Identification of Amino Acid Residues of Matrix Metalloproteinase-7 Essential for Binding to Cholesterol Sulfate

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Matrix metalloproteinase-7 (MMP-7; matrilysin) induces homotypic adhesion of colon cancer cells by cleaving cell surface protein(s) and enhances their metastatic potential. Our previous study (Yamamoto, K., Higashi, S., Kioi, M., Tsunezumi, J., Honke, K., and Miyazaki, K. (2006) J. Biol. Chem. 281, 9170–9180) demonstrated that binding of MMP-7 to cell surface cholesterol sulfate (CS) is essential for the cell membrane-associated proteolytic action of the protease. To determine the region of MMP-7 essential for binding to CS, we constructed chimeric proteases consisting of various parts of MMP-7 and those of the catalytic domain of MMP-2; the latter protease does not have an affinity for CS. Studies of these chimeric proteases and other mutants of MMP-7 revealed that Ile29, Arg33, Arg51, and Trp55, in the internal sequence, and the C-terminal three residues corresponding to residues 171–173 of MMP-7 are essential for binding to CS. An MMP-7 mutant, which had the internal 4 residues at positions 29, 33, 51, and 55 of MMP-7 replaced with the corresponding residues of MMP-2 and the C-terminal 3 residues deleted, had essentially no affinity for CS. This mutant and wild-type MMP-7 showed similar proteolytic activity toward fibronectin, whereas the mutant lacked the ability to induce the colon cancer cell aggregation. In the three-dimensional structure of MMP-7, the residues essential for binding to CS are located on the molecular surface in the opposite side of the catalytic cleft of the protease. Therefore, it is assumed that the active site of MMP-7 bound to cell surface is directed outside. We speculate that the direction of the cell-bound MMP-7 makes it feasible for the protease to cleave its substrates on cell surface.

The matrix metalloproteinases (MMPs) comprise a family of zinc-dependent endopeptidases that degrade components of extracellular matrix (ECM) and are believed to play pivotal roles in tissue remodeling under physiological and pathological conditions such as morphogenesis, angiogenesis, tissue repair, and tumor invasion (1–4). However, it has recently been suggested that several MMPs proteolytically modulate the biological functions of various cell surface proteins, including growth factor precursors, growth factor receptors, or cell adhesion molecules (4); such regulation as well as MMP-catalyzed degradation of ECM is important for tumor growth, invasion, metastasis, and progression. A typical MMP consists of an N-terminal propeptide of about 80 amino acids, a catalytic domain of about 170 amino acids, and a C-terminal hemopexin-like domain of about 200 amino acids. Membrane type MMPs further have a transmembrane domain or a glycosylphosphatidylinositol anchor on the C-terminal side of the hemopexin-like domain and are thus localized on the cell surface. It has been reported that membrane type 1 MMP cleaves several cell surface proteins, such as syndecan-1 (5), β-amylloid precursor protein (6), integrin αv subunit (7), low density lipoprotein receptor-related protein (8), CD44 (9), and CD147 (10). The anchoring of this MMP with the cell membrane is probably a benefit in its interaction with cell surface substrates.

MMP-7 (matrilysin) is the smallest member of the MMP family, of which the pro-form consists of only a propeptide and a catalytic domain and lacks the C-terminal hemopexin-like domain. Although various MMPs are overexpressed both in stromal and tumor cells in cancer tissues, MMP-7 has been detected specifically in tumor cells but not in stromal cells (11). Expression of MMP-7 is correlated well with malignancy and metastasis of cancers, especially in liver metastasis of colon cancer (12). Although MMP-7 has neither transmembrane domain nor glycosylphosphatidylinositol anchor, this protease processes several cell surface proteins, such as Fas-ligand (13), pro-tumor necrosis factor-α (14), syndecan-1 (15), and E-cadherin (16). Recent studies further suggest that MMP-7-catalyzed cleavage of Notch on the cell surface leads to dedifferentiation of pancreatic acinar cells by activating the Notch signaling pathway (17). We previously reported that active MMP-7 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 mouse model (18). More recently, we identified cholesterol sulfate (CS) as a major cell surface substance to which active MMP-7 binds (19) and demonstrated that binding of MMP-7 to CS is...
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essential for its membrane-associated proteolytic action and induction of the cell aggregation.

Although mode of interaction between MMP-7 and liposome consisting of anionic or cationic lipids has been suggested (20), detailed amino acid residues of the protease contributing to its interaction with CS have not been identified. In this study, we constructed various mutants of MMP-7 and identified residues of the protease essential for binding to CS. Clarification of the interaction between MMP-7 and CS provides the potential to develop MMP-7-targeted novel anti-cancer drugs that specifically block the membrane-associated proteolytic action of this MMP.

EXPERIMENTAL PROCEDURES

Materials—The sources of materials used are as follows: L-arginine, cholesterol (CL), CS, and pFLAG-CTC vector from Sigma; cDNA of human proMMP-2 cloned into pCMV6 vector from OriGene Technologies, Inc. (Rockville, MD); p-amino-phenyl mercuric acetate from Tokyo Kasei (Tokyo, Japan); human recombinant MMP-7 from Wako Pure Chemical Industries (Osaka, Japan); the synthetic substrate for MMPs, (7-methoxycoumarin-4-yl)-acetyl-Pro-Leu-Gly-Leu-[Np-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg amide (3163v) from Peptide Institute, Inc. (Osaka, Japan); dinitrophenyl-Arg-Pro-Leu-Ala-Trp-Arg-Ser-OH (DNP-RPLALWRS) from Merck Calbiochem (Darmstadt, Germany). cDNA of human proMMP-7 cloned into the pAPR vector was provided by Dr. T. Tanaka, (Nagahama Institute of Oriental Yeast Co., Shiga, Japan). The anti-human MMP-7 monoclonal antibody 11B4G was a gift from Dr. Y. Matsuo (Oriental Yeast). All custom oligo-DNA primers were provided by Rikaken Co., Ltd. (Tokyo, Japan). All other chemicals were of analytical grade or the highest quality commercially available.

Construction of Expression Vector for MMP Mutants—Gene constructions carried out in this study are described in the supplemental material.

Expression and Purification of MMP Mutants—The expression vectors of various MMP mutants were transfected separately into the Escherichia coli strain DH5α. The transformants were cultured in 2× YT medium (0.08% (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.25% (w/v) NaCl) at 37 °C, and recombinant proteins were induced by the addition of 1.0 mM isopropl-β-D-thiogalactopyranoside. After a 5-h induction, E. coli cells were broken in 50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl and 5 mM EDTA with a sonicator, and the resultant inclusion bodies were collected by centrifugation. The inclusion bodies were solubilized in 50 mM Tris-HCl (pH 8.0) containing 6 mM guanidine HCl and 100 mM dithiothreitol with gentle stirring for 2 h at 25 °C. The solubilized sample was first clarified by centrifugation and then applied to a Cosmosyl 5C4 column (4.6 × 100 mm) and eluted with a linear gradient of 0–80% acetonitrile containing 0.1% trifluoracetic acid for 70 min at a flow rate of 0.5 ml/min. The column effluent was monitored at 280 nm. The recombinant proteins eluted at about 56–62% acetonitrile were collected. One mg each of the collected proteins was freeze-dried and dissolved again with 200 μl of 50 mM Tris-HCl (pH 8.0) containing 6 mM guanidine HCl and 100 mM dithiothreitol. The dissolved samples were then refolded by the rapid dilution method using a refolding buffer containing 1.0 M arginine, as described previously (21). The refolded proteins were dialyzed extensively against 50 mM sodium HEPES (pH 7.5) containing 150 mM NaCl and 10 mM CaCl2, and concentrated using a Centricon YM-10 ultrafiltration device (Millipore Corp., Bedford, MA). The concentrations of the purified proteins were determined by Bradford’s dye method with a BioRad protein assay kit, using bovine serum albumin as a standard.

Assay of Effect of CS on Peptidolytic Activities of MMP-7 and Its Variants—Pro-forms of the MMP mutants (1 μM) were activated by incubation with 1 mM p-aminophenyl mercuric acetate at 37 °C for 2 h, as described previously (22). As described in the supplemental material, we also constructed MMP-7 mutants that have a spacer sequence between the first methionine residue and N terminus of the catalytic domain instead of the propeptide sequence. This spacer sequence is designed to contain the C-terminal 9 residues of the propeptide of MMP-7 and a peptide bond susceptible to autocatalytic cleavage (Fig. 1B). The N-terminal spacer region of each mutant was removed autocatalytically during the preparation described above. Active forms of the MMP mutants were first measured for their activities toward a synthetic substrate 3163v, as described previously (22). The peptidolytic activities of the representative mutants are listed in supplemental Table S2. MMP-7 (2.0 nm) and the mutants, of which concentrations were adjusted to give the activity equivalent to that of 2.0 nm MMP-7, were each incubated with various concentrations of CS or an equimolar mixture of CS and CL (CS/CL) in 190 μl of 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 10 mM CaCl2, and 0.01% bovine serum albumin at 25 °C for 15 min. Then 10 μl of 1 mM 3163v was added to the mixture, and the incubation was further continued at 37 °C for 30 min. The reaction was terminated by adding 20 μl of 0.5 mM EDTA (pH 8.0). The amounts of the synthetic substrate hydrolyzed by proteases were measured fluorometrically with excitation at 326 nm and emission at 400 nm. The amount of the substrate hydrolyzed without enzyme was subtracted from the total amount of the hydrolyzed substrate.

Separation of Free and CS-bound Forms of MMP-7 or Its Mutant—MMP-7 and its mutant were each incubated with various concentrations of CS in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 10 mM CaCl2, and 0.05% polyethylene glycol 4000 at 25 °C for 15 min. The sample was then centrifuged at 21,000 × g for 15 min, and the supernatant was collected. The resultant precipitate containing the CS-bound form of proteases was resuspended in the assay buffer. The peptidolytic activities in the supernatant and the resuspended precipitate were measured as described above.

Cell Lines and Culture Conditions—Human colon cancer cell line Colo201 was obtained from the Japanese Cancer Resources Bank. This cell line was maintained in Dulbecco’s modified Eagle’s/Ham’s F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum.

Assay of MMP-7 Variant Binding to Cell Surface—Assay of the binding of MMP-7 variants to the surface of Colo201 cells was carried out as described previously (19). The MMP-7 vari-
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Figure 1. Effect of deletion of C-terminal residues of MMP-7 on its affinity for CS. A, the constructions of pro-MMP-7 and its C-terminal amino acid residues-deleted variants, described under “Experimental Procedures,” are schematically represented. The amino acid sequence covering from the C-terminal region of propeptide (boldface letters) to the N-terminal region of the catalytic domain (normal letters) of pro-MMP-7 or the C-terminal sequence of each variant is shown at the bottom of the scheme. The numbers shown at the top of these sequences represent the amino acid residue numbers in active MMP-7 numbering. The spacer sequence between the first methionine residue (M) and the N terminus of the catalytic domain is schematically represented. The spacer sequence is shown at the bottom of the scheme, and the sequence of the C-terminal part of propeptide included in the spacer sequence is represented by boldface letters. INS, the inserted spacer region. C, active forms of MMP-7 (2.0 nM; ●), MMP-7ΔC3 (1.3 nM; ○), MMP-7ΔC8 (1.5 nM; △), and MMP-2-cat-FLAG (0.33 nM; △) were incubated with 50 μM 3163v at 37 °C for 30 min in the presence of the indicated concentrations of CS. All of the reaction mixtures contained 50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl₂, and 0.01% bovine serum albumin. The amount of 3163v hydrolyzed by each enzyme in the absence of CS was taken as 100%. The enzyme activity on the ordinate is shown as the relative amount of 3163v hydrolyzed by the enzyme.

The Effect of Deletion of C-terminal Residues of MMP-7 on Its Affinity for CS—It has been reported that MMP-7 has an affinity for CS, whereas MMP-2 and MMP-3 have no affinity with this acidic lipid (19). To examine whether the interaction between MMP-7 and CS affects the activity of MMP-7, we measured the peptidolytic activity of MMP-7 in the presence of various concentrations of CS. As shown in Fig. 1C, the activity of MMP-7 was reduced with increasing concentrations of CS and reached constant in the presence of 6.3 μM or higher concentrations of CS. The activity of MMP-7 in the presence of saturating concentrations of CS was about 20% of that in the absence of CS. The IC₅₀ value, which is defined in this study to be a concentration of CS giving half-maximal reduction of the MMP-7 activity, was 0.65 μM. CS did not affect the peptidolytic activity of the catalytic domain of MMP-2 (Fig. 1C), probably reflecting the inability of this protease to bind to CS. The mechanism of the CS-mediated reduction of the peptidolytic activity of MMP-7 is discussed below. To evaluate the CS binding affinities of MMP-7 and its variants, we compared the IC₅₀ values for the CS-mediated reduction of their peptidolytic activities in the following studies.

We previously constructed a FLAG-tagged MMP-7 (22), which has an 8-residue FLAG epitope peptide on the C-terminal side of MMP-7. When the FLAG-tagged MMP-7 and wild-type MMP-7 were compared for their affinities for CS, the FLAG-tagged MMP-7 showed a slightly lower affinity for CS (data not shown). Therefore, the CS-binding site of MMP-7 may locate near the C terminus of the protease. To examine whether the C-terminal residues of MMP-7 contribute to the interaction with CS, we constructed MMP-7 mutants, named MMP-7ΔC3 and MMP-7ΔC8, that had the C-terminal 3 and 8 residues of MMP-7 deleted, respectively (Fig. 1A), and the effects of CS on the activities of these mutants were compared. As shown in Fig. 1C, the peptidolytic activities of MMP-7ΔC3 (IC₅₀ = 3.1 μM) and MMP-7ΔC8 (IC₅₀ = 4.0 μM) were reduced by CS with similar IC₅₀ values. These IC₅₀ values are about 5-fold higher than the value for the CS-mediated reduction of MMP-7 activity (0.65 μM), suggesting that the C-terminal 3 residues of MMP-7 partially contribute to its interaction with CS. Because the artificially added FLAG tag may obstruct the interaction between MMP-7 and CS, we constructed MMP-7 and its variants without adding the FLAG tag (Fig. 1A and B). Since we could not use an anti-FLAG antibody to purify the variants of MMP-7, we purified them before the refolding, using reversed-phase high-performance liquid chromatography, as described under “Experimental Procedures.”

Effect of CS on Peptidolytic Activities of Chimeric MMP Mutants Consisting of Various Parts of MMP-7 and Those of...
**MMP-2 Catalytic Domain**—The data shown in Fig. 1C suggest that some regions of MMP-7 other than its C terminus also contribute to the high affinity interaction between the protease and CS, because the C-terminally deleted mutants of MMP-7 still had affinities for CS. To explore the regions of MMP-7 contributing to the affinity for CS, we constructed chimeric MMP mutants that consist of various N-terminal parts of the catalytic domain of MMP-2 and remaining C-terminal parts of MMP-7 (Fig. 2A), and effects of CS on their activities were compared. To facilitate understanding, we use active MMP-7 numbering for the amino acid residue numbers of the mutants corresponding to the structural difference represented by the arrows in A. C, MMP-7 and M2n-37-M7c were incubated, separately, with the indicated concentrations of CS at 25 °C for 15 min. The sample was then centrifuged. The region corresponding to residues 11–37 of MMP-7 may contribute partially to the interaction with CS. On the other hand, M2n-56-M7c, M2n-83-M7c, and M2n-156-M7c reached less than 10% (Fig. 2B). This suggests that the region corresponding to residues 11–55 of MMP-7 contributes partially to the interaction with CS. Similar to the case of MMP-7, the activity of M2n-11-M7c in the presence of saturating concentrations of CS was about 20% of that in the absence of CS, whereas the activities of M2n-37-M7c, M2n-56-M7c, M2n-83-M7c, and M2n-156-M7c reached less than 10% (Fig. 2B). The region corresponding to residues 11–37 of MMP-7 may correlate with the level of activity of MMP-7 upon its complex formation with CS.

To verify that the observed activities of MMP-7 and its mutants in the presence of saturating concentrations of CS are those of enzyme-CS complexes but are not caused by a partial dissociation of the complexes, MMP-7 and M2n-37-M7c were first incubated with various concentrations of CS, and then free and CS-bound forms of the enzymes were separated by centrifugation. As shown in Fig. 2C, the activity of MMP-7 in the supernatant disappeared as the concentration of CS was
increased to 7.0 μM or higher. The activity of MMP-7 in the precipitate was increased with the increasing concentrations of CS and reached constant together with the disappearance of the activity in the supernatant. The activity that reached constant was about 20% of that in the absence of CS. When M2n-37-M7c was incubated with the saturating concentrations of CS, the activity of the mutant in the supernatant also disappeared, and its activity in the precipitate reached 3% of that in the absence of CS. These data suggest that CS-bound forms of MMP-7 and M2n-56-M7c have the peptidolytic activities, although their activities are much smaller than those of their free forms.

**CL Enhances CS Binding Affinities of MMP-7 and Its Variants**—We previously reported that CS is localized in rafts or caveolae on the cancer cell surface (19). Since these microdomains are rich in CL, CS may form a complex with CL on the cell surface. To examine whether CL affects the affinity between MMP-7 and CS, we tested the effect of an equimolar mixture CS/CL on the affinity for CS. Because CL alone did not affect the affinity. When all of the Ile29, Arg33, Arg51, and Trp55 of MMP-7 did not affect the affinity significantly. When all of the residues 24–31, 32–36, and 49–55 of MMP-7 led to 5.8-, 3.1-, and 4.6-fold reductions of the affinity for CS/CL, respectively (Fig. 4), whereas that of residues 13–23 and 37–48 did not affect the affinity significantly. When all of the residues 24–36 and 49–55 of MMP-7 were replaced with the corresponding residues of MMP-2, the resultant mutant, named MMP-7(24–36,49–55/M2), showed 420-fold reduced affinity for CS/CL as compared with MMP-7. In the regions corresponding to the residues 24–36 and 49–55 of MMP-7, 13 residues are different between MMP-7 and MMP-2 (Fig. 5). To examine which residues contribute the affinity for CS/CL, we next replaced individual residues in these regions of MMP-7 with the corresponding residues of MMP-2 as schematically represented in Fig. 4. We found that the replacements of residues 24–31, 32–36, and 49–55 of MMP-7 led to 5.8-, 3.1-, and 4.6-fold reductions of the affinity for CS/CL, respectively (Fig. 4), whereas that of residues 13–23 and 37–48 did not affect the affinity significantly. When all of the residues 24–36 and 49–55 of MMP-7 were replaced with the corresponding residues of MMP-2, the resultant mutant, named MMP-7(24–36,49–55/M2), showed 420-fold reduced affinity for CS/CL as compared with MMP-7. In the regions corresponding to the residues 24–36 and 49–55 of MMP-7, 13 residues are different between MMP-7 and MMP-2 (Fig. 5). To examine which residues contribute the affinity for CS/CL, we next replaced individual residues in these regions of MMP-7 with the corresponding residues of MMP-2, as shown in Fig. 5. We found that the replacements of the pair Ile29 and Arg33 and the pair Arg51 and Trp55 of MMP-7 each led to a 10-fold reduction of the affinity for CS/CL. Other replacements, including that of Arg51 or Trp55, also reduced the affinity for CS/CL, whereas the replacements of Arg3, Pro27, His28, Leu34, and Val56 of MMP-7 did not affect the affinity. When all of the Ile29, Arg33, Arg51, and Trp55 of MMP-7 were replaced with the corresponding residues of MMP-2, the resultant mutant, named MMP-7(29,33,51,55/M2), showed 420-fold reduced affinity for CS/CL as compared with MMP-7. Since MMP-7(29,33,51,55/M2) and MMP-7(24–36,49–55/M2) had almost the same affinities for

**Contributions of Amino Acid Residues Located in a Region Corresponding to Residues 11–55 of MMP-7 to Its Interaction with CS**—The data shown in Figs. 2 and 3 strongly suggest that the region corresponding to residues 11–55 of MMP-7 contains amino acid residues contributing to the affinity for CS. To explore the residues, we first divided the region of MMP-7 into five sections, and the residues in the each section were replaced with the corresponding residues of MMP-2 as schematically represented in Fig. 4. We found that the replacements of residues 24–31, 32–36, and 49–55 of MMP-7 led to 5.8-, 3.1-, and 4.6-fold reductions of the affinity for CS/CL, respectively (Fig. 4), whereas that of residues 13–23 and 37–48 did not affect the affinity significantly. When all of the residues 24–36 and 49–55 of MMP-7 were replaced with the corresponding residues of MMP-2, the resultant mutant, named MMP-7(24–36,49–55/M2), showed 420-fold reduced affinity for CS/CL as compared with MMP-7. In the regions corresponding to the residues 24–36 and 49–55 of MMP-7, 13 residues are different between MMP-7 and MMP-2 (Fig. 5). To examine which residues contribute the affinity for CS/CL, we next replaced individual residues in these regions of MMP-7 with the corresponding residues of MMP-2, as shown in Fig. 5. We found that the replacements of the pair Ile29 and Arg33 and the pair Arg51 and Trp55 of MMP-7 each led to a 10-fold reduction of the affinity for CS/CL. Other replacements, including that of Arg51 or Trp55, also reduced the affinity for CS/CL, whereas the replacements of Arg3, Pro27, His28, Leu34, and Val56 of MMP-7 did not affect the affinity. When all of the Ile29, Arg33, Arg51, and Trp55 of MMP-7 were replaced with the corresponding residues of MMP-2, the resultant mutant, named MMP-7(29,33,51,55/M2), showed 420-fold reduced affinity for CS/CL as compared with MMP-7. Since MMP-7(29,33,51,55/M2) and MMP-7(24–36,49–55/M2) had almost the same affinities for

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5-fold each the CS binding affinities of wild-type MMP-7, M2n-11-M7c, and M2n-37-M7c, whereas it did not enhance the affinities of M2n-56-M7c, M2n-83-M7c, and M2n-156-M7c, suggesting that the region corresponding to residues 37–55 of MMP-7 correlates with the CL-enhanced CS binding affinity of this protease. We also found that the regions corresponding to residues 11–36 and 37–55 of MMP-7 contribute, respectively, to its high affinity interaction with CS/CL (Fig. 3B). As compared with the CS binding affinity of MMP-7, its affinity for CS/CL was reduced more prominently upon the replacement of residue 11–36. We further analyzed the CL-enhanced CS binding affinities of other variants of MMP-7 in the following studies, because the analysis was thought to be a benefit
CS/CL, it is likely that in the regions of MMP-7 and MMP-2, corresponding to the residues 24–36 and 49–55, only the 4 residues at positions 29, 33, 51, and 55 are responsible for the difference in their CS/CL binding affinities. Collectively, except for the C-terminal 3 residues, the internal Ile29, Arg33, Arg34, and Trp55 of MMP-7 mainly contributed to the affinity for CS.

**Effects of Modifications of MMP-7 on Its CS Binding Affinity, Cell Binding Ability, or Fibronectin Cleaving Activity**—To verify that the internal 4 residues of MMP-7 contributing to the affinity for CS/CL are also important for its interaction with CS, we measured the peptidolytic activity of MMP-7(29,33,51,55/M2)∆C3 in the presence of various concentrations of CS. As shown in Fig. 6A, the activity of the MMP-7 mutant was reduced by CS with a high IC50 value (50 µM), suggesting that the internal 4 residues also contribute significantly to the affinity for CS. When the C-terminal 3 residues of this mutant were deleted, the resultant mutant, named MMP-7(29,33,51,55/M2)∆C3, showed essentially no affinity with CS (Fig. 6A).

We previously demonstrated that CS located on the cell surface mainly mediates the binding of MMP-7 to colon cancer cell line Colo201 (19). To examine whether the internal 4 residues and the C-terminal 3 residues of MMP-7 also contribute to the interaction between the protease and CS on the cell surface, we tested the abilities of the MMP-7 mutants to bind to Colo201 cells. As shown in Fig. 6B, wild-type MMP-7 bound effectively to the cells, whereas MMP-7AC3 bound to them with significantly reduced affinity, suggesting that the C-terminal 3 residues contribute to the cell binding ability of MMP-7. We could not detect the binding of MMP-7(29,33,51,55/M2) or MMP-7(29,33,51,55/M2)∆C3 to the cells by Western blotting analysis, although these mutants and wild-type MMP-7 were similarly immunoreactive (Fig. 6B). These data are consistent with the view that the internal 4 residues are very important for the cell binding affinity of MMP-7, and the replacement of these residues reduces the affinity into the undetectable level.

We found that neither the replacement of the internal 4 residues nor the deletion of the C-terminal 3 residues of MMP-7 affected significantly the peptidolytic activity of this protease (Table S2). However, these modifications of MMP-7 may alter the activity toward macromolecular substrates. To test this possibility, cleavages of fibronectin catalyzed by MMP-7 and its variants were compared. As shown in Fig. 6C, MMP-7 and MMP-7(29,33,51,55/M2)∆C3 similarly cleaved fibronectin and produced almost the same fragments, suggesting that the modifications of MMP-7 does not significantly alter the activity toward macromolecular substrates.

Cancer Cell Aggregation-inducing Abilities of MMP-7 Variants Having Low CS Binding Affinity—It has been reported that binding of MMP-7 to CS on cell surface is essential for its induction of cancer cell aggregation (19). To examine whether the loss of the CS binding affinity of MMP-7 upon the deletion or replacement of its residues also leads to loss of the ability of
the protease to induce the cell aggregation, MMP-7 and its variants having low affinities for CS were incubated with Colo201 cells, and their cell aggregation-inducing abilities were compared. As shown in Fig. 7, wild-type MMP-7 rapidly induced the cell aggregation, and 90% of Colo201 cells were aggregated within 30 min. The rates of the cell aggregation induced by MMP-7ΔC3 and MMP-7(29,33,51,55/M2) were relatively slow, and 80 and 40% of the cells were aggregated after the 60-min incubation, respectively (Fig. 7B). In contrast, MMP-7(29,33,51,55/M2)ΔC3 did not induce the cell aggregation even after the 60-min incubation. Collectively, the variants of MMP-7 having low CS binding affinity also had low potency to induce the cell aggregation.

**Enzyme Kinetics**—To investigate the mechanism of the CS-mediated reduction of MMP-7 activity, the kinetic parameters of MMP-7, MMP-7ΔC3, MMP-7(29,33,51,55/M2), and their CS-bound forms toward a synthetic peptide substrate DNP-RPLALWRS were measured. As shown in Fig. 8A, $V_{\text{max}}$ value was practically equal for MMP-7 and its CS-bound form, whereas the $K_m$ value of the enzyme was about one-fifth of that of the CS-bound one, suggesting that the mode of CS-mediated reduction of the MMP-7 activity is a competitive inhibition. Similar to the case of MMP-7, $V_{\text{max}}$ values of MMP-7ΔC3 and MMP-7(29,33,51,55/M2) were not affected by CS, whereas $K_m$ values of the mutants were enhanced about 5-fold upon the binding of CS.

**DISCUSSION**

We explored the amino acid residues of MMP-7 contributing to its high affinity interaction with CS and found that residues Ile${}^{29}$, Arg${}^{33}$, Arg${}^{51}$, and Trp${}^{55}$ in the internal sequence and Arg${}^{171}$, Lys${}^{172}$ and Lys${}^{173}$ in the C terminus of MMP-7 were essential for the interaction. In the three-dimensional structure of MMP-7 (23), the internal 4 residues were located on the molecular surface in the opposite side of the catalytic cleft (Fig. 9A). On the other hand, structure of the C-terminal regions of MMP-7, including the Arg${}^{171}$, Lys${}^{172}$, and Lys${}^{173}$, had not been determined in the x-ray analysis, probably because of high flexibility of this region. Considering that the flexible C-termi-
nal strand of MMP-7 is long enough to bring the C-terminal 3 residues close to the internal 4 residues (Fig. 9A), all of the 7 residues essential for binding to CS may form a CS-binding patch on the molecular surface of MMP-7 upon its binding to CS. It should be noted that the structure in Fig. 9A is the active site-inhibited form of MMP-7. As described below, the CS binding affinity of MMP-7 is reduced significantly upon its binding to inhibitors. Therefore, the inhibitor-bound structure of MMP-7 may be different from its conformational state that has high affinity for CS. Prediction of the effect of inhibitor on the CS-binding residues of MMP-7 has been difficult thus far, because no three-dimensional structure of the inhibitor-free form of MMP-7 has been determined. Comparison of the three-dimensional structures of inhibitor-free and inhibitor-bound forms of the catalytic domain of MMP-1 (Protein Data Bank codes 1AYK and 4AYK, respectively) suggests, however, that the residues of the protease corresponding to Ile29, Arg33, Arg51, and Trp55 of MMP-7 do not move significantly upon inhibitor binding. Subtle changes in the location or orientation of the residues of MMP-7 induced by inhibitors may lead to dramatic reduction of the CS binding affinity. Since 5 of the 7 residues of MMP-7 essential for binding to CS are basic ones, electrostatic interaction between the positive charge of these residues and negative charge of a sulfate group of CS is probably a main force stabilizing the high affinity interaction between the protease and the lipid. Considering that 5 basic residues of MMP-7 contribute to the interaction, several CS molecules probably interact with one molecule of the protease. Like other lipids, CS molecules probably form clusters in aqueous solution. We speculate that MMP-7 binds to the clustered CS molecules in the solution. CL may enhance the apparent affinity between MMP-7 and CS by facilitating the cluster formation of CS molecules. However, further studies are needed to clarify the mechanism. The remaining 2 essential residues of MMP-7 were hydrophobic ones that may interact with a hydrophobic portion of CS. A recent report suggested that two basic residues and one hydrophobic residue of factor C, a horseshoe crab serine protease, contribute to its recognition of acidic lipid lipopolysaccharide on the Gram-negative bacteria surface (24). Use of both basic residues and hydrophobic ones may be a common strategy for proteins to recognize acidic lipids. Since the residues of MMP-7 essential for binding to CS are located on the molecular surface in the opposite side of the active site, it is assumed that the active site of MMP-7 is directed outside when the protease binds to CS on the cell surface. We speculate that the direction of the cell-bound MMP-7 makes it feasible for the protease to cleave its substrates on cell surface.

**FIGURE 8.** Mechanism of CS-mediated reduction of peptidolytic activities of MMP-7 and its mutants. A, MMP-7 (4.0 nM, ○) or MMP-7ΔC3 (3.5 nM, ◇) was incubated with various concentrations of DNP-RPLALWRS at 37 °C for 30 min. The amount of DNP-RPLALWRS hydrolyzed was then measured as described under “Experimental Procedures,” and the results were plotted with 1/v versus 1/[S]. The kinetic parameters obtained from the results are indicated near each fitting curve. B, a model for the equilibria among various states of MMP-7 (E) in the presence of substrate (S) and CS (I) is shown schematically (left). Km, and K′m represent the Michaelis constants for the substrate hydrolysis catalyzed by MMP-7 and MMP-7-CS complex, respectively. K and K′i represent dissociation constants for binding of CS to MMP-7 and that of CS to MMP-7-substrate complex, respectively. P, the products of enzyme reaction; kcat, the catalytic rate constant. Equations pertaining to the cyclic equilibria (left) are given on the right as Equations 1–5 (Eq. 1 to Eq. 6). The relationship among velocity (v) of the steady-state enzyme reaction and kinetic parameters in this model is given as Equation 6 (Eq. 6) in a Lineweaver-Burk style equation.
MMP-7, and the CS-binding site of this proteinase is allosterically altered. The data in the present study support the latter possibility, because the CS-binding site was in the opposite side of the catalytic cleft of MMP-7. An allosteric linkage between the substrate-binding site and ligand-binding site is well known in some serine proteases. For instance, covalent 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 (27). Tissue factor-binding, on the contrary, accelerates the activity of factor VIIa.

Although the kinetic data (Fig. 8A) suggest that the mode of CS-mediated reduction of the MMP-7 activity is a competitive inhibition, it is unlikely that CS competes directly with substrate for the catalytic site of MMP-7, because MMP-7-CS complex was found to have the peptidolytic activity (Fig. 2C). Considering that active site-directed inhibitors reduce the affinity between MMP-7 and CS and, inversely, CS reduces the affinity between the substrate and the enzyme, we present in Fig. 8B a model for CS-mediated reduction of MMP-7 activity. In this model, the MMP-7-CS complex and free MMP-7 catalyze the reaction with the same catalytic rate constant ($k_\text{cat}$). If $K_m$ of MMP-7 and MMP-7-CS complex, respectively, obtained from the result in Fig. 8A, are applied to Equation 5 (Eq. S) in Fig. 8B, the ratio of $K_i/K$ is calculated to be 0.22, indicating that the affinity between MMP-7 and CS is reduced about 5-fold upon the occupation of the catalytic site of MMP-7 by substrate. Equation 6 (Eq. 6) in Fig. 8B indicates that this model is categorized to be the competitive inhibition. Therefore, the model is in good agreement with the nature of CS-mediated reduction of MMP-7 activity and that of interaction among MMP-7, CS, and the catalytic site-occupying molecules, such as substrates and substrate-mimetic inhibitors.

The association of MMPs with cancer cell invasion and metastasis has suggested that these proteases represent attractive targets for the development of novel anti-tumor therapies. However, to date, no MMP inhibitor has been developed successfully as anti-tumor drugs mainly because of deleterious side effects. Considering that MMPs play essential roles under both physiological and pathological conditions, inhibition of activities of MMPs other than the target MMPs probably causes the side effects. The broad specificity of the MMP inhibitors so far designed must be a stiff obstacle for developing safe and effective drugs. We have recently identified a $\beta$-amyloid precursor protein-derived decapeptide having the ISYGNDALMP...
sequence as an MMP-2-selective inhibitor, which interacts with the active site of MMP-2 (28). It has also been reported that the reactive site-modified tissue inhibitor of metalloprotease-2 specifically blocks the membrane type-1 MMP-catalyzed activation of pro-MMP-2 (29). However, no MMP-7-selective inhibitor has been identified thus far. Moreover, MMP-7 also has physiological roles besides the pathological ones; the data from the MMP-7-deficient mice suggest that this protease is responsible for the activation of prodefensins and thereby participates in innate host defense. Therefore, resistance to infection may be affected by inhibition of MMP-7 activity. On the other hand, MMP-7 induces aggregation of colon cancer cells by cleaving cell surface protein(s) and enhances their metastatic potential (18). This protease also cleaves Notch on the cell surface and promotes the dedifferentiation of pancreatic acinar cells by activating the Notch signaling pathway (17); the dedifferentiation of the pancreatic cells is associated with an increased risk for tumorigenesis. Therefore, it seems likely that MMP-7-catalyzed processing of cell surface proteins associates preferentially with pathological stages. We showed that the variants of MMP-7 having low CS binding affinity also had low potency to induce the cell aggregation (Fig. 7), suggesting that binding of MMP-7 to the cell membrane facilitates MMP-7-catalyzed processing of cell surface proteins. These data also suggest that the cell membrane-associated proteolytic action of MMP-7 and its proteolytic activities toward ECM substrates can be uncoupled by blocking the CS-binding site of MMP-7. For instance, anti-MMP-7 antibodies that recognize the CS-binding site probably block the binding of MMP-7 to the cell surface, MMP-7-catalyzed processing of cell surface proteins, and MMP-7 stimulation of cancer metastasis without preventing its production of defensins. Since modifications of the residues forming the CS-binding site of MMP-7 did not affect its fibronectin-cleaving activity, this protease is unlikely to use the CS-binding site as the substrate-binding exosite to cleave this ECM protein. Therefore, blocking of the CS-binding site of MMP-7 may not affect the ECM-degrading activity of the protease. Taken together, our finding provides the potential to develop MMP-7-targeted novel anti-cancer drugs that block specifically the membrane-associated proteolytic action of this MMP, thereby having restricted side effects.

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