Membrane-type Matrix Metalloproteinase-1 (MT1-MMP) Is a Processing Enzyme for Human Laminin γ2 Chain*

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Processing of the laminin-5 (Ln-5) γ2 chain by membrane-type-1 matrix metalloproteinases (MT1-MMP) promotes migration and invasion of epithelial and tumor cells. We previously demonstrated that MT1-MMP cleaves the rat γ2 chain at two sites, producing two major C-terminal fragments of 100 (γ2γ) and 80 (γ2x) kDa and releasing a 30-kDa fragment containing epidermal growth factor (EGF)-like motifs (domain III (DIII) fragment). The DIII fragment bound the EGF receptor (EGF-R) and stimulated cell scattering and migration. However, it is not yet clear whether human Ln-5 is processed in a similar fashion to rat Ln-5 because of the two MT1-MMP cleavage sites present in rat γ2 is not found in human γ2. To identify the exact cleavage site for MT1-MMP in human Ln-5, we purified both the whole molecule as well as a monomeric form of human γ2 that is frequently expressed by malignant tumor cells. Like rat Ln-5, both the monomer of γ2, as well as the γ2 derived from intact Ln-5, were cleaved by MT1-MMP in vitro, generating C-terminal γ2′ (100 kDa) and γ2x (85 kDa) fragments and releasing DIII fragments (25 and 27k Da). In addition to the conserved first cleavage site used to generate γ2′, two adjacent cleavage sites (Gly435–Asp436 and Gly579–Ser580) were found that could generate the γ2x and DIII fragments. Two of the three EGF-like motifs present in the rat DIII fragment are present in the 27-kDa human fragment, and like the rat DIII, this fragment can promote breast carcinoma cell migration by engaging the EGF-R. These results suggest that MT1-MMP processing of Ln-5 in human tumors may stimulate the EGF-R, resulting in increased tumor cell scattering and migration that could possibly increase their metastatic potential.

Laminin-5 (Ln-5),† a major component of the basement membrane, is a heterotrimer composed of α3, β3, and γ2 subunits (1, 2). Migration and scattering of epithelial and tumor cells are induced by proteolytic processing of the γ2 chain of Ln-5. The γ2 chain is a 140-kDa polypeptide and forms a triple helix with the other subunits at its C-terminal (see Fig. 1A) (3, 4). Processing of the γ2 chain occurs at the N terminus generating two major C-terminal fragments of 100 (γ2γ) and 80 (γ2x) kDa (see Fig. 1A), and this processing has been observed in different species including humans and rodents (3, 4). Because of the limited availability of purified Ln-5, most biochemical studies in this area have been carried out using the rat protein. MT1-MMP and MMP-2 were identified as the proteases responsible for the second cleavage of the N terminus generating the γ2x fragment (3, 4). In contrast, only MT1-MMP cleaved the first site to generate the γ2′ fragment (5). The two cleavage sites on the rat γ2 chain were identified as Gly435–Asp435 for γ2′ and Ala586–Leu587 for γ2x (3, 6).

Processing of the rat γ2 chain at these two sites releases an internal fragment containing three of the four EGF-like motifs in domain III (DIII) (7). Although Ln-5 does not stimulate the EGF receptor (EGF-R), the DIII fragment released has the ability to bind EGF-R and induce its phosphorylation (8). Inhibition of the processing using MMP inhibitors or inhibition of EGF-R activity using specific kinase inhibitors abolished cell migration on Ln-5 (8). Thus, the DIII fragment generated by this processing appears to play a major role in the observed biological effect of Ln-5 and MT1-MMP. This system seems to function in vivo as well because MT1-MMP-deficient mice showed significantly reduced processing of the γ2 chain in the kidney, resulting in abnormalities terminal differentiation of the tubular epithelium (5).

Processing of the γ2 chain by MT1-MMP appears to play a critical role in tumor growth and progression because the γ2 chain is frequently expressed as a monomer in malignant tumors that express MT1-MMP (9, 10). In addition, aggressive melanoma cells are known to form vascular-like networks (vascular mimicry) requiring expression of MT1-MMP and the γ2 chain (11). Treatment of these cells with a neutralizing antibody against MT1-MMP or antisense oligonucleotide against the γ2 gene abrogated the mimicry (11). It has also been reported that the γ2′ and γ2x fragments in humans are similar in size to those in the rat (3, 4), suggesting that the processing of the human γ2 chain by MT1-MMP is similar to that of the rat.

However, a comparison of rat and human γ2 sequences reveals that although the first site (Gly434–Asp435) is conserved in humans, the second cleavage site of rat (Ala586–Leu587) is not. Thus, it is not known whether MT1-MMP directly cleaves pansulfonic acid; mAb, monoclonal antibody; HPLC, high pressure liquid chromatography.
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the second site in addition to the first and generates γ2x and the DIII fragment. Further confusion has been raised by two contradictory reports on this issue. Veitch et al. (12) reported that the human γ2 chain cannot be processed by MT1-MMP even at the first site, which is conserved between rodents and humans. Instead of MT1-MMP, they reported that the astacin family of proteases, such as bone morphogenetic protein-1 (BMP-1) and mammalian Tolloid-like metalloproteinases, cleave the γ2 chain (12, 13). On the other hand, Gilles et al. (14) reported that recombinant MT1-MMP induces processing of Ln-5 deposited in the extracellular matrix, although the cleavage sites were not identified, and it was not clear whether a DIII-like fragment was generated as a result of the processing. In this manuscript, we have attempted to settle this controversy by identifying the cleavage sites on the human γ2 chain cut for MT1-MMP. To this end, we purified the γ2 chain either as a monomer or as a heterotrimer (Ln-5) from human cancer cell lines. Incubation of the purified γ2 chain and Ln-5 with a recombinant catalytic fragment of MT1-MMP generated the two C-terminal fragments (γ2′ and γ2x) and released DIII-like fragments functionally. By purifying the γ2x fragment, two adjacent cleavage sites were determined. In addition, the γ2 monomeric chain that is expressed in human malignant tumors show greater sensitivity to MT1-MMP than the heterotrimer form of Ln-5.

MATERIALS AND METHODS

Cells and Cell Culture—MKN45, a human gastric carcinoma cell line, was provided by the Japanese Collection of Research Bioresources (Tokyo, Japan). Mum2B, a human melanoma cell line, was a gift from Professor Mary Hendrix, University of Iowa, Iowa City, IA. STKM-1, a human gastric carcinoma cell line, was obtained from Dr. Shunsuke Yanoma, Kanagawa Cancer Center, Research Institute, Yokohama, Japan. All cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO2, 95% air. Dulbecco’s modified Eagle’s medium or RPMI 1640 medium (Sigma) supplemented with 10% FCS, 1.2 mg/ml of NaHCO3, and 2 mM glutamate was used as basal medium and supplemented with 10% fetal bovine serum (Irvine Scientific, Irvine, CA).

SDS-PAGE and Western Blotting—The method for SDS-PAGE was described in our previous report (4). The Western blotting was performed as reported (4). Serum-free conditioned medium was collected from confluent cultures of cancer cells incubated for 2 days in serum-free medium. The serum-free conditioned medium was concentrated with a 4–20% gradient on SDS-PAGE gel under reducing conditions, cut for MT1-MMP. To this end, we purified the recombinant protein using Transwell chambers as described in our previous report (5). Briefly, the filter undersides were coated with human Ln-5 (500 ng/ml) overnight at 4 °C, blocked with 5% milk, phosphate-buffered saline, 0.05% Tween 20 for 2 h at room temperature, washed with phosphate-buffered saline twice, and used for cell migration assays. MDA-MB-231 cells were resuspended in Dulbecco’s modified Eagle’s medium and 0.1% bovine serum albumin in the absence or presence of human rDIII protein (1.3 μM) and were seeded into the upper chamber at 20,000 cells/Transwell chamber. After an 8-h incubation with/without an anti-human EGF-R neutralizing antibody (10 μg/ml) (LA-1) (Upstate Group, Lake Placid, NY), cells on the upper filter were scrapped and washed with phosphate-buffered saline three times, and then migrating cells on the lower filter were fixed with 100% MeOH and stained with 0.25% crystal violet, 20% MeOH for 15 and 30 min, respectively. Each bar represents the mean ± S.D. for cell migration in three wells.

RESULTS

Purification of Human Laminin-5 and Laminin-γ2 Monomer—The human cancer cell lines Mum2B and STKM-1 express the γ2 chain as both a monomer and a heterotrimer, Ln-5, respectively. Serum-free conditioned medium of the cells was used to purify both forms of the γ2 chain using an immunoaffinity column conjugated with a monoclonal antibody (4D5B) against the DIII domain of rat γ2. The purified preparation from Mum2B cells contained one major (140 kDa) and two minor (280 and 100 kDa) polypeptides as detected by silver staining in Fig. 1B. Based on previous studies, the 140-kDa protein corresponded to the intact γ2 chain, and the 280-kDa protein is presumably a dimer. The smaller band (100 kDa) corresponded to γ2 cleaved at the first site, and the very weak band (85 kDa) corresponded to γ2x cleaved at the second site (Fig. 1, A and B). On the other hand, the preparation from STKM-1 cells contained α3 (160-kDa) and β3 (153-kDa) chains in addition to γ2 (140 and 100 kDa). However, most of the γ2 chain was detected as γ2′, and the amount of intact γ2 chain was negligible (Fig. 1B). A very faint band corresponding to γ2x was also detected.

Processing of Human Ln-γ2 Chain by MT1-MMP—The Ln-γ2 chain and γ2′ fragment were also detected by Western blotting using a polyclonal antibody against the rat DIII domain (Fig. 2, A and B). A fully processed γ2x fragment was weakly detectable by the antibody, although the amount of DIII fragment was negligible in these preparations (Fig. 2, see MT1-MMP 0 nM). The results presumably indicate that the mAb 4D5B used for the preparation recognizes the C-terminal fragment of the DIII domain, and this portion is not included in the clipped DIII fragment as illustrated in Fig. 2C. To examine whether MT1-MMP cleaves γ2 and γ2′, purified protein samples were incubated with increasing amounts of a catalytic fragment of human MT1-MMP. The amount of γ2 monomer decreased and that of γ2x and the DIII fragment increased dependent on the concentration of MT1-MMP (Fig. 2A, from 4 to 20 nM). Thus,
the human Ln γ2 chain can be cleaved by MT1-MMP like its rat counterpart. The amount of γ2' did not change significantly presumably because of the balance between production and further processing. The γ2 chain in Ln-5 was also processed into γ2x by MT1-MMP, and the DII fragment was generated (Fig. 2B). However, it is of note that the processing of the γ2 chain in Ln-5 requires 10 times more MT1-MMP than the processing of the single chain form. Thus, the two forms of the γ2 chain differ in their susceptibility to MT1-MMP.

Since the first cleavage site identified for the rat γ2 chain is conserved, along with its flanking sequences, in humans (Fig. 2C), it is most likely that cleavage to generate the γ2' fragment occurs at this site. Thus, we tried to identify the second cleavage site that generates γ2x. The γ2x fragment was extracted from the polyacrylamide gel and sequenced. Two N-terminal sequences were identified in the γ2x preparation, Asp-Pro-Leu-Ala and Ser-Glu-Pro-Val. Thus, the two cleavage sites were identified as Gly\(^{559}\)-Asp\(^{560}\) and Gly\(^{579}\)-Ser\(^{580}\) (Fig. 2C). Although the Gly\(^{559}\)-Asp\(^{560}\) sequence is not conserved in rats, Gly\(^{579}\)-Ser\(^{580}\) is present in its flanking sequences (Fig. 2C), which, although unreported, may be a cleavage site for the rat γ2 chain.

The human DIII fragments are calculated to be 152 and 145 amino acids long, respectively, and presumably correspond to the 27- and 25-kDa bands in Fig. 2, A and B. Thus, human γ2 fragments are generated by MT1-MMP as in rodents.

**Human DIII Fragment Stimulates Carcinoma Cell Migration**—We previously described that the rat DIII fragment contained three EGF-like motifs and promoted cell migration by engaging the EGF-R (8). As the human DIII fragment only contained two of the three motifs (Figs. 1A and 2C), we attempted to confirm whether the human DIII fragment (Asp\(^{435}\)-Gly\(^{579}\)) retained its activity to stimulate EGF-R. To do this, we expressed a secreted form of the recombinant DIII protein with a FLAG tag at the C terminus in COS-7 cells. The DIII fragment was purified from the serum-free conditioned medium using anti-FLAG antibody and subsequently by anion-exchange HPLC. The final preparation contained a 27-kDa single protein band as detected by silver staining after SDS-PAGE (Fig. 3A, left), and the band reacted with anti-FLAG M2 antibody (Fig. 3A, right).

The effect of the DIII on migration of MDA-MB-231 cells was analyzed using a Transwell chamber. Addition of human rDIII (1.3 μM) resulted in a 1.5-fold increase in migration on Ln-5 (Fig. 3B) and this effect was completely abolished by a neutralizing antibody against EGF-R, LA-1. Thus, the two EGF-like motifs retained in the human DIII fragment appear to be sufficient to induce EGF-R-dependent cell migration.

**Cell-mediated Processing of the Human Ln γ2 Chain by MT1-MMP**—To examine whether MT1-MMP is responsible for cell-mediated processing of the human γ2 chain, MT1-MMP was transiently expressed into Mum2B cells, and conditioned media was analyzed by Western blotting (Fig. 4A). Untransfected Mum2B cells express MT1-MMP at a low level and constitutively release C-terminal fragments of γ2 as well. Forced expression of MT1-MMP increased the amount of γ2' and γ2x fragments. To confirm that the γ2 chain of Ln-5 is processed from the intact Ln-5 molecule by MT1-MMP in a cell-mediated manner, we used human gastric carcinoma STKM-1 cells. STKM-1 produces Ln-5 (85 kDa) were detected and are indicated by arrowheads.
Cleavage of Human Laminin \( \gamma_2 \) by MT1-MMP

**FIG. 2.** Cleavage of human Ln \( \gamma_2 \) by MT1-MMP. A and B, purified human Ln \( \gamma_2 \) monomer (0.5 \( \mu \)g) (A) or Ln-5 (1.5 \( \mu \)g) (B) was digested with a recombinant human MT1-MMP at the indicated concentrations (mole/mole; 0 to 20 or 100 nM, respectively) for 18 h at 37 °C in vitro. The proteolytic fragments were then analyzed by SDS-PAGE with a 4–20% gradient under reducing conditions and detected by Western blotting using a polyclonal antibody against domain III of Ln \( \gamma_2 \) (2778). The positions of \( \gamma_2 \) (140-kDa), \( \gamma_2' \) (100-kDa), and \( \gamma_2x \) (85-kDa) chains are indicated. C, schematic illustration of human Ln \( \gamma_2 \) and the cleavage sites. MT1-MMP cleaved at three different sites on the short arm of human Ln \( \gamma_2 \). The first cleavage site and flanking sequences are conserved completely, as indicated in the left box. Cleavage occurs at the site indicated by an arrow (Gly434–Asp435). Two cleavage sites (Gly559–Asp560 and Gly579–Ser580) identified in human Ln \( \gamma_2x \) are also indicated in the right box. The sequences in the rat Ln \( \gamma_2 \) corresponding to the first and second cleavage sites of human Ln \( \gamma_2 \) are also shown. The predicted reactive sites of both \( \gamma_2 \) antibodies (2778 and D4B5) are indicated below.

**FIG. 3.** Stimulation of cell migration by human recombinant DIII fragment. A, purified human rDIII was analyzed by SDS-PAGE, and protein was detected by silver staining (left lane). After the separated protein was transferred to a nitrocellulose membrane, Western blotting (WB) was carried out using anti-FLAG M2 antibody (right lane). B, the human rDIII fragment was tested to determine whether it promotes MDA-MB-231 cell migration. Cells were seeded on the Ln-5-coated Transwell membrane and incubated for 8 h at 37 °C (control (Cont)). The cells that migrated to the lower surface of the membrane were counted as described under “Materials and Methods.” The rDIII protein (1.3 \( \mu \)g), a neutralizing antibody (LA-1) against EGF-R (10 \( \mu \)g/ml), or both were added to the lower chamber and incubated for 8 h. Results were presented as percentage of the control.
detected at least under these conditions (Fig. 4A). The processing was inhibited by 10 μM MMP inhibitor BB94 (data not shown). Thus, the cell-associated form of MT1-MMP promotes the processing of both forms of the human γ2 chain.

To determine whether there was a correlation between the expression levels of processing enzymes and Ln-5 processing, we assessed whether there was a correlation between endogenous MT1-MMP and the amount of cleavage observed. Ln-5 produced by STKM-1 cells and Ln-5 produced by Mum2B cells were both found in the cleaved form, and the Ln-5 in the STKM-1 cells contained only a small amount of intact γ2 chain, whereas most of the γ2 chains were detected as processed forms (Fig. 1B). All three cell lines were analyzed by Western blotting with an antibody to MT1-MMP. MT1-MMP was detected in Mum2B cells but not in STKM-1 or MKN45 cells (Fig. 4C). As BMP-1 has also been reported as a potential processing enzyme for Ln-5 (13), we determined whether it was found in the cells. STKM-1 (Fig. 4C) cells were the only line to express this enzyme. Thus, BMP-1 appears to be responsible for the processing by the cells, and MT1-MMP appears to be responsible for that by Mum2B cells.

DISCUSSION

This study was performed due to the debate over the ability of MT1-MMP to cleave the human Ln γ2 chain. We demonstrated that when monomeric or Ln-5 forms of the human γ2 chain were purified and incubated with MT1-MMP, two major C-terminal fragments, γ2' and γ2x, were formed as reported for rat Ln-5. The first cleavage, which generates γ2', presumably occurs at the same site as rat γ2 because the cutting site, including flanking sequences, is well conserved between the two species. At the location of the second cleavage sites, which generates the γ2x fragment, we identified two adjacent sites (Gly559–Asp560 and Gly579–Ser580). Since the sites are close together, the two γ2x bands could not be separated clearly by SDS-PAGE (Fig. 2, A and B). This cleavage at the two different
The γ2 chain is frequently expressed in malignant human tumors. Using the human cell line Mum2B, we purified a monomeric form of γ2 and compared the processing of the monomer by MT1-MMP with that of Ln-5. Interestingly, the monomer was 10 times more sensitive to MT1-MMP than the Ln-5 form. Since the γ2 monomer is frequently expressed together with MT1-MMP in malignant tumors, a similar processing of the γ2 chain may be occurring in tumors. This result supports the previous observations that both γ2 and MT1-MMP were required for melanoma cells to show vascular mimicry (11). We have also found that rat Ln-5 showed greater sensitivity to MT1-MMP than human Ln-5 (data not shown). The relatively resistant nature of human Ln-5 may be why Veitch et al. (12) could not detect its processing by MT1-MMP. It is not clear whether the difference in sensitivity affects the biological outcome mediated by Ln-5 and MT1-MMP in humans significantly.

The second cleavage site, which generates the rat γ2x fragment, is not conserved in humans (3, 6), and two new cleavage sites were identified for human γ2x. Although one site (Gly558–Asp600) is not conserved in the rat sequence, the other site (Gly579–Ser680) is. Thus, in addition to the rat γ2x site (Ala586–Leu587) reported previously, the conserved second site (Gly558–Ser559) in rat Ln-γ2 may also be cleaved by MT1-MMP. Cleavage at the first and second sites in human Ln-γ2 generates shorter DIII fragments than in the rat (7 and 27 amino acids, respectively). Although the 27-kDa human DIII fragment retains only two of the three EGF-like motifs present in the rat DIII fragment (7, 8), it induces EGF-R-dependent carcinoma cell migration and invasion through the action of the released DIII fragment (8).

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