Novel Processing of β-Amyloid Precursor Protein Catalyzed by Membrane Type 1 Matrix Metalloproteinase Releases a Fragment Lacking the Inhibitor Domain against Gelatinase A†

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Received November 1, 2002; Revised Manuscript Received March 10, 2003

ABSTRACT: In various mammalian cell lines, β-amyloid precursor protein (APP) is proteolytically processed to release its NH_2-terminal extracellular domain as a soluble APP (sAPP) that contains the inhibitor domain against gelatinase A. To investigate roles of sAPP in the regulation of gelatinase A activity, we examined the correlation between the activation of progelatinase A and processing of APP. We found that stimulation of HT1080 fibrosarcoma cells with concanavalin A led to an activation of endogenous progelatinase A and to a novel processing of APP, which releases a COOH-terminally truncated form of sAPP (sAPPtrc) into the culture medium. Reverse zymographic analysis showed that sAPPtrc lacked an inhibitory activity against gelatinase A. Analyses of production of sAPPtrc in the presence of various metalloproteinase inhibitors showed that membrane type 1 matrix metalloproteinase (MT1-MMP), an activator of progelatinase A, is most likely responsible for the production of sAPPtrc. When the concanavalin A-stimulated HT1080 cells were cultured in the condition that inhibited MT1-MMP activity, sAPP and APP were associated with the extracellular matrix deposited by the cells, whereas these gelatinase A inhibitors in the matrix were displaced by sAPPtrc after exertion of MT1-MMP activity. Taken together, these data support a model in which MT1-MMP-catalyzed release of sAPPtrc leads to reduction of the extracellular matrix-associated gelatinase A inhibitor, sAPP, thus making it feasible for gelatinase A to exert proteolytic activity only near its activator, MT1-MMP.

The β-amyloid precursor protein (APP)[1] is a type I integral membrane protein, which was initially identified as a precursor of β-amyloid (Aβ) peptide, the principal component of extracellular deposits in senile plaques observed in Alzheimer's disease brain (*1*). In cultured cells, APP synthesized and maturated through the constitutive secretory pathway is proteolytically cleaved at the cell surface within the Aβ sequence (Figure 1), and the extracellular domain of APP is released as a soluble APP (sAPP) into the culture medium (*2*, *3*). An unidentified protease called α-secretase catalyzes the processing of APP. Alternative proteolysis of APP by β- and γ-secretases leads to production of Aβ peptide (Figure 1). As the processing of APP through the α-secretase pathway precludes the production of Aβ peptide, the enzyme possessing the α-secretase activity has been explored to clarify the mechanism of pathogenesis of Alzheimer's disease. These studies suggested that α-secretase is a plasma-membrane-associated metalloproteinase(s), the activity of which is readily inhibited by some hydroxamate-based synthetic inhibitors (*4*, *5*). Furthermore, it has been reported that the release of sAPP is extremely diminished in cells lacking a gene of tumor necrosis factor α converting enzyme that belongs to a disintegrin and metalloproteinase (ADAM) family (*6*). Therefore, this metalloproteinase is a prime candidate for the enzyme responsible for the α-secretase activity. In vivo, sAPP is detected in human plasma and the cerebrospinal fluid (*7*, *8*). Although the biological significance of the processing of APP is not well known, sAPP containing the Kunitz-type protease inhibitor (KPI) domain, also called protease nexin II, may act as an inhibitor of coagulation factor XIa (*9*) or other extracellular serine proteases (*10*). Both the soluble form and membrane-bound APP have also been suggested to participate in cell adhesion (*11*, *12*), neurite outgrowth (*13*, *14*) and synaptic plasticity (*15*). We previously reported (*16*) that sAPP contains an inhibitor domain against gelatinase A (MMP-2), a member of the matrix metalloproteinase (MMP) family, and suggested that sAPP is involved in the regulation of the degradation of extracellular matrix (ECM). The extracellular domain of APP has a heparin-binding property and an ability to associate with ECM, probably via heparan sulfate-containing proteoglycans (*17*, *18*). Regions of sAPP that are essential for binding to laminin (*19*), type I collagen (*20*), and fibulin-1 (*21*) have also been identified. Interaction between sAPP and ECM via several binding sites may be of benefit in protecting ECM from degradation catalyzed by gelatinase A.

† This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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[1] Abbreviations: APP, β-amyloid precursor protein; Aβ, β-amyloid; sAPP, soluble APP; MMP(s), matrix metalloproteinase(s); KPI, Kunitz-type protease inhibitor; ECM, extracellular matrix; TIMP, tissue inhibitor of metalloproteinases; MT1-MMP, membrane type 1−MMP; Con A, concanavalin A; PBS, phosphate-buffered saline; CM, conditioned medium; sAPPtrc, truncated sAPP.

10.1021/bi020643m CCC: $25.00
Published on Web 05/08/2003

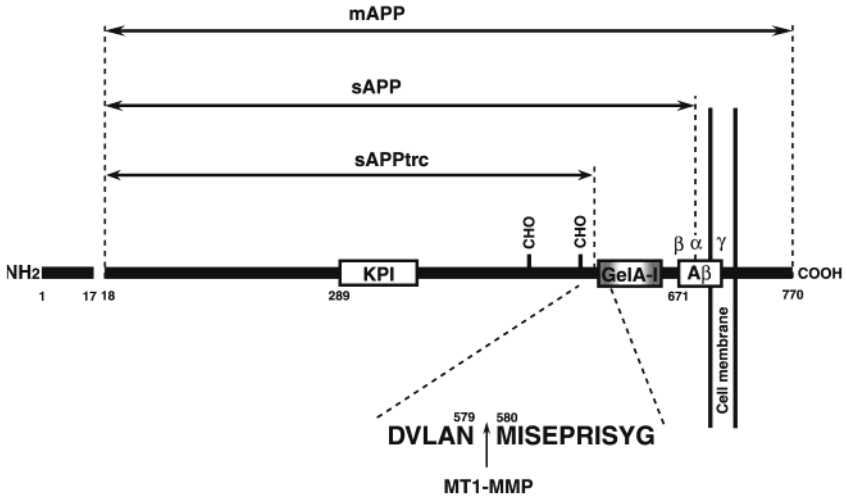

FIGURE 1: Schematic diagram of APP and various processing sites.The synthesized APP, comprising 770 amino acids, is maturated by removal of a 17-residue signal peptide and addition of carbohydrate chains through the constitutive secretory pathway. The maturated APP (mAPP) is then proteolytically cleaved by α-secretase within the Aβ sequence, and the NH_2-terminal extracellular domain of APP is released as sAPP. Alternative proteolysis of mAPP by β- and γ-secretases leads to the production of Aβ peptide. The present study suggests that MT1-MMP also cleaves mAPP to release sAPPtrc, a COOH-terminally truncated form of sAPP. The MT1-MMP-catalyzed cleavage of the Asn^{579}−Met^{580} bond of sAPP also produces the sAPPtrc-like fragment, which lacks the gelatinase A inhibitor domain (Gel A-I) identified in the previous study (*16*). α, β, and γ represent the sites of cleavage by α-, β-, and γ-secretases, respectively. CHO, potential Asn-linked glycosylation site; KPI, the KPI domain.

MMPs are zinc-dependent endopeptidases that degrade components of the extracellular matrix and play an essential role in tissue remodeling under physiological and pathological conditions, such as morphogenesis, angiogenesis, tissue repair, and tumor invasion (*22*−*24*). Among the MMP family, gelatinase A and gelatinase B (MMP-9) are critical in the invasion of tumor cells across basement membranes because of their strong activity against type IV collagen, a major component of basement membranes (*25*−*27*). Involvement of gelatinase A activity in angiogenesis has also been suggested (*28*). In this process, gelatinase A activity probably supports the invasive behavior of new blood vessels. Most MMPs are secreted as inactive zymogens and are activated by serine proteases or some activated MMPs. Unlike other zymogen of MMPs, activation of progelatinase A is catalyzed specifically by a novel type of MMP that has a transmembrane domain and is thus localized on the cell surface (*29*). So far, six members of membrane-type MMP have been identified, and membrane type 1 MMP (MT1-MMP)-catalyzed progelatinase A activation has been well characterized (*30*−*32*). In the activation of progelatinase A, the protease zymogen is first recruited on the cell surface via MT1-MMP complexed with tissue inhibitor of metalloproteinase-2 (TIMP-2), and then a TIMP-2-free, noninhibited form of MT1-MMP also present on the cell surface cleaves the propeptide of progelatinase A to initiate the activation (*29*, *33*). After the activation, the gelatinase A retained on the cell surface appears to be involved in the proteolytic maturation of the intermediate form of gelatinase A (*34*). The cell-associated active form of gelatinase A also plays a role in the local degradation of extracellular matrix. We showed previously that gelatinase A has the ability to hydrolyze the Aβ peptide at the peptidyl bond between Lys^{16} and Leu^{17}; the corresponding peptidyl bond in APP is also cleaved by α-secretase (*16*). This finding prompted us to examine whether cell-associated gelatinase A or MT1-MMP cleaves the membrane-bound form of APP. In this study, we found that the cell-mediated activation of progelatinase A is accompanied by a novel processing of APP, which is catalyzed by MT1-MMP. This processing releases a soluble fragment of APP that lacks the inhibitor domain against gelatinase A. Possible roles of the novel processing of APP in the regulation of gelatinase A-catalyzed degradation of ECM are discussed.

EXPERIMENTAL PROCEDURES

Materials. The sources of materials used are as follows: the plant lectin concanavalin A (Con A, type IV, substantially free of carbohydrates) from Sigma (St. Louis, MO); sodium monensin, sodium heparin, and potassium cyanate from Wako Pure Chemical Industries (Osaka, Japan); Affi-Gel 10 from Bio-Rad (Hercules, CA); gelatin from Difco (Detroit, MI); mouse monoclonal antibody 22C11 from Roche Diagnostics Co. (Indianapolis, IN); anhydrotrypsin−agarose from Takara Shuzo Co. (Kyoto, Japan); bovine placenta type IV collagen from Koken (Tokyo, Japan); recombinant catalytic domain of MT1-MMP from Chemicon International Inc. (Temecula, CA); and 3163v (7-methoxycoumarin-4-yl) acetyl-L-prolyl-L-leucyl-glycyl-L-leucyl-[N^{β}-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-alanyl-L-arginine amide) from Peptide Institute, Inc. (Osaka, Japan). A synthetic hydroxamate-based inhibitor for MMPs, KB-8301 [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-methylsuccinyl]-L-3-(5,6,7,8-tetrahydro-1-naphthyl)alanine-*N*-methylamide], was a generous gift from Dr. K. Yoshino, Kanebo Institute for Cancer Research (Osaka, Japna). All other chemicals were of analytical grade or the highest quality commercially available.

Proteins. TIMP-1 (*35*) and sAPP (*16*) were purified from the conditioned medium (CM) of the human bladder carcinoma cell line EJ-1, as described previously. TIMP-2-free and TIMP-2-bound forms of progelatinase A were separately purified from the CM of the human glioblastoma cell line T98G, as described previously (*36*). TIMP-2 was purified from the TIMP-2-bound progelatinase A using a SynChropak RP-4 reverse-phase column (SynChrom, Lafay-

ette, IN) according to the method of Collier et al. (*26*). The NH_2-terminally modified TIMP-2, the reactive site of which had been destroyed by carbamylation of Cys^1, was prepared as described previously (*34*). A mouse anti-Aβ monoclonal antibody and mouse anti-sAPP monoclonal antibody 278 were raised against a synthetic peptide of human Aβ 1−40 and the purified sAPP, respectively.

Cell Culture and Preparation of CM, Cell Lysate, and ECM. HT1080 fibrosarcoma cell line was grown to semiconfluency in a 1:1 mixture of Dulbecco's modified Eagles's medium and Ham's F12 medium (Gibco, Grand Island, NY), DME/F12, supplemented with 10% fetal calf serum. The cells were rinsed three times with serum-free DME/F12, and the culture was further continued in the presence of various concentrations of inhibitors and a fixed concentration of Con A (0 or 100 μg/mL) in serum-free DME/F12. After incubation, the resultant CM was collected, clarified by centrifugation, and dialyzed against distilled water at 4 °C. The sample was then lyophilized and dissolved in a small volume of a sodium docecyl suflate (SDS) buffer consisting of 50 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol. By these procedures, the initial CM was concentrated 20-fold. To prepare cell lysates, the cells were rinsed three times with phosphate-buffered saline (PBS) and then dissolved in a small volume of the SDS-sampling buffer. For preparation of ECMs, the cells on plastic dishes were first rinsed three times with PBS and then dissolved in PBS containing 10 mM EDTA and 1% Triton X-100 at 4 °C. The cells remaining on the dishes were detached completely by pipetting, and the lysates were removed. The dishes were sequentially washed three times with the lysis buffer and twice with PBS containing 10 mM EDTA. The ECM remaining on the surface of the dishes was extracted with the SDS-sampling buffer at room temperature.

Preparation of Membrane-Associated Proteins. HT1080 cells cultured as described above were first rinsed three times with PBS and then dissolved in PBS containing 10 mM EDTA and 1% Triton X-114 at 4 °C. The resultant lysates were collected, clarified by centrifugation, and then incubated at 37 °C for 5 min. After incubation, the samples were centrifuged at 25 °C to collect the Triton X-114-condensed bottom phase in which membrane-associated proteins are concentrated. After removal of the upper phase, each sample was cooled in a container of ice water and mixed with 10 vol of ice-cold PBS containing 10 mM EDTA. The homogenized samples were incubated again at 37 °C for 5 min and centrifuged to collect the bottom phase. By the repeated phase separation, almost all soluble proteins in the detergent-condensed phase were removed. To collect the membrane-associated proteins in the detergent-condensed phase, the bottom phase of each sample was then mixed with 10 vol of ice-cold acetone and allowed to stand for 1 h at −20 °C. The resultant protein precipitate was collected by centrifugation, dried, and then dissolved in the SDS-sampling buffer

Gelatin Zymography and Trypsin Reverse Zymography. Zymography (*36*), trypsin reverse zymography (*37*), and gelatinase A reverse zymography (*16*) were carried out on 10% polyacrylamide gels containing 1 mg/mL gelatin, as described previously.

Western Blotting Analysis. CMs, membrane-associated proteins, or digested sAPP were subjected to SDS−PAGE. After electrophoresis, the proteins on the gel were transferred onto a nitrocellulose membrane using a Bio-Rad Mini Trans-Blot apparatus. The membrane was blocked with PBS containing 5% skim milk at 37 °C for 2 h, washed with PBS containing 0.05% Tween 20 and 0.1% bovine serum albumin (PBS-Tween), and then incubated at room temperature with a monoclonal antibody 22C11 (0.2 μg/mL), an anti-Aβ monoclonal antibody (0.2 μg/mL), or an anti-sAPP monoclonal antibody 278 (2.0 μg/mL) in PBS-Tween. After 12 h of incubation, the membrane was washed with PBS-Tween and incubated for 1 h with a biotinylated anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA), which had been diluted 1000-fold with PBS-Tween. After being washed with PBS-Tween, the membrane was incubated with 1000-fold diluted avidin−alkaline phosphatase (Vector) at room temperature for 1 h. The membrane was washed extensively and then incubated in a reaction mixture containing 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium to develop a colored product on the membrane.

Partial Purification of Trypsin Inhibitors Released from Concanavalin A-Stimulated HT1080 Cells. HT1080 cells were grown to confluency in roller bottles, rinsed, and incubated for 48 h in serum-free medium with 100 μg/mL Con A as described above. The resultant CM was clarified by centrifugation, added with ammonium sulfate to a final saturation of 80%, and allowed to stand overnight at 4 °C. The resultant protein precipitate was collected by centrifugation and then dissolved in and dialyzed against 50 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl and 0.01% (w/v) Brij-35. The dialyzed sample was applied to an anhydrotrypsin−agarose column equilibrated with the same buffer. The column was washed extensively with the equilibration buffer, and then the adsorbed proteins were eluted with 0.1 M formic acid. The eluted sample was immediately neutralized by adding same volume of 2 M Tris-HCl (pH 8.0) and dialyzed against 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl. By this procedure, 85- and 90-kDa trypsin inhibitors and sAPP were copurified. The partially purified inhibitors were separated by SDS−PAGE under nonreduced conditions. After electrophoresis, the proteins on the gel were transferred onto a polyvinylidene difluoride membrane, stained with Coomassie Brilliant Blue R-250, and separately subjected to NH_2-terminal sequence analysis.

Amino-Terminal Sequence Analysis. Samples were analyzed on an Applied Biosystems 477A gas-phase sequencer. Phenylthiohydantoin derivatives were detected using an Applied Biosystems 120A PTH analyzer with an online system.

Preparation of APP-Accumulated HT1080 Cells and Proteolytic Liberation of APP Fragments from the Cells. HT1080 cells were first incubated for 48 h in serum-free medium with 10 μM KB-8301 and 100 μg/mL Con A. This treatment allowed the cells to accumulate the mature form of APP on the surface (see Figure 4B, below). After incubation, the culture medium was added with sodium monensin to a final concentration of 10 μM, followed by incubation for 3 h. After the monensin treatment, the cells were rinsed three times with serum-free medium to remove KB-8301 and secreted proteins, and they were further incubated with or without metalloproteinase inhibitors in serum-free medium containing 100 μg/mL Con A and 10 μM sodium monensin. CMs and cell lysates were prepared from the incubated cells as described above.

Digestion of sAPP with the Catalytic Domain of MT1-MMP. Twenty microliters of 400 nM sAPP was incubated with various concentrations of the recombinant catalytic domain of MT1-MMP in 50 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$ and 0.01% Brij-35 at 37 °C for 4 h. The enzyme reaction was terminated by adding of 10 μL of 0.1 M EDTA (pH 7.5). The resultant digests were subjected to Western blotting analysis or gelatinase A reverse zymography.

Determination of the MT1-MMP Cleavage Site in sAPP. sAPP (120 μg) was incubated with 0.2 M KNCO in 200 μL of 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl at 37 °C for 6 h. The sAPP sample was further incubated with 0.2 M hydroxylamine hydrochloride at 25 °C for 1 h and then dialyzed extensively against 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and 10 mM $CaCl_2$. This treatment appeared to result in complete carbamylation of the α-amino group of NH_2-terminal Leu^{18}, because no phenylthiohydantoin derivative was detected in the NH_2-terminal sequence analysis on the modified sAPP. The modified sAPP (2 μM) was then incubated with the catalytic domain of MT1-MMP (200 nM) in 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and 10 mM $CaCl_2$ at 37 °C for 4 h. The resultant digest was blotted onto a polyvinylidene difluoride membrane, using an Atto Immunodot apparatus (Tokyo, Japan). The membrane was washed extensively with pure water and then stained with Coomassie Brilliant Blue R-250. The stained spot was subjected to NH_2-terminal sequence analysis.

Assay of Gelatinase A Inhibitory Activity of sAPP. TIMP-2-free form of progelatinase A was activated by incubation with 1 mM APMA at 37 °C for 1 h as described previously (*35*). The activated gelatinase A (0.61 nM) was incubated with various concentrations of sAPP and a fixed concentration of heparin (0 or 105 μg/mL), or with various concentrations of heparin and a fixed concentration of sAPP (0 or 126 nM) in 190 μL of 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 10 mM $CaCl_2$, 0.01% Brij 35, and 0.01% bovine serum albumin at 37 °C for 15 min. To the mixtures were added 10 μL of 1 mM 3163v (synthetic substrate), and the mixtures were further incubated for 40 min. The reaction was terminated by adding 20 μL of 0.5 M EDTA (pH 7.5). The amounts of 3163v hydrolyzed by gelatinase A were measured fluorometrically with excitation at 360 nm and emission at 460 nm. The amount of 3163v hydrolyzed without enzyme was subtracted from the total amount of the hydrolyzed substrate.

Assay of Binding of Active Gelatinase A with Immobilized sAPP. Thirty microliters of the TIMP-2-free form of progelatinase A (930 nM) was incubated with 1 mM APMA at 37 °C for 4 h in 50 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$ and 0.01% Brij 35. After incubation, the sample was transferred to an ice water container, and 270 μL of ice-cold buffer consisting of 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl, 0.01% Brij 35, and 0.1% Nonidet P-40 was added to quench the activation reaction. One hundred microliters each of the preparation was mixed with 20 μL of sAPP−Affi-Gel 10 beads (1.0 mg of sAPP was coupled to 1 mL of Affi-Gel 10) or unconjugated Affi-Gel 10 beads and incubated in the absence or presence of 1.0 μM KB-8301 or 100 nM TIMP-2 at 4 °C for 5 h with rotation. After the beads were washed with the incubation buffer, the adsorbed sample was extracted with the SDS-sampling buffer consisting of 50 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol. The active gelatinase A forms in the extract were analyzed by gelatin zymography. The gelatinase A samples prepared as described above but incubated without beads were also subjected to the zymographic analysis.

RESULTS

Novel Processing of APP in Con A-Stimulated HT1080 Cells. It has been reported that stimulation of fibroblastic cells with Con A leads to activation of endogenous progelatinase A (*38*). Various tumor cell lines release an extracellular part of the membrane-bound Aβ precursor protein APP into culture medium by proteolytic processing (*10*). Since the majority of the APP fragments (sAPP) contain the KPI domain (see the scheme in Figure 1), they can be analyzed by reverse zymography with trypsin. To examine the correlation between the activation of progelatinase A and the proteolytic processing of APP, we stimulated HT1080 cells with Con A, and KPI domain-containing APP fragments released into culture medium were analyzed by trypsin reverse zymography. As shown in Figure 2, nonstimulated HT1080 cells were found to secrete progelatinase A and a 105-kDa trypsin inhibitor, which has been identified as sAPP in previous studies (*10*, *16*). When the cells were stimulated with Con A, activation of endogenous progelatinase A (Figure 2A) and release of 85- and 90-kDa trypsin inhibitors in addition to sAPP (Figure 2B) were observed. The stimulation also reduced the cell-bound mature form of APP (Figure 2C), suggesting that proteolytic liberation of APP was facilitated in the stimulated cells. On the other hand, both the release of trypsin inhibitors from the Con A-stimulated HT1080 cells and that of sAPP from nonstimulated cells were inhibited in the presence of a hydroxamate-based metalloproteinase inhibitor, KB-8301. This metalloproteinase inhibitor also inhibited the activation of progelatinase A in Con A-stimulated HT1080 cells (Figure 2A). We also examined the effect of 12-*O*-tetradecanoylphorbol-13-acetate on the processing of APP and activation of progelatinase A in HT1080 cells. Similarly to Con A, the phorbol ester stimulated both activation of progelatinase A and release of 85- and 90-kDa trypsin inhibitors (data not shown).

Structural Analyses of Trypsin Inhibitors Released from Con A-Stimulated HT1080 Cells. To determine whether the trypsin inhibitors released from Con A-stimulated HT1080 cells are derived from APP, interaction of these inhibitors with the monoclonal antibody 22C11, which recognizes an NH_2-terminal epitope (residues 66−81) of APP, was tested by Western blotting analysis. Since intramolecular disulfied bonds affect the electrophoretic mobility of proteins, SDS−PAGE and Western blotting are generally carried out with reduced samples. However, gelatin zymography and reverese zymography used in this study must be carried out with nonreduced samples. Therefore, in Figure 3, nonreducing and reducing SDS−PAGE was done to compare the mobilities of soluble APP fragments between the two conditions. As shown in Figure 3A, both the 85- and 90-kDa nonreduced inhibitors and sAPP (105 kDa) were detected in the analysis, suggesting that all inhibitors detected in the trypsin reverse zymography are fragments derived from APP. We also tested

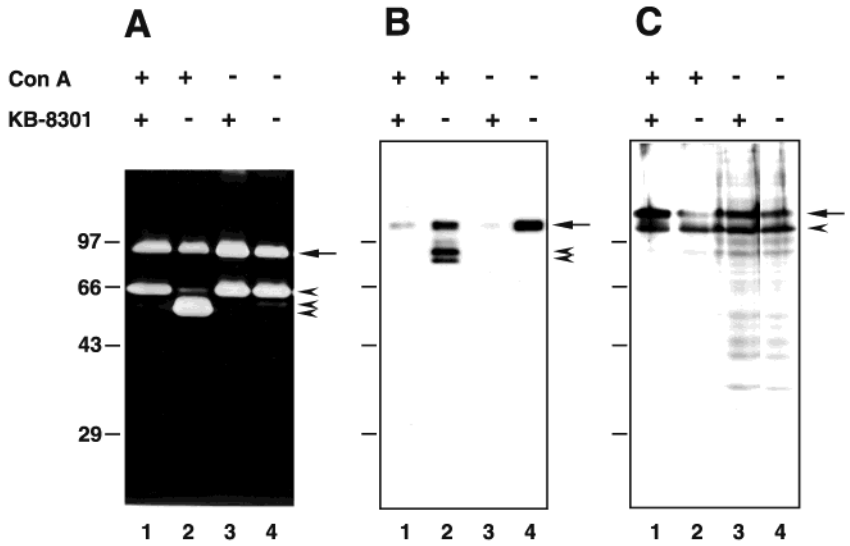

FIGURE 2: Effects of Con A stimulation and KB-8301 treatment on proteolytic processing of APP in HT1080 cells. HT1080 cells were incubated for 24 h in serum-free medium with both 10 μM KB-8301 and 100 μg/mL Con A (lane 1), with Con A only (lane 2), with KB-8301 only (lane 3), or without them (lane 4). CMs (A,B) and cell lysates (C) were prepared from the incubated cells as described in the Experimental Procedures and subjected to gelatin zymography (A) and trypsin reverse zymography (B,C). The samples loaded were equivalent to 200 and 40 μL of nonconcentrated CMs for gelatin zymography and trypsin reverse zymography, respectively, and cell lyzates equivalent to 1×10^5 cells. In panel A, the arrow at 90 kDa indicates a gelatinolytic band of progelatinase B. The arrowheads indicate the gelatinolytic bands of progelatinase A at 66 kDa (upper), the intermediate form of gelatinaze A at 59 kDa (center), and the active form of gelatinase A at 57 kDa (lower). In panel B, the arrow at 105 kDa indicates the inhibitor band of sAPP. The arrowheads indicate the bands of 90- and 85-kDa trypsin inhibitors. In panel C, the arrow at 115 kDa and the arrowhead at 103 kDa indicate the inhibitor bands of the mature and the immature forms of APP, respectively. Con A, concanavalin A. The ordinate gives molecular size in kilodaltons.

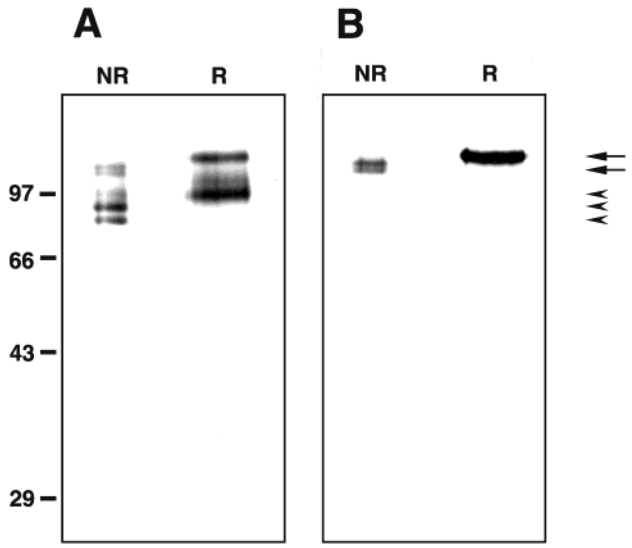

FIGURE 3: Western blotting analysis of APP fragments released from Con A-stimulated HT1080 cells. HT1080 cells were incubated for 24 h in serum-free medium with 100 μg/mL Con A. The resultant CM was subjected to Western blotting analysis using the monoclonal antibody 22C11 (A) and an anti-Aβ monoclonal antibody (B), which recognize NH_2- and COOH-terminal epitopes of sAPP, respectively. NR represents nonreduced conditions, R reduced conditions. The arrows indicate 110-kDa reduced sAPP (upper) and its 105-kDa nonreduced form (lower). The arrowheads indicate 95-kDa reduced sAPPtrc (upper) and its 90- (center) and 85-kDa (lower) nonreduced forms. The ordinate gives molecular size in kilodaltons.

the interaction of these APP-derived fragments with a monoclonal antibody against Aβ, which recognizes the NH_2 terminus of Aβ, and found that only sAPP reacted with the antibody (Figure 3B). As a part of Aβ structure locates in the COOH terminus of sAPP, the lack of immuno-reactivity suggests that the 85- and 90-kDa fragments are COOH-terminally truncated forms of sAPP. When these fragments were analyzed after reduction, the monoclonal antibody 22C11 reacted mainly with 95- and 110-kDa fragments, whereas the anti-Aβ monoclonal antibody reacted only with the 110-kDa fragment, indicating that the 110-kDa fragment is a reduced form of sAPP and the 95-kDa fragment is that of the COOH-terminally truncated sAPP. On the basis of these results, the 95-kDa fragment of APP was named "truncated sAPP" (sAPPtrc). We also analyzed NH_2-terminal sequences of the 85- and 90-kDa forms of nonreduced sAPPtrc and sAPP as described in the Experimental Procedures. The same LEVPTDGNAG sequence, corresponding to the 18th to 27th amino acid residues of APP770, was determined in the analyses of all the APP fragments, indicating that the NH_2 terminus of APP remains uncleaved in both sAPPtrc and sAPP.

Effect of Concentration of KB-8301 on Processing of APP. The data shown in Figure 2 suggest that KB-8301 inhibits both the α-secretase and an enzyme responsible for the production of sAPPtrc. To examine the inhibitor susceptibilities of these two enzymes, Con A-stimulated HT1080 cells were cultured in the presence of various concentrations of KB-8301, and the processing of APP was analyzed. As shown in Figure 4A, the release of sAPPtrc was diminished in the presence of 10^{-7} M or higher concentrations of KB-8301. On the other hand, the concentration of KB-8301 required for inhibition of the sAPP release was over 10^{-5} M. Therefore, the sAPPtrc-producing reaction was more susceptible to KB-8301 than the sAPP-releasing reaction. The activation of progelatinase A was also inhibited in the presence of 10^{-7} M or higher concentrations of KB-8301 (Figure 4C), indicating that the activator of progelatinase A and the sAPPtrc-producing enzyme have similar inhibitor

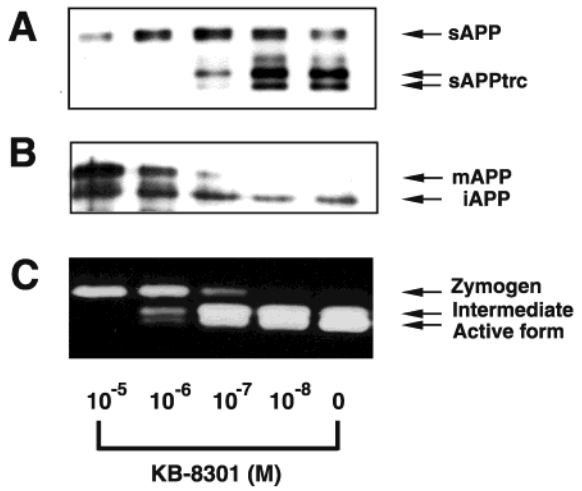

FIGURE 4: Effects of concentration of KB-8301 on processing of APP in Con A-stimulated HT1080 cells. HT1080 cells were incubated for 24 h in serum-free medium with the indicated concentrations of KB-8301 and a fixed concentration of Con A (100 μg/mL). CMs (A,C) and cell lysates (B) were prepared from the incubated cells and subjected to trypsin reverse zymography (A,B) or gelatin zymography (C). The samples loaded were equivalent to 200 and 40 μL of nonconcentrated CMs for geratin zymography and trypsin reverse zymography, respectively, and cell lyzates equivalent to 1×10^5 cells. mAPP, the mature form of APP; iAPP, the immature form of APP; zymogen, progelatinase A; intermediate, the intermediate form of gelatinase A; active form, the active form of gelatinase A.

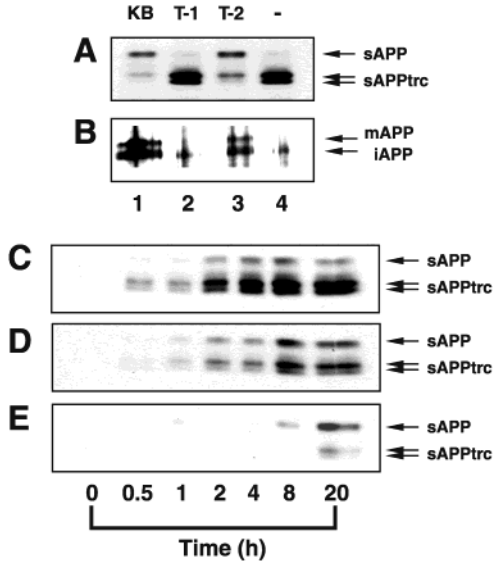

FIGURE 5: Effects of metalloproteinase inhibitors on proteolytic liberation of APP fragments from APP-accumulated HT1080 cells. The APP-accumulated HT1080 cells were first prepared, and then the release of APP fragments from the cells was examined as described in the Experimental Procedures. (A,B) The APP-accumulated cells were incubated for 20 h with 10 μM KB-8301 (KB, lane 1), with 100 nM TIMP-1 (T-1, lane 2), with 100 nM TIMP-2 (T-2, lane 3), or without inhibitor (−, lane 4) in serum-free medium containing 10 μM sodium monensin and 100 μg/mL Con A. Alternatively, the APP-accumulated cells were incubated for the indicated lengths of time without inhibitor (C) or with 100 nM TIMP-2 (D) or 10 μM KB-8301 (E) in serum-free medium containing 10 μM sodium monensin and 100 μg/mL Con A. CMs (A,C,D,E) and cell lysates (B) were prepared from the incubated cells and subjected to trypsin reverse zymography. The samples loaded were equivalent to 200 μL of nonconcentrated CMs and cell lyzates equivalent to 1×10^5 cells. mAPP, the mature form of APP; iAPP, the immature form of APP.

susceptibilities. In the presence of 10^{-5} M KB-8301, accumulation of the mature form of APP in cell lysate was observed (Figure 4B).

Effect of Inhibitors on Proteolytic Liberation of APP Fragments from APP-Accumulated Cells. There are two possible pathways for production of sAPPtrc. In the first mechanism, the cell-bound APP is cleaved directly to release sAPPtrc. In the second one, sAPP released by α-secretase is subsequently converted to sAPPtrc. To examine these possibilities, we first prepared APP-accumulated HT1080 cells, and then the proteolytic liberation of APP fragments from the cells was analyzed as described in the Experimental Procedures. After 20 h of incubation of the APP-accumulated cells without inhibitor, sAPPtrc was found to be the major fragment of APP released into CM (Figure 5A), and the mature form of APP remaining in the cell lysate was hardly observed (Figure 5B). On the other hand, TIMP-2 and KB-8301, but not TIMP-1, effectively inhibited the release of sAPPtrc into CM (Figure 5A), and the mature form of APP remaining in cell lysate was observed (Figure 5B). We further examined the time course of release of the APP fragments from the APP-accumulated cells. In the absence of inhibitor, sAPPtrc appeared after 30 min of incubation, and both the release of sAPPtrc and that of sAPP were saturated after 4 h (Figure 5C). Considering that the mature form of APP was absent in the lysate from the cells incubated for 20 h, it is likely that almost all APP molecules were converted to sAPP or sAPPtrc during the first 4 h of incubation, and therefore further incubation did not cause the increase of the fragments. Up to 4 h of incubation, the rate of release of sAPPtrc was reduced in the presence of TIMP-2, but that of sAPP was not changed (Figure 5D), suggesting that TIMP-2 specifically inhibits the sAPPtrc-producing enzyme but does not inhibit α-secretase activity. Since the reduction of the production of sAPPtrc did not increase the amount of sAPP in the initial phase of the reaction, it is unlikely that a major part of sAPP is cleaved to produce sAPPtrc. Constant appearance of sAPPtrc and sAPP during 4−20 h of incubation without inhibitor (Figure 5C) also suggests that the conversion of sAPP to sAPPtrc is very slow or negligible, if any. In contrast to the effects of TIMP-2, the releasing rates of both sAPPtrc and sAPP were significantly reduced in the presence of KB-8301 (Figure 5E).

Analysis of COOH-Terminal Fragment of APP Remaining on Cell Surface after Release of sAPPtrc. The data shown in Figure 5 suggest that the direct cleavage of membrane-bound APP is the major pathway of the production of sAPPtrc. To confirm this, we analyzed the COOH-terminal fragment of APP remaining on the cell surface after the release of sAPPtrc. For this analysis, we first prepared the fraction of membrane-associated proteins from the HT1080 cells, which had been stimulated with Con A for various time periods, and the fractions were then subjected to Western blotting analysis with a monoclonal antibody 278 against sAPP. Since the monoclonal antibody 278 does not react with sAPPtrc (data not shown), this antibody is expected to recognize the COOH-terminal epitope of sAPP. As shown in Figure 6, 38-, 36-, and 30-kDa fragments which react with the antibody appeared after 1 h of stimulation with Con A, and the band intensities of all the fragments reached their maximum after 2 h of stimulation. Further incubation

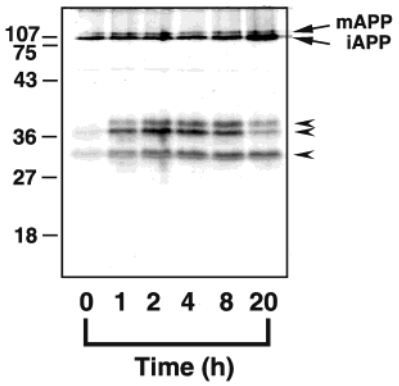

FIGURE 6: Analysis of COOH-terminal fragment of APP remaining on the cell surface after release of sAPPtrc. HT1080 cells were incubated for the indicated lengths of time in serum-free medium with 100 μg/mL Con A. The fractions of membrane-associated proteins were prepared from the incubated cells as described in the Experimental Procedures and subjected to Western blotting analysis with the anti-sAPP monoclonal antibody 278. The samples loaded were the fractions of membrane-associated proteins equivalent to 1×10^6 cells. The arrowheads indicate the 38-, 36-, and 30-kDa cell-associated fragments which have the COOH-terminal epitope of sAPP. The ordinate gives molecular size in kilodaltons.

did not cause an accumulation of these fragments, suggesting that the produced fragments are unstable and are catabolized during the incubation.

Effect of Reactive-Site-Modified TIMP-2 on Production of sAPPtrc. The data shown in Figure 5 also suggest that a cell-associated, TIMP-2-sensitive metalloproteinase is responsible for the production of sAPPtrc. As both gelatinase A and MT1-MMP are active metalloproteinases associated with the surface of Con A-stimulated HT1080 cells and both are also susceptible to TIMP-2 inhibition, these two enzymes are candidates for the sAPPtrc-producing enzyme. We previously reported that a chemically modified TIMP-2, the reactive site of which has been destroyed by carbamylation, has an ability to inhibit the cell-mediated activation of progelatinase A, but it does not inhibit the catalytic activity of MT1-MMP (*34*). To determine which metalloproteinase is the responsible enzyme, we examined the effect of the reactive-site-modified TIMP-2 on production of sAPPtrc in Con A-stimulated HT1080 cells. As shown in Figure 7B, the active form of gelatinase A was diminished with increasing concentrations of the reactive-site-modified TIMP-2. The active form of gelatinase A almost disappeared as the concentration of the modified TIMP-2 was increased to 36 nM or higher, while sAPPtrc in CM was not diminished at all, even when the concentration of the modified TIMP-2 was increased to 72 nM (Figure 7A). We also found that the cell-associated active form of gelatinase A was starved in the presence of 36 nM or higher concentrations of the modified TIMP-2 (Figure 7C). The release of sAPPtrc independent of the cell-associated gelatinase A suggests that a metalloproteinase other than gelatinase A acts as the sAPPtrc-producing enzyme. In contrast to the effects of reactive-site-modified TIMP-2, unmodified TIMP-2 at concentrations higher than 18 nM strongly inhibited both the activation of progelatinase A (Figure 7B) and the release of sAPPtrc (Figure 7A). The disappearance of the active form of gelatinase A was in parallel with that of sAPPtrc in CM. Taken together, these data suggest that MT1-MMP, a cell-associated activator of progelatinase A, is most likely to be the sAPPtrc-producing enzyme.

Gelatinase A Inhibitory Activity of sAPPtrc. Since a gelatinase A inhibitor domain has been reported to locate in the COOH-terminal region of sAPP (*16*), the processing of APP that produces sAPPtrc may affect the inhibitory activity. To examine this possibility, the inhibitory activity of sAPPtrc against gelatinase A was tested by reverse zymography. As shown in Figure 8, sAPP had an ability to inhibit the gelatinolytic activity of gelatinase A, but sAPPtrc did not. This indicates that sAPPtrc lacks the inhibitor domain against gelatinase A.

Production of sAPPtrc-like Fragment by Recombinant MT1-MMP. To confirm that sAPPtrc is produced by MT1-MMP, we digested purified sAPP with the recombinant catalytic domain of MT1-MMP and assayed the gelatinase A-inhibitory activities of the resultant fragments. We found that the digestion of sAPP yielded fragments with molecular masses similar to those of the nonreduced forms of sAPPtrc (Figure 9B). None of the fragments other than sAPP remaining undigested was found to have inhibitory activity toward gelatinase A (Figure 9A), suggesting that the inhibitor domain of sAPP is readily removed by the MT1-MMP cleavage. These sAPPtrc-like fragments showed the trypsin-inhibitory activity on reverse zymography (data not shown). To determine the site of cleavage, we tried to separate the sAPP-derived fragments by SDS−PAGE. However, isolation of the fragment derived from a COOH-terminal region of sAPP was not feasible because the fragment was hardly stained with Coomassie Brilliant Blue R-250. We then first treated sAPP with KNCO to mask its NH_2 terminus by carbamylation and then digested the modified sAPP with the recombinant catalytic domain of MT1-MMP. The NH_2 terminus, newly exposed by the MT1-MMP cleavage, was analyzed as described in the Experimental Procedures. We found that the treatment of sAPP with KNCO did not affect the inhibitory activity against gelatinase A and that the catalytic domain of MT1-MMP also cleaved the modified sAPP to yield the noninhibitory fragments with molecular masses similar to those of the nonreduced forms of sAPPtrc (data not shown). The MISEPRISYG sequence corresponding to residues 580−589 of APP_{770} was determined in the NH_2-terminal sequence analysis on the digest of the modified sAPP, indicating that the MT1-MMP cleavage occurs at the peptidyl bond between Asn^{579} and Met^{580} of the modified sAPP (Figure 1).

Effect of KB-8301 or Heparin on APP-Derived Fragments Associated with ECM. The extracellular domain of APP has been reported to have affinities for some components of ECM (*17*−*21*). To examine whether the fragments derived from APP associate with the ECM deposited by the Con A-stimulated HT1080 cells, the fraction of ECM was prepared as described in the Experimental Procedures, and APP fragments in this fraction were analyzed. As shown in Figure 10A, sAPPtrc was found to be the major fragment in the ECM fraction when the cells had been incubated without inhibitor. With increasing concentrations of KB-8301, the ECM-associated sAPPtrc was diminished, with a concomitant increase of sAPP and APP in the ECM fraction. We found that the 105-kDa trypsin inhibitor in the ECM fraction was sAPP, not the immature form of membrane-bound APP, because this fragment did not react with the polyclonal

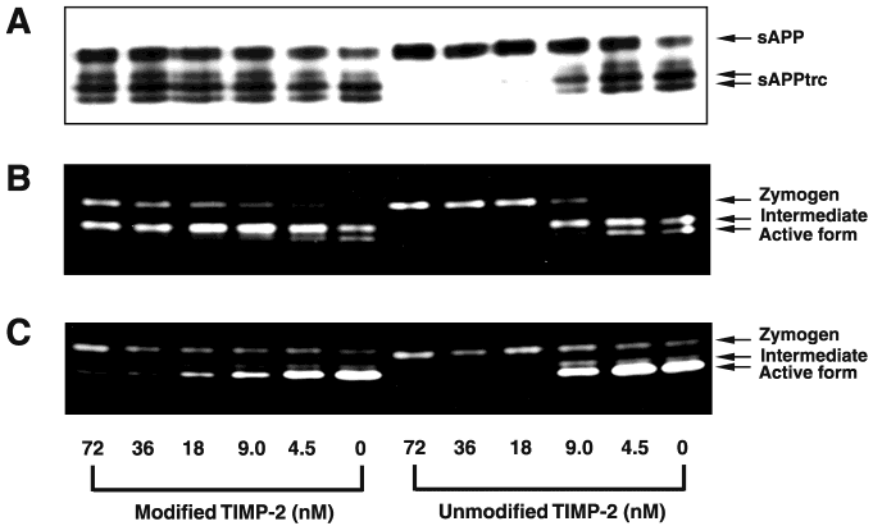

FIGURE 7: Effects of NH_2-terminally modified and unmodified TIMP-2 on processing of APP in Con A-stimulated HT1080 cells. HT1080 cells were incubated for 24 h in serum-free medium supplemented with the indicated concentrations of NH_2-terminally modified TIMP-2 (modified TIMP-2) and unmodified TIMP-2 and a fixed concentration of Con A (100 μg/mL). CMs (A,B) and cell lysates (C) were prepared from the incubated cells and subjected to trypsin reverse zymography (A) and gelatin zymography (B,C). The samples loaded were equivalent to 50 and 40 μL of nonconcentrated CMs for geratin zymography and trypsin reverse zymography, respectively, and cell lyzates equivalent to 1×10^5 cells. Zymogen, progelatinase A; intermediate, the intermediate form of gelatinase A; active form, the active form of gelatinase A.

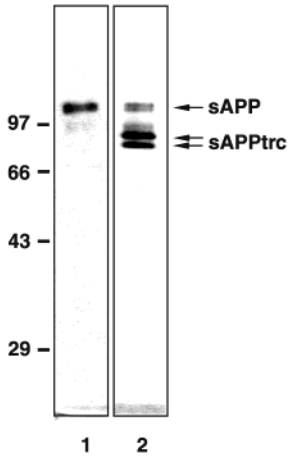

FIGURE 8: Gelatinase-inhibitory activity of sAPP and sAPPtrc on reverse zymography. A partially purified sAPPtrc fraction that contained both sAPPtrc and sAPP (see Experimental Procedures) was subjected to gelatinase A reverse zymography (lane 1). The same sample was also analyzed by Western blotting using the monoclonal antibody 22C11 (lane 2). The ordinate gives molecular size in kilodaltons.

antibody APP C-terminus (Chemicon International Inc.) that reacts with the cytoplasmic region of APP (data not shown). Since the sites of sAPPtrc essential for interaction with ECM also exist in sAPP or APP, these molecules are likely to compete for the limiting affinity sites of ECM. To examine this possibility, the Con A-stimulated cells were first incubated with 10^{-6} M KB-8301 for 6 h to allow the cells to produce sAPP and APP (see Figure 4), and then the incubation was continued for 12 h without the inhibitor. As shown in Figure 10B, sAPP and APP that accumulated in the ECM fraction during the first incubation were diminished, and sAPPtrc appeared after the second incubation. In contrast, when the cells were first incubated without KB-8301, the subsequent incubation with the inhibitor led to a decrease of sAPPtrc and to an increase of the gelatinase A inhibitor-containing derivatives of APP in the ECM fraction. These results suggest that sAPP and APP associated with ECM can be displaced by sAPPtrc and vice versa. It has been reported that APP interacts with the ECM obtained from Engelbreth−Holm−Swarm tumors via heparan sulfate proteoglycans, and heparin effectively inhibits the interaction (*18*). We further examined the effect of heparin on the interaction between the ECM deposited by the Con A-stimulated HT1080 cells and the derivatives of APP. As shown in Figure 10C, all of the sAPPtrc, sAPP, and APP in the ECM fraction decreased in amount with increasing concentrations of heparin, and a concomitant increase of sAPPtrc and sAPP in the CM was observed (Figure 10D). These data are consistent with the view that heparin prevents the interaction between the ECM and APP fragments, thus liberating the fragments from ECM into CM.

Effect of Heparin on Gelatinase A Inhibitor Activity of sAPP. The interaction of heparin with some serine protease inhibitors enhances their inhibitory activities not only by altering the inhibitor conformation but also by concentrating both inhibitor and protease molecules on the same heparin chain (*39*, *40*). As both sAPP and gelatinase A have heparin-binding properties, heparin may enhance the gelatinase A inhibitory activity of sAPP. We tested this possibility. As shown in Figure 11A, sAPP inhibited gelatinase A-catalyzed hydrolysis of a synthetic peptide substrate with the IC_{50} value of 320 nM, where IC_{50} represents the concentration of sAPP that gives a 50% inhibition of the gelatinase A activity. As it has been reported that sAPP inhibits the gelatinase A-catalyzed hydrolysis of a peptide substrate in a competitive manner (*16*), eq 1 can be derived from the Michaelis−Menten equation (*41*), where $[I]_{50}$ represents the concentration of the free inhibitor that gives a 50% inhibition, K_i is the inhibition constant, [S] is the concentration of free substrate, and K_m is the Michaelis constant for the gelatinase A-catalyzed hydrolysis of the substrate. Considering that the

$$[I]_{50} = K_i(1 + [S]/K_m) \quad (1)$$

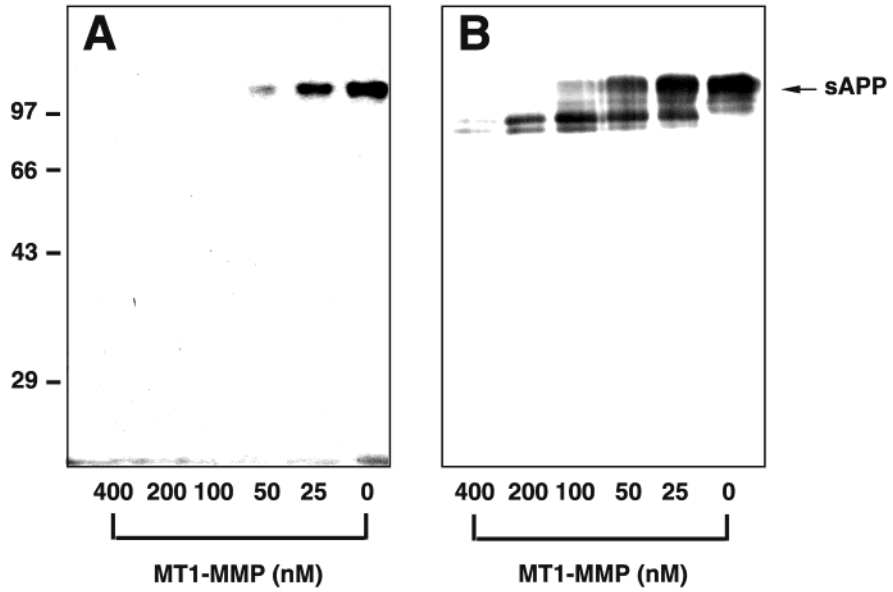

FIGURE 9: Effect of MT1-MMP cleavage on inhibitory activity of sAPP toward gelatinase A. (A) 400 nM sAPP was digested with the indicated concentrations of the catalytic domain of MT1-MMP at 37 °C for 4 h, and the resultant digests were subjected to gelatinase A reverse zymography. (B) The digests were also analyzed by Western blotting using the monoclonal antibody 22C11. The ordinate gives molecular size in kilodaltons.

IC_{50} value obtained in Figure 11A was much higher than the concentration of gelatinase A (0.58 nM) in the assay system, the IC_{50} value can be assumed to be close to $[I]_{50}$. When the concentration of the synthetic substrate is lower than the K_m value, one can assume that the $[I]_{50}$ value given by eq 1 is not much different from the K_i value. We found that the IC_{50} value was not changed when the concentration of the substrate was reduced from 50 to 25 μM (data not shown), suggesting that these substrate concentrations are sufficiently lower than the K_m value, and the IC_{50} value (320 nM) is close to the K_i value. In the presence of 100 μg/mL heparin, the IC_{50} value was reduced to 80 nM, indicating that the apparent affinity between sAPP and gelatinase A is enhanced. To examine the enhancing mechanism, effect of various concentrations of heparin on the sAPP inhibition of gelatinase A activity was tested. As shown in Figure 11B, heparin did not change the activity of gelatinase A, whereas the inhibition of gelatinase A activity by sAPP was changed, depending on the heparin concentration. The shape of the curve shown in Figure 11B is consistent with the ternary complex model of heparin action (*39*), in which heparin at lower concentrations binds both protease and inhibitor, thereby bringing them closer in proximity. At higher concentrations, heparin acts to sequester the reactants. These results suggest that the gelatinase A inhibitory activity of sAPP can be potentiated in the presence of ECM components such as heparan sulfate-containing proteoglycans.

Effect of sAPP on Gelatinase A-Catalyzed Degradation of Type IV Collagen. We tested the gelatinase A inhibitory activity of sAPP, using type IV collagen as a substrate. As shown in Figure 12A, the chains of type IV collagen were converted into low-molecular-weight fragments during the incubation with gelatinase A. The degradation of type IV collagen was effectively inhibited in the presence of 1.5 μM sAPP (Figure 12B).

Ability of sAPP to Bind to Active Forms of Gelatinase A. To examine the interaction between the active gelatinase A and sAPP, progelatinase A was incubated with APMA for 4

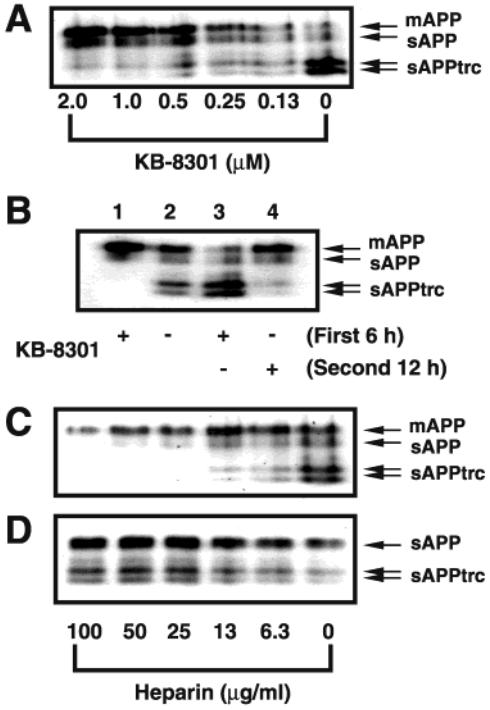

FIGURE 10: Effect of KB-8301 or heparin on ECM-associated derivatives of APP. (A) HT1080 cells were incubated for 20 h in serum-free medium with the indicated concentrations of KB-8301 and a fixed concentration of Con A (100 μg/mL). (B) HT1080 cells were incubated for 6 h in serum-free medium with 1 μM KB-8301 (lane 1) or without the inhibitor (lane 2). Alternatively, the cells first incubated for 6 h with or without 1 μM KB-8301 were further incubated for 12 h, respectively, without (lane 3) or with 1 μM KB-8301 (lane 4) after exchanging the medium. All the culture media contained 100 μg/mL Con A throughout the incubation. (C,D) HT1080 cells were incubated for 12 h in serum-free medium with the indicated concentrations of heparin and a fixed concentration of Con A (100 μg/mL). ECMs (A−C) and CMs (D) were prepared from the incubated cells and subjected to trypsin reverse zymography. The samples loaded were equivalent to 40 μL of nonconcentrated CMs and ECMs deposited by 1×10^6 cells.

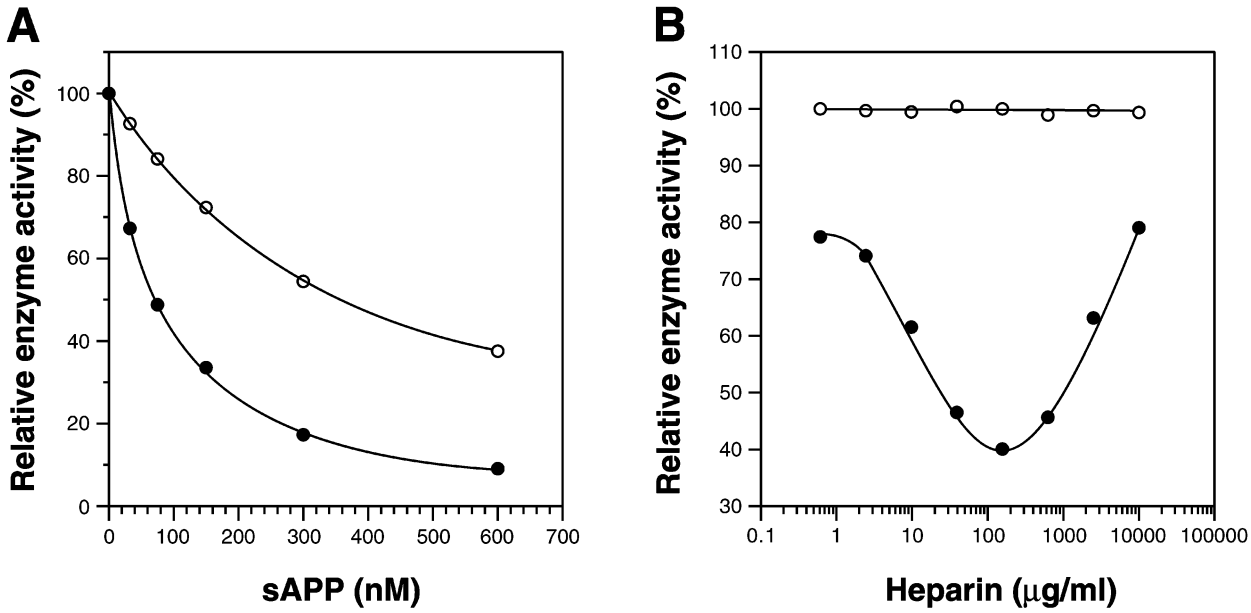

FIGURE 11: Effect of heparin on gelatinase A inhibitory activity of sAPP. (A) APMA-activated gelatinase A (0.58 nM) was incubated with 50 μM 3163v at 37 °C for 40 min in the presence of various concentrations of sAPP with (●) or without (○) a fixed concentration of heparin (100 μg/mL). (B) APMA-activated gelatinase A (0.58 nM) was incubated with 50 μM 3163v at 37 °C for 40 min in the presence of various concentrations of heparin with (●) or without (○) a fixed concentration of sAPP (120 nM). All the reaction mixtures contained 50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, 0.01% Brij 35, and 0.01% bovine serum albumin. The amount of 3163v hydrolyzed in the absence of sAPP and heparin was taken as 100%. The enzyme activity is shown as the relative amount of 3163v hydrolyzed by the enzyme on the ordinate.

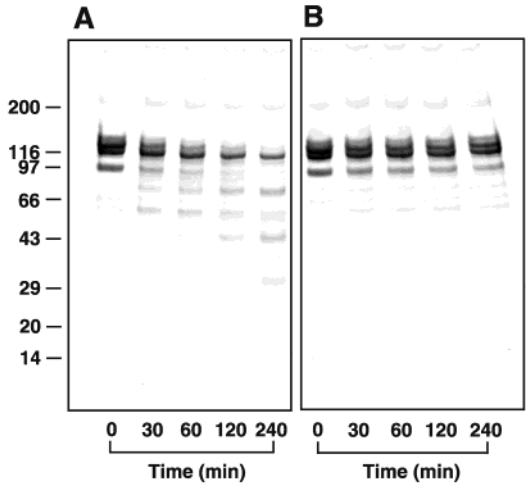

FIGURE 12: Effect of sAPP on gelatinase A-catalyzed degradation of type IV collagen. Type IV collagen (6 μg) was incubated with 7 nM APMA-activated gelatinase A at 37 °C for the indicated length of time in the absence (A) or presence (B) of 1.5 μM sAPP in 10 μL of 50 mM Tris-HCl containing 150 mM NaCl, 10 mM $CaCl_2$, and 0.01% Brij 35. The resultant digests were subjected to SDS−PAGE. The protein bands of fragments derived from type IV collagen were visualized by Coomassie Brilliant Blue R-250 staining. The ordinate gives molecular size in kilodaltons.

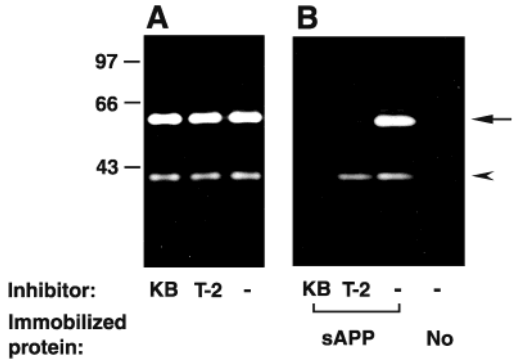

FIGURE 13: Ability of active gelatinase A to bind to immobilized sAPP. Progelatinase A was incubated with 1 mM APMA at 37 °C for 4 h in 50 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$ and 0.01% Brij 35. After treatment with APMA, the sample was incubated with sAPP coupled to Affi-Gel 10 beads or unconjugated beads (No) in the absence (−) or presence of 1.0 μM KB-8301 (KB) or 100 nM TIMP-2 (T-2) at 4 °C for 5 h with rotation. The gelatinase A forms adsorbed into the beads were analyzed by gelatin zymography (B) as described in the Experimental Procedures. The gelatinase A samples, prepared as described above but incubated without beads, were also subjected to the zymographic analysis (A). The arrow and arrowhead indicate the gelatinolytic bands of the 57- and 41-kDa forms of active gelatinase A, respectively. The ordinate gives molecular size in kilodaltons.

h, and the resultant active forms of gelatinase A were tested for their abilities to bind to immobilized sAPP, as described in the Experimental Procedures. As shown in Figure 13A, both the 41-kDa form, which is reported to be a hemopexin-like domain-less form of active gelatinase A (*42*), and the 57-kDa active form of gelatinase A were produced during the 4-h incubation with APMA. We found that both of the active forms of gelatinase A (57- and 41-kDa forms) had an ability to bind to the immobilized sAPP, but neither of the active forms was adsorbed into the unconjugated beads (Figure 13B). When the hydroxamate-based inhibitor KB-8301 was added to the reaction mixture, the binding of the active forms with the immobilized inhibitor was completely inhibited (Figure 13B). We also found that TIMP-2 inhibited the binding of the 57-kDa form with the immobilized sAPP, but it failed to prevent the interaction between the 41-kDa form and sAPP (Figure 13B). This result is consistent with the previous study (*43*) that showed that the 41-kDa hemopexin-like domain-less form of active gelatinase A is resistant to TIMP-2 inhibition. Neither TIMP-2 nor KB-8301

hampered the zymographic analysis of the active forms of gelatinase A (Figure 13A). These results suggest that the unoccupied active site of gelatinase A is required for the interaction between gelatinase A and sAPP, but the hemopexin-like domain of gelatinase A is not necessary for the interaction.

DISCUSSION

To investigate the APP-mediated regulation of gelatinase A activity, we examined the correlation between the cell-mediated activation of progelatinase A and the processing of APP and showed that the activation of the MMP zymogen is accompanied by a novel processing of APP. In this processing, a COOH-terminally truncated form of sAPP, named sAPPtrc, is released into the culture medium. Several lines of evidence presented here support the conclusion that MT1-MMP, an activator of progelatinase A, cleaves the membrane-bound form of APP to release sAPPtrc. First, sAPPtrc was produced in Con A-stimulated HT1080 cells, but not in the nonstimulated cells (Figure 2). The lectin stimulation also led to activation of endogenous progelatinase A, suggesting that the active form of MT1-MMP is induced only in the stimulated cells. Moreover, both the activation of progelatinase A and the production of sAPPtrc were inhibited in the presence of KB-8301 (Figure 4) or TIMP-2 (Figure 7), and the disappearance of the active form of gelatinase A was always in parallel with that of sAPPtrc. These results are consistent with the view that MT1-MMP catalyzes both the processing of APP and the activation of progelatinase A. Second, we showed that sAPPtrc is released from the APP-accumulated HT1080 cells (Figure 5A). As soluble proteases were absent in the medium, it is likely that a protease on the cell surface is responsible for producing sAPPtrc. We also showed that the production of sAPPtrc is inhibited by TIMP-2, but not by TIMP-1 (Figure 5A). To our knowledge, no metalloproteinase other than those from the MMP family has been reported to be susceptible to TIMP-2 inhibition. Therefore, the sAPPtrc-producing enzyme seems to be a cell-associated MMP. On the other hand, almost all MMPs are susceptible to both TIMP-1 and TIMP-2. Unlike other MMPs, MT1-MMP is reported to be susceptible to TIMP-2 inhibition but not to TIMP-1 inhibition (*44*); this characteristic inhibitor susceptibility of MT1-MMP activity is similar to that of the sAPPtrc-producing enzyme. The proportion of sAPPtrc to sAPP released from the APP-accumulated cells (Figure 5A) was significantly higher than that released from the Con A-stimulated cells (Figure 2B). It has been reported that treatment of cells expressing MT1-MMP with a hydroxamate-based inhibitor leads to starvation of the cell-bound TIMP-2 (*45*). The synthetic inhibitor occupies the active site of MT1-MMP, thus preventing formation of the complex between MT1-MMP and TIMP-2. A hydroxamate-based inhibitor also appears to protect MT1-MMP from autolysis (*46*). The facilitated release of sAPPtrc from the APP-accumulated cells may reflect an accumulation of the TIMP-2-free form of MT1-MMP on the surface of the cells during the treatment with KB-8301. The time course of release of APP fragments from the APP-accumulated cells showed that the initial rate of sAPPtrc production was much higher than that of sAPP (Figure 5C). TIMP-2 reduced the rate of production of sAPPtrc, but it did not change that of sAPP. Since the reduction of sAPPtrc production did not lead to an increase of sAPP in the initial phase of the reaction, it is unlikely that sAPP is a major precursor of sAPPtrc. Moreover, the Con A-stimulation-dependent appearance of the membrane-bound fragments of APP that contain the COOH-terminal epitope of sAPP (Figure 6) strongly suggests that the membrane-bound APP is cleaved to release sAPPtrc. Third, we demonstrated that the reactive-site-modified TIMP-2 prevented the activation of progelatinase A in the Con A-stimulated HT1080 cells, but it failed to inhibit the production of sAPPtrc (Figure 7). Considering that modified TIMP-2 does not have an ability to inhibit the catalytic activity of MT1-MMP, this membrane-type MMP is thought to be the sole cell-associated, active MMP after the disappearance of active gelatinase A and, therefore, is a prime candidate for the sAPPtrc-producing enzyme. It should be noted that MT2-MMP and MT3-MMP are hardly detectable in Con A-stimulated HT1080 cells (*47*). Finally, A549 lung adenocarcinoma and Hela S3 cervix epithelioid carcinoma cell lines, which do not express MT1-MMP mRNA (*47*), were found to secrete sAPP, but production of sAPPtrc in these cell lines before or after Con A stimulation was not observed (data not shown). In contrast to these cell lines, T24 urinary bladder carcinoma, T98G glioblastoma, and G361 malignant melanoma, as well as HT1080 fibrosarcoma cell lines, all of which are known to express MT1-MMP mRNA (*47*), were also found to secrete sAPPtrc after Con A stimulation (data not shown). None of these data is inconsistent with the conclusion that MT1-MMP is responsible for the sAPPtrc production.

The present study also demonstrates that sAPPtrc lacks the gelatinase A inhibitor domain of APP. In vitro, a gelatinase A inhibitor domain-less fragment similar to sAPPtrc was also produced during incubation of sAPP with the catalytic domain of MT1-MMP (Figure 9). The MT1-MMP-catalyzed cleavage of the Asn^{579}-Met^{580} peptidyl bond of sAPP led to production of the noninhibitory fragment. We speculate that the production of sAPPtrc upon expression of MT1-MMP activity is important for the regulated degradation of extracellular matrix. Unlike other soluble MMPs, expression of gelatinase A activity is strictly regulated by several mechanisms. For example, activation of progelatinase A is limited on a cell surface that contains optimum concentrations of free MT1-MMP and TIMP-2-inhibited MT1-MMP (*33*). It is also thought that both the cell-associated active gelatinase A and MT1-MMP are recruited into invadopodia, thus limiting proteolysis to the site of cell invasion (*48*). These mechanisms involve all of (pro)gelatinase A, TIMP-2, and MT1-MMP, and local concentrations of these factors determine the cellular proteolytic profile. In general, activities of MMPs are primarily regulated by TIMPs, which form tight stoichiometric complexes with MMPs. The K_i value for the inhibition of gelatinase A activity by TIMP-1 is reported to be less than 2 pM (*49*). Therefore, MMPs can exert their proteolytic activity only in the environment where the local concentration of the enzyme is in excess of that of TIMPs. Focusing of the active gelatinase A and MT1-MMP at the cell-ECM interface may be an effective strategy for enhancing the local concentration of the MMPs relative to that of TIMPs. Although sAPP was found to inhibit the gelatinase A activity with a relatively high IC_{50} value (320 nM) in solution, we speculate that this molecule can be a secondary regulator of

the TIMP-uninhibited gelatinase A at the cell−ECM interface, where sAPP can be concentrated via its interaction with ECM. We demonstrated that type IV collagen was effectively protected from the gelatinase A-catalyzed degradation in the presence of 1.5 μM sAPP (Figure 12); this concentration of sAPP may be accomplished at the interface. It is reported that the concentration of sAPP/protease nexin-2 in plasma is less than 60 pM and increases to 30 nM upon platelet activation (*50*). We found that the CM of HT1080 cells contained about 10 nM sAPP. As these concentrations of sAPP are much lower than the IC_{50} value for the sAPP inhibition of the gelatinase A activity, sAPP may not be an effective gelatinase inhibitor in solution. We also speculate that the MT1-MMP-catalyzed processing of APP contributes to limiting the gelatinase A activity near the active MT1-MMP in a following manner. On the cell surface where the local concentration of active MT1-MMP is low, APP is liberated mainly as sAPP that has an ability to inhibit the activity of gelatinase A. The released sAPP or the membrane-bound form of APP associates with the ECM; the interaction may lead to an enhancement of the gelatinase A inhibitory activity of the derivatives of APP (Figure 11). Therefore, ECM near the cell surface is protected from the gelatinase A-catalyzed degradation. In contrast, a high concentration of active MT1-MMP converts APP to sAPPtrc, which lacks an ability to inhibit gelatinase A. The released sAPPtrc displaces ECM-associated sAPP or APP, thereby removing the gelatinase A inhibitor from ECM. The removal of the inhibitor from ECM makes it feasible for activated gelatinase A to exert proteolytic activity. Together with the MT1-MMP-catalyzed activation of progelatinase A, removal of gelatinase A inhibitor from ECM makes a sharp contrast between the ECM degradation in the vicinity of active MT1-MMP-rich region and that in other regions. Recently, MT1-MMP was reported to be down-regulated by autolysis (*46*). Therefore, a high local concentration of active MT1-MMP is thought to cause a rapid disappearance of cell-surface MT1-MMP (*51*). The disappearance of MT1-MMP may be needed to terminate the activation of progelatinase A. So far, the mechanism for clearance of active gelatinase A has not been elucidated. We speculate that the degradation of ECM by gelatinase A is terminated after the disappearance of cell-surface MT1-MMP, because sAPP or APP produced in the absence of active MT1-MMP begins to protect the ECM. It should be noted that sAPP or APP also has an ability to displace sAPPtrc associated with ECM (Figure 10B). The APP-involved mechanism may be also important for temporal degradation of ECM in tissue-remodeling processes.

Recently, we found that the APP-derived inhibitor has a high selectivity toward gelatinase A (*52*). Therefore, APP can be assumed to be a specific regulator for the MT1-MMP−gelatinase A pathway. We also found that the gelatinase A inhibitor domain is localized within the ISYGNDALMP sequence, corresponding to residues 586−595 of APP_{770} (*52*). The inhibitor region is located just in the COOH-terminal parts of the MT1-MMP-cleavage site in APP. The location of the inhibitor domain is probably of benefit in switching the APP function, because the protease cleavage separates the inhibitor domain from the NH_2-terminal ECM-binding regions of APP. We could not rule out the possibility that the inhibitor domain is released by the sequential MT1-MMP and α-secretase cleavages of APP (Figure 1). Considering that no fragment other than sAPP in the CM of Con A-stimulated HT1080 cells was detectable with anti-Aβ monoclonal antibody or anti-sAPP monoclonal antibody 278 (data not shown), the inhibitor-containing COOH-terminal fragment of APP may be unstable, even if it is released into CM. The assumed inhibitor-containing fragment also may not be a potent protector of ECM because it is expected to lack most of the sites essential for interaction with ECM.

ACKNOWLEDGMENT

We thank M. Isaji and K. Hoshida (Biosciences Research Laboratory, Mochida Pharmaceutical Co., Ltd., Tokyo) for amino acid sequence analyses. We also thank J. Tsunezumi and K. Moriyama for technical support. We are grateful to Dr. K. Yoshino (Kanebo Institute for Cancer Research, Japan) for providing the synthetic metalloproteinase inhibitor KB-8301.

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BI020643M