The plasma membrane-bound tissue inhibitor of metalloproteinases (TIMP)-2 specifically inhibits matrix metalloproteinase 2 (gelatinase A) activated on the cell surface*

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The cell-surface activation of pro-matrix metalloproteinase 2 (pro-MMP-2) is considered to be critical for cell migration and invasion. Treatment of human uterine cervical fibroblasts with concanavalin A activates pro-MMP-2 on the cell surface by converting it to the 65-kDa form with a minor form of 45 kDa. However, the 65-kDa MMP-2 was inactivated by tissue inhibitor of metalloproteinases (TIMP)-2 that was bound to the plasma membrane upon concanavalin A treatment. TIMP-2 binds to the plasma membrane through its N-terminal domain by two different modes of interaction as follows: one is sensitive to a hydroxamate (HXM) inhibitor of MMPs and the other is HXM-insensitive. TIMP-2 bound to the membrane in a HXM-insensitive manner, comprising about 40–50% of TIMP-2 on the membrane, is the inhibitor of the cell surface-activated MMP-2. It, however, does not inhibit MMP-3, MMP-9, and the 45-kDa MMP-2 lacking the C-terminal domain. The inhibition of the 65-kDa MMP-2 by TIMP-2 is initiated by the interaction of their C-terminal domains. Subsequently, the MMP-2-TIMP-2 complex is released from the membrane, and the activity of MMP-2 is blocked by TIMP-2. In the presence of collagen types I, II, III, V, or gelatin, the rate of inhibition of the 65-kDa MMP-2 by the membrane-bound TIMP-2 decreased considerably. These results suggest that the pericellular activity of MMP-2 is tightly regulated by membrane-bound TIMP-2 and surrounding extracellular matrix components.

Matrix metalloproteinases (MMPs), also called matrixins

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 Dedicated to Dr. J. Frederick Woessner, Jr., on the occasion of his 70th birthday.
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The abbreviations used are: MMP, matrix metalloproteinase; APMA, 4-aminophenylmercuric acetate; TIMP, tissue inhibitor of metalloproteinases; ConA, concanavalin A; α2-M, α2-macroglobulin; HXM, hydroxamic acid MMP inhibitor; E-64, trans-epoxysuccinyl-l-leucylamido(4-guanidino)-butane; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; HBSS, Hank’s balanced salt solution; Mca, (7-methoxycoumarin-4-yl)acetyl; Dpa, 3-(2,4-dinitrophenyl)-1,2,3-diaminopropionyl.
nism of pro-MMP-2 activation and the fate of the TIMP-2 upon activation of pro-MMP-2 are not clearly understood.

In this report, we have investigated the activation processes of pro-MMP-2 using human uterine cervical fibroblasts treated with ConA. This treatment induced the activation of pro-MMP-2 and accumulation of TIMP-2 on the cell surface. The TIMP-2 bound to the plasma membrane specifically inhibits MMP-2 upon activation by the same membrane. We also report that there are at least two modes of binding of TIMP-2 to the cell surface, i.e., hydroxamate inhibitor of MMP (HXM)-sensitive and -insensitive binding. The TIMP-2 bound to the plasma membrane in an HXM-insensitive manner specifically inhibits MMP-2 upon activation. Our study suggests that the TIMP-2 bound to the membrane plays an important role in controlling the pericellular activity of MMP-2.

**EXPERIMENTAL PROCEDURES**

**Materials—**ConA (type VI), diisopropyl phosphorofluoridate, trans-epoxysuccinyl-l-leucylamido(4-guanidino)-butane (E-64), N-(alpha-amino- nopyranosylxoy-hydroxophosphoryl)-Leu-Trp (phosphoramidon), collagen types II (bovine nasal septum), III (human placenta), V (human placenta), gelatín (porcine skin), and trypsin (bovine) were from Sigma. Dulbecco’s modified Eagle’s medium (DMEM), Hank’s balanced salt solution (HBSS), antibiotics, fetal calf serum, and lactalbumin hydrolysate were from Life Technologies, Inc. Fluorogenic substrate, (7-methoxyxoumarin-4-yl)acetyl-Pro-Leu-Glu-Leu-(3,2,4-dinitrophenyl)-L-2,3-diaminopropanionyl-Ala-Arg-NH₂ (Mca-Pro-Leu-Glu-Lys-Arg-Ala-Arg-NH₂) (23) was from Bachem Bioscience Inc., King of Prussia, PA. Type I collagen was purified from guinea pig skin by the standard method (24). Anti-human MT1-MMP antibody was raised against synthetic peptide (159FREVPYAYTREGHEKQ174) of the catalytic domain in rabbit. Matlystatin A was prepared as described previously (25). GM6001-X (HONHCOCH₂CH(isobutyl)CO-Tyr(O-Me)-NHMe) (26) was a generous gift from Dr. J. Oloksyzyn of OsteoArthritis Sciences, Inc. N-Ac-NH₂ (Nan) was kindly provided by Dr. J. E. Engfeldt at Duke University Medical Center, Durham, NC. Escherichia coli-expressed N-terminal domain of TIMP-2 (N-TIMP-2) was kindly provided by Dr. D. S. Waugh at Hoffmann-La Roche, Nutley, NJ. Human uterine cervical fibroblasts were prepared as described previously (27).

**Purification of Pro-MMP-2 and TIMP-2—**Pro-MMP-2 free from TIMP-2 was purified from the conditioned medium of human uterine cervical fibroblasts by gelatin-Sepharose 4B and, subsequently, by gel permeation chromatography on Sephacryl S-200 as described previously (28). TIMP-2 was isolated from the pro-MMP-2-TIMP-2 complex by dialysing the complex with 20 mM EDTA and 8 mM urea, and subsequent gel permeation chromatography on Sephacryl S-200 equilibrated with 8 mM urea containing 50 mM Tris-HCl (pH 7.5), 1 mM NaCl, 20 mM EDTA, 0.02% NaN₃, 0.02% NaN₃, 0.02% Na₂SO₄, 0.02% NaCl, 10 mM CaCl₂, 0.02% NaN₃ and further purified by gel permeation chromatography on Sephacryl S-200 equilibrated with TNC buffer.

**Purification of Active MMP-3 and MMP-9—**Recombinant pro-MMP-3 was purified from the culture medium of Chinese hamster ovary cells transfected with human MMP-3 cDNA as described by Benbow et al. (29). Purified pro-MMP-3 was then activated with chymotrypsin, and the 45-kDa MMP-3 was isolated according to Ogata et al. (30). To obtain active pro-MMP-3, pro-MMP-P9-TIMP-1 complex was purified from the conditioned medium of HT-1080 cells according to Ogata et al. (31). The purified complex was activated by a 2 mM excess of active MMP-3 at 37 °C for 2 h. The active MMP-9 was then separated by passing the sample through anti-TIMP-1 affinity and anti-MMP-3 affinity columns and by gel permeation chromatography on Sephacryl S-200. The concentration of these active MMPs was determined by titrating with a known amount of purified TIMP-1.

**Preparation of Plasma Membrane—**Human uterine cervical fibroblasts were cultured in DMEM containing 10% fetal calf serum. After confluence, the cells were washed and treated with 50 μg/ml ConA in the serum-free DMEM supplemented with 0.2% lactalbumin hydrolysate for 36 h. The cells were then scraped with a rubber policeman and used for the plasma membrane preparation as described previously (14, 16) with modifications. Briefly, the cells were suspended in chilled 250 mM sucrose, 100 mM Tris-HCl (pH 8.0), 0.02% NaN₃, 2 mM diisopropyl phosphorofluoridate, 10 μM E-64, then homogenized on ice, and the whole lysate was centrifuged at 16,000 × g for 20 min at 4 °C. The supernatant was then centrifuged at 100,000 × g for 1 h at 4 °C. Precipitates were washed with TNC buffer and centrifuged again at 100,000 × g for 1 h at 4 °C. Precipitates were then suspended in TNC buffer and kept at -20 °C until used for experiments. The concentration of the membrane was determined by measuring the A₅₃₀ of the sample dissolved in 50 mM NaOH taking A₅₃₀ of 0.1 value as 10.

**Zymography and Reverse Zymography—**Zymography was conducted with SDS-polyacrylamide gel containing gelatin (0.8 mg/ml) (32). The samples were mixed with SDS-PAGE sample buffer without reducing agent and subjected to electrophoresis at room temperature. After electrophoresis, gels were washed with the buffer containing 25% Triton X-100 to reconstitute pro-MMP-2 in the gels by removal of SDS. The activity of TIMPs was visualized as inhibition of gelatin lysis by MMP-2; thus, positive staining with Coomassie Brilliant Blue R-250 is observed where TIMPs locate. The intensity of positive staining is linear in the range of 0.1–1 ng of TIMP-2 per lane.

**Purification of Active MMP-3 and MMP-9—**The TIMP-2 bound to the cell surface was purified by gelatin-Sepharose 4B and, subsequently, by gel permeation chromatography on Sephacryl S-200 equilibrated with 8 M urea, and subsequent gel permeation chromatography on Sephacryl S-200. The concentration of these active MMPs was determined by titrating with a known amount of purified TIMP-1.

**Results of the Membrane Preparation—**The failure of the membrane preparation contained a readily detectable amount of MMP-2 but not TIMP-1 (Fig. 1B). However, TIMP-2 was not found in the membrane of unstimulated control cells (Fig. 3). The failure of the membrane preparation contained a readily detectable amount of TIMP-2 but not TIMP-1 (Fig. 1B). However, TIMP-2 was not found in the membrane of unstimulated control cells (Fig. 3).
TIMP-2 Controls MMP-2 Activity on the Cell Surface

**Fig. 1.** Zymographic analysis of pro-MMP-2 treated with the plasma membrane from ConA-treated human uterine cervical fibroblasts. Pro-MMP-2 (final concentration of 1 µg/ml) prepared from the ConA-treated human cervical fibroblasts (Cont.) or pro-MMP-2 alone. The samples were then divided into two portions; one portion was further incubated with TNC buffer as indicated. Plasma membrane (Mb) alone was run as control (lanes 5 and 6). The samples were then analyzed by zymography (A) and reverse zymography (B) under nonreducing conditions.

**Fig. 2.** Inhibition of the APMA-activated MMP-2 by the plasma membrane. A 20-µl portion of pro-MMP-2 (7 nm) was activated with 1 mM APMA at 37 °C for 1 h and incubated with a different amount of the plasma membrane prepared from nontreated control cells ([]) or ConA-treated cells (O,◆), and the residual MMP-2 activity was measured against the synthetic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$ in the presence of 10 µM phosphoramidon. Two different preparations of active MMP-2 (O,◆) were tested against the membrane from ConA-treated cells to show different degrees of the inhibition. ◆, MMP-2 contained about 10–15% of 45-kDa form; O, MMP-2 contained about 30–40% of 45-kDa form as judged by zymography.

The amount of TIMP-2 in the supernatant also increased in a time-dependent manner, suggesting that TIMP-2 bound to the membrane specifically reacts with the cell surface-activated MMP-2. A small portion of the 65-kDa MMP-2 was associated with the membrane after 4 h. This treatment also generated the 45-kDa MMP-2. A small portion of the 65-kDa MMP-2 was associated with the membrane at a 1-h time point, but most MMP-2 was released from the membrane after 4 h. The 45-kDa MMP-2 was never found in the membrane fraction. When MMP-2 activity in the supernatant was measured against Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$, the enzyme activity correlated with the formation of the 45-kDa MMP-2 with the highest activity at 4 h. The intensity of the 65-kDa form in the supernatant and in the total mixture did not change even after a 20-h incubation. The amount of TIMP-2 in the supernatant also increased in a time-dependent manner, suggesting that TIMP-2 bound to the membrane specifically reacts with the cell surface-activated MMP-2.

Cell Surface-bound TIMP-2 Does Not Inhibit the 45-kDa

Orientation of TIMP-2 on the Plasma Membrane and the Specific Inhibition of MMP-2 by the Membrane-bound TIMP-2—We then examined whether the TIMP-2 in the plasma membrane inhibits APMA-activated MMP-2 and other MMPs. Fig. 2 shows a dose-dependent inhibition of two different preparations of MMP-2 by membranes prepared from ConA-treated cells but not by those from control cells. It is notable, however, that the inhibition of MMP-2 was observed up to about 60–80%, but the rest of 20–40% was resistant to inhibition even with an increased amount of membrane. The TIMP-2-resistant activity was due to the presence of the 45-kDa MMP-2 in the APMA-activated MMP-2 (see below). Thus, two different preparations of active MMP-2 gave a slightly different inhibition curve depending on the amount of the 45-kDa MMP-2 generated.

In contrast, TIMP-2 on the membrane failed to inhibit MMP-3 and MMP-9, whereas free TIMP-2 in solution inhibited MMP-3 (Table I). These results suggest that the N-terminal inhibitory domain of TIMP-2 is not readily available to other MMPs when it is bound to the membrane. If so, the reaction of MMP-2 and the membrane-bound TIMP-2 is likely to be triggered by their initial interaction through their unique C-terminal domains. To determine the orientation of TIMP-2 in the membrane, the membrane was dot-blotted on a nitrocellulose filter without denaturation and reacted with monoclonal antibodies raised against the peptide (Asp$^{10}$–Gln$^{44}$) in the N-terminal domain (clone 68–6H4) and the C-terminal tail (Tyr$^{178}$–Asp$^{193}$) of TIMP-2 (clone 67–4H11) (36). As shown in Fig. 3, the ConA-membrane preparation reacted with monoclonal antibody 67–4H11 but not with 68–6H4. Isolated TIMP-2 reacted with both antibodies, whereas TIMP-2 of the pro-MMP-2/TIMP-2 complex reacted only with 68–6H4. The control membrane did not react with either antibody. These data suggest that TIMP-2 binds to the membrane mainly through its N-terminal domain.

To investigate further the possible interaction of the N-terminal domain of TIMP-2 to the membrane, the binding experiments of full-length TIMP-2 and the C-terminal truncated recombinant TIMP-2 (N-TIMP-2) to the cell surface were conducted. Scatchard plot analysis (data not shown) indicated that both TIMP-2 and N-TIMP-2 bound to ConA-stimulated cells with a similar affinity with $K_d$ values of 1.4 and 1.6 nM, respectively. The numbers of the binding sites of two TIMP-2 species were $1.92 \times 10^4$/cell and $1.91 \times 10^4$/cell, respectively, almost identical to each other.
MMP-9 (3 nM) was reacted with the plasma membrane (2 mg/ml) at 37 °C for 24 h. The plasma membrane at a protein concentration of 0.8 mg/ml contains 7 nM TIMP-2 as judged by reversed zymography. The residual MMP-3 activity was measured using S^3H-carboxymethylated transferrin (33). MMP-9 (3 nM) was reacted with the plasma membrane (2 mg/ml) at 37 °C for 1 h. The residual activity was measured using synthetic peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ as described under "Experimental Procedures."

| Enzyme   | Treatment            | Enzymic activity (%) |
|----------|----------------------|----------------------|
| MMP-3 (7 nM) | + TNC buffer (control) | 100                  |
|          | + TIMP-2 3.5 nM  | 55                   |
|          | + TIMP-2 7.0 nM  | 11                   |
|          | + TIMP-2 14.0 nM | 12                   |
|          | + Membrane 0.4 mg/ml (3.5 nM) | 99               |
|          | + Membrane 0.8 mg/ml (7.0 nM) | 97               |
|          | + Membrane 1.6 mg/ml (14 nM) | 95               |
| Membrane alone | + Membrane 2.4 mg/ml (21 nM) | 90               |
| MMP-9 (3 nM) | + TNC Buffer (control) | 100                  |
|          | + TIMP-2 2.3 nM  | 9                    |
|          | + Membrane 2.0 mg/ml (17.5 nM) | 81               |

* Estimated concentration of TIMP-2.

**TABLE I**

Lack of inhibition of MMP-3 and MMP-9 by the membrane-bound TIMP-2

The 45-kDa active MMP-3 (7 nM) was reacted with various concentrations of the isolated TIMP-2 or the plasma membrane prepared from human uterine fibroblasts treated with ConA at 37 °C for 24 h. The plasma membrane at a protein concentration of 0.8 mg/ml contains 7 nM TIMP-2 as judged by reversed zymography. The residual MMP-3 activity was measured using S^3H-carboxymethylated transferrin (33). MMP-9 (3 nM) was reacted with the plasma membrane (2 mg/ml) at 37 °C for 1 h. The residual activity was measured using synthetic peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ as described under "Experimental Procedures."

MMP-2—Although the activation of pro-MMP-2 by the membrane generates predominantly the 65-kDa form, a small amount of the 45-kDa MMP-2 is also generated, and only the 45-kDa species bound to α₂M (see Fig. 1). The 45-kDa MMP-2 lacks the C-terminal domain (37), which may be the reason why the membrane-bound TIMP-2 cannot inhibit the 45-kDa MMP-2. To test this further, MMP-2 containing a large portion of 45-kDa MMP-2 was prepared by activating pro-MMP-2 with APMA in the presence of 5% Me₂SO at 37 °C for 36 h to generate the 65- and 45-kDa active MMP-2. The MMP-2 was then diluted to 3 nM and reacted with or without an equal volume of the plasma membrane (2 mg/ml containing 17.5 nM TIMP-2) at 37 °C for 30 min and then reacted with α₂M (100 μg/ml). The samples with or without α₂M were analyzed by zymography. Relative activity of MMP-2 against Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ before the reaction with α₂M is indicated.

**Fig. 3. Immuno-slot blot analysis of the plasma membrane with anti-TIMP-2 antibodies.** Plasma membranes (20 μg) from the untreated control cells (Control Mb) or ConA-treated cells (ConA Mb) were applied to immuno-slot blot analysis using monoclonal antibodies raised against the peptide (Asp^50-Glu^54) in the N-terminal domain of human TIMP-2 (68-6H4) and the C-terminal tail (Tyr^178-Asp^193) of TIMP-2 (67-4H11). Purified TIMP-2 (200 ng) and purified pro-MMP-2/TIMP-2 complex (800 ng) were also applied as standards.

**Fig. 4. Release of the MMP-2/TIMP-2 complex from the plasma membrane.** Pro-MMP-2 (final concentration of 1 μg/ml) was mixed with the plasma membrane from ConA-treated fibroblasts (final concentration of 3.5 mg/ml) (+ Mb) and incubated at 37 °C for the indicated periods. Portions of the samples were then centrifuged at 100,000 × g for 1 h to separate the supernatant (Sup) and the membranes (Mb). The samples before and after separation were analyzed by zymography (A) and reverse zymography (B). Pro-MMP-2 treated with 1 mM APMA for 1 h at 37 °C is shown as a standard. The activity of MMP-2 in the supernatant was measured against Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂, and the relative activities are indicated taking the APMA-activated MMP-2 as 100%. Membrane alone exhibited a small amount of MMP-2 activity in zymography but not against the peptide substrate, suggesting that it was inhibited by TIMP-2.

**Fig. 5. Inability of the membrane-bound TIMP-2 to inhibit the 45-kDa MMP-2.** Pro-MMP-2 (1 μg) was activated with 1 mM APMA in the presence of 5% MeSO at 37 °C for 36 h to generate the 65- and 45-kDa active MMP-2. The MMP-2 was then diluted to 3 nM and reacted with or without an equal volume of the plasma membrane (2 mg/ml containing 17.5 nM TIMP-2) at 37 °C for 30 min and then reacted with α₂M (100 μg/ml). The samples with or without α₂M were analyzed by zymography. Relative activity of MMP-2 against Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ before the reaction with α₂M is indicated.

is through the active site of the enzyme, a peptidyl hydroxamic acid inhibitor of the MMPs (HXM) should compete with the binding of TIMP-2 (38). We thus tested the effect of two synthetic inhibitors, Matlystatin A (25) and GM6001X (26). As
shown in Fig. 6, ConA treatment of the cells increased the specific binding of TIMP-2 6.8-fold compared with nontreated control cells. When the binding experiments were performed in the presence of an HXM inhibitor, the binding of TIMP-2 to the cells was inhibited in a dose-dependent manner down to the level of control cells. This suggests that the exogenously added TIMP-2 binds to the active MMPs on the cell surface, presumably to MT1-MMP whose expression is induced upon the ConA treatment of the cells.

The specific binding of TIMP-2 to the ConA-treated cell surface was also examined by reverse zymography. As shown in Fig. 7A, both TIMP-1 and TIMP-2 were found in the culture medium. Upon ConA treatment of the cells the amount of TIMP-2 in the medium, but not that of TIMP-1, decreased, and in turn a significant amount of TIMP-2 was found in the cell fraction. Although 50 \( \mu \)M Matlystatin A reduced TIMP-2 binding to the ConA-treated cell to the level of control cells (Fig. 6), reverse zymography revealed that the cellular fraction obtained from the cells cultured in the presence of ConA and Matlystatin A for 30 h retained a considerable amount of TIMP-2 (Fig. 7A). Western blotting and its densitometric analyses for TIMP-2 in the two plasma membrane preparations indicated that about 50% TIMP-2 was associated with the membrane even after culturing in the presence of 10 \( \mu \)M GM6001-X. These results indicate that TIMP-2 binds to plasma membrane by two different mechanisms as follows: one is an HXM-sensitive manner and the other is HXM-insensitive.

Both ConA-treated membranes and ConA/GM6001-X-treated membranes contained a very similar amount of MT1-MMP (see Fig. 8, inset). The blocking of TIMP-2 binding to the plasma membrane by HXM increased the peptidase activity of the membrane against Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH\(_2\) about 1.4-fold. This peptidase activity was inhibited by TIMP-2 in a dose-dependent manner, but not by TIMP-1, suggesting the activity is derived from MT1-MMP. Thus, the increase of this peptidase activity is most likely due to an increased amount of active MT1-MMP on the membrane. Nonetheless, the ability of the membrane from the ConA/GM6001-X-treated cells to activate pro-MMP-2 was approximately 50% that of the ConA membrane (Fig. 8B). These results indicate that an increase of MT1-MMP activity alone does not correlate with the activation of pro-MMP-2.

TIMP-2 Bound to the Membrane in an HXM-insensitive Manner Inhibits the 65-kDa MMP-2—Plasma membranes were prepared from the ConA-treated cells and the ConA/GM6001-X-treated cells, and their ability to inhibit MMP-2 was compared. Fig. 9 shows that both membrane preparations inhibited MMP-2 in an identical fashion. This indicates that only the TIMP-2 bound to the membrane in an HXM-insensitive man-
A 20-mer peptidase was used to determine the ability of procedures to inhibit the activity of MMP-2. TIMP-1 or TIMP-2 were tested. Mean values ± S.D. from triplicate samples are shown. A 40-mer peptidase was also tested. Mean values ± S.D. from triplicate samples are shown. A 40-mer peptidase was also tested. Mean values ± S.D. from triplicate samples are shown.

**Pro-MMP-2 (7 nM) was activated with 1 mM APMA at 37 °C for 1 h and the mixture was measured against Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ in the presence of 10 μM phosphoramidon.**

**FIG. 8. Increase of Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ peptidase activity in the plasma membrane from ConA/HXM-treated cells. A, the plasma membranes were prepared from ConA-treated or ConA/GM6001-X-treated cells as described under “Experimental Procedures,” and the peptidase activity was measured against Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂. The ability of TIMP-1 or TIMP-2 to inhibit the activity was also tested. Mean values ± S.D. from triplicate samples are shown. A 40-μg portion of each membrane preparation was subjected to Western blotting using rabbit anti-(human MT1-MMP) antibody (inset). B, pro-MMP-2 (1 μg/ml) was reacted with the above membranes (0.5 mg/ml) at 37 °C for 2 h. The samples were subjected to zymographic analysis under nonreducing conditions. Cont., pro-MMP-2 alone.**

**FIG. 9. Inhibition of MMP-2 by the TIMP-2 bound to the membrane in the presence of a Matrix Protein—An interaction of the activated 65-kDa MMP-2 with membrane-bound TIMP-2 has suggested that MMP-2 may not exhibit full proteolytic activity in vivo when activated on the cell surface. On the other hand, MMP-2 binds to extracellular matrix components such as collagens through the fibronectin type II-like domain (39, 40) and to fibronectin and heparin through the C-terminal domain of the enzyme (41). Therefore, we examined whether the reaction rate of MMP-2 with membrane-bound TIMP-2 decreases in the presence of matrix components. Pro-MMP-2 was reacted with the ConA-treated cell membrane in the presence of gelatin, collagen I, or fibronectin for various periods, and MMP-2 activity of the mixture was measured against Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (Fig. 10A). When pro-MMP-2 was reacted with the membrane without matrix components, the activity reached a maximum after a 4-h incubation and then gradually decreased. In the presence of gelatin or collagen I, the activity detected was about twice as much as the control throughout the incubation time. Fibronectin did not show any effect on the activity. Therefore, we conclude that the enhancing effect on the apparent MMP-2 activity is specific for proteins that interact with fibronectin type II-like domains of MMP-2. Similar results were observed when pro-MMP-2 was treated with APMA in the presence of gelatin or collagen I. A higher, stable MMP-2 activity was observed for the first 4 h, whereas APMA alone rapidly inactivated MMP-2 (Fig. 10B). It is notable that 50–63% of the full activity still remained even after a 20-h incubation at 37 °C when gelatin or collagen I was present. Fibronectin was not effective.

When the membrane-activated products were analyzed by zymography, it became evident that the decrease of activity was not due to autodegradation but due to inhibition by TIMP-2. As shown in Fig. 10C, pro-MMP-2 was completely converted to 65 kDa by the membrane within 4 h in all cases in a very similar manner, suggesting that none of the matrix components influenced the activation process. Importantly, although a higher activity was detected in the presence of gelatin or collagen I, there were no significant differences in the intensity of 65- and 45-kDa bands at 1- and 4-h time points in these experiments. This suggests that binding of MMP-2 to gelatin or collagen I slows down the rate of inactivation of MMP-2 by TIMP-2 on the membrane. Similarly, collagens II, III, and V also reduced the rate of TIMP-2-MMP-2 interaction in a similar manner (data not shown). At later time points, compared with controls, a higher intensity of the 45-kDa band was noted in the presence of these extracellular matrix components. Since this species of the enzyme is not inhibited by the membrane-bound TIMP-2, the elevated enzymic activity in the presence of gelatin and collagen I may be in part attributed to this 45-kDa species.

On the other hand, when pro-MMP-2 was activated with APMA in the presence of gelatin or collagen I, the 65- and 45-kDa bands were more intense in zymography, suggesting that these matrix components stabilize the activated forms of MMP-2 and prevent them from autodegradation. Fibronectin (Fig. 10) and bovine serum albumin (data not shown) had no effect on the pro-MMP-2 activation and autolysis by either APMA or the membrane, further supporting that this event was specific to the components that react with the fibronectin type II-like domains of MMP-2 such as gelatin and collagens.

**DISCUSSION**

The results presented here demonstrate that fibroblasts treated with ConA accumulate TIMP-2 on the cell surface and that TIMP-2 on the membrane specifically inhibits MMP-2 activated on the cell surface. We also have shown that TIMP-2 binds to the plasma membrane by at least two mechanisms, i.e., an HXM-sensitive and an HXM-insensitive manner, and that the level of bound TIMP-2 by two different interaction modes are similar in the case of human uterine cervical fibroblasts. HXM-sensitive binding suggests that TIMP-2 binds to the active site of MMP, presumably MT1-MMP on the cell surface by interacting through its inhibitory domain. Recently, Zucker et
al. (42) and Butler et al. (43) reported that the binding of TIMP-2 to the membrane was through an interaction between the inhibitory site of TIMP-2 and the catalytic site of MT1-MMP. However, TIMP-2 that is bound to active MT1-MMP is unable to inhibit MMP-2 (Ref. 44 and this work). In this report we have shown that the TIMP-2 bound to the membrane in an HXM-insensitive manner has the ability to interact with and inhibit MMP-2 on the cell surface and regulates pericellular MMP-2 activity. This inhibition is specific for the 65-kDa form of MMP-2. The membrane-anchored TIMP-2 could not inhibit MMP-3 and MMP-9 and most likely other matrixins as well. The reaction of the membrane-bound TIMP-2 and the 65-kDa MMP-2 probably takes place in at least three steps as follows: step 1, the interaction through C-terminal domains of both MMP-2 and TIMP-2; step 2, the dissociation of TIMP-2 from the binding molecule located on the membrane, which exposes the N-terminal domain of TIMP-2 for inhibition; and step 3, the binding of the inhibitory domain of TIMP-2 and the catalytic domain of MMP-2. The specific interaction of the C-terminal domains of MMP-2 and TIMP-2 is critical for this reaction. Thus, the 45-kDa MMP-2 that lacks the C-terminal domain and other matrixins are not inhibited by the membrane-bound TIMP-2.

Whereas the TIMP-2 bound to the plasma membrane is an important regulator of MMP-2 activated on the cell surface, the rate of the reaction slows down considerably in the presence of collagen I or other collagen types but not fibronectin. This protection is due to the interaction of these macromolecules with the fibronectin type II-like domains of MMP-2, which are located near the end of the active site cleft, close to the S1' pocket of the enzyme. They are therefore thought to be crucial for a proper orientation of the substrate in relation to the catalytic site of the enzyme. Although the three-dimensional structure of MMP-2 is not known, it may be postulated that the binding of collagen or gelatin to these domains may influence the rate of interaction between MMP-2 and TIMP-2. Furthermore, our activation studies of pro-MMP-2 by APMA in the presence of collagen I or gelatin have provided some insights into the stabilization of the activated MMP-2. When pro-MMP-2 is activated by APMA, the activated MMP-2 is rapidly degraded, but in the presence of macromolecules that interact with the fibronectin-like domains of MMP-2 about 50–60% of MMP-2 remains active even after 20 h at 37 °C. In our activation assay system gelatin in the activation mixture was completely degraded into small peptides after 8 h, but the activity of MMP-2 was sustained beyond this point, suggesting that small peptides derived from gelatin are sufficient to stabilize the MMP-2 molecule. Thus, the half-life of MMP-2 in collagen-rich tissues is probably much longer than what has been postulated from the in vitro activation study with APMA.

Our present study does not address whether or not the interaction of pro-MMP-2 and TIMP-2 bound to the cell surface in an HXM-insensitive manner is critical for the activation of pro-MMP-2. However, it is notable that removing TIMP-2 from MT1-MMP by HXM significantly decreased the activation activity of the membrane by 50%, although it increased TIMP-2-sensitive peptidase activity that is thought to be MT1-MMP (Fig. 8). These data suggest that an increase in MT1-MMP activity does not necessarily enhance the activation of pro-MMP-2. A certain amount of TIMP-2 bound to MT1-MMP appears to be required for efficient activation of pro-MMP-2.

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Footnote: 2 Y. Itoh, and H. Nagase, unpublished observations.
TIMP-2 Controls MMP-2 Activity on the Cell Surface

This supposition agrees with the hypothesis by Strongin et al. (21) that the formation of a ternary complex of MT1-MMP/TIMP-2-MMP-2 on the cell surface is critical for the activation of pro-MMP-2. It is not clear, however, from our experiments that the TIMP-2 bound to the membrane in an HXM-insensitive way participates in the activation of pro-MMP-2. Nonetheless, our studies indicate that TIMP-2 plays an important role in regulating MMP-2 activity on or near the cell surface. The activated 65-kDa MMP-2 can be further converted to the 45-kDa form by removing the C-terminal domain autolytically or by other proteinases, and this reaction is likely to be enhanced in the presence of extracellular matrices as shown in our in vitro experiments. Once this takes place, the membrane-bound TIMP-2 can no longer inhibit the activity of MMP-2. Olson et al. (46) have recently reported that when active MMP-2 loses its C-terminal domain, TIMP-1 can no longer regulate the 45-kDa form by removing the C-terminal domain autolytically or by other proteinases, and this reaction is likely to be enhanced in the presence of extracellular matrices as shown in our in vitro experiments. In this case, increases in the K

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