, D. J. (1996) J. Biol. Chem. 271, 8443–8451. Boland, K., Behrens, M. D., Bode, W., Manias, K., and Pielmutter, D. H. (1996) J. Biol. Chem. 271, 18032–18044. Narita, M., Holtzman, D. M., Schwartz, A. L., and Bu, G. (1997) J. Neurochem. 69, 1904–1911. Du, Y., Ni, B., Gilim, M., Dodel, R. C., Bales, K. R., Zhang, Z., Hyslop, P. A., and Paul, S. M. (1997) J. Neurochem. 69, 299–305. Yan, S. D., Chen, X., Fu, J., Chen, M., Zhu, H., Roher, A., Slattery, T., Zhao, L., Nagahama, M., Morser, J., Migheli, A., Nawrath, P., Stern, D., and Smith, A. M. (1996) Nature 382, 685–691. Conn, K. J., Pietropaolo, M., Ju, S. T., and Abraham, C. R. (1996) J. Neurochem. 66, 2011–2018. Kim, S. K., Wen, G. Y., Bancher, C., Chen, C. M. J., Sapienza, V. J., Hong, H., and Wisniewski, H. M. (1990) Neurosci. Resum. 7, 113–121. Harada, S., Smith, R. M., Smith, J. A., and Jaret, L. (1995) Endocrinology 132, 2295–2298. Affholter, J. A., Hsieh, C. L., Franke, U., and Roth, A. R. (1990) Mol. Endocrinol. 4, 1125–1135. Ishii, C., Chevallier, N., Delaere, P., Dournaud, P., Epelebaum, J., Haan, J. W., Vincent, P. J., and Cheecier, F. (1994) J. Neurochem. 62, 645–655. Thompson, A., Grueninger-Leitch, F., Huber, G., and Malherbe, P. (1997) Brain Res. Mol. Brain Res. 48, 206–214. Brown, A. M., Tumolo, D. M., Spruyt, M. A., Jacobsen, J. S., and Sonnenberg-Ankmann, D. M., Dive, V., and Cheecier, F. (1997) Br. J. Pharmacol. 121, 556–562. Healy, D. P., and Orlovski, M. (1992) Brain Res. 571, 121–128. Waters, S. M., and Davis, T. P. (1997) Ann. N. Y. Acad. Sci. 814, 30–39. Dauch, P., Masuo, Y., Vincent, J. P., and Checler, F. (1993) J. Neurochem. 60, 2364–2446. Chevallier, N., Jiracek, J., Vincent, B., Baur, C. P., Spillantini, M. G., Goedert, M., Dive, V., and Cheecier, F. (1997) Br. J. Pharmacol. 121, 556–562. Healy, D. P., and Orlovski, M. (1992) Brain Res. 571, 121–128. Waters, S. M., and Davis, T. P. (1997) Ann. N. Y. Acad. Sci. 814, 30–39. Dauch, P., Masuo, Y., Vincent, J. P., and Cheecier, F. (1993) Peptides 14, 435–599. Bai, J. P., Hong, H. J., Rothenberger, D. A., Wong, W. D., and Buls, J. G. (1996) J. Pharm. Pharmacol. 48, 1180–1184. Potter, H., Abraham, C. R., and Dressler, D. (1991) in Alzheimer’s Disease: Basic Mechanisms, Diagnosis and Therapeutic Strategies (Libal, K., McLauchlan, D., Winblad, B., and Wisniewski, H., eds) pp. 275–279, J. Wiley & Sons Ltd., New York. Janicki-Fromkine, S., Rubin, H., Lukacs, C. M., and Wright, H. T. (1998) J. Biol. Chem. 273, 28360–28364. Orlovski, M., Michaud, C., and Chu, T. G. (1983) Eur. J. Biochem. 135, 81–88. Tsujik, U. (1990) Biol. Chem. Hoppe-Seyler 374, 91–109.