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Cleavage of Syndecan-1 by Membrane Type Matrix Metalloproteinase-1 Stimulates Cell Migration*

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The transmembrane heparan sulfate proteoglycan syndecan-1 was identified from a human placenta cDNA library by the expression cloning method as a gene product that interacts with membrane type matrix metalloproteinase-1 (MT1-MMP). Co-expression of MT1-MMP with syndecan-1 in HEK293T cells promoted syndecan-1 shedding, and concentration of cell-associated syndecan-1 was reduced. Treatment of cells with MMP inhibitor BB-94 or tissue inhibitor of MMP (TIMP)-2 but not TIMP-1 interfered with the syndecan-1 shedding promoted by MT1-MMP expression. In contrast, syndecan-1 shedding induced by 12-*O*-tetradecanoylphorbol-13-acetate treatment was inhibited by BB-94 but not by either TIMP-1 or TIMP-2. Shedding of syndecan-1 was also induced by MT3-MMP but not by other MT-MMPs. Recombinant syndecan-1 core protein was shown to be cleaved by recombinant MT1-MMP or MT3-MMP preferentially at the Gly^{245}-Leu^{246} peptide bond. HT1080 fibrosarcoma cells stably transfected with the syndecan-1 cDNA (HT1080/SDC), which express endogenous MT1-MMP, spontaneously shed syndecan-1. Migration of HT1080/SDC cells on collagen-coated dishes was significantly slower than that of control HT1080 cells. Treatment of HT1080/SDC cells with BB-94 or TIMP-2 induced accumulation of syndecan-1 on the cell surface, concomitant with further retardation of cell migration. Substitution of Gly^{245} of syndecan-1 with Leu significantly reduced shedding from HT1080/SDC cells and cell migration. These results suggest that the shedding of syndecan-1 promoted by MT1-MMP through the preferential cleavage of Gly^{245}-Leu^{246} peptide bond stimulates cell migration.

Syndecans are transmembrane heparan sulfate proteoglycans expressed on all adherent cells (1, 2) and have been proposed to play an important role in tissue morphogenesis by virtue of their ability to bind, via their covalently attached glycosaminoglycan chains, to a variety of extracellular adhesive molecules including fibronectin, thrombospondin, various collagens, and heparin-binding growth-associated molecules and growth factors such as basic fibroblast growth factor (3–8). Since the expression of syndecans appears to be controlled during both development and the progression of tumor cells to the metastatic phenotype, it has been proposed that syndecans are important regulators of the migratory and invasive behaviors of both normal and transformed cells (9, 10). The syndecan family is composed of four closely related proteins (syndecan-1, -2, -3, and -4) encoded by four different genes. Syndecan-1 is abundant in normal epithelial cells and tissues, localizing to both basal and suprabasal cell layers (1). Disruption of syndecan-1 expression in cultured cells leads to an epithelial mesenchymal transformation, with associated changes in cell polarity and cell-cell adhesion and altered epithelium-specific gene expression (7, 11).

The intact ectodomain of each syndecan is constitutively shed from cultured cells (12, 13) as part of normal cell surface heparan sulfate proteoglycan turnover (14). Ectodomain shedding appears to contribute to diverse pathophysiological events such as host defense, wound healing, arthritis, and Alzheimer's disease, but how shedding is regulated remains largely unknown (15–17).

Matrix metalloproteinases (MMPs)[1] are a family of Zn^{2+}-dependent enzymes that are known to cleave extracellular matrix proteins in normal and pathological conditions (18–20). To date, more than 20 mammalian MMPs have been identified by cDNA cloning, and they can be subgrouped into soluble type and membrane type MMPs (MT-MMPs) (20, 21). MMPs are overexpressed in various human malignancies and have been thought to contribute to tumor invasion and metastasis by degrading extracellular matrix components (18, 22). Thus, the level of MMP expression correlates with the invasiveness or malignancy of tumors (23, 24). Particularly, MT1-MMP, MMP-2, MMP-7, and MMP-9 have been reported to be most closely associated with tumor invasion and metastasis. Whereas degradation of extracellular matrix is an important aspect of MMP biology, growing evidence has demonstrated specific processing/activation or degradation of cell surface receptors and ligands. Fas ligand (25), tumor necrosis factor-α (26), the ectodomain of the fibroblast growth factor receptor-1 (27), the heparin-binding epidermal growth factor (28), and interleukin-8 (29) were reported to be released or activated by MMPs. MMPs also cleave and inactivate interleukin-1β (30), insulin-like growth factor-binding proteins (31), fibrinogen and factor XII (32), the CC chemokine MCP-3 (33), and the CXC chemokines stromal cell-derived factor-1 α and β (34, 35). Re-

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[1] The abbreviations used are: MMP, matrix metalloproteinase; GST, glutathione *S*-transferase; MT-MMP, membrane-type matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BB-94, [4-(*N*-hydroxyamino)-2*R*-iso-butyl-3-*S*-(thienylthiomethyl)-succinyl]-L-phenylalanine-*N*-methylamide.

cently, matrilysin (MMP-7) was shown to mediate shedding of syndecan-1/a CXC chemokine (KC) complex from the mucosal surface, which directs and confines neutrophil influx to active sites in injured lungs (36).

Previously, we have developed an expression cloning method to screen genes, the products of which not only modulate pro-MMP-2 activation mediated by MT1-MMP but also serve as substrates of MT1-MMP (37–39). In this study, we have identified syndecan-1 as a substrate of MT1-MMP and demonstrated that shedding of syndecan-1 by MT1-MMP stimulates cell migration.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium was from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). 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). Recombinant MT1-MMP and MT3-MMP catalytic domains tagged with FLAG epitope at the COOH terminus were prepared as described previously (40, 41). Recombinant TIMP-2 and anti-MT2-MMP monoclonal antibody 162-4E3 were from Daiichi Fine Chemical Co. Ltd. (Takaoka, Japan). Monoclonal antibodies against FLAG and His_6 epitope were purchased from Sigma and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. [4-(*N*-hydroxyamino)-2*R*-isobutyl-3-*S*-(thienylthiomethyl)-succinyl]-L-phenylalanine-*N*-methylamide (BB-94) was a gift from Kotobuki Pharmaceutical Co., Ltd. (Nagano, Japan).

Cell Culture—Human embryonic kidney HEK293T and fibrosarcoma HT1080 cells were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum.

Expression Cloning—Expression cloning to identify genes, the products of which interact with MMP-2, MMP-9, or MT1-MMP, was performed as described previously (37). Western blotting and slot blotting For Western blot analysis, an expression plasmid for syndecan-1 (1 μg), was co-transfected with either MT1-MMP or control plasmid (4 μg) into 293T cells cultured in 10-cm dishes using TransIT LT1 transfection reagent according to the manufacturer's instructions (Mirus, Madison, WI). At 24 h after transfection, culture medium was replaced with serum-free medium, and cells were incubated for a further 24 h. Syndecan-1 was enriched from the culture supernatants or cell lysates by DEAE-Sepharose beads (Amersham Biosciences) as described previously (42, 43). Enriched syndecan-1 was separated on 7% SDS-PAGE and blotted onto Hybond N^+ membrane (Amersham Biosciences). The membrane was fixed with 0.05% glutaraldehyde in phosphate-buffered saline for 30 min and then pretreated with 10% skim milk in phosphate-buffered saline for 2 h. Syndecan-1 was detected with anti-syndecan-1 MI15 antibody (Dako, Glostrup, Denmark) as a first antibody and goat anti-mouse IgG antibody conjugated with Alexa Fluor 680 (Molecular Probes, Inc., Eugene, OR) as a second antibody in phosphate-buffered saline containing 3% skim milk. The signal was monitored by LI-COR Odyssey™ infrared imaging system (Lincoln, NE).

For slot blot analysis, an expression plasmid for syndecan-1 (100 ng) was co-transfected with either MT1-MMP or control plasmid (400 ng) into 293T cells cultured in 24-well microplates as described above. The culture supernatants and cell lysates were blotted onto Hybond N^+ by the method described previously (17, 44), and syndecan-1 was detected as above. HT1080 cells stably expressing syndecan-1 (HT1080/SDC) were established by selecting cells transfected with syndecan-1 cDNA cloned in pEAK8 vector (EdgeBio Systems) in culture medium containing 0.5 μg/ml puromycin.

Immunostaining—Expression plasmids for syndecan-1 (0.2 μg) and MT1-MMP (0.8 μg) were co-transfected with TIMP or control plasmid (1 μg) into 293T cells cultured in 35-mm diameter dishes with glass bottoms coated with type I collagen (Matsunami Glass Industry Ltd., Tokyo, Japan). Immunostaining for syndecan-1 was performed 48 h after transfection using anti-syndecan-1 MI15 antibody and goat anti-mouse IgG conjugated with Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) as described previously (37). F-actin was visualized with rhodamine-phalloidin (Molecular Probes). Fluorescence was monitored using inverted confocal laser microscopy (Zeiss).

Preparation of Recombinant Syndecan-1 Protein—Syndecan-1 cDNA fragment encoding amino acids 23–251 was generated by PCR using a flanking forward primer with an extra *Xho*I site (underlined) starting at nucleotide 272 (GenBank™ accession number J05392) (GGCCCTCGAGCAAATTGTGGCTACTAATTT) and a flanking reverse primer with an extra *Bgl*II site (underlined) starting at nucleotide 958 (CCAGATCTCTCTTTCCTGTCCAGGAGGCCC). A cDNA fragment encoding a syndecan-1 mutant protein in which Gly^{245} was substituted with Leu was generated by PCR amplification of syndecan-1 cDNA using mutagenesis primers (sense, GGGCCTCACAGCTCCTCCTGGACAGGAAAG; antisense, CTTTCCTGTCCAGGAGGAGCTGTGAGGCCC), which contain mutated nucleotides (underlined). The amplified DNA fragment was digested with *Xho*I and *Bgl*II and inserted into the *Xho*I and *Bgl*II sites of His_6-CTC plasmid (39). Expression plasmid for syndecan-1-glutathione *S*-transferase (GST) fusion protein was generated by inserting a cDNA fragment encoding GST at the *Bgl*II site of the above plasmid as described previously (39). The BL20 *Escherichia coli* strain was transformed with these plasmids, and the protein expression was induced by 0.5 mM isopropyl-β-thiogalactopyranoside. Cells were collected and then sonicated in phosphate-buffered saline containing 0.5% Triton X-100. Syndecan-1 protein tagged with His_6 was purified from the supernatant by a Ni^{2+}-chelating Sepharose (Amersham Biosciences). Syndecan-1 GST fusion proteins were purified using glutathione-Sepharose beads according to the manufacturer's instructions (Amersham Biosciences).

Determination of the Cleavage Site of Syndecan-1 Protein—Recombinant syndecan-1 fusion protein (200 ng) was incubated with recombinant MT1-MMP catalytic domain (20 ng) in 50 μl of TNC buffer at 37 °C for 3 h, and generated fragments were separated on 12% SDS-PAGE and blotted to polyvinylidene difluoride membrane (Milipore Corp., Bedford, MA). The NH_2-terminal amino acid sequence of each fragment was determined using the Beckman Coulter LF300 amino acid sequencer.

Wound-induced Migration Assay—Mock-transfected HT1080 cells or HT/SDC cells were plated onto 35-mm diameter dishes coated with collagen (1×10^6 cells/plate). Semiconfluent monolayers formed 2 h after plating were scraped with a plastic tip, rinsed in medium to avoid cells resettling, and cultured in fresh medium for further 12 h. Migrating cells were photographed under microscopy before and after incubation at 10 points for each culture (39).

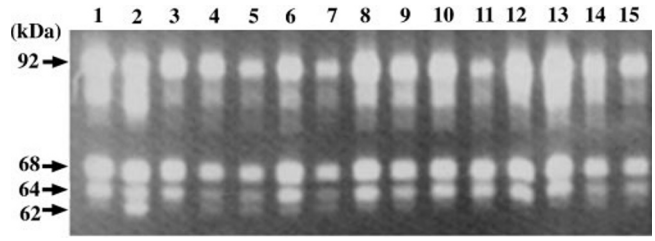

FIG. 1. **Expression cloning.** Plasmid DNA aliquots from the human placenta cDNA library was co-transfected with MMP-9, MMP-2, and MT1-MMP into 293T cells cultured in 96-well microplates as described under "Experimental Procedures," and cell lysates were subjected to gelatin zymography at 48 h post-transfection. Note that processing of pro-MMP-2 to the active form (62 kDa) was enhanced in *lane 2*.

RESULTS

Screening of Human Placenta cDNA Library—Plasmid DNA from the human placenta cDNA library was co-transfected with MMP-2, MMP-9, and MT1-MMP cDNA into 293T cells, and cell lysates were analyzed by gelatin zymography (Fig. 1). Transfection with MMP-2, MMP-9, and MT1-MMP cDNA into 293T cells generated a 92-kDa gelatinolytic band of latent pro-MMP-9, a 68-kDa band of latent pro-MMP-2, and a 64-kDa band of MMP-2 intermediate form. Transfection of a pool of cDNA partially stimulated processing of MMP-2 to the 62-kDa active form (*lane 2*). Five cDNA clones of 24 clones from this cDNA pool were isolated by a second screening, transfection of which induced partial MMP-2 processing to the active form (data not shown). The size of all five cDNA fragments was 2.4 kb, and the nucleotide sequence of the cDNA fragment was determined. Homology search analysis revealed that this cDNA encodes the transmembrane heparan sulfate proteoglycan syndecan-1 (GenBank™ accession number J05392).

Shedding of Syndecan-1 by MT1-MMP—Although stimulation of MMP-2 activation mediated by MT1-MMP by the expression of syndecan-1 was weak, it suggested a possible interaction between MT1-MMP and syndecan-1. Thus, we examined

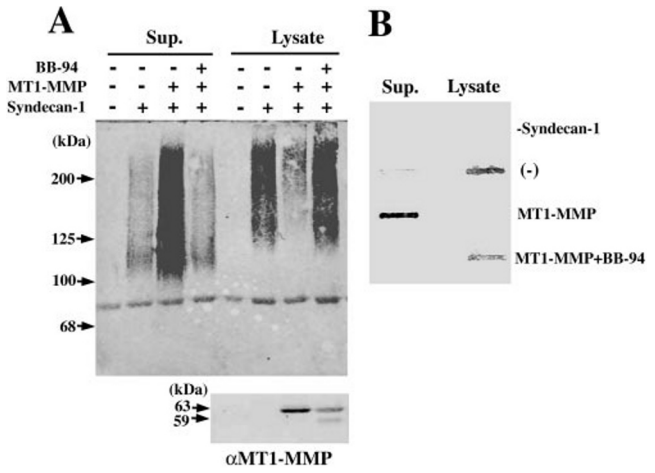

FIG. 2. **Syndecan-1 shedding is enhanced by MT1-MMP.** *A*, syndecan-1 (1 μg) and/or MT1-MMP (4 μg) plasmid were transfected into 293T cells cultured in 10-cm diameter dishes. Total plasmid DNA was adjusted to 5 μg/dish with pSG5 plasmid. At 24 h after transfection, the culture medium was replaced with serum-free medium with or without 0.1 μM BB-94, and cells were incubated further for 24 h. Culture supernatants and cell lysates were then analyzed by Western blotting using anti-syndecan-1 antibody as described under "Experimental Procedures" (*upper panel*). Expression of MT1-MMP was examined by Western blotting of cell lysates using anti-MT1-MMP antibody (*lower panel*). *B*, control or syndecan-1 plasmid (100 ng) was co-transfected with MT1-MMP plasmid (400 ng) into 293T cells cultured in 24-well culture plates. Total plasmid DNA was adjusted to 500 ng/well with pSG5 plasmid. At 24 h after transfection, culture medium was replaced with serum-free medium with or without 0.1 μM BB-94. At 24 h after incubation, culture supernatants and cell lysates were analyzed by slot blotting using anti-syndecan-1 antibody as described under "Experimental Procedures."

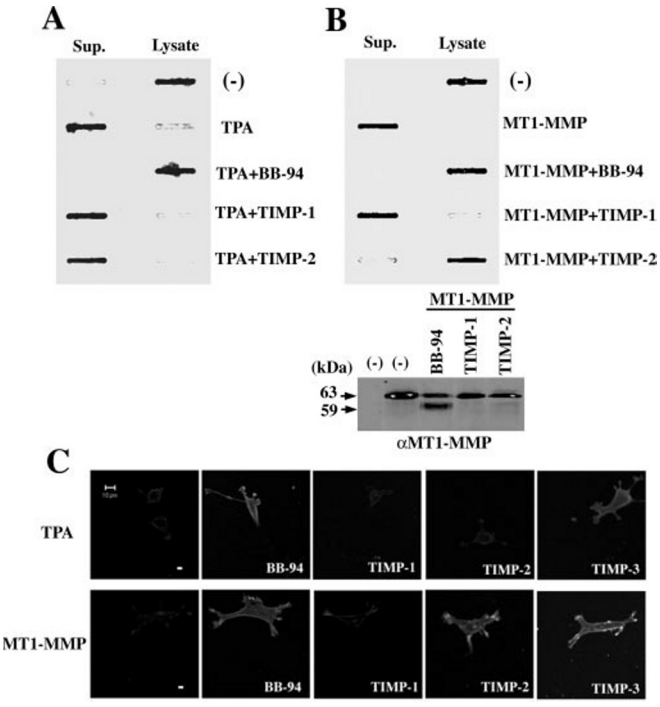

FIG. 3. **Effects of MMP inhibitors on syndecan-1 shedding.** *A*, syndecan-1 plasmid (100 ng) was transfected into 293T cells cultured in 24-well culture plates. Total plasmid DNA was adjusted to 500 ng/well with pSG5 plasmid. At 24 h after transfection, culture medium was replaced with serum-free medium with or without 50 ng/ml TPA as indicated. BB-94 (0.1 μM), recombinant TIMP-1 or TIMP-2 protein (2 μg/ml, respectively) was included in TPA-containing medium as indicated. At 24 h after incubation, culture supernatants and cell lysates were analyzed by slot blotting using anti-syndecan-1 antibody as described under "Experimental Procedures." *B*, syndecan-1 plasmid (100 ng) was co-transfected with control plasmid (*lane* −) or MT1-MMP plasmid (400 ng) (*lanes MT1-MMP*) into 293T cells cultured in 24-well culture plates, and cells were incubated and analyzed as described above (*upper panel*). Expression of MT1-MMP was examined by Western blotting of cell lysates using anti-MT1-MMP antibody (*lower panel*). *C*, syndecan-1 plasmid (200 ng) was co-transfected with TIMP-1, TIMP-2, or TIMP-3 plasmid (1 μg) into 293T cells cultured in 35-mm diameter collagen-coated glass bottom dishes. Total plasmid DNA was adjusted to 2 μg/dish with pSG5 plasmid. Cells were incubated in the medium containing TPA in the presence or absence of BB-94 for 24 h (*upper panels*). Syndecan-1 (200 ng) and MT1-MMP plasmids (800 ng) were co-transfected with TIMP-1, TIMP-2, or TIMP-3 plasmid (1 μg) into 293T cells, and cells were incubated in the presence or absence of BB-94 for 24 h as described above (*lower panels*). Cells were immunostained with anti-syndecan-1 antibody and observed under confocal laser microscopy.

whether syndecan-1 serves as a substrate of MT1-MMP. Syndecan-1 was co-expressed with MT1-MMP in 293T cells, and the shedding of syndecan-1 into culture medium or its association with cells was examined by Western blotting and slot blotting using anti-syndecan-1 antibody (Fig. 2). Syndecan-1 was spontaneously shed slightly into culture medium from 293T cells transfected with the syndecan-1 cDNA, detected as a smear band ranging from 100 to more than 200 kDa. Most of the syndecan-1 was observed in cell lysate, which migrated rather slower than that in the culture medium. Co-expression of MT1-MMP accelerated syndecan-1 shedding and reduced its level in the cell layer. Treatment of cells with the MMP inhibitor BB-94 blocked the shedding of syndecan-1 stimulated by MT1-MMP expression.

Treatment of syndecan-1-transfected cells with TPA also stimulated shedding of syndecan-1 (Fig. 3*A*) as reported previously (17, 45). To compare syndecan-1 shedding induced by TPA and MT1-MMP, effects of MMP inhibitors BB-94, TIMP-1, and TIMP-2 were examined (Fig. 3, *A* and *B*). Shedding of syndecan-1 induced by either TPA treatment or MT1-MMP expression was inhibited by the addition of BB-94, but TIMP-1 had no effect. In contrast, the addition of recombinant TIMP-2 blocked syndecan-1 shedding induced by MT1-MMP expression but not that by TPA treatment. These results indicate that TPA induces a BB-94-sensitive endogenous protease capable of syndecan-1 shedding in 293T cells that is different from MT1-MMP.

Shedding of syndecan-1 induced by TPA and MT1-MMP was also compared by immunostaining of cells using anti-syndecan-1 antibody (Fig. 3*C*). The surface of cells transfected with the syndecan-1 cDNA was only faintly immunostained for syndecan-1 when treated with TPA. Syndecan-1 staining was also weak on the surface of cells co-transfected with the MT1-MMP gene. Treatment with BB-94 recovered a strong syndecan-1 staining on cells treated with TPA or co-transfected with MT1-MMP. Co-expression of TIMP-2 or TIMP-3 recovered syndecan-1 staining of cells co-transfected with the MT1-MMP gene, but co-expression of TIMP-1 had no effect. Reduction of syndecan-1 staining by TPA treatment was reversed by the expression of TIMP-3 but not by the expression of TIMP-1 or TIMP-2. These results further confirmed that the TPA-induced syndecan-1 shedding is due to protease(s) different from MT1-MMP.

MT1-MMP mutants were compared with wild type for their capacity to cause shedding of syndecan-1 (Fig. 4). MT1-MMP mutant defective in catalytic domain (*MT1-EA*) did not induce syndecan-1 shedding. Deletion of the transmembrane/cytoplasmic domain (Δ535 or Δ335) abolished shedding, but substitution of the transmembrane/cytoplasmic domain with that of interleukin-2 receptor (*MT1/IL2R*) did not affect it. MT1-MMP deletion mutant lacking hemopexin domain (Δ*Pex*) also failed to induce syndecan-1 shedding. These results suggest that MT1-MMP may interact with syndecan-1 through its he-

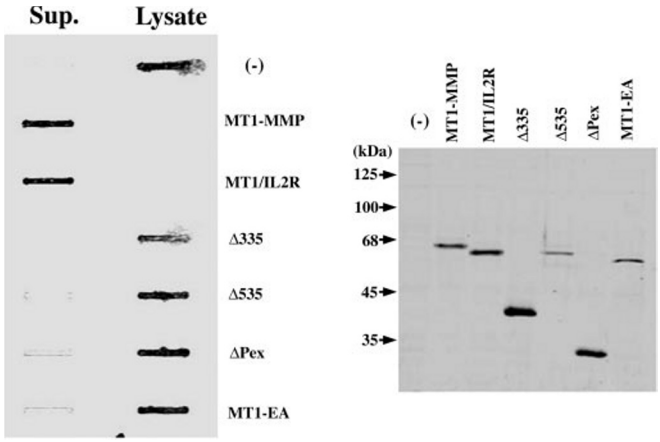

FIG. 4. **Shedding of syndecan-1 by MT1-MMP mutants.** Syndecan-1 plasmid (100 ng) was co-transfected with control plasmid (*lane −*) or expression plasmid for MT1-MMP or its mutant as indicated (400 ng) into 293T cells cultured in 24-well plates. At 24 h after transfection, culture medium was replaced with serum-free medium, and cells were incubated further for 24 h. Then culture supernatants and cell lysates were analyzed by slot blotting using anti-syndecan-1 antibody as described under "Experimental Procedures" (*left panel*). Expression of MT1-MMP was examined by Western blotting of cell lysates using anti-MT1-MMP antibody (*right panel*).

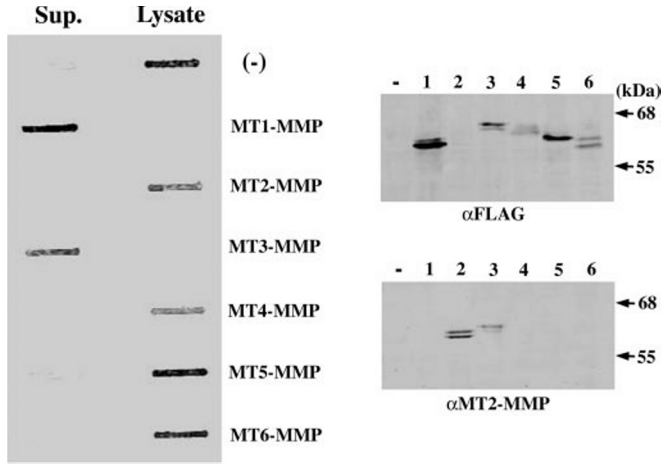

FIG. 5. **Shedding of syndecan-1 by MT-MMPs.** Syndecan-1 plasmid (100 ng) was co-transfected with control plasmid (*lane −*) or expression plasmid for MT-MMP family members as indicated (400 ng) (*left panel*) into 293T cells cultured in 24-well plates. At 24 h after transfection, culture medium was replaced with serum-free medium, and cells were incubated further for 24 h. Then culture supernatants and cell lysates were analyzed by slot blotting using anti-syndecan-1 antibody as described under "Experimental Procedures" (*left panel*). MT-MMPs except for MT2-MMP were tagged with FLAG epitope and were examined by Western blotting of cell lysates using anti-FLAG M2 antibody (*panel αFLAG*). MT2-MMP was detected with anti-MT2-MMP monoclonal antibody 162–4E3, which cross-reacted with MT3-MMP (*panel αMT2-MMP*).

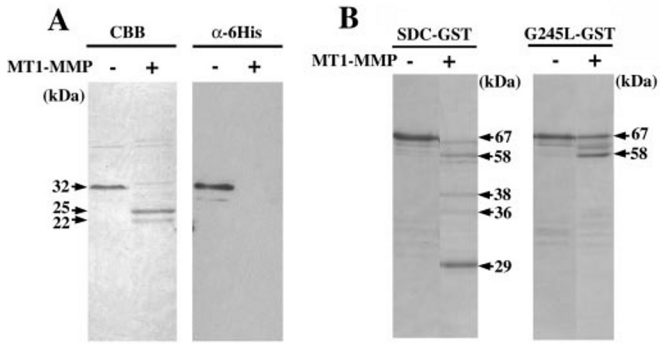

FIG. 6. **Cleavage of syndecan-1 protein by MT1-MMP.** *A*, recombinant syndecan-1 core protein tagged with His_6 epitope (200 ng) was incubated with or without recombinant MT1-MMP catalytic domain (20 ng) in TNC buffer at 37 °C for 3 h and analyzed on 12% SDS-PAGE. After electrophoresis, gel was either stained with Coomassie Brilliant Blue or immunoblotted with antibody against His_6 epitope. *B*, recombinant SDC-GST or G245L-GST fusion protein (200 ng) was incubated with or without recombinant MT1-MMP catalytic domain (20 ng) for 3 h and analyzed on 12% SDS-PAGE.

mopexin domain and that cell surface localization of MT1-MMP is essential for the shedding of syndecan-1.

Next, other members of the MT-MMP family were compared for their capacity to cause shedding of syndecan-1 (Fig. 5). Among the six members, only MT1-MMP and MT3-MMP promoted syndecan-1 shedding with a comparable efficiency.

Cleavage of Syndecan-1 by MT1-MMP—Recombinant syndecan-1 ectodomain protein tagged with His_6 epitope at the carboxyl terminus was incubated with recombinant MT1-MMP and was analyzed on SDS-PAGE (Fig. 6*A*). The major digestion product of 25 kDa and a minor fragment of 23 kDa were observed; however, none of them was detected by the antibody against the His_6 epitope. This indicates that syndecan-1 ectodomain protein tagged with His_6 epitope at the carboxyl terminus is preferentially cleaved at the site adjacent to the carboxyl terminus. To identify the cleavage site, recombinant syndecan-1 ectodomain protein fused to GST was synthesized and incubated with recombinant MT1-MMP (Fig. 6*B*). The major digestion product was a 29-kDa fragment, the NH_2-terminal sequence of which demonstrated the cleavage of Gly^{245}-Leu^{246} peptide bond of syndecan-1. Next, syndecan-1-GST mutant protein in which Gly^{245} was substituted with Leu (G245L-GST) was incubated with recombinant MT1-MMP, which did not generate a 29-kDa fragment but a major fragment of 58 kDa. The NH_2-terminal sequence analysis of the 58-kDa fragment generated by digestion of syndecan-1-GST or G245L-GST showed the cleavage of Gly^{82}-Leu^{83} peptide bond of syndecan-1 by MT1-MMP. Incubation of syndecan-1-GST fusion protein with MT3-MMP generated a similar digestion pattern including other minor fragments (data not shown).

Syndecan-1 Shedding Enhances Cell Motility—The syndecan-1 cDNA was stably transfected into HT1080 fibrosarcoma cells (HT1080/SDC), which express high levels of endogenous MT1-MMP (46), and syndecan-1 shed into culture medium or associated with cells was monitored by Western and slot blotting using anti-syndecan-1 antibody (Fig. 7). HT1080/SDC cells continuously shed a moderate level of syndecan-1, which was detected by Western blotting as a smear band migrating slower that that from 293T cells, and the syndecan-1 concentration in cell lysate was low (Fig. 7*A*). TPA treatment of HT1080/SDC cells induced further syndecan-1 shedding and reduced the level of cell-associated syndecan-1. In contrast, treatment of cells with BB-94 blocked syndecan-1 shedding, resulting in syndecan-1 accumulation in the cells. Spontaneous shedding of syndecan-1 from HT1080/SDC cells was also blocked by TIMP-2 but not by TIMP-1 as shown by slot blotting (Fig. 7*B*). TPA-induced shedding from HT1080/SDC cells was inhibited only by BB-94.

HT1080/SDC cells were also examined by immunostaining for syndecan-1 localization (Fig. 7*C*). Syndecan-1 was weakly stained on HT1080/SDC cell surface, and treatment with BB-94 induced accumulation of syndecan-1 on the cell surface. Formation of actin stress fibers was frequently observed in BB-94-treated HT1080/SDC cells but not in mock-transfected cells (data not shown). The addition of TIMP-2 but not TIMP-1 significantly enhanced cell surface staining of syndecan-1.

Motility of HT1080/SDC cells on collagen-coated dishes was examined in the wound-induced migration assay (Fig. 7*D*).

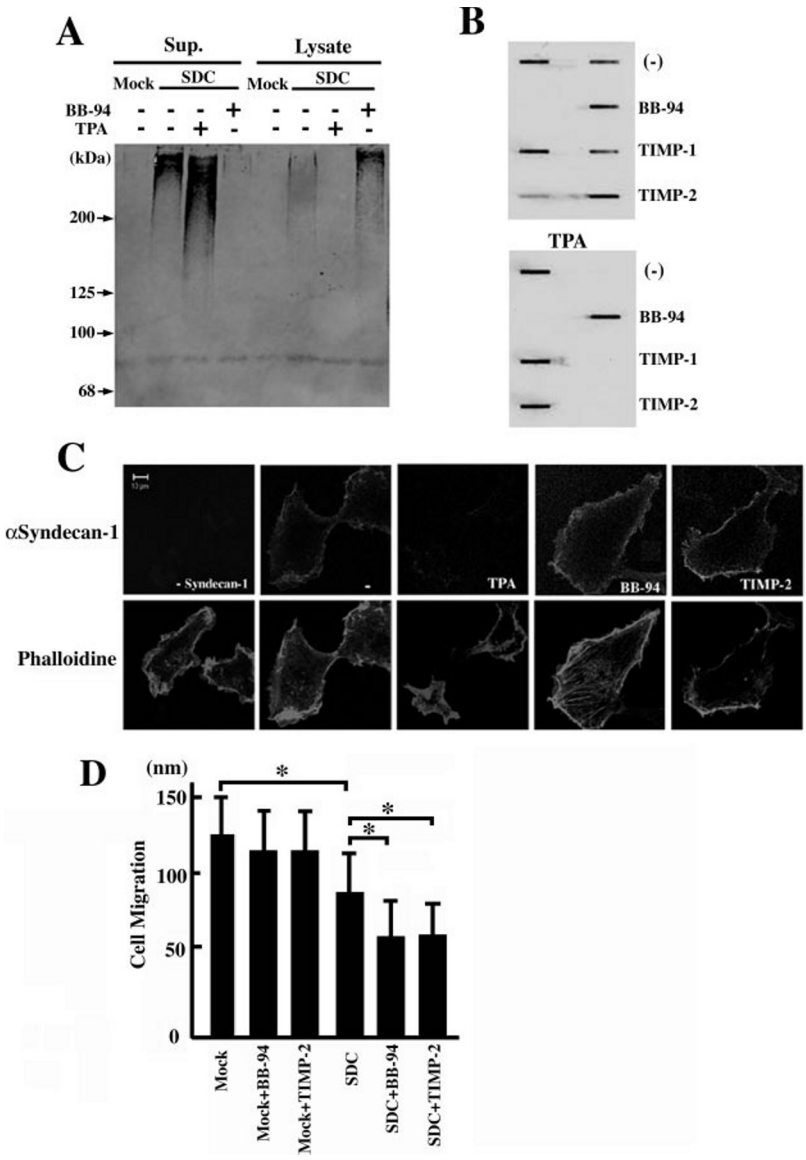

FIG. 7. **Shedding of syndecan-1 from HT1080/SDC cells.** *A*, confluent monolayers of mock-transfected HT1080 (*Mock lanes*) or HT1080/SDC (*SDC lanes*) cells cultured in 10-cm diameter dishes were incubated in serum-free medium with or without 50 ng/ml TPA or 0.1 μM BB-94 as indicated for 24 h, and culture supernatants or cell lysates were analyzed by Western blotting using anti-syndecan-1 antibody as described under "Experimental Procedures." *B*, culture supernatants and cell lysates prepared from HT1080/SDC cells cultured in 24-well plates with serum-free medium containing 0.1 μM BB-94 and 2 μg/ml recombinant TIMP-1 or TIMP-2 in the absence (*upper panel*) or presence of TPA (*lower panel*) were analyzed by slot blotting using anti-syndecan-1 antibody as described under "Experimental Procedures." *C*, mock-transfected HT1080 cells (panel *−Syndecan-1*) or HT1080/SDC cells (*panel −*) cells were plated onto 35-mm diameter dishes with collagen-coated glass bottom. HT1080/SDC cells were cultured in medium containing 50 ng/ml TPA, 0.1 μM BB-94, or 2 μg/ml recombinant TIMP-2 (*panels TPA*, *BB-94*, or *TIMP-2*, respectively) for 24 h were stained with anti-syndecan-1 antibody (*upper panels*) and phalloidin (*lower panels*). *D*, mock-transfected HT1080 (*Mock lanes*) or HT1080/SDC (*SDC lanes*) cells (1×10^6 cells/plate) were plated onto 35-mm dishes coated with collagen. At 2 h after plating, semi-confluent monolayers of cells were scraped with a plastic tip and were cultured for 12 h in medium containing 0.1 μM BB-94 or 2 μg/ml TIMP-2 protein as described under "Experimental Procedures." *, $p < 0.01$.

Motility of mock-transfected HT1080 cells was slightly inhibited by the addition of BB-94 or TIMP-2 (93 and 94% of mock-treated cells, respectively). Migration of HT1080/SDC cells was 78% of mock-transfected cells. Treatment of HT1080/SDC cells with BB-94 or TIMP-2 further suppressed migration to 70 and 71% of the untreated HT1080/SDC cells, respectively.

Syndecan-1 Shedding through the Cleavage of Gly^{245}-Leu^{246} Peptide Bond—Wild-type syndecan-1 and its mutant with an amino acid substitution of Gly^{245} with Leu (G245L) were compared for shedding accelerated by MT1-MMP expression (Fig. 8*A*). Shedding of G245L mutant induced by MT1-MMP expression or TPA treatment was significantly less effective than that of wild-type syndecan-1. Spontaneous shedding from HT1080 cells stably expressing G245L (HT1080/G245L) was less effective than that from HT1080/SDC cells, and the level of cell-associated syndecan-1 in HT1080/G245L cells was higher than that of HT1080/SDC cells (Fig. 8*B*). The concentration of syndecan-1 accumulated in these cells by BB-94 treatment was at a comparable level. Consistent with the level of cell-associated syndecan-1, migration of HT1080/G245L cells was significantly slower than that of HT1080/SDC cells (Fig. 8*C*). As described above, BB-94 suppressed migration of HT1080/SDC cells, but migration of HT1080/G245L was not significantly affected by it.

DISCUSSION

Previously, we have identified claudin-5, N-Tes/testican-3, and KiSS-1/metastin as molecules that interact with MMPs by the expression cloning strategy (37–39). In the present study, we identified that expression of the syndecan-1 gene also promoted conversion of the MMP-2-activated intermediate form to the fully active form in the process mediated by MT1-MMP in 293T cells. Since endogenous TIMP-2 concentrations in 293T cells are suboptimal for pro-MMP-2 activation through the formation of ternary complex between pro-MMP-2, TIMP-2, and MT1-MMP, focal concentration of the complex by syndecan-1 may promote this process (37). However, the effect of syndecan-1 expression on pro-MMP-2 activation by MT1-MMP was weak compared with that of claudin-5 expression (data not shown) (37). CD44, a major receptor for hyaluronan, not only directs MT1-MMP to lamellipodia but also serves as a substrate for MT1-MMP (47, 48), which led us to examine weather syndecan-1 is also cleaved by MT1-MMP. Co-expression of MT1-MMP with syndecan-1 promoted shedding of syndecan-1, and the concentration of cell surface syndecan-1 was reduced. The promotion of syndecan-1 shedding was also observed with MT3-MMP, but not with other MT-MMPs. Mutation analysis of MT1-MMP suggested that its cell surface localization is essen-

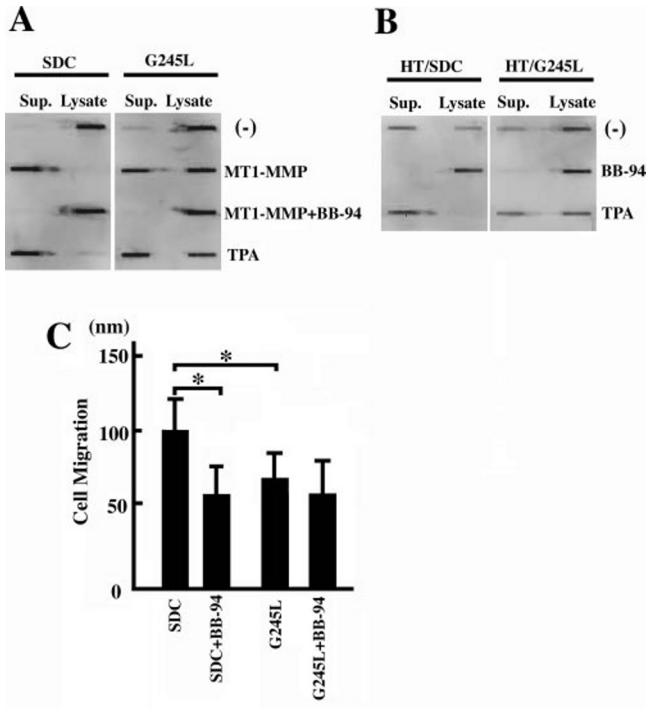

FIG. 8. **Syndecan-1 shedding through the cleavage of Gly^{245}-Leu^{246} peptide bond.** *A*, syndecan-1 (*SDC lanes*) or G245L plasmid (*G245L lanes*) was co-transfected into 293T cells with either control plasmid or MT1-MMP plasmid. At 24 h after transfection, the culture medium was replaced with serum-free medium with or without 0.1 μM BB-94 or 50 ng/ml TPA, and cells were incubated further for 24 h. Culture supernatants and cell lysates were then analyzed by slot blotting using anti-syndecan-1 antibody as described in the legend to Fig. 2. *B*, confluent monolayers of HT1080/SDC (*HT/SDC lanes*) or HT1080/G245L cells (*HT/G245 lanes*) were analyzed by slot blotting using anti-syndecan-1 antibody as described in the legend to Fig. 7. *C*, HT1080/SDC (*SDC lanes*) or HT1080/G245L cells (*G245 lanes*) were subjected to the migration assay in the presence or absence of 0.1 μM BB-94 as described in the legend to Fig. 7. *, $p < 0.01$.

tial for the shedding of syndecan-1. Deletion of the hemopexin-like domain of MT1-MMP abolished syndecan-1 shedding, which suggests the interaction of MT1-MMP with syndecan-1 through this domain.

Syndecan-1 ectodomain shedding was also accelerated by TPA. Syndecan-1 shedding accelerated by either MT1-MMP or TPA was inhibited by both BB-94 and TIMP-3; however, TIMP-2 selectively blocked syndecan-1 shedding promoted by MT1-MMP, and neither were inhibited by TIMP-1. These results indicate that syndecan-1 shedding induced by TPA is mediated by metalloproteinase(s) other than MT1-MMP. TPA-accelerated shedding of mouse syndecan-1 ectodomain results from cleavage at a juxtamembrane site in core protein (17). One of the potential cleavage sites of syndecan-1 by MT1-MMP or MT3-MMP determined using recombinant proteins was the Gly^{245}-Leu^{246} peptide bond within a juxtamembrane site. Indeed, substitution of Gly^{245} with Leu significantly reduced shedding promoted by MT1-MMP expression or TPA treatment. This indicates that the Gly^{245}-Leu^{246} peptide bond is one of the major cleavage sites for shedding promoted by MT1-MMP expression or TPA treatment. Gly^{245}-Leu^{246} of human syndecan-1 corresponds to Ser^{246}-Leu^{247} in mouse syndecan-1. It still remains to be examined whether mouse syndecan-1 is cleaved at this site by MT1-MMP.

Syndecan-mediated cellular attachment to extracellular matrix proteins including collagen and fibronectin regulates the migratory and invasive phenotype of both normal and transformed cells (9, 10). For example, exogenous expression of syndecan-1 in epithelial mammary tumor cells restored an epithelial morphology and growth characteristics (49). In this study, syndecan-1 expression was shown to down-regulate migration of HT1080 cells, and treatment of cells with MMP inhibitors increased cell surface syndecan-1 concentrations concomitant with formation of actin stress fibers, which resulted in further retardation of cell migration. HT1080 cells are known to produce relatively high levels of MMPs, including MT1-MMP, MMP-2, and MMP-9. HT1080/SDC cells constitutively shed syndecan-1 ectodomain, and this could be blocked by TIMP-2 or BB-94 but not by TIMP-1. The selective inhibition suggests that endogenous MT1-MMP in HT1080 cells is mainly involved in syndecan-1 shedding. Matrilysin (MMP-7) was also recently reported to mediate syndecan-1 shedding (36); however, matrilysin appears not be involved in shedding from HT1080 cells, because TIMP-1 did not block the shedding. High cancer cell syndecan-1 expression in tissue biopsies has been found to be associated with a favorable outcome in head and neck cancer (50, 51), squamous cell lung cancer (52), and mesothelioma (53), and low syndecan-1 expression in immunostaining of cancer tissue is associated with poor histological grade of differentiation in squamous cell lung carcinoma (52), increasing aggressiveness of basal cell carcinoma (54), and low grade of differentiation and presence of metastases in hepatocellular carcinoma (55). High serum syndecan-1 levels at diagnosis were reported to be associated with poor outcome in lung cancer (56). It would be hypothesized that syndecan-1 ectodomain might be shed by MT1-MMP and/or matrilysin expressed in tumor cells, because expression levels of MT1-MMP and matrilysin are also associated with the malignancy of tumors including lung cancer (24, 57–60).

Syndecans are also known to bind to various growth factors and cytokines via glycosaminoglycan chains and consequently regulate signal transductions (5, 6, 8). Thus, shedding of syndecan-1 complexed with these factors and the interaction of shed products with these factors may be one of the important steps for the regulation of their activities in various pathophysiological situations. Recently, matrilysin was shown to mediate shedding of a syndecan-1/a CXC chemokine (KC) complex from the mucosal surface, which directs and confines neutrophil influx to sites of injury in injured lungs (36). MT1-MMP is also expressed in diverse pathophysiological conditions and may contribute to the regulation of signals from growth factors and cytokines by shedding syndecan-1 complexed with them.

In conclusion, we have shown that MT1-MMP cleaves and sheds syndecan-1 ectodomain, which enhances cell motility on collagen. Since syndecan-1 shedding is involved in diverse pathological events such as tumor progression, wound healing, arthritis, and Alzheimer's disease, the identification of shedding proteases may contribute to the development of diagnostic and therapeutic strategies.

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