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Cleavage of Amyloid-β Precursor Protein (APP) by Membrane-Type Matrix Metalloproteinases

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Amyloid-β precursor protein (APP) was identified on expression cloning from a human placenta cDNA library as a gene product that modulates the activity of membrane-type matrix metalloproteinase-1 (MT1-MMP). Co-expression of MT1-MMP with APP in HEK293T cells induced cleavage and shedding of the APP ectodomain when co-expressed with APP adaptor protein Fe65. Among the MT-MMPs tested, MT3-MMP and MT5-MMP also caused efficient APP shedding. The recombinant APP protein was cleaved by MT3-MMP *in vitro* at the A^{463}-M^{464}, N^{579}-M^{580}, H^{622}-S^{623}, and H^{685}-Q^{686} peptide bonds, which included a cleavage site within the amyloid β peptide region known to produce a C-terminal fragment. The Swedish-type mutant of APP, which produces a high level of amyloid β peptide, was more effectively cleaved by MT3-MMP than wild-type APP in both the presence and absence of Fe65; however, amyloid β peptide production was not affected by MT3-MMP expression. Expression of MT3-MMP enhanced Fe65-dependent transactivation by APP fused to the Gal4 DNA–binding and transactivation domains. These results suggest that MT1-MMP, MT3-MMP and MT5-MMP should play an important role in the regulation of APP functions in tissues including the central nervous system.

Key words: amyloid-β precursor protein, cleavage, MMP, MT-MMP.

Abbreviations: Aβ, amyloid β peptide; APP, amyloid-β precursor protein; BB94, [4-(*N*-hydroxyamino)-2*R*-isobutyl-3-*S*-(thienylthiomethyl)-succinyl]-L-phenylalanine-*N*-methylamide; CTF, carboxyl-terminal fragment; DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)-*S*-phenylglycine-*t*-butyl ester]; GST, glutathione S-transferase; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

Amyloid-β precursor protein (APP) is a type I transmembrane glycoprotein constitutively expressed in many types of mammalian cells. As its name implies, APP was initially identified as the precursor of amyloid β peptide (Aβ), the principal component of amyloid plaques in the pathogenesis of Alzheimer's disease (*1*). Under physiological conditions, APP is processed through a series of proteolytic cleavages by enzymes referred to as secretases (*2*, *3*). This results in shedding of the large N-terminal domain, the generation of 40 or 42-residue long Aβ peptides, and the release of the C-terminal fragment (CTF). Recently, accumulating evidence has emphasized the potential importance of APP CTFs, including their involvement in gene transactivation. In this case, the sequential proteolytic processing of APP is strikingly similar to that of other proteins that undergo regulated intramembrane proteolysis, whereby upon release, the cytoplasmic tail enters the nucleus with Fe65 as its transcriptional coactivator and regulates gene transactivation (*4–6*).

Matrix metalloproteinases (MMPs) comprise a family of Zn^{2+}-dependent enzymes that are known to cleave extracellular matrix proteins under normal and pathological conditions (*7–9*). Presently, at least 28 mammalian MMPs have been identified by cDNA cloning, and they can be subgrouped into soluble type MMPs and membrane type MMPs (MT-MMPs) (*9*, *10*). MMPs are overexpressed in various human malignancies and have been thought to contribute to tumor invasion and metastasis by degrading extracellular matrix components (*7*, *11*). Thus, the level of MMP expression correlates with the invasiveness or malignancy of tumors (*12*, *13*). In particular, MT1-MMP, MMP-2, MMP-7 and MMP-9 have been reported to be most closely associated with tumor invasion and metastasis. Although degradation of the extracellular matrix is an important aspect of MMP biology, growing evidence has demonstrated the involvement of MMPs in specific processing resulting in activation or degradation of cell surface receptors and ligands. Fas ligand (*14*), tumor necrosis factor-α (*15*), the ectodomain of fibroblast growth factor receptor-1 (*16*), the heparin binding epidermal growth factor (*17*), and interleukin-8 (*18*) have been reported to be released or activated by MMPs. MMPs also cleave and inactivate interleukin-1β (*19*), insulin-like growth factor–binding proteins (*20*), fibrinogen and factor XII (*21*), the CC chemokine MCP-3 (*22*), the CXC chemokines stromal cell–derived factor-1α and β (*23*, *24*), metastasis suppressor

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gene product KiSS-1/metastin (*25*), heparin sulfate proteoglycan syndecan-1 (*26*), small leucine-rich proteogycan lumican (*27*), and apolipoprotein E (*28*).

We previously developed an expression cloning method for the screening of genes that modulate pro-MMP-2 activation mediated by MT1-MMP, and also for gene products that serve as substrates for MT1-MMP (*25–30*). In this study, we demonstrated that in addition to the most abundant MT-MMP (MT1-MMP), MT3-MMP and MT5-MMP, whose expression is the highest in the brain, caused cleavage and shedding of the APP ectodomain to generate CTFs that may contribute to Fe65-dependent transactivation.

MATERIALS AND METHODS

Materials—Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma (St Louis, MO). Primers were synthesized by Genset (Kyoto, Japan). A human placenta cDNA library constructed in the pEAK8 expression vector was obtained from EdgeBio Systems (Gaithersburg, MD). The recombinant MT3-MMP catalytic domain tagged with the FLAG epitope at the C-terminus was prepared as described previously (*31*, *32*). Monoclonal antibodies against the FLAG and HA epitopes were purchased from Sigma. The anti–MT1-MMP (113-5B7), MT2-MMP (162-4E3), and MT3-MMP (117-4E1) monoclonal antibodies were from Daiichi Fine Chemical Co., Ltd. (Takaoka, Japan). The APP monoclonal antibodies 6E10 and 4G8, specific for Aβ peptide residues 1–17 (APP amino acid residues 672–688) and 17–24 (688–695), respectively, were from Signet (Dedham, MA). The synthetic Aβ40 peptide was synthesized by Genset.

Cell Culture—Human embryonic kidney HEK293T and COS-1 cells were obtained from ATCC and cultured in DMEM supplemented with 5% fetal calf serum.

Construction of Expression Plasmids—Expression plasmids for MMP-2, MMP-9, MT1-MMP, MT3-MMP and MT-MMPs with the FLAG epitope following the furin cleavage sequence were prepared as described previously (*33–36*). An expression plasmid for APP tagged with the FLAG epitope at the C-terminus (APP-F) was constructed by PCR using an APP reverse primer with an extra *Bgl*II site (underlined) starting at nucleotide 2483 of the APP gene (GeneBank™ accession number NM_000484) (GGAGATCTGTTCTGCATCTGCTCAAAGAAC), as described previously (*29*). An expression plasmid for APP tagged with the FLAG epitope 4 amino-acid residues downstream from the signal sequence (F-APP) was constructed by inserting a double stranded DNA oligonucleotide encoding the FLAG epitope (5′: TC GAC TAC AAG GAC GAC GAT GAC AAG GTA C; 3′: CTT GTC ATC GTC GTC CTT GTA GTC GA GTAC) at the *Kpn*I site (nucleotide 251 of the APP gene) of the APP cDNA fragment in the pEAK8 vector. A cDNA fragment encoding the Swedish-type mutant APP, in which Lys^{670}-Met^{671} were substituted by Asn-Leu, was generated by PCR amplification of F-APP cDNA using a mutagenesis primer starting at nucleotide 2185 (AGGAGATCTCTGAAGTGAACTTGGATGCAG), which contains mutated nucleotides (underlined). The pCMV.SPORT6-Fe65 plasmid was purchased from Invitrogen (Carlsbad, CA). An expression plasmid for Fe65 tagged with the HA epitope at the C-terminus (Fe65-HA) was constructed by inserting an Fe65 cDNA fragment into the pEAK-HA plasmid, which was amplified by PCR using an Fe65 reverse primer with an extra *Xba*I site (underlined) starting at nucleotide 2198 of the Fe65 gene (GeneBankTM accession number BC010854) (AGTCTAGATGGGGTATGGGCCCCCAGCCGT) (*29*).

A bacterial expression plasmid for APP-GST was constructed as follows. An APP cDNA fragment encoding amino acids 379 to 700 was generated by introducing a *Bgl*II site at nucleotide 2294 by PCR using a mutation-containing primer with an extra *Bgl*II sequence (underlined) (ATAGATCTTCTTTGCAGAAGATGTGGGTTC). The fragment was cut with *Xho*I and *Bgl*II, and then inserted into the *Xho*I/*Bgl*II site of the GST-CTC plasmid as described previously (*25*).

An expression plasmid for APP-Gal4 fusion protein was constructed as follows. A cDNA fragment encoding the Gal4-DNA binding domain was PCR amplified using a forward primer with an extra *Bgl*II site (underlined) starting at nucleotide 13717 of the Gal4 gene (GeneBankTM accession number Z67751) (AGAGATCT AAGCTACTGTCTTCTATCGAAC) and a reverse primer with an extra *Bam*HI site (AGGGATCCCGATACAGTCAACTGTCTTTGA). The amplified fragment was digested with *Bam*HI and *Bgl*II, and then inserted at the *Bgl*II site, which was created at the 3′ end of the APP cDNA fragment in the pSG-APP-FLAG plasmid.

Expression Cloning—Expression cloning to identify candidate genes whose products interact with MMP-2, MMP-9 or MT1-MMP was carried out as described previously (*29*).

Western Blotting—Transfection into HEK293T or COS-1 cells was performed with TransIT LT1 transfection reagent (Mirus, Madison, WI). Cell lysates and proteins precipitated from conditioned medium with 10% trichloroacetic acid were analyzed by Western blotting using the indicated antibodies. A goat anti–mouse IgG antibody conjugated with Alexa Fluor 680 (Molecular Probes Inc., Eugene, OR) was used as a second antibody. The signal was monitored using a LI-COR Odyssey IR imaging system (Lincoln, NE). For the detection of APP CTFs, cell lysates and immunoprecipitated samples were separated by 4%/10%/16% Tris-Tricine SDS-PAGE, and then transferred to polyvinylidene difluoride (PVDF) membranes (Milipore, Bedford, MA) at 1.5 mA/cm^2 per gel, under semidry conditions, using a Bio Craft model BE-320 electroblotter (Tokyo, Japan). The blots were analyzed as described above. For detection of the Aβ peptide, culture supernatants concentrated with trichloroacetic acid were separated on 15% Bicine/Tris SDS-PAGE gels containing 8 M urea, and then transferred to PVDF membranes. The Aβ peptide was detected with an anti–APP 6E10 antibody as the primary antibody.

Cell Surface Biotinylation—HEK293T cells stably expressing MT1-MMP or MT3-MMP were transfected with expression plasmids for FLAG-APP and Fe65. Forty-eight hours after transfection, the cells were washed with cold PBS, and then incubated with PBS containing 0.5 mg/ml Biotin Sulfo-OSu (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 30 min at 4°C. Unreacted excess biotin was eliminated by washing with 25 mM lysine in PBS. The cells were then lysed and then subjected to immunoprecipitation with anti–FLAG M2

antibody–conjugated agarose beads as described previously (*30*). The immunoprecipitated material was separated by 10% SDS-PAGE, and then blotted with IRDye™800-conjugated streptavidin (Rockland, Gilbertsville, PA) and Alexa Fluor 680–conjugated FLAG M2 antibodies. IRDye™800 and Alexa Fluor 680 were detected with a LI-COR Odyssey scanner as described above.

Determination of the Cleavage Sites of the APP Protein—The recombinant APP-GST fusion protein was purified as described previously (*28*). The APP-GST protein (5 μg) was incubated with the recombinant MT3-MMP catalytic domain (0.5 μg) in 30 μl of TNC buffer containing 0.03% Brij at 37°C for 3 h. The generated fragments were separated by 12% SDS-PAGE and then blotted onto a PVDF membrane. The N-terminal amino acid sequence of each fragment was determined with a Beckman Coulter LF300 amino acid sequencer.

Luciferase Transactivation Assay—COS-1 cells were plated on 12-well plates 24 h before transfection. For the luciferase transactivation assay, the pFR-Luc trans-reporter plasmid (Stratagene, La Jolla, CA), renilla luciferase control plasmid (pRL-SV40) (Promega, Madison, WI), and pSG-APP-Gal4 were transfected with a control plasmid or the MT3-MMP plasmid in the presence or absence of APP transcriptional cofactor/coactivator Fe65. The cells were harvested 2 days post-transfection, and then analyzed with a Dual Luciferase Assay kit (Promega) and a Lumat LB 9507 luminometer (EG&G Berthold, Germany) following the manufacturer's protocol. Assays were performed at least three times and the luciferase activity levels were normalized as to that of renilla luciferase.

RESULTS

Screening of a Human Placenta cDNA Library—Aliquots of plasmid DNA from a human placenta cDNA library were cotransfected with MMP-2, MMP-9 and MT1-MMP cDNA into HEK293T cells, and cell lysates were analyzed by gelatin zymography (Fig. 1). A latent form of pro-MMP-9, a latent form of pro-MMP-2, and an activated intermediate form of MMP-2 were detected as 92-, 68-, and 64-kDa bands, respectively (Fig. 1A). Processing of pro-MMP-2 to generate the 62-kDa active form was found to be more significant in lane 19 as compared with the other lanes. From this positive pool of plasmid DNA, single clones were prepared and each clone was transfected into cells for second screening (Fig. 1B). Among 21 cDNA clones, two were found to stimulate the processing of pro-MMP-2 to its active form (lanes 3 and 17). The size of both cDNA fragments was 3.5 kb, and their nucleotide sequences were determined. Homology search analysis revealed that both cDNAs encoded the integral type I transmembrane glycoprotein APP (GenBank™ accession no. NM_000484).

APP Promotes Pro-MMP-2 Processing by MT1-MMP—The processing of pro-MMP-2 to an activated intermediate form was enhanced by the expression of MT1-MMP in HEK293T cells. Expression of APP alone did not have any effect on pro-MMP-2 processing, but the fully active form of MMP-2 was observed when APP was cotransfected with MT1-MMP (Fig. 2A). This finding on gelatin zymography clearly demonstrated that APP stimulated the activation of MMP-2 mediated by MT1-MMP. Therefore, we next examined the effect of APP on MT1-MMP expression to determine the basis for this stimulation. Immunoblotting analysis confirmed that expression of APP enhanced generation of the MT1-MMP active form, as detected using both anti–Flag M2 and M1 antibodies (Fig. 2B).

Cleavage and Shedding of APP by MT-MMPs—We previously showed that the type I transmembrane protein syndecan-1 not only stimulates MT1-MMP–mediated pro-MMP-2 processing, but also serves as a substrate for MT1-MMP (*26*). This led us to examine whether APP could be a substrate for MT1-MMP. To monitor the cleavage and shedding of the APP ectodomain, an expression plasmid

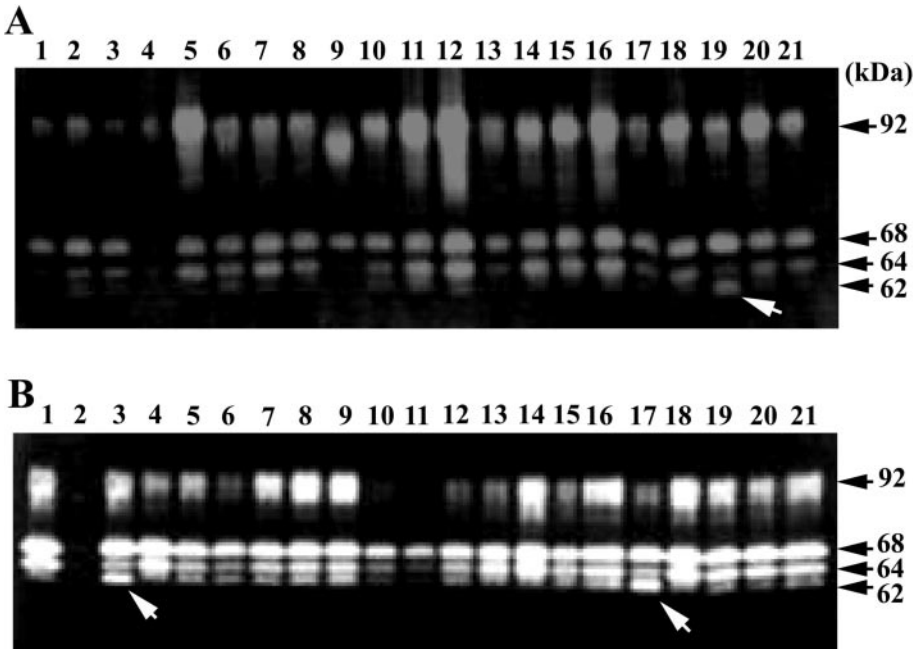

Fig. 1. **Expression cloning.** (A) Aliquots of plasmid DNA from a human placenta cDNA library were cotransfected with MMP-9, MMP-2 and MT1-MMP into HEK293T cells cultured in 96-well microplates as described under "EXPERIMENTAL PROCEDURES," and cell lysates were subjected to gelatin zymography at 48 h post-transfection. Note that processing of pro-MMP-2 to the active form (62 kDa) was enhanced in lane 19, as indicated by the arrow. (B) Single clones of plasmid DNA indicated to be positive on the first screening were analyzed as described above. Note that pro-MMP-2 processing was enhanced in lanes 3 and 17, as indicated by the arrows.

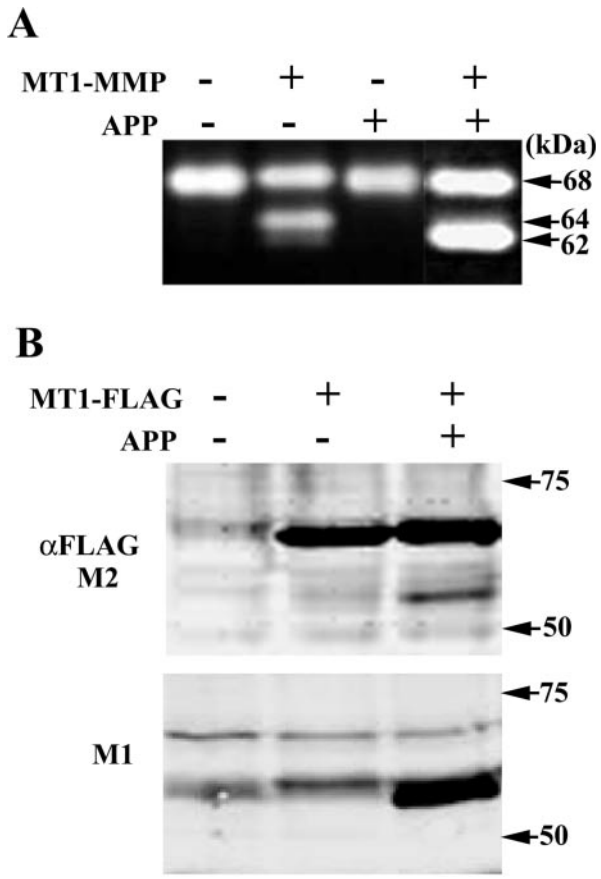

Fig. 2. **Effects of APP on pro-MMP-2 processing and MT1-MMP expression.** (A) Pro-MMP-2 plasmid (80 ng) was cotransfected into HEK293T cells cultured in 24-well culture plates with either the MT1-MMP plasmid (220 ng), APP plasmid (200 ng), or both. At 48 h post-transfection, cell lysates were analyzed by gelatin zymography. (B) MT1-FLAG plasmid (200 ng) was cotransfected with a control or the APP plasmid (300 ng) into HEK293T cells cultured in 24-well plates. Cells were harvested 48 h after transfection, and then subjected to Western blotting analysis with anti–FLAG M1 and M2 antibodies as described under "EXPERIMENTAL PROCEDURES." Note that the FLAG M1 antibody preferentially recognizes the active form MT1-FLAG, which has a FLAG epitope at the N-terminus, and the M2 antibody recognizes both the latent and active forms of MT1-FLAG.

for F-APP was transfected into HEK293T cells, and the shed APP fragments were detected with anti–FLAG M2 antibody (Fig. 3A). Secretion of a 120 kDa APP fragment was induced by co-expression of APP adaptor protein Fe65. Co-transfection of MT1-MMP or Fe65 alone slightly promoted shedding of the major 56 kDa and 120 kDa APP fragments into the culture medium, and the shedding was dramatically enhanced by co-expression of MT1-MMP and Fe65. Co-transfection of the MT3-MMP gene, the expression of which is highest in the brain (*33*), stimulated the shedding of APP fragments more than co-transfection of the MT1-MMP gene in the presence of Fe65. Although minor bands of around 90 kDa were more apparent for the supernatant of MT3-MMP–transfected cells than that of MT1-MMP–transfected cells, the APP fragmentation patterns of both seemed to be identical. Shedding of a 120 kDa APP fragment in the presence of Fe65, detected with an antibody against the Aβ sequence (6E10), was not affected by the expression of MT1-MMP or MT3-MMP. These results suggest that MT1-MMP and MT3-MMP mediate effective cleavage of APP at multiple sites in the presence of Fe65, and that the shed APP fragments do not contain the Aβ sequence recognized by the 6E10 antibody. The MT1-MMP active form was detected in cells expressing MT1-MMP and APP, as can be seen in Fig. 2A, but was decreased with co-expression of Fe65.

Other MT-MMP family members were next compared as to their ability to stimulate the shedding of APP in the presence of Fe65 (Fig. 3B). Among the six members of the MT-MMP family examined, MT3-MMP induced the cleavage and shedding of APP most efficiently, followed by MT1-MMP and MT5-MMP. The fragmentation patterns of APP produced by these MT-MMPs were almost identical. MT2-MMP, MT4-MMP and MT6-MMP did not cause effective shedding of APP even in the presence of Fe65. MT5-MMP induced shedding only when co-expressed with Fe65 (data not shown).

To examine the cell-surface localization of APP in cells expressing MT-MMP, APP was co-expressed with Fe65 in HEK293T cells stably expressing MT1-MMP or MT3-MMP in the presence or absence of the MMP inhibitor BB94, and then the levels of cell-surface APP were compared (Fig. 4A). APP on the cell-surface was detected as a broad 120 kDa band for control cells, and treatment of the cells with BB94 increased the cell-surface APP concentration by 2- to 3-fold. Co-expression of Fe65 with APP was essential for the cell-surface localization of APP (data not shown). The level of cell-surface APP in cells expressing MT1-MMP or MT3-MMP was quite low, and was restored to the level in control cells by BB94 treatment. Consistent with the biotin-labeled cell-surface APP levels, cells expressing MT1-MMP or MT3-MMP shed APP effectively in the absence of BB94 (Fig. 4B).

Cleavage of the APP Protein by MT3-MMP—To confirm the direct cleavage of APP by MT3-MMP and to identify the cleavage sites, a recombinant APP protein consisting of amino acids 379 to 700 fused to GST was incubated with recombinant MT3-MMP, and then analyzed by SDS-PAGE (Fig. 5). The digestion produced five major fragments, the N-terminal sequences of which showed cleavage of the A^{463}-M^{464}, N^{579}-M^{580}, H^{622}-S^{623}, and H^{685}-Q^{686} peptide bonds of APP, respectively. These results indicate that MT3-MMP cleaves the APP ectodomain at multiple sites, including the sequence within the Aβ domain (H^{685}-Q^{686}).

Cleavage within the Amyloid β Peptide Domain by MT3-MMP and Production of CTFs—To identify the CTFs produced in intact cells via cleavage of APP by MT3-MMP, APP tagged with the FLAG epitope at the C-terminus (APP-F) was co-expressed with Fe65 and MT3-MMP, and then CTFs were analyzed by Western blotting with antibodies against the FLAG epitope, Aβ peptide residues 1–17 (APP amino acid residues 672–688, 6E10), and 17–24 (688–695, 4G8), respectively (Fig. 6). Production of CTFs by MT3-MMP was stimulated by Fe65 expression. CTFs detected with the antibody against the FLAG epitope were also detected with antibody 4G8, but antibody 6E10 failed to detect the shortest CTF. The results of *in vitro* digestion and detection with site-specific antibodies (with epitopes flanking the cleavage point on both sides) suggest that

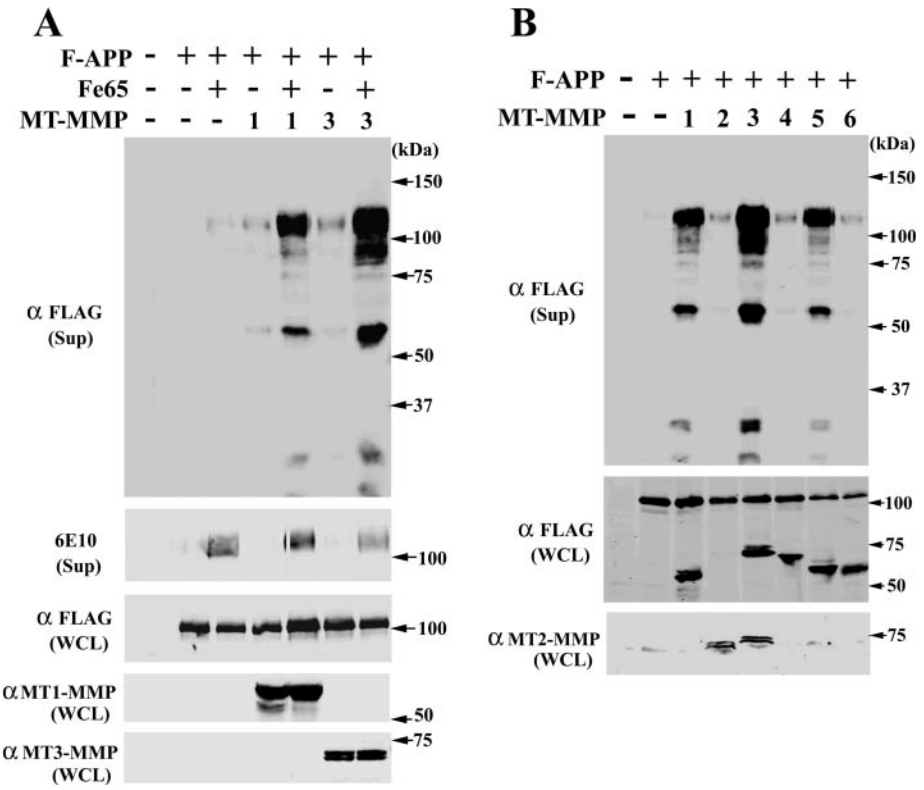

Fig. 3. **Stimulation of APP shedding by MT-MMP.** (A) An expression plasmid for APP tagged with the FLAG epitope adjacent to the N-terminus (F-APP) (0.5 μg) or the empty pSG5 plasmid (–) was cotransfected with 1.0 μg of a control plasmid (–), or an expression plasmid for MT1-MMP or MT3-MMP (as indicated by the number above the gel image) with or without the Fe65-HA plasmid (0.5 μg) into HEK293T cells cultured in 35-mm diameter dishes. Forty-eight hours after transfection, the culture medium was replaced with serum-free medium, and the cells were incubated further for 24 h. Culture supernatants (Sup) or whole cell lysates (WCL) were examined by Western blotting using the anti–FLAG M2 antibody, anti-HA antibody, anti–Aβ peptide antibody 6E10, anti–MT1-MMP antibody 113–5B7 or anti–MT3-MMP 117-4E1 antibody. (B) The expression plasmid for F-APP (0.5 μg) and the Fe65 plasmid (0.5 μg) were co-transfected with 1 μg of the control plasmid (–) or the expression plasmid for a MT-MMP family member, as indicated, into HEK293T cells. Culture supernatants were analyzed for the shedding of F-APP, and whole cell lysates for the expression of APP and MT-MMP as described above. MT-MMPs except for MT2-MMP were tagged with the FLAG epitope and were detected with the anti-FLAG M2 antibody together with the APP protein. MT2-MMP was detected with anti–MT2-MMP antibody 162-4E3, which cross reacted with MT3-MMP (bottom panel).

MT3-MMP cleaves the H^{685}-Q^{686} peptide bond within the Aβ sequence.

Effect of MT3-MMP Expression on Aβ Peptide Production—The Swedish-type mutant of F-APP (F-APP/SW), which is known to generate the Aβ peptide more effectively than the wild-type APP, was examined for susceptibility to MT3-MMP (Fig. 7A). Spontaneous shedding of APP/SW was more vigorous than for the wild-type APP, and was not significantly affected by Fe65 expression. Expression of MT3-MMP resulted in effective shedding and fragmentation of F-APP/SW in the presence and absence of Fe65. Shedding of F-APP/SW detected with an antibody against the Aβ peptide domain 6E10 was not significantly affected by the expression of MT3-MMP. Next, the effect of MT3-MMP expression on Aβ peptide production was examined. The Aβ peptide produced by cells transfected with F-APP/SW accumulated with time, and MT3-MMP expression did not have a significant effect on Aβ peptide accumulation (Fig. 7B).

Cleavage of APP by MT3-MMP Induces Fe65-Dependent Transactivation—There have been an increasing number of reports on the involvement of the APP cytoplasmic domain in transcription regulation via the formation of a complex with adaptor protein Fe65 (*37*, *38*). Therefore, we decided to examine whether MT3-MMP affects APP-Fe65 complex–dependent transcriptional activation. A chimeric protein in which the Gal4 DNA–binding domain was fused to the C-terminus of APP (APP-Gal4) was expressed in the luciferase assay system using COS-1 cells. Significant transactivation was observed when APP-Gal4 was co-expressed with Fe65 (Fig. 8). Co-expression of MT3-MMP with APP-Gal4 and Fe65 caused a significant increase in the transactivation activity. The addition of γ-secretase inhibitor DAPT significantly suppressed the transactivation induced by not only Fe65 alone but also MT3-MMP/Fe65.

DISCUSSION

Using the expression cloning strategy, we previously identified at least six molecules that exhibit a significant interaction with MMPs (*25–30*). In the present study, we discovered that the expression of APP in HEK293T cells promoted conversion of the MMP-2 activated intermediate form to the fully active form in the process mediated by MT1-MMP. Expression of APP was shown to increase the

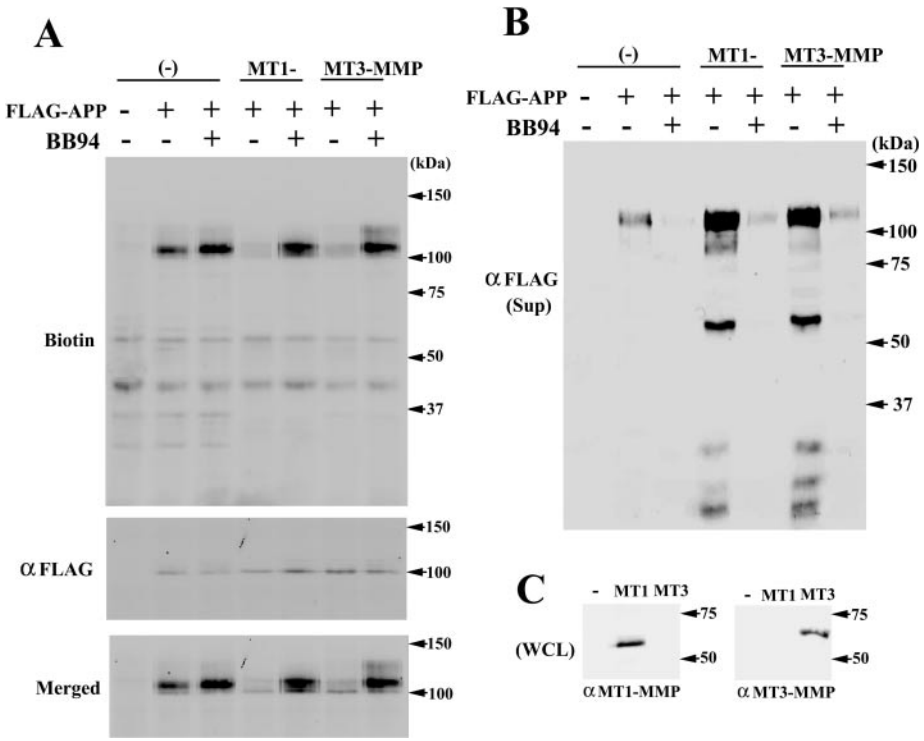

Fig. 4. **Detection of cell-surface APP.** (A) An expression plasmid for FLAG-APP (0.5 μg) was co-transfected with the Fe65 plasmid (0.5 μg) into HEK293T cells stably transfected with the empty vector (–), or the MT1-MMP or MT3-MMP plasmid cultured in 8-well plates. Twenty-four hours after transfection, 1 μM BB94 was added to the indicated cultures, and the cells were incubated further for 24 h. The cells were then labeled with biotin, and analyzed as described under "EXPERIMENTAL PROCEDURES." Cell surface APP labeled with biotin was detected with IRDye™800-conjugated streptavidin (upper panel), while APP core protein expression was detected with an anti–FLAG M2 antibody labeled with Alexa Fluor 680 (middle panel). Merged 800 and 680 signals reflected the total APP (lower panel). (B) HEK293T cells that had been mock transfected (–), or stably expressing MT1-MMP or MT3-MMP were transfected with the FLAG-APP and Fe65 plasmids, cultured for 48 h as above, and then incubated further in serum-free medium for 24 h. BB94 was included in the serum-free medium of the indicated cultures, and supernatants were analyzed for shed F-APP fragments using the anti–FLAG M2 antibody. (C) Whole cell lysates of HEK293T cells stably transfected with the empty pIREShyg vector, pIRES/MT1 or pIRES/MT3 were subjected to Western blotting with anti–MT1-MMP antibody 113-5B7 or anti–MT3-MMP 117-4E1 as indicated.

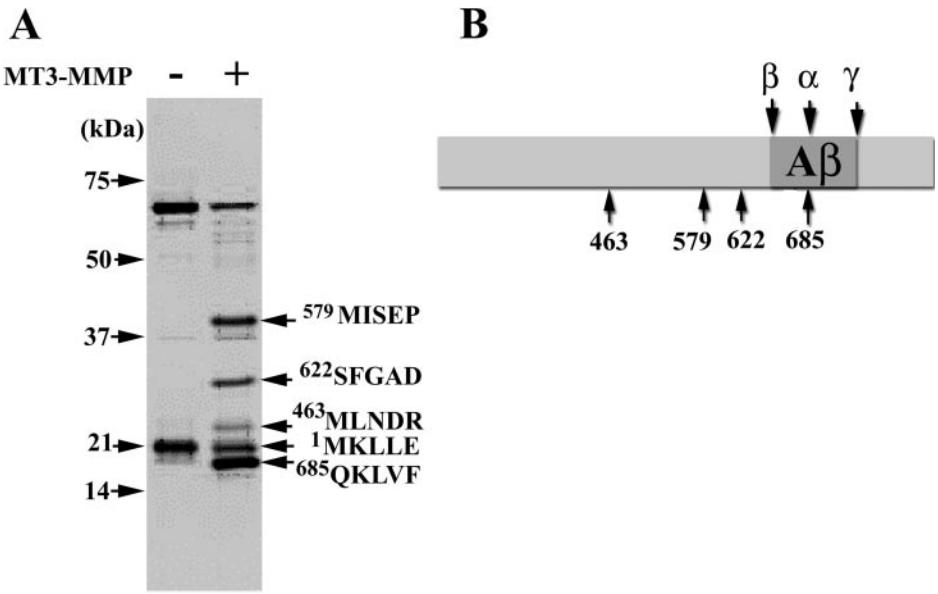

Fig. 5. **Cleavage of recombinant APP protein by MT3-MMP.** (A) The recombinant APP protein (5 μg) was incubated with or without the recombinant MT3-MMP catalytic domain (0.5 μg) for 3 h, separated by 12% SDS-PAGE, and then stained with Coomassie Brilliant Blue. The N-terminal amino acid sequence of each fragment was determined as described under "EXPERIMENTAL PROCEDURES." Note that the 21 kDa species co-purified with 70 kDa species was also cleaved to generate the smallest fragment. (B) The MT3-MMP cleavage sites are indicated on full length APP in relation to the cleavage sites for α-, β- and γ-secretases.

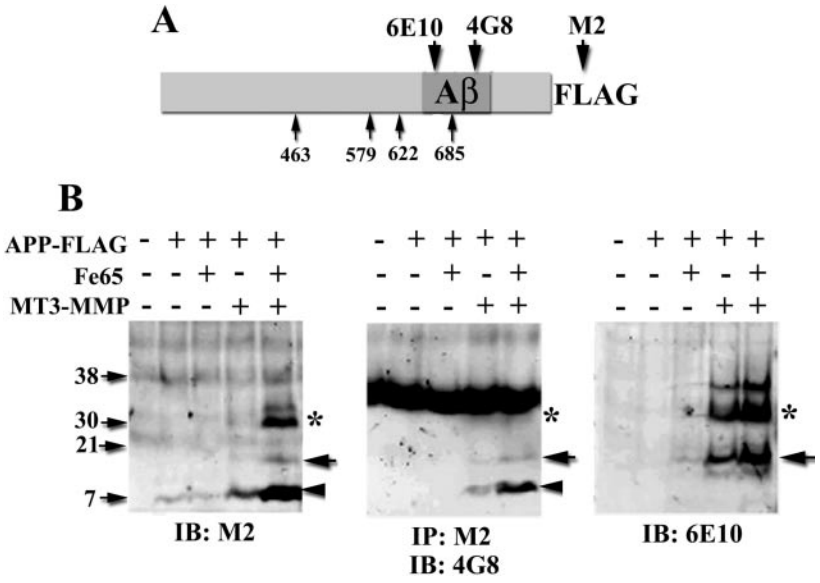

Fig. 6. **Cleavage within the amyloid β peptide domain by MT3-MMP.** (A) The recognition sites for the three antibodies used to detect APP CTFs are indicated on APP. Anti–APP 6E10 and 4G8 recognize epitopes on opposite sides of the MT3-MMP cleavage point within the Aβ domain. (B) The APP plasmid (1.6 μμg) tagged with the FLAG epitope at the C-terminus (APP-F) was cotransfected with a control plasmid (–), Fe65 (0.8 μg) or MT3-MMP (2.4 μg), or both into COS-1 cells cultured in 60 mm diameter dishes. At 48 h post-transfection, cells were lysed in 400 μl of lysis buffer. A 20-μl aliquot of each lysate was separated by 4%/10%/16% Tris-Tricine SDS-PAGE and then immunoblotted with the 6E10 or FLAG M2 antibody, the remainder of each lysate being subjected to immunoprecipitation with anti–FLAG M2 antibody–conjugated agarose beads followed by detection with 4G8 as described under "EXPERIMENTAL PROCEDURES." Bands with the same mobility in different lanes are indicated by arrowheads, arrows and asterisks, respectively. Note that the band indicated by the asterisk in the blot of the immunoprecipitated sample overlapped with the immunoglobulin light chain band (panel IP:M2/IB:4G8).

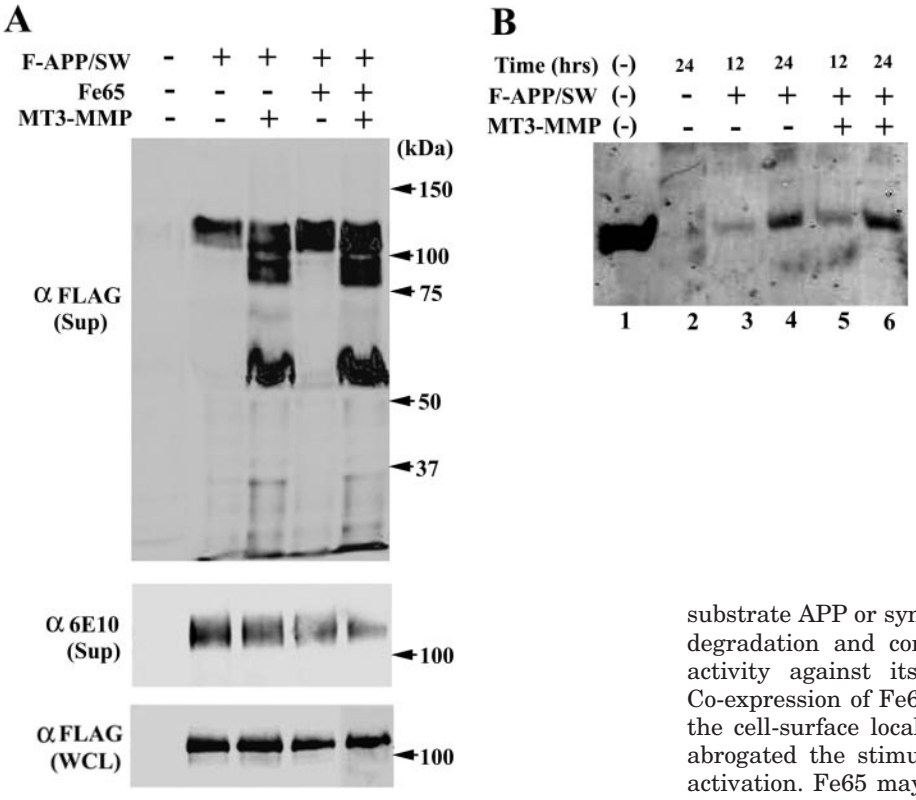

Fig. 7. **Effect of MT3-MMP expression on Aβ peptide production.** (A) An expression plasmid for the Swedish-type mutant form of F-APP (F-APP/SW) was co-transfected with a control plasmid or MT3-MMP with or without the Fe65 plasmid into COS-1 cells, and then culture supernatants (Sup) or whole cell lysates were examined by Western blotting as described above. (B) The control plasmid or expression plasmid for F-APP/SW was co-transfected with the empty pSG5 or MT3-MMP plasmid into COS-1 cells, and the culture medium was replaced with serum-free medium 48 h after transfection. The culture medium collected after 12 h or 24 h incubation was examined for Aβ peptide production as described under "EXPERIMENTAL PROCEDURES" (lanes 2–6). The Aβ40 peptide (20 ng) was used as a standard sample (lane 1).

concentration of the cell-surface MT1-MMP active form, which could account for the promotion of pro-MMP-2 activation. Previously we identified type I transmembrane protein syndecan-1 as an MT1-MMP substrate that also stimulates MT1-MMP–mediated pro-MMP-2 processing. Here we also showed that APP acts as a substrate for MT1-MMP. Since auto-degradation is the most critical regulatory step of MT1-MMP activity, expression of excess substrate APP or syndecan-1 may interfere with the auto-degradation and consequently augment the MT1-MMP activity against its favourable substrate pro-MMP-2. Co-expression of Fe65 with APP, which greatly enhances the cell-surface localization of APP did not enhance but abrogated the stimulatory effect of APP on pro-MMP-2 activation. Fe65 may affect not only the cell-surface concentration but also the structure of APP, and APP is more susceptible to MT1-MMP in the presence of Fe65, which in turn cancels the stimulatory effect of APP on MT1-MMP activity (data not shown).

Higashi and Miyazaki (*39*) reported that endogenous MT1-MMP is most likely involved in the cleavage and shedding of APP in HT1080 cells, and identified a cleavage site (N^{579}-M^{580} peptide bond) using an APP fragment and a recombinant MT1-MMP protein. Here we demonstrated for the first time that the expression of either

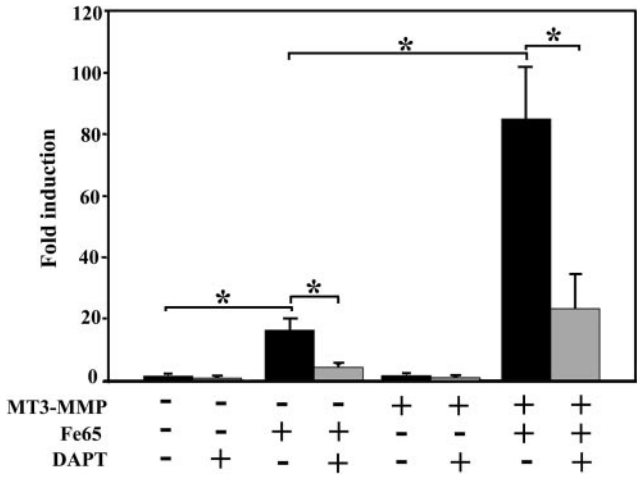

Fig. 8. **Transactivation by APP.** The APP-Gal4 plasmid (200 ng), luciferase reporter plasmid (pFR-Luc) (100 ng), and renilla luciferase control plasmid (pRL-SV40) (1 ng) were cotransfected with a control plasmid or the MT3-MMP plasmid (100 ng) in the presence or absence of the Fe65 plasmid (500 ng), as indicated into COS-1 cells cultured in 12-well plates. Cells were cultured for 2 days in the presence or absence of 2 μM γ-secretase inhibitor DAPT, and then harvested for the luciferase assay as described under "EXPERIMENTAL PROCEDURES." *$p < 0.01$.

MT1-MMP, MT3-MMP or MT5-MMP causes cleavage of APP on the cell surface and shedding of the ectodomain. We have focused on MT3-MMP as it showed the strongest effect on APP processing, although it should be noted that expression of MT5-MMP is neuron-specific (*40*, *41*). Four cleavage sites were identified within APP as a novel substrate for MT3-MMP using recombinant MT3-MMP and APP proteins, including the site identified previously (N^{579}-M^{580}) (*39*). Another MT3-MMP–cleavage site was mapped within the Aβ peptide sequence (H^{685}-Q^{686}), indicating that MT-MMP could be an α-secretase. Cleavage of APP within the Aβ domain was confirmed by analysis of CTFs generated by cells expressing MT3-MMP and Fe65. Since the APP fragmentation seen after cleavage by MT1-MMP, MT3-MMP and MT5-MMP was almost identical, MT1-MMP and MT5-MMP may cleave APP at the same sites as MT3-MMP. Zn^{2+}-dependent metalloproteases including ADAM (a disintegrin and metalloprotease) 9, ADAM 10 and ADAM 17 were reported to act as α-secretases for APP (*42*). ADAMs are also referred to as MDCs (metalloproteinases, disintegrin, cysteine-rich), and among these α-secretases, MDC9 also cleaves the APP H^{685}-Q^{686} peptide bond (*43*). As cleavage of APP by an α-secretase destroys the Aβ sequence, it is generally thought that the α-secretase pathway mitigates amyloid formation, although this has not been demonstrated unequivocally. Aβ has been shown to be generated in both the secretory and endocytic pathways in transfected cell lines and cultured neurons (*44–46*). It is hypothesized that Aβ is first generated in the *trans*-Golgi network. Remaining unprocessed APP is then transported to the cell surface where it is either cleaved by the nonamyloidogenic α-secretase or reinternalized into the endosomes. It was also shown that an α-secretase competes with a β-secretase for the cleavage of APP in the *trans*-Golgi network (*47*). In contrast to secreted Aβ, intracellular Aβ is generated in the endoplasmic reticulum/intermediate compartment (*48–51*). Our present study demonstrated that although expression of MT3-MMP induced the shedding of cell-surface APP through cleavage of APP within the Aβ domain, it did not significantly affect Aβ peptide production (Fig. 7). This finding is consistent with the above observations that Aβ is produced in intracellular compartments. Furthermore, the Aβ peptide itself was not cleaved by recombinant MT3-MMP or by cells expressing MT3-MMP (data not shown).

Co-expression of Fe65 with wild-type APP dramatically enhanced MT-MMP–mediated cleavage and shedding of APP, however, it had a negligible effect on the Swedish-type mutant of APP (Fig. 7A). Fe65 is known to increase the trafficking of APP to the cell surface (*52*), where MT-MMPs function as secretases. The Swedish-type mutant APP appeared on the cell surface effectively without the requirement of Fe65 (data not shown).

From the high degree of evolutionary conservation of the endo- and ectodomains of APP and their widespread tissue expression, APP has been expected to be involved in a variety of cellular processes and events. APP and Fe65 are known to be involved in cell motility, which may be directly related to the proteolytic processing of APP (*52*). Since MT1-MMP plays an important role in cell movement on an extracellular matrix, cleavage of APP by MT1-MMP may at least in part contribute to the regulation of it. MT1-MMP expression is highest in the placenta (*36*). The MT3-MMP gene, whose expression is highest in the brain and second highest in the placenta, has been cloned from a placenta cDNA library (*33*). In this study we isolated an APP gene from a human placenta cDNA library by expression cloning. The results may suggest that cleavage of APP by MT1-MMP and MT3-MMP plays a role in placenta tissue formation or its function.

γ-Secretase cleavage not only leads to the generation of Aβ but also results in the liberation of an intracellular domain of APP, which has been suggested to potentially function in nuclear signaling in conjunction with Fe65. Although a γ-secretase is incapable of processing the full length forms of its substrates, it efficiently cleaves membrane-anchored truncated C-terminal derivatives produced on ectodomain shedding (*53*). MT3-MMP cleaves APP to generate membrane-anchored CTFs (Fig. 6), which might be susceptible to further cleavage by a γ-secretase. This may account for the enhancement of APP-Gal4–mediated transactivation by MT3-MMP. Suppression of MT3-MMP–induced transactivation by γ-secretase inhibitor DAPT may support this hypothesis.

In summary, in addition to the most abundant MT1-MMP, MT3-MMP and MT5-MMP were shown to cleave the APP ectodomain at multiple sites including the Aβ sequence, and to induce Fe65-dependent transactivation. Although the physiological functions of APP remain to be fully elucidated, MT-MMPs should play an important role in the regulation of APP functions in tissues including the central nervous system.

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