Membrane Type 1 Matrix Metalloproteinase (MT1-MMP/MMP-14) Cleaves and Releases a 22-kDa Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) Fragment from Tumor Cells*

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Proteolytic shedding is an important step in the functional down-regulation and turnover of most membrane proteins at the cell surface. Extracellular matrix metalloproteinase inducer (EMMPRIN) is a multifunctional glycoprotein that has two Ig-like domains in its extracellular portion and functions in cell adhesion as an inducer of matrix metalloproteinase (MMP) expression in surrounding cells. Although the shedding of EMMPRIN is reportedly because of cleavage by metalloproteinases, the responsible proteases, cleavage sites, and stimulants are not yet known. In this study, we found that human tumor HT1080 and A431 cells shed a 22-kDa EMMPRIN fragment into the culture medium. The shedding was enhanced by phorbol 12-myristate 13-acetate and inhibited by TIMP-2 but not by TIMP-1, suggesting the involvement of membrane-type MMPs (MT-MMPs). Indeed, down-regulation of the MT1-MMP expression in A431 cells using small interfering RNA inhibited the shedding. The 22-kDa fragment was purified, and the C-terminal amino acid was determined. A synthetic peptide spanning the cutting site was cleaved by MT1-MMP in vitro. The cleavage site is located in the linker region connecting the two Ig-like domains. The N-terminal Ig-like domain is important for the MMP inducing activity of EMMPRIN and for cell-cell interactions, presumably through its ability to engage in homophilic interactions, and the 22-kDa fragment retained the ability to augment MMP-2 expression in human fibroblasts. Thus, the MT1-MMP-dependent cleavage eliminates the functional N-terminal domain of EMMPRIN from the cell surface, which is expected to down-regulate its function. At the same time, the released 22-kDa fragment may mediate the expression of MMPs in tumor tissues.

The extracellular matrix metalloproteinase inducer (EMMPRIN)‡, also known as CD147, tumor collagenase-stimulating factor, basigin, and M6) is a multifunctional glycoprotein that belongs to the immunoglobulin superfamily (1–4). EMMPRIN-null mice are sterile and have defects in spermatogenesis, fertilization, sensory and memory functions, and mixed lymphocyte responses (5–7). However, the exact mechanisms underlying the observed defects are still largely unknown.

The protein backbone of EMMPRIN is 28 kDa, but the molecular mass of the glycosylated form varies between 44 and 66 kDa (4). The extracellular portion of EMMPRIN contains two Ig-like motifs and three potential N-glycosylation sites (8). EMMPRIN is expressed at high levels in many types of tumors and stromal cells (9–14), and the N-terminal Ig-like domain, which can form homodimers (15, 16), may mediate cell-cell interactions within tumor tissues or during metastasis. EMMPRIN is released from tumor cells and acts as an inducer of collagenase (MMP-1) expression in the surrounding stroma and tumor cells (17–19).

Matrix metalloproteinases (MMPs) are zinc-binding endopeptidases responsible for the turnover of many proteins in the extracellular space, including those that compose the extracellular matrix (ECM), cell adhesion molecules, cytokines, growth factors, and receptors (20). Most tumor types and the surrounding stromal cells overexpress multiple MMPs, which are important players in promoting tumor growth, invasion, and metastasis (20). EMMPRIN also induces MMP-2, -3, and -9, MT1-MMP, and MT2-MMP in addition to MMP-1 (9, 21–25); only glycosylated EMMPRIN is able to induce these MMPs (26), and the N-terminal Ig-like domain is also indispensable for the MMP inducing activity (26), as well as for the homophilic interactions of the protein (26). Therefore, EMMPRIN potentially mediates the excessive production of MMPs in tumor tissue and is expected to act as a modulator of ECM in tumor tissues through the activity of the MMPs that it induces.

MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; mAb, monoclonal antibody; MT-MMP, membrane-type MMP; pAb, polyclonal antibody; PMA, phorbol 12-myristate 13-acetate; siRNA, small interfering RNA; TGL, total cell lysate; TIMP, tissue inhibitor of metalloproteinase; Dox, docycycline; MS/MS, tandem mass spectrometry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline.

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2The abbreviations used are: EMMPRIN, extracellular matrix metalloproteinase inducer; MMP, matrix metalloproteinase; CM, conditioned medium; ECM, extracellular matrix; HPLC, high performance liquid chromatography;
According to the literature, EMMPRIN is released from tumor cells in at least two different ways. A significant amount of intact EMMPRIN is released from tumor cells (27, 28), possibly contained within membrane vesicles (microvesicles) that some tumor cells release upon stimulation (28). The other pathway is proteolytic shedding. In addition to inducing MMPs, EMMPRIN is likely to be cleaved and shed by MMPs or other metalloproteinases, because the shedding of EMMPRIN is inhibited by zinc chelators (25, 29). Supporting this, MMP-1 and MMP-2 can cleave EMMPRIN at the membrane-proximal region, at least in vitro (29). The proteolytic shedding of EMMPRIN from the cell surface is not only important for regulating EMMPRIN function at the cell surface but is also a mechanism to release a soluble MMP inducer. However, the details of the proteolytic processing of EMMPRIN, such as the responsible proteinases, cleavage sites, and stimulants, is very limited.

In this context, MT1-MMP (MMP-14) is an interesting candidate for the EMMPRIN shedding as an integral membrane protease responsible for pericellular proteolysis (30, 31). MT1-MMP is expressed in human tumors, and its potential substrates are ECM proteins, such as collagens, fibronectin, laminins, vitronectin, and aggrecan (30, 31). Other functional proteins that can be cleaved by MT1-MMP include pro-MMPs, CD44, the integrin αv chain, low density lipoprotein receptor-related protein, interleukin 8, and pro-tumor necrosis factor 

The gastric cancer cell line TMK-1 was a gift from Prof. E. Tahara (Hiroshima University, Hiroshima, Japan). Human primary fibroblast cells were obtained from nonlesional dermis around nodular fasciitis according to the guideline for clinical sample handling in Fukuoka University Hospital. A431, HT1080, COS-7, and human normal fibroblast cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% fetal bovine serum (Hyclone, Logan, UT), 100 μg/ml streptomycin, and 100 units/ml penicillin (Sigma). TMK-1 and A375 cells were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum, streptomycin, and penicillin. MT1-MMP-inducible expression cell lines were established using the Tet-Off gene expression system (Takara Bio Inc., Otsu, Japan) as described previously (39). The cells were seeded in 6-well plates (1.5 × 10⁵ cells/well) and cultured for 16 h before transfection. Expression plasmids were transfected using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. TIMP-1 and TIMP-2 were kindly gifted from Daiichi Fine Chemical (Takaoka, Japan).

Construction of Expression Vectors—FLAG-tagged MT-MMP constructs (MT1, MT2, MT3, MT4, MT5, and MT6) were prepared as described previously (34, 40). The other cDNAs were subcloned into eENTR/D-TOPO vectors (Invitrogen) using the Gateway System (Invitrogen). We amplified a cDNA sequence corresponding to the open reading frame of human EMMPRIN using the PCR, and then generated expression constructs for a Flag-tagged form (NM-EMMPRIN) or a FLAG-tagged form (NF-EMMPRIN) in which each tag was inserted downstream of its signal sequence by a PCR-based method. We also generated an expression construct for a C-terminally FLAG-tagged EMMPRIN fragment (from Met¹ to Thr¹²¹) containing the N-terminal Ig-like domain (D1) by a PCR-based method.

Establishment of Stable Transfectants—Cells expressing EMMPRIN or MT1-MMP were established using the ViralPower lentiviral expression system (Invitrogen) following the manufacturer’s instructions. The pLenti6 vectors for mock, NF-EMMPRIN, MT1-MMP, or D1 and the packaging plasmid mixture were introduced into 293FT packaging cells provided minimally FLAG-tagged EMMPRIN fragment (from Met¹ to Thr¹²¹) containing the N-terminal Ig-like domain (D1) with 10% fetal bovine serum (Hyclone, Logan, UT), 100 μg/ml streptomycin, and 100 units/ml penicillin. The recombinant lentiviruses in the conditioned medium were harvested and titrated using A431 cells. Transductions were performed at a multiplicity of infection of 5, and the cells were propagated and maintained in the presence of blasticidin (5 μg/ml; Invitrogen).

Western Blot Analysis—To detect proteins in the conditioned medium (CM), proteins in the CM were precipitated with 10% trichloroacetic acid, and precipitates were collected and dissolved in SDS-PAGE sample buffer. Total cell lysates (TCL) and proteins in the CM were separated by SDS-PAGE (10 and 14% gels, respectively) under reducing conditions, and proteins separated in the gel were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). After blocking the membrane with 5% fat-free dry milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20, the membrane was probed with a primary antibody specific to each protein, including an anti-EMMPRIN rabbit polyclonal antibody (pAb, Invitrogen), an anti-actin goat polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), an anti-FAS ligand

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—The human fibrosarcoma cell line HT1080, human epidermoid carcinoma cell line A431, and human melanoma cell line A375 were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and the African green monkey kidney cell line COS-7 was obtained from Health Science Research Resources Bank (Osaka, Japan).

3 N. Egawa, N. Koshikawa, T. Tomari, K. Nabeshima, T. Isobe, and M. Seiki, unpublished results.
mouse monoclonal antibody (BD Biosciences), an anti-FLAG mouse monoclonal antibody (Sigma), and an anti-c-Myc mouse monoclonal antibody (Roche Applied Science). The reacted antibodies were treated with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare) and detected with the Western Lightning chemiluminescence reagent (PerkinElmer Life Sciences). An alkaline phosphatase (ALP)-conjugated anti-goat IgG secondary antibody (Sigma) in developing solution (8.25 mg of nitro blue tetrazolium, 2.1 mg of 5-bromo-4-chloro-3-indolyl phosphosphate in 25 ml of 100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl₂, pH 8.6) was used to detect actin. Densitometry analysis was performed using ImageJ (available online).

Detection of Cell Surface EMMPRIN by FACS Analysis—A431 cells that express MT1-MMP in a doxycycline (Dox)-inducible manner were used. The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 3 days in the presence or absence of 1 μg/ml doxycycline (Sigma). The cells were trypsinized, collected, and stained with an anti-EMMPRIN mAb (R&D Systems, Minneapolis, MN). An Alexa 488-conjugated goat anti-mouse IgG (Invitrogen) was used as a secondary antibody. The amount of cell-surface EMMPRIN was evaluated with a FACSCalibur cytofluorimeter (BD Biosciences).

Temporary Knockdown of MT1-MMP and MT2-MMP by RNA Interference—Small interfering RNAs (siRNAs) targeting the MT1-MMP and MT2-MMP mRNAs were designed and prepared by B-Bridge (Sunnyvale, CA), and transfection was performed using Lipofectamine 2000 (Invitrogen) and the Smart cycler system (Takara Bio). The specific primer pairs used were 5’-GAGCGTATGTCTTTGTATC-3’ and 5’-AGTGTGACCTGAACTGC-3’ for MT1-MMP, 5’-GAGCGTATGTCTTTGTATC-3’ and 5’-AGTGTGACCTGAACTGC-3’ for MT2-MMP, and 5’-GGTGTGACCTGAACTGC-3’ and 5’-AGTGTGACCTGAACTGC-3’ for GAPDH. After initial denaturation (95 °C, 15 s), the PCRs were cycled 40 times with the following parameters: denaturation 95 °C, 1 s; annealing 65 °C, 4 s; and extension at 72 °C, 6 s.

Purification of EMMPRIN Fragments from the Culture Medium—A375-derived cells that express NF-EMMPRIN stably and MT1-MMP in a Dox-inducible manner were used to isolate the EMMPRIN fragments shed by MT1-MMP, and cells that express D1 stably were used to isolate the C-terminally FLAG-tagged EMMPRIN fragment containing N-terminal Ig-like domain. The cells (5 × 10⁹) cultured in Dox-free induction medium were further cultured without serum for 72 h to obtain CM. The CM containing the FLAG-tagged EMMPRIN fragment was purified on an affinity column filled with agarose beads conjugated to an anti-FLAG M2 mouse monoclonal antibody (Sigma). After washing with a buffer containing 25 mM HEPES, 1.0 mM NaCl, 1% Triton X-100, pH 7.4, the EMMPRIN fragment retained in the column was eluted with the FLAG peptide (200 μg/ml). Then the eluate was applied to a gel permeation column (Superdex 75 10/300 GL; GE Healthcare), and fractionation was carried out using an AKTA Explorer 105 (GE Healthcare) at a flow rate of 0.5 ml/min. Fractions containing the EMMPRIN fragment were collected, dialyzed against MilliQ water (Millipore), and freeze-dried. The purity of the EMMPRIN fragment was checked with SDS-PAGE and silver staining. The concentration of the fragment was determined by ELISA using an anti-EMMPRIN mAb and a BCA protein assay kit (Pierce). Deglycosylation was performed using N-glycosidase F (2 units/100 μl; Roche Applied Science) in a reaction buffer (200 mM Na₂PO₄, 10 mM EDTA, 0.5 μl Triton X-100, 0.1% SDS, pH 8.0) at 37 °C for 16 h.

Determination of the C-terminal Amino Acid of the EMMPRIN Fragment by Mass Spectrometry—The purified NF-EMMPRIN fragment was digested with 2 pmol of V8 peptidase (Roche Applied Science) in 25 μl of digestion buffer (25 mM ammonium carbonate, 5% acetonitrile, pH 7.8) for 18 h at 25 °C. The reaction mixture was applied to a C18 reverse-phase HPLC (Hi-Q-Sil C18-3; Kya Tech, Hachioji, Japan) and eluted by acetonitrile with a linear gradient from 5 to 90%. Each peak fraction was collected and applied to a sample plate (Applied Biosystems, Foster City, CA) with 1 μl of matrix solution and analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (4700 Proteomics Analyzer, Applied Biosystems). Ions were then used to interrogate human protein sequences in the NCBI nr data base using the MASCOT database search algorithms (available online).

Digestion of the EMMPRIN Peptide by MT1-MMP in Vitro—An EMMPRIN peptide (FLPENMGTHANIQLHGPRVK) was incubated with the catalytic fragment of human MT1-MMP (rMT1; 10 nm) (34) in a digestion buffer (50 mM Tris-HCl, 50 mM NaCl, 10 mM CaCl₂, pH 7.5) in the presence or absence of 10 μM MM270 (a synthetic hydroxyxamic MMP inhibitor, a kind gift from Novartis Pharma AG, Basel, Switzerland). After incubation at 37 °C for 8 h, the digest was directly subjected to MALDI-TOF mass spectrometry analysis.

Induction of MMP-2 by the EMMPRIN Fragment in Normal Fibroblasts—Normal human fibroblast cells were cultured in a 24-well plate (5 × 10⁶ cells/well) for 3 days. After the culture medium was changed to serum-free Dulbecco’s modified Eagle’s medium containing the purified EMMPRIN fragment (0, 0.1, 1.0, or 2.0 μg/ml), it was cultured for 24 or 72 h. The amount of MMP-2 in the CM was measured using an MPP-2 quantitative assay kit (Daiichi Fine Chemical) according to the instructions in the product manual.

Immunoprecipitation—A431 cells (2 × 10⁶ cells) expressing FLAG-tagged MT1-MMP were lysed in lysis buffer (25 mM HEPES, 1% Triton X-100, 150 mM NaCl, protease inhibitor mixture set I (Calbiochem), pH 7.5) at 4 °C for 1 h. Cell lysates were clarified by centrifugation at 15,000 rpm for 30 min at 4 °C, and the supernatant was collected and incubated with anti-FLAG M2 mAb-conjugated agarose beads at 4 °C for 12 h. The beads were washed with lysis buffer four times, and the materials bound to the beads were eluted with lysis buffer containing 200 μg/ml FLAG peptide. The samples were then subjected to Western blot analysis.
Immunocytochemistry and Immunohistochemistry—A431 cells expressing FLAG-tagged MT1-MMP were cultured on glass coverslips for 16 h in the presence of 10 μM MMI270 and then fixed with 4% paraformaldehyde in PBS for 5 min. After a blocking step using 5% goat serum, 3% bovine serum albumin in PBS, the cells were treated with a rabbit anti-FLAG polyclonal antibody (Sigma) or a mouse anti-EMMPRIN mAb. An Alexa 488-conjugated goat anti-mouse IgG and an Alexa 568-conjugated goat anti-rabbit IgG (Invitrogen) were used as secondary antibodies. The signals were analyzed with a confocal laser microscope (Bio-Rad).

Formalin-fixed, paraffin-embedded tissue sections of human ovarian carcinoma were kindly provided by Prof. Y. Okada at Keio University (Tokyo, Japan). Sections were deparaffinized and dehydrated. Endogenous peroxidase was quenched with 3% H2O2 in methanol for 15 min. The antigen was activated by microwaving it for 10 min in 10 mM citric acid, pH 6.0. Blocking was performed using a Histofine blocking kit (Nichirei, Tokyo, Japan) for 10 min. Samples were treated with an anti-MT1-MMP mAb (222-1D8, a kind gift of Daiichi Fine Chemical) or anti-EMMPRIN mAb overnight at 4 °C. After three washes with PBS, biotinylated rabbit anti-mouse immunoglobulins were used as secondary antibodies, and they were reacted with a horseradish peroxidase-streptavidin complex. Peroxidase activity was detected by a solution of dianamobenzidine containing H2O2 in a Tris-HCl buffer, which develops a brown color.

RESULTS

Tumor Cells Shed EMMPRIN in an MMP-mediated Manner—EMMPRIN is expressed in most human tumor cell lines. We used three such cell lines for our study, the human fibrosarcoma line HT1080, the human epidermoid carcinoma line A431, and the human gastric carcinoma line TMK-1. We examined the expression of EMMPRIN in cell lysates and its shedding into the CM with Western blotting using anti-EMMPRIN pAb (Fig. 1). The cells were also treated with PMA, because PMA often stimulates the expression and activity of MMPs. All cell lines expressed EMMPRIN with a molecular mass between 44 and 66 kDa, as reported previously, and the peak of the distribution appeared to be at 58 kDa (Fig. 1, upper panels). A smaller 37-kDa band that presumably represents an intermediately glycosylated form (41) was also present. Stimulation of the cells with PMA did not affect the expression levels or the band patterns in the cells.

EMMPRIN of almost the same size as the cellular form was detected in the CM (Fig. 1, lower panels). This is consistent with previous reports that the intact form of EMMPRIN is released into the CM, although PMA did not stimulate this shedding significantly. In addition to the intact form, a small 22-kDa fragment that had not been reported previously was also shed into the CM of HT1080 and A431 cells, but not TMK-1 cells. PMA treatment enhanced the shedding of the 22-kDa fragment from both cell types, and it was completely inhibited by a broad spectrum synthetic MMP inhibitor, MMI270. However, the shedding is not an immediate response to PMA treatment, because the 22-kDa fragment began to appear more than 4 h after stim-

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**FIGURE 1.** Tumor cells shed EMMPRIN in an MMP-mediated manner. HT1080, A431, and TMK-1 cells were cultured and treated with 100 nM PMA in the presence or absence of 10 μM MMI270 for 8 h in serum-free medium. The TCL was analyzed by Western blotting using an anti-EMMPRIN pAb (upper panel) and an anti-actin pAb (middle panel). The CM was collected and analyzed with an anti-EMMPRIN pAb (lower panel). Arrows in the upper and lower panels indicate the intact form of EMMPRIN. The arrowhead in the lower panel indicates the 22-kDa EMMPRIN fragment. The asterisk indicates a presumed intermediately glycosylated form of EMMPRIN. Molecular weights deduced from marker proteins are indicated on the left.

immunization (data not sown). The shedding was not affected by PMA treatment or by MMI270.

Identification of the Protease Responsible for TMK-1 cells

To test the effects of MT-MMPs on EMMPRIN shedding, each of six MT-MMPs was expressed as a FLAG-tagged form in COS-7 cells, and EMMPRIN was expressed as a Myc-tagged form (NM-EMMPRIN). Expression levels of MT-MMPs were confirmed by Western blotting (Fig. 2B, lower panel). Although the expression levels of the MT-MMPs varied widely, with MT2-MMP and MT3-MMP expressed at much lower levels than the others, the 22-kDa EMMPRIN fragment was detected in the culture medium of cells that expressed either MT1-MMP or MT2-MMP (Fig. 2B, upper panel). Thus, at least MT1-MMP and MT2-MMP can induce the shedding of the 22-kDa EMMPRIN fragment. Then the cell lysate used for Fig. 1 was analyzed by Western blotting using either anti-MT1-MMP or anti-MT2-MMP. HT1080 and A431 cells but not TMK-1 cells expressed MT1-MMP (Fig. 2C). MT2-MMP was undetectable...
in all three cell lines (data not shown). Shedding of the 22-kDa fragment from the cell surface in the presence of MT1-MMP decreased the amount of cell-associated EMMPRIN, as analyzed by FACS analysis (Fig. 2D).

A real time reverse transcription-PCR analysis showed that mRNAs for both MT1-MMP and MT2-MMP were detectable in A431 cells and that their expression was increased about three times upon treatment of the cells with PMA (Fig. 3A). To evaluate the role of each MT-MMP on the EMMPRIN shedding, we performed knockdown experiments using siRNAs. The siRNA for MT1-MMP (siMT1) specifically reduced MT1-MMP mRNA by 80% and that for MT2-MMP (siMT2) specifically reduced the mRNA for MT2-MMP by 90% (Fig. 3A). An siRNA for the luciferase gene (siLuc) used as a control did not affect the MT-MMP mRNA levels. We then tested the effect of the siRNAs on shedding from A431 cells stimulated with PMA. Down-regulation of MT1-MMP strongly inhibited shedding of the 22-kDa fragment (Fig. 3B, CM), and down-regulation of MT2-MMP slightly inhibited the shedding. Combining the siRNAs for MT1-MMP and MT2-MMP reduced the level more efficiently (Fig. 3B, CM). Thus, MT1-MMP appears to be a major protease leading to EMMPRIN shedding from A431 cells, although MT2-MMP also has some effect.

Identification of the Cleavage Site for the 22-kDa EMMPRIN Fragment—To characterize the 22-kDa EMMPRIN fragment, we established A375 cells that express MT1-MMP under the control of a Dox-inducible promoter. Depletion of Dox from the culture medium induced expression of MT1-MMP (Fig. 4A). The induction was accompanied by the generation of an auto-degraded 43-kDa fragment, which can be inhibited by MMI270 (42). The expression levels of EMMPRIN were not affected by Dox (Fig. 4B, TCL). Induction of MT1-MMP clearly augmented the shedding of the 22-kDa fragment, which again was inhibited almost completely by MMI270 (Fig. 4B, CM).

To make it easy to purify the 22-kDa fragment, the A375 cells were modified to express EMMPRIN with a FLAG tag at its N terminus (NF-EMMPRIN). The FLAG-tagged 22-kDa fragment was shed upon expression of MT1-MMP (Fig. 4C). The 22-kDa fragment appears to retain the intact N terminus of the full-length EMMPRIN, because it was detected by the anti-FLAG mAb (Fig. 4C). The CM of the A375 cells was collected, and the FLAG-tagged EMMPRIN fragment was purified with a combination of an affinity column using an anti-FLAG mAb and a gel permeation column. When the EMMPRIN fragment was incubated with N-glycosidase F to eliminate the sugar moiety, the molecular weight shifted to a relatively sharp 10-kDa band (Fig. 4D).

To determine the C-terminal amino acid sequence of the purified fragment, the 22-kDa fragment was digested with V8 pepti-
Recombinant MT1-MMP Cleaves EMMPRIN at the Same Site—To confirm whether MT1-MMP cleaves EMMPRIN at the expected site, a 20-mer synthetic polypeptide (FLPEPVQGIRSHMLGEMTGANIQHGPRVKA) spanning the expected cutting site was prepared and incubated with the catalytic fragment of MT1-MMP (rMT1). The reaction products were then analyzed using MALDI-TOF mass spectrometry. Two peaks matched the EMMPRIN peptides spanning amino acids (aa) 74–84 (1324.55 Da) and aa 85–98 (1584.69 Da), respectively (Fig. 5A, underlined sequences). The amino acid sequences of the two fragments were further analyzed with the tandem MS/MS mode. Peaks obtained from the laser-generated fragments completely matched the sequences of aa 74–84 (bn ion series for 2–10 and yn ion series for 3, 4, and 7–9; data not shown) and aa 85–98 (bn ion series for 2, 5–8, and 10–13, and yn ion series for 6 and 8–13; see Fig. 5B), respectively. Because the V8 peptidase generates Glu at the C terminus, the peptide 85–98 is not the product cleaved by V8 peptidase at the C terminus. Thus, we expected Asn98 to be the C terminus of the 22-kDa fragment (Fig. 5A, arrowhead). The fragment deduced from the expected cleavage site was calculated to be 9.3 kDa.

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Characterization of the 22-kDa EMMPRIN fragment shed by MT1-MMP. A, A375 cells with inducible MT1-MMP (as in Fig. 2C) were cultured for 3 days in the presence (MT1−) or absence (MT1+) of Dox and with or without MMI (MMI270). Expression of MT1-MMP was monitored by Western blotting using an anti-MT1-MMP mAb (upper panel). Actin was detected with an anti-actin mAb as a standard (lower panel). B, EMMPRIN in the TCL (left panel) and the CM (right panel) was examined by Western blotting analysis using an anti-EMMPRIN pAb. The arrow indicates the intact form of EMMPRIN, and the arrowhead indicates the 22-kDa EMMPRIN fragment. C, A375 cells were modified further to stably express NF-EMMPRIN. The cells were cultured and analyzed similarly to B except that an anti-FLAG mAb was used for detection. D, NF-EMMPRIN fragment shed by MT1-MMP was purified from the serum-free medium using a column conjugated with an anti-FLAG M2 mAb and was then subjected to gel filtration chromatography. The purified NF-EMMPRIN fragment was incubated with or without N-glycosidase F (N-gly, 2 units/100 μl). An anti-FLAG mAb was used to check the fragment by Western blotting. The black arrow indicates the untreated form of the EMMPRIN fragment, and the white arrow indicates the unglycosylated form of the fragment.
manner (NF) showed multiple bands presumably reflecting differences in its glycosylation status. The concentrations of the obtained fragments were determined by a single ELISA and a BCA protein assay kit and confirmed further by Western blotting (Fig. 7B).

The activity of the purified fragments in inducing expression of MMP-2 was assessed using normal human fibroblast cells. The fibroblasts were cultured in the presence of NF or D1 for 3 days, and MMP-2 secreted into the CM was measured by ELISA. MMP-2 was expressed constitutively in cells, and its expression was enhanced in a dose-dependent manner (Fig. 7C). The fragment shed from NF stimulated production of MMP-2 to a similar extent. Thus, the 22-kDa fragment retains the MMP inducing activity.

Expression of MT1-MMP and EMMPRIN in Cancer Cells and Tissue—In order for the cleavage reaction to occur, MT1-MMP and EMMPRIN would be expected to localize in the same area of the cells. Indeed, separate experiments have reported that these proteins localize at lamellipodia (30, 43), but their co-localization has not yet been confirmed. We used immunostaining of A431 cells stably expressing the FLAG-tagged form of MT1-MMP to confirm the co-localization of the proteins, as shown in the representative picture in Fig. 8A. A similar co-localization pattern was also observed with HT1080 cells (data not shown). Co-localization of the proteins was further supported by the observation that immunoprecipitation of MT1-MMP also precipitated EMMPRIN from the cell lysate (Fig. 8B). Under the same conditions, FAS-L, used as a negative control, was not precipitated.

EMMPRIN and MT1-MMP have each been reported to be overexpressed in a variety of human tumors. To confirm whether the proteins are co-expressed in the same tumors, we prepared serial sections of human ovarian carcinomas and subjected them to immunostaining. A pair of representative pictures is shown in Fig. 8C. Prominent expression of MT1-MMP was detected in carcinoma cells, some fibroblast-like cells, and endothelial cells (Fig. 8C, left). EMMPRIN was detected in most of the carcinoma cells (Fig. 8C, right), primarily at the tumor cell border. Although EMMPRIN signals differed from the MT1-MMP staining pattern in being weak in endothelial cells and...
fibroblasts in the stroma, both MT1-MMP and EMMPRIN are at least expressed in carcinoma cells derived from the same tumor.

**DISCUSSION**

Cell-surface EMMPRIN is shed through the release of microvesicles or by proteolytic cleavage. In this study, we first confirmed the release of the previously reported intact form (27, 28) and identified a new EMMPRIN fragment in the CM of two tumor cell lines (HT1080 and A431). Shedding of the 22-kDa fragment was enhanced by PMA whereas that of the intact form was not. In contrast, another tumor cell line, TMK-1, did not shed the 22-kDa fragment at all, although these cells released the intact form of EMMPRIN. We hypothesized that MT1-MMP is the proteinase responsible for the shedding of the 22-kDa fragment for the following reasons. First, we had noticed from previous studies that HT1080 and A431 cells express MT1-MMP, but TMK-1 cells do not (44, 45). Second, the TIMP-2-sensitive and TIMP-1-insensitive nature of the shedding fits well with the profile of MT-MMPs (30). Third, the expression and activity of MT1-MMP are reported to be enhanced by PMA (42). Fourth, we identified EMMPRIN as a protein possibly associated with MT1-MMP in a proteomic analysis (data not shown). MT1-MMP was confirmed to be expressed in A431 cells, and knockdown of the expression using siRNA inhibited the shedding substantially. MT2-MMP may also contribute to the shedding because knockdown of both MT1-MMP and MT2-MMP expression produced slightly

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**FIGURE 7.** Induction of MMP-2 expression in fibroblasts by the EMMPRIN fragment. A, schematic illustration of the two forms of EMMPRIN expressed in the cells. IgD1, the first Ig-like domain; IgD2, the second Ig-like domain; TM, transmembrane domain; CP, cytoplasmic domain. Black boxes represent FLAG sequences. NF is the N-terminal EMMPRIN fragment generated by the MT1-MMP-dependent cleavage. D1 represents C-terminally FLAG-tagged EMMPRIN fragment containing the IgD1. B, A375 cells were stably transfected with expression plasmids for the two forms of EMMPRIN. The conditioned media were collected, and EMMPRIN fragments were purified. After the concentration of the purified fragments was determined using an anti-FLAG mAb, the control is the fraction obtained from mock-transfected cells. C, indicated proteins were incubated with normal human fibroblast cells for 24 or 72 h in serum-free medium. Then the amount of MMP-2 in the CM was measured using an ELISA kit. Closed circle, in the presence of NF for 24 h; closed square, in the presence of D1 for 24 h; open circle, in the presence of NF for 72 h; opened square, in the presence of D1 for 72 h. Each point represents the average of three independent experiments; error bars, S.D.

**FIGURE 8.** Co-localization of EMMPRIN and MT1-MMP. A, A431 cells stably expressing FLAG-tagged MT1-MMP and endogenous EMMPRIN were used for immunostaining. Cells were cultured on slide glasses in the presence or absence of 10 μM MIII270. MT1-MMP was detected with an anti-FLAG pAb (left panel, red) and EMMPRIN with an anti-EMMPRIN mAb (center panel, green). A merged image is presented in the right panel. Because MII270 did not affect the localization pattern of the proteins, a single representative picture of MIIIPI-treated cells is presented. Arrows indicate signals. Bar, 20 μm. B, FLAG-tagged MT1-MMP was expressed in A431 cells, and immunoprecipitation was carried out using anti-FLAG antibody. Precipitates were analyzed by Western blotting. Antibodies used for blotting are indicated to the left. C, representative pictures of ovarian carcinoma tissue expressing MT1-MMP and EMMPRIN. Serial sections of an ovarian carcinoma were stained for MT1-MMP (left panel) and EMMPRIN (right panel), as described under the “Experimental Procedures,” and observed under a microscope (×200). Arrows indicate typical signals.
MT1-MMP Cleaves EMMPRIN

greater inhibition (Fig. 3). Similar results were obtained with HT1080 cells (data not shown). Thus, MT1-MMP appears to be a major enzyme leading to the cell-mediated shedding of the 22-kDa EMMPRIN fragment.

In previous in vitro studies, proteolytic cleavage leading to the shedding of EMMPRIN was suggested to occur at the region of the molecule proximal to the plasma membrane (25, 29), although the actual fragments released into the CM had not been characterized clearly. The 22-kDa fragment we detected had not been detected in previous studies, perhaps because the 22-kDa fragment cannot be detected by antibodies that recognize the membrane-proximal region of EMMPRIN. We identified the C-terminal amino acid of the 22-kDa fragment as Asn98 (Fig. 5), and MT1-MMP cleaved at this sequence even in an in vitro digestion (Fig. 6). Although an additional cleavage site was observed in the digest with MT1-MMP in vitro, the corresponding fragment was not identified by mass spectrometry analysis of the fragment purified from the CM. Cleavage of EMMPRIN by MT1-MMP significantly decreased the total amount of cell-surface EMMPRIN as demonstrated by FACS analysis (Fig. 2C). Thus, the shedding of EMMPRIN by MT1-MMP appears to be a mechanism for protein turnover at the cell surface.

The Asn98–Ile scissile bond is located in the linker sequence connecting the two Ig-like domains, so that the 22-kDa EMMPRIN fragment contains the distal Ig-like domain. This domain has been reported to be indispensable for the MMP inducing activity and for homophilic interactions (26). Thus, the shedding may down-regulate the cellular functions mediated by EMMPRIN, because MT1-MMP cleaves off the important N-terminal Ig-like domain. This regulation may be particularly important at the ruffling edge because both of the proteins co-localize there (Fig. 8A). The purified 22-kDa fragment retained the MMP inducing activity (Fig. 7), suggesting the intriguing possibility that MT1-MMP itself acts as a trigger to promote MMP expression in tumor stromata by releasing the 22-kDa EMMPRIN fragment.

In addition to shedding the 22-kDa EMMPRIN fragment, all three tumor cell lines tested released the intact form of EMMPRIN, presumably integrated with microvesicles released from the cells (Fig. 1). This intact form is also expected to retain the MMP inducing activity. We do not know at this time whether these two forms of the MMP inducer have some difference in activity, efficiency, or fate. The intact form of EMMPRIN is heavily glycosylated compared with the 22-kDa fragment, and the microvesicles containing EMMPRIN are bigger than the fragment. Thus, it may be difficult for the intact form of EMMPRIN to reach distant target cells that can be reached by the 22-kDa fragment.

There is a possibility that the proteinases responsible for shedding differ depending on the cell type and the stimulants employed. For example, tumor cells co-cultured with fibroblasts have been reported to release a 50-kDa soluble EMMPRIN (25), although we have not been able to detect this fragment in the cells both in the monoculture and co-culture system (data not shown). How EMMPRIN induces expression of MMP remains unclear. In our experiments, relatively high doses of EMMPRIN fragment were required for substantial induction of MMP-2 expression. This may suggest that the MMP inducing activity of the 22-kDa fragment does not have biological significance or that the inducer becomes active only in the presence of co-stimulators. The shedding of EMMPRIN in response to MT1-MMP stimulation may be more important as a mechanism to down-regulate EMMPRIN function on the cell surface than as an MMP inducer.

In summary, we identified MT1-MMP as the enzyme responsible for the cell-mediated shedding of EMMPRIN. MT1-MMP cleaved off the N-terminal Ig-like domain that is important for MMP induction and cell-cell interactions. If the released 22-kDa fragment indeed acts as an MMP inducer, MT1-MMP may play an intriguing role in the regulation of MMP expression in tumor tissue.

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