Binding of Active Matrilysin to Cell Surface Cholesterol Sulfate Is Essential for Its Membrane-associated Proteolytic Action and Induction of Homotypic Cell Adhesion*

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Regulation of cell surface molecules by matrix metalloproteinases (MMPs), as well as MMPs-catalyzed degradation of extracellular matrix, is important for tumor invasion and metastasis. Our previous study (Kioi, M., Yamamoto, K., Higashi, S., Koshikawa, N., Fujita, K., and Miyazaki, K. (2003) Oncogene 22, 8662–8670) demonstrated that active matrilysin specifically binds to the surface of colon cancer cells and induces notable cell aggregation due to processing of the cell membrane protein(s). Furthermore, these aggregated cells showed a dramatically enhanced metastatic potential. To elucidate the mechanism of matrilysin-induced cell aggregation, we attempted to identify the matrilysin-binding substance on the cell surface. Here, we demonstrate that cholesterol sulfate on the cell surface is a major matrilysin-binding substance. We found that active matrilysin bound to the cell membrane and cholesterol sulfate incorporated into liposomes with similar affinities. Treatment of colon cancer cells with β-cyclodextrin significantly reduced not only matrilysin binding to the cell surface but also matrilysin-dependent proteolysis and cell aggregation. Interestingly, replenishment of cholesterol sulfate, but not cholesterol, neutralized the effects of β-cyclodextrin. Taken together, it is likely that binding of matrilysin to cholesterol sulfate facilitates the matrilysin-catalyzed modulation of cell surface proteins, thus inducing the cancer cell aggregation.

Matrix metalloproteinases (MMPs)² are zinc-dependent endopeptidases that degrade components of the extracellular matrix and play essential roles in tissue remodeling in physiological and pathological processes such as morphogenesis, differentiation, angiogenesis, tissue remodeling, and tumor invasion (1, 2). The association of MMPs with cancer cell invasion and metastasis has suggested that these proteinases represent attractive targets for the development of novel anti-tumor therapies.

Recently, it has been suggested that the biological functions of various cell surface proteins are proteolytically modulated by several MMPs (1, 3, 4), including MT-MMPs, gelatinase A (MMP-2), gelatinase B (MMP-9), stromelysin (MMP-3), and matrilysin (MMP-7). These metalloproteinases are thought to regulate cellular functions by activating, inactivating, or releasing membrane proteins. Such regulation of cell surface molecules as well as MMP-catalyzed degradation of the extracellular matrix is important for tumor invasion and metastasis.

Matrilysin is the smallest member of the MMP family, which lacks the C-terminal hemopexin-like domain. In cancer tissues, various MMPs are overexpressed both in stromal and tumor cells. In contrast, matrilysin has been detected specifically in tumor cells but not in stromal cells (5, 6). Among many MMPs, matrilysin appears to be one of the most important MMPs in human colon cancers because it is almost always overexpressed in colon cancers. Expression of matrilysin is also correlated well with malignancy and metastasis of cancers, especially in liver metastasis of colon cancer (7).

It has recently been reported that matrilysin contributes to tumor malignancy by cleaving cell surface proteins (8), such as Fas ligand. Another study suggested that degradation of IgG by matrilysin protects tumor cells from the immune system (9). More recently, we reported that active matrilysin efficiently binds to the surface of human colon cancer cells and induces E-cadherin-mediated cell aggregation by processing a cell membrane protein(s). The aggregated cells showed dramatically enhanced metastatic potential in the nude mice model (10). The advantage of aggregation of colon cancer cells in their metastasis is also supported by other studies (11). We speculate the following three possibilities to explain how cell aggregation facilitates the liver metastasis: first, the aggregated colon cancer cells are able to survive in the bloodstream due to E-cadherin-mediated intracellular signals, which are required for anchorage-independent survival and growth of tumor cells (12). Second, aggregated cancer cells are easily trapped in microvascular vessels, which facilitates their extravasation and subsequent invasion into tissues. Third, formation of cell aggregation protects the cells from the immune system.

It is believed that localization of secreted MMPs on the cell surface is required for processing or degradation of cell surface molecules (13, 14). The anchoring of the soluble MMPs with the cell membrane is probably a benefit not only in preventing rapid diffusion of the proteinases but also in their efficient interaction with cell surface substrates. The C-terminal, hemopexin-like domains of MMPs are thought to be possible sites for cell association (15). However, we reported that matrilysin, which lacks the C-terminal, hemopexin-like domain, efficiently binds to the cell surface. Previous studies suggested that rat matrilysin binds to heparan sulfate proteoglycans on cells in rat uterine tissue (16). Therefore, it is speculated that optimal interaction between matrilysin and its cell surface substrates are mediated by the third molecules, such as heparan sulfate proteoglycans.

In this study, we tried to identify the matrilysin-binding substance on the cell surface to elucidate the mechanism of matrilysin-induced cell aggregation. We demonstrate that cell surface cholesterol sulfate, but
not heparan sulfate proteoglycans, is the major and specific substance to which human active matrilysin binds. Moreover, binding of matrilysin to cholesterol sulfate was found to be essential for matrilysin-catalyzed modulation of cell surface protein(s) that is involved in the induction of prominent cell aggregation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—Human colon cancer cell lines Colo201, WiDr, DLD-1, and Lovo, human gastric cancer cell line STKM-1, human oral cancer cell line HSC-4, human bladder cancer cell line EJ-1, and human fibrosarcoma cell line HT1080 were obtained from the Japanese Cancer Resources Bank. These cell lines were maintained in Dulbecco’s modified Eagle’s/Ham’s F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum.

**Antibodies and Other Reagents**—The sources of materials used were as follows: the anti-FLAG M2 monoclonal antibody, heparinase, heparitinase, chondroitinase ABC, sodium selenate, fumonisin B1, the anti-FLAG M2 monoclonal antibody-conjugated agarose column. The purified ant pro-matrilysin and pro-E215A were purified, using an anti-FLAG M2 monoclonal antibody-conjugated agarose column. The purified pro-matrilysin was activated by incubation with 1 mM p-aminophenyl mercuric acetate at 37 °C for 1 h. To remove the dehydrase S-transferase region, the refolded proteins were incubated with thrombin, and the purified M2 monoclonal antibody-conjugated agarose column. The purified pro-matrilysin was activated by incubation with 1 mM p-aminophenyl mercuric acetate at 37 °C for 1 h. To remove the porcine pro-matrilysin and pro-E215A were purified, using an anti-FLAG M2 monoclonal antibody-conjugated agarose column. The purified pro-matrilysin was activated by incubation with 1 mM p-aminophenyl mercuric acetate at 37 °C for 1 h. To remove the propeptide from the E215A mutant, the mutant pro-matrilysin was mixed with active matrilysin-conjugated Sepharose 4B beads and incubated in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl (Tris-buffered saline; TBS), containing 10 mM CaCl2 (TBSC) and 1 mM p-aminophenyl mercuric acetate at 4 °C overnight with rotation, and then, the mature form of the mutant was collected by filtration.

**Western Blotting Analysis and Immunofluorescence Staining**—Western blotting analysis and immunofluorescence staining were carried out using the anti-matrilysin antibody 11B4G as described previously (10, 21).

**Assay of Matrilysin-binding Ability of Cell Membrane**—Each cell line (1 × 10⁵) in serum-free Dulbecco’s modified Eagle’s/Ham’s F-12 medium was incubated without or with 50 nM matrilysin on polyhydroxyethyl methacrylate-coated 35-mm dishes at 37 °C for 3 h. After incubation, cells were collected, washed three times with serum-free Dulbecco’s modified Eagle’s/Ham’s F-12 medium, and then homogenized in 1 ml of 20 mM HEPES buffer (pH 7.5) containing 250 mM sucrose by a Potter-Elvehjem-type homogenizer. The homogenates were centrifuged at 800 × g for 7 min to remove nuclei and cellular debris. The postnuclear supernatant was centrifuged at 21,000 × g for 30 min, and the resultant precipitate was used as the cell membrane. The membrane fraction was dissolved in a SDS sample buffer consisting of 50 mM Tris-HCl (pH 6.8), 2% 2-mercaptoethanol, 2% SDS, and 10% glycerol and subjected to SDS-PAGE. Matrilysin bound to the cell membrane was detected by Western blotting with the anti-matrilysin antibody 11B4G.

**Phase Separation of Membrane-associated Substances in Triton X-114 Solution**—To separate membrane-associated substances from soluble ones, we used phase separation of the Triton X-114 solution, according to Brusa and Radolf (22). Then, the binding of matrilysin to the membrane-associated substance was examined. Briefly, the membrane fraction of WiDr cells dissolved in TBSC containing 1% Triton X-114 was incubated with 100 nM active matrilysin at 4 °C for 3 h. The temperature of the sample was then transferred to 37 °C and the sample was further incubated for 3 min. The resultant detergent and aqueous phases were separated by centrifugation at 25 °C. Matrilysin partitioned in the aqueous and detergent phases were separately precipitated by adding an excess 10-fold volume of cold acetone. The precipitate was collected by centrifugation, dried, and subjected to Western blotting analysis.
Sucrose Density Gradient Ultracentrifugation—WiDr cells (1 × 10⁶) were lysed in 1 ml of TBS containing 1% Triton X-100 at 4 °C. The lysate was centrifuged at 1,500 × g for 5 min to remove nuclei. The resultant supernatant (1 ml) was then mixed with 1 ml of TBS containing 80% sucrose. A linear sucrose gradient (5–30% in 10 ml of TBS) was layered on the mixture. The sample was centrifuged at 200,000 × g at 4 °C for 18 h in a Beckman SW41 rotor and fractionated from the bottom (1 ml each, total 12 fractions). Aliquots of each fraction were dialyzed with water (30:60:8). Each fraction was desalted using a Sep-Pak C18 cartridge consisting of 27 ml of a test sample, respectively.

(1) Control represent the number of single cells in the presence or absence of a substance(s) capable of specifically binding to active matrilysin is present on the cell surface. In this study, to analyze the cell-bound matrilysin, we prepared membrane fractions of WiDr cells by the differential centrifugation method as described under "Experimental Procedures." We first examined the distribution of matrilysin endogenously produced by WiDr cells because WiDr cells express the highest amounts of matrilysin in our tested cells (data not shown). As shown in Fig. 1A, when cells were incubated for 36 h, pro-matrilysin was detected only in the conditioned medium but not in other fractions. When the cells were incubated for 3 h, we could not detect (pro)matrilysin in any fraction. To analyze the binding of active matrilysin to the cell surface, WiDr cells were incubated with active matrilysin for 3 h. After incubation, cells were washed and fractionated. We found that active matrilysin was detected in the membrane fraction (Fig. 1A). As the cells did not produce a detectable amount of (pro)matrilysin during the 3-h incubation, it is unlikely that endogenous matrilysin was detected in the fraction. E-cadherin, which is a cell membrane protein, was mainly detected in the membrane fraction. Lamin and enolase, which are nuclear membrane and cytoplasmic proteins, were mainly detected in the nucleus-rich and cytoplasm fractions, respectively. As expected, these results suggest that exogenously added active matrilysin bound to the cell surface and was fractionated into the membrane fraction.

Next, we examined the matrilysin-binding abilities of various cell lines, including Colo201, WiDr, DLD-1, Lovo, STKM-1, HSC-4, EJ-1, and HT1080. When these cells were incubated with active matrilysin,
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FIGURE 1. Binding of matrilysin to various cancer cell lines. A, WiDr cells in the suspended condition were incubated with or without active matrilysin (50 nM) in serum-free medium at 37 °C for 3 or 36 h. Cells were homogenized and then fractionated by differential centrifugation as described under “Experimental Procedures.” B, the indicated cells in the suspended condition were incubated with active matrilysin (50 nM) for 3 h. WiDr cells were incubated with 50 nM matrilysin in the absence or presence of 2 μM TAPI-1, 2 μM KB-8301, or 1 mg/ml heparin for 3 h. E, WiDr cells were incubated with 50 nM FLAG-tagged matrilysin or FLAG-tagged E215A matrilysin in the absence or presence of 2 μM TAPI-1 for 3 h. Matrilysin in each fraction (A) and that in the membrane fraction (B, D, and E) were detected by Western blotting with the anti-matrilysin monoclonal antibody 11B4G. Matrilysin bound to WiDr cells was visualized by immunofluorescent staining (C) with the anti-FLAG monoclonal antibody as described under “Experimental Procedures.” MAT, matrilysin; WT, wild type.

Localization of the Matrilysin-binding Substance on the Cell Surface—To identify the matrilysin-binding substance, we first attempted to solubilize cell-bound matrilysin using the non-ionic detergent Triton X-100. As shown in Fig. 2A, matrilysin was partitioned into an insoluble fraction when cells were lysed with 1% Triton X-100 at 4 °C. On the other hand, matrilysin was efficiently solubilized when cells were lysed at 37 °C. These results suggest that the matrilysin-binding substance is localized in distinctive microdomains such as rafts, because rafts are isolated based on their insolubility in the Triton X-100-containing buffer at 4 °C. Localization of the matrilysin-binding substance in the lipid rafts was further examined by sucrose density gradient centrifugation, which separates detergent-resistant membranes from the bulk of cellular membranes and cytosolic proteins (Fig. 2B). The matrilysin-binding activity was detected in low density fractions of the sucrose density gradient centrifugation, which separates detergent-resistant membranes from the bulk of cellular membranes and cytosolic proteins (Fig. 2B). The matrilysin-binding activity was detected in low density fractions of the sucrose density gradient centrifugation, which separates detergent-resistant membranes from the bulk of cellular membranes and cytosolic proteins (Fig. 2B). The matrilysin-binding activity was detected in low density fractions of the sucrose density gradient centrifugation, which separates detergent-resistant membranes from the bulk of cellular membranes and cytosolic proteins (Fig. 2B). The matrilysin-binding activity was detected in low density fractions of the sucrose density gradient centrifugation, which separates detergent-resistant membranes from the bulk of cellular membranes and cytosolic proteins (Fig. 2B). 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Effect of β-CD Treatment of Cells on Matrilysin Binding and Modulation of Cell Surface Molecules—We investigated the effect of β-CD treatment of cells on matrilysin-binding ability and cell aggregation. When WiDr cells were treated with various concentrations of β-CD, the binding of matrilysin to cells was decreased in a dose-dependent manner (Fig. 3A). The significant reduction of the matrilysin-binding ability of cells by β-CD treatment was also observed in Colo201 cells (data not shown) and in other cancer cell lines (Fig. 3B). Concomitant with the reduction of cell-bound matrilysin, cell aggregation induced by matrilysin was also inhibited by β-CD treatment (Fig. 3, C and D). When WiDr cells were treated with lovastatin, an inhibitor of biosynthesis of cholesterol, binding of matrilysin to cells was also decreased significantly (data not shown).

Extraction and Characterization of the Matrilysin-binding Substance—To characterize the matrilysin-binding substance, we prepared a membrane fraction of WiDr cells and then treated it with various reagents. After treatment, the matrilysin-binding activity of the membrane was tested. As shown in Fig. 4A, treatments with heparanase, heparitinase, chondroitinase ABC, trypsin, and chymotrypsin, and heating (97 °C for 30 min) had no effect on the matrilysin-binding ability, suggesting that neither heparan sulfate proteoglycan nor protein is the matrilysin-binding substance. To isolate the matrilysin-binding substance from the membrane fraction, we tried to extract the substance with various volatile solvents (data not shown). Comparing the matrilysin-binding activity of each extract, we found that the matrilysin-binding substance was efficiently extracted by chloroform/methanol (2:1), which is conventionally used for lipid extraction. These results gave rise to the possibility that the matrilysin-binding substance is a lipid component. After evaporation of the solvent, the extract was incorporated into liposomes consisting of phosphatidylcholine, cholesterol, and phosphatidylglycerol, and the binding of matrilysin to the liposome was examined. As shown in Fig. 4B, the liposome combined with the extract, but not the liposome alone, showed matrilysin-binding ability, and the binding was inhibited by TAPI-1, suggesting that the characteristic matrilysin-binding activity of the cell membrane is retained after the liposome incorporation.

To characterize the lipid component with an affinity for matrilysin, we separated the crude extract on a DEAE-Toyopearl anion-exchange column. We found that the column-bound fraction had matrilysin-binding activity, suggesting that the matrilysin-binding substance is an acidic lipid (data not shown). To further classify the active lipid, we treated WiDr cells with fumonisin B1 and selenate, which are inhibitors for the biosynthesis of sphingolipids and sulfated lipids, respectively (25, 26). When cells were incubated with fumonisin B1, binding of matrilysin to the cell surface was not affected. In contrast, selenate treatment of
the cells led to a significant decrease of the cell-bound matrilysin (Fig. 4C), suggesting that the matrilysin-binding substance is a sulfated lipid.

Isolation and Identification of the Matrilysin-binding Substance—To isolate the matrilysin-binding substance, the crude lipid extract of WiDr cells was separated by anion-exchange chromatography, using a TSK-GEL DEAE-2SW column. Acidic lipids were eluted with a linear gradient of sodium acetate. Eluted lipids were analyzed by HPTLC, employing neutral solvent system I and iodine vapor visualization (Fig. 5A). Matrilysin-binding activity of each fraction was also analyzed by the liposome binding assay (Fig. 5B). Fractions 10 to 12 had matrilysin-binding activity and fraction 11 showed the highest activity. As shown in Fig. 6A, fraction 11 showed a faint band, but there is no detectable lipid band in fraction 12, which showed matrilysin-binding activity. We also tried to visualize lipid molecules in fractions 10, 11, and 12 with an azure A reagent, which efficiently stains sulfated lipids and cardiolipin (Fig. 6A, right panel). It was found that one of the three bands in fraction 10 and one band in fraction 11 visualized with iodine vapor were also detected with azure A. In addition, fraction 12 contained a lipid that was efficiently stained only with azure A. The lipids in fractions 10, 11, and 12 showed different Rf values, respectively, suggesting that different kinds of acidic lipids have matrilysin-binding activities. These lipids were also subjected to HPTLC analysis with acidic solvent system II, which is known to separate sulfated glycolipids with high resolution, and detected by the azure A staining (Fig. 6B). The acidic lipid in fraction 10 co-migrated with SM3, and the lipids in fractions 11 and 12 co-migrated with cholesterol sulfate and cardiolipin, respectively. To verify the identities, the cell-derived lipids were mixed with standard lipids, and then subjected to HPTLC analysis with solvent systems I or II. As shown in Fig. 6C, the migration rates of the lipids in fractions 10 and 12 coincided with those of cardiolipin and cholesterol sulfate, respectively.

We next examined the matrilysin-binding activities of standard lipids by the liposome binding assay. As shown in Fig. 6D, matrilysin bound to the liposomes combined with cardiolipin, SM3, SM4, and cholesterol sulfate, respectively. The bindings of matrilysin to cholesterol sulfate (Fig. 6D), cardiolipin, SM3, and SM4 (data not shown) were significantly inhibited by TAPI-1 but not by o-phenanthroline. Taken together, sulfated lipids and cardiolipin were thought to be important candidates for the matrilysin-binding substance on cell surface.

Effect of Replenishment of Cholesterol or Cholesterol Sulfate on Matrilysin-mediated Cell Aggregation—Because of the size of hydrophobic cavity, β-CD stimulates efflux of cholesterol sulfate as well as cholesterol from the cell membrane (27). In fact, when the liposomes combined with cholesterol sulfate, SM3, or cardiolipin were incubated with β-CD, the amount of cholesterol sulfate incorporated in liposome was significantly decreased. In contrast, the amount of SM3 and cardiolipin incorporated in liposomes were not changed (Fig. 7A), indicating that β-CD stimulates the efflux of cholesterol sulfate but not SM3 and cardiolipin. We further examined the effect of β-CD treatment on the level of SM3 expressed on the surface of WiDr cells by flow cytometry. We found that WiDr cells expressed SM3 antigen and the expression level of SM3 was not affected by β-CD treatment (data not shown).

The observed reduction of matrilysin binding after β-CD treatment of cells can be explained in two alternative ways. One explanation is that the depletion of cholesterol by β-CD leads to destruction of the raft domain, thus reducing the matrilysin-binding sites on the cell surface. The other explanation is that cholesterol sulfate is a major substance for the matrilysin binding, and hence its depletion directly leads to loss of the matrilysin-binding ability. To test these possibilities, WiDr cells were first treated with β-CD, and then replenished with cholesterol and/or cholesterol sulfate. After treatment, cells were tested for their matrilysin-binding abilities. As shown in Fig. 7B, the matrilysin-binding ability was recovered completely when cells were replenished with cholesterol sulfate. In contrast, replenishment of cholesterol alone did not recover the matrilysin-binding ability. Similar results were observed in Colo201 cells (data not shown). Based on these results, it is likely that cholesterol sulfate is a major matrilysin-binding substance, and depletion of this molecule from the cell surface directly leads to loss of the matrilysin-binding ability. We also examined the effect of replenishment of cholesterol sulfate on cell aggregation. As shown in Fig. 7,
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FIGURE 6. Identification of the matrilysin-binding substance. A, fractions 10–12 obtained in Fig. 6 were applied to a HPTLC plate, and then developed with solvent system I and stained with vapor of iodine (left) or azure A reagent (right). B, fractions 10–12 and standard lipids were analyzed by HPTLC with solvent system II. The separated lipids were visualized by azure A staining. C, fractions 10–12 were mixed with SM3, cardiolipin, and cholesterol sulfate (Cho. Sul.), respectively. These mixtures were analyzed by HPTLC with solvent I (I) or solvent II (II). The separated lipids were visualized by azure A staining. Arrowheads represent lipids derived from cells and arrows represent standard lipids. D, matrilysin-binding activity of standard lipids. The indicated lipids were incorporated into liposome, and then matrilysin-binding activity of each liposome and cholesterol sulfate incorporated into liposome in the presence of 10 μM TAPI-1 or 1 mM o-phenanthroline (o-phe.) was analyzed as described under “Experimental Procedures.”

As SM3 and cardiolipin showed affinities for matrilysin, these lipids may have roles in the matrilysin-induced cell aggregation. We next tested this possibility. As expected, matrilysin-binding ability was enhanced when β-CD-treated WiDr cells were supplied with SM3 or cardiolipin (Fig. 7E). Concomitant with the increment of cell-bound matrilysin after the supplement of SM3, cell aggregation induced by matrilysin was also stimulated. However, matrilysin-induced cell aggregation was not stimulated after supplement of cardiolipin (Fig. 7F and G).

Determination of Dissociation Constants for Binding of Matrilysin to Cell Membrane or Standard Lipids—To determine the apparent dissociation constants (K_{d(app)}) for binding of matrilysin to the cell surface, cholesterol sulfate, SM3, and cardiolipin, WiDr cells, and each lipid-incorporated liposome were incubated with various concentrations of matrilysin, and cell- or lipid-bound matrilysin was determined by Western blotting followed by imaging analysis. The data were subjected to Woolf-Augustsson-Hofstee plot analysis. As shown in Fig. 8, matrilysin bound to WiDr cells (K_{d(app)} = 22 nM) and cholesterol sulfate incorporated into liposome (K_{d(app)} = 16 nM) with similar affinities. SM3 (K_{d(app)} = 75 nM) showed relatively lower affinity for matrilysin. On the other hand, the incubated matrilysin was completely adsorbed to the cardiolipin-incorporated liposome. Therefore, it was not feasible to measure the concentrations of free matrilysin to determine the K_{d} value. This result also indicates that the affinity of matrilysin for cardiolipin is much higher than that for cholesterol sulfate or SM3.

Binding of Other Active MMPs to Cholesterol Sulfate—To test whether cholesterol sulfate has such an enzyme specificity, we examined the cholesterol sulfate-binding abilities of the active forms of gelatinase A andstromelysin by the liposome binding assay. As shown in Fig. 9, only matrilysin bound to the liposome combined with cholesterol sulfate.

Binding of Matrilysin to Cholesterol Sulfate Is Required for Matrilysin-catalyzed Modulation of Certain Cell Membrane Proteins—Our previous study suggested that membrane-bound matrilysin retained proteolytic activity and this activity is essential for the induction of cell aggregation. Furthermore, membrane-bound matrilysin catalyzes cleavage of some membrane proteins (10). To investigate the relevance of the interaction between matrilysin and cholesterol sulfate on the cell surface, we examined effects of depletion and/or replenishment of cholesterol sulfate on matrilysin-catalyzed modulation of cell surface proteins. Surface proteins of WiDr cells were first biotinylated and then the cells were incubated with or without β-CD. The β-CD-treated cells were then replenished with cholesterol sulfate or cholesterol. When the control WiDr cells, which had been incubated without β-CD, were treated with matrilysin, several biotinylated proteins were released into the culture medium (Fig. 10). A similar pattern of release of biotinylated proteins was observed when the β-CD-treated and cholesterol sulfate-replenished cells were incubated with matrilysin. In contrast, the proteins specifically released from the control cells upon
Matrilysin treatment were not released from the $\beta$-CD-treated and cholesterol-replenished cells. Similar results were observed in Colo201 cells (data not shown). These results suggest that binding of active matrilysin to cholesterol sulfate on the cell surface plays crucial roles in the proteolytic regulation of cell surface proteins, such as an unidentified protein that is involved in the induction of cell aggregation.

**DISCUSSION**

In this study we explored a matrilysin-binding substance on the cell surface. We found that matrilysin-binding activity in the membrane fraction resisted heating or treatment with without 10 mM $\beta$-CD for 30 min. The total lipids extracted from liposomes were analyzed by HPTLC with solvent system I. Cholesterol sulfate, SM3, and cardiolipin were visualized by azure A staining. Phosphatidylcholine (PC) was visualized by iodine vapor staining. WiDr cells were pretreated without (control) or with 10 mM $\beta$-CD for 30 min, and then incubated without (none) or with 250 nM $\beta$-CD saturated with cholesterol (Cho), cholesterol sulfate (Cho.Sul), or and an equimolar mixture of cholesterol and cholesterol sulfate (Cho./Cho.Sul). The cells treated as described above were then incubated without (−) or with 50 nM matrilysin at 37 °C for 3 h. B, the cell-bound matrilysin (top) and F-actin in the cell lysate (bottom) were detected by Western blotting. C, the cells incubated without or with matrilysin were photographed. D, the degree of cell aggregation was quantified as described under “Experimental Procedures.” Bars represent standard deviations. WiDr cells were pretreated with 10 mM $\beta$-CD for 30 min, and then incubated without (none), with 100 mM SM3, or with 100 mM cardiolipin. The cells treated as described above were then incubated without (−) or with 50 nM matrilysin at 37 °C for 3 h. E, cell-bound matrilysin was detected by Western blotting. F, the incubated cells were photographed. G, the degree of cell aggregation was quantified as described under “Experimental Procedures.” Bars represent standard deviations.

matrilysin treatment were not released from the $\beta$-CD-treated and cholesterol-replenished cells. Similar results were observed in Colo201 cells (data not shown). These results suggest that binding of active matrilysin to cholesterol sulfate on the cell surface plays crucial roles in the proteolytic regulation of cell surface proteins, such as an unidentified protein that is involved in the induction of cell aggregation.
Matrilysin Binds to Cell Surface Cholesterol Sulfate

Matrilysin binds not only to cholesterol sulfate but also to cardiolipin and sulfatides SM3 and SM4. Furthermore, the matrilysin-binding ability of these sulfatides is significantly elevated in clinical conditions such as liver cirrhosis and recessive X-linked ichthyosis (28). Although the correlation between matrilysin and cholesterol sulfate under these pathologic conditions is unknown, our present study provides a new clue to elucidate their roles in pathological processes.

Sulfatides, which belong to a group of acidic glycolipids, are components of the cell membrane. Previous reports demonstrated that sulfatides are increased in many human cancer tissues, including lung, stomach, colon, ovary, and kidney (30). However, the pathologic functions of sulfatides in these cancer tissues remain unclear. On the other hand, cardiolipin is a major mitochondrial membrane phospholipid, which is important for the regulation of mitochondrial function. Our present study demonstrated that matrilysin binds not only to cholesterol sulfate but also to cardiolipin and sulfatides SM3 and SM4. Furthermore, the matrilysin-binding ability of cells was enhanced when β-CD-treated cells were supplied with SM3 or cardiolipin. Concomitant with the increment of cell-bound matrilysin after supplement of SM3, cell aggregation was also stimulated. However, matrilysin-induced cell aggregation was not stimulated by the supplement of cardiolipin. Matrilysin probably binds to SM3 and cholesterol sulfate in a similar manner, but it binds to cardiolipin in a distinct mechanism. Although the major matrilysin-binding substance was shown to be cholesterol sulfate in the tested cells, it is also possible that sulfatides and cardiolipin become alternative matrilysin-binding substances in other cell lines or under certain conditions.

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It has been reported that rat matrilysin binds to heparan sulfate proteoglycans on the surface of rat uterine cells (16, 23). In the present study, however, neither addition of excess heparin in the culture medium nor digestion of heparan sulfate on the cell surface affected binding of matrilysin to the cell surface. Therefore, it is unlikely that cell surface proteoglycans are the major sites for the matrilysin-binding. The discrepancy between the earlier and present studies is probably because of differences between experimental approaches. For instance, the earlier studies reported that matrilysin efficiently binds to the surface of CD44-transfected cells, whereas we did not test the proteoglycan-transfected cells. Another difference is that the earlier studies showed the binding of rat pro- and active matrilysin to the cell surface, whereas we examined that of the human active enzyme. Rat pro-matrilysin is reported to bind to heparin more tightly than the active one because the propeptide region contains a high affinity site for heparin (16). On the other hand, we showed that only active matrilysin, but not pro-matrilysin, efficiently binds to the cell surface. Therefore, human active matrilysin probably binds to cholesteryl sulfate via a site different from the putative heparin-binding site of rat pro-matrilysin.

Interestingly, the binding of matrilysin to cholesterol sulfate was abrogated by hydroxamate-based MMP inhibitors, such as TAPI-1 and KB-8301. We also found that the catalytically inactive E215A mutant of matrilysin still had the cell-binding ability, suggesting that the proteolytic activity of matrilysin is not required for the binding. These data also suggest that the unoccupied substrate-binding site of matrilysin is required for its interaction with cholesteryl sulfate. Therefore, we speculate two possibilities to explain how the hydroxamate inhibitor blocks the interaction between matrilysin and cholesteryl sulfate. One possibility is that the cholesterol sulfate-binding site and the inhibitor-binding site of matrilysin are overlapping or are in close proximity, thus the binding of the hydroxamate inhibitor competitively blocks the interaction between matrilysin and cholesteryl sulfate by steric hindrance. As hydroxamate inhibitors interact mainly with the prime subsite of MMPs (31), cholesteryl sulfate may bind to a site within or near the substrates of matrilysin. The other possibility is that the binding of the hydroxamate inhibitor induces the conformational change of matrilysin, and the cholesteryl sulfate-binding site of this proteinase is allosterically altered. Such an allosteric linkage between the substrate-binding site and ligand-binding site is well known in some serine proteases. For instance, incorporation of peptide-mimetic small inhibitors into the active site of blood coagulation factor VIIa induces a conformational change in its cofactor-binding site and enhances the affinity between the protease and its cofactor tissue factor (32). Tissue factor-binding vice versa accelerates the activity of factor VIIa. Although the cofactor for MMPs has not been found, so far, cholesteryl sulfate may modify the activity of matrilysin. The site of matrilysin essential for binding to cholesteryl sulfate and the mode of interaction are currently under investigation in our laboratory.

We have shown that active matrilysin induces homotypic adhesion of human colon cancer cells and enhances their metastatic potential in a nude mouse model (10). It was also demonstrated that the aggregation inducing effect apparently depends on the proteolytic activity of matrilysin, because MMP inhibitors completely neutralize the effect. On the other hand, cholesteryl sulfate can regulate the activities of various proteinases. For example, cholesteryl sulfate facilitates the activation of factor XII and prekallikrein (33). In contrast, activities of trypsin and chymotrypsin are inhibited by cholesteryl sulfate (28). We examined whether the membrane-bound matrilysin retains its proteolytic activity or not. When colon cancer cells were treated with active matrilysin, some membrane proteins were released into culture medium. Treatment of cells with β-CD led not only to significant reduction of matrilysin-binding but also to abrogation of release of several proteins. Interestingly, replenishment of the cells with cholesteryl sulfate, but not cholesteryl alone, neutralized the effects of β-CD, suggesting that the cholesterol sulfate-bound matrilysin retains its proteolytic activity.

Recently, many hydroxamate-based inhibitors or other synthetic inhibitors of MMPs have been designed to develop drugs for the treatment of diseases in which MMPs are involved (34, 35). However, none of them is a specific inhibitor of individual MMPs. Our study demonstrates the novel interaction between matrilysin and cholesteryl sulfate on the cell surface. Cholesteryl sulfate is likely to provide specific sites for matrilysin to modulate appropriate membrane-associated proteins. As compared with the active site-directed MMP inhibitors, application of mechanism-based function blockers, such as the isolated hemopexin-like domain of gelatinase A (36) or reactive site modified tissue inhibitor of metalloproteinases-2 (18), is probably of benefit in developing specific regulators for individual MMPs. Therefore, our finding provides the potential to design novel anti-cancer drugs that block specific processes of tumor metastasis by inhibiting the binding of matrilysin to cholesterol sulfate.

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