High Affinity Binding of Latent Matrix Metalloproteinase-9 to the α2(IV) Chain of Collagen IV*

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Association of matrix metalloproteinases (MMPs) with the cell surface and with areas of cell-matrix contacts is critical for extracellular matrix degradation. Previously, we showed the surface association of pro-MMP-9 in human breast epithelial MCF10A cells. Here, we have characterized the binding parameters of pro-MMP-9 and show that the enzyme binds with high affinity (Kd=22 nm) to MCF10A cells and other cell lines. Binding of pro-MMP-9 to MCF10A cells does not result in zymogen activation and is not followed by ligand internalization, even after complex formation with tissue inhibitor of metalloproteinase-1 (TIMP-1). A 190-kDa cell surface protein was identified by ligand blot analysis and affinity purification with immobilized pro-MMP-9. Microsequencing and immuno blot analysis revealed that the 190-kDa protein is the α2(IV) chain of collagen IV. Specific pro-MMP-9 surface binding was competed with purified α2(IV) and was significantly reduced after treatment of the cells with active MMP-9 before the binding assay since α2(IV) is hydrolyzed by MMP-9. A pro-MMP-9-TIMP-1 complex and MMP-9 bind to α2(IV), suggesting that neither the C-terminal nor the N-terminal domain of the enzyme is directly involved in α2(IV) binding. The closely related pro-MMP-2 exhibits a weaker affinity for α2(IV) compared with that of pro-MMP-9, suggesting that sites other than the gelatin-binding domain may be involved in the binding of α2(IV) to pro-MMP-9. Although pro-MMP-9 forms a complex with α2(IV), the proenzyme does not bind to triple-helical collagen IV. These studies suggest a unique interaction between pro-MMP-9 and α2(IV) that may play a role in targeting the zymogen to cell-matrix contacts and in the degradation of the collagen IV network.

The degradation of ECM components is partly achieved by proteolytic enzymes closely associated with discrete areas of cell-matrix contacts. These include the plasminogen/plasmin system (1), cathepsins (2), and MMPs (3–6). The gelatinases A (MMP-2) and B (MMP-9) are two members of the MMP family that have been shown to play a central role in many normal and pathological conditions involving ECM degradation, including wound healing, angiogenesis, embryogenesis, arthritis, and tumor metastasis (3–6). Like other members of the MMP family, the gelatinases are produced in a latent form (pro-MMP) that requires activation to become proteolytically active. Thus, activation is a critical step in the regulation of MMP-dependent proteolytic activity. Considerable evidence has associated the gelatinases with the ability of tumor cells to metastasize due to their ability to degrade basement membrane collagen IV and to their elevated expression in malignant tumors (5, 6). Previous studies have shown that tumor cells contain gelatinase activity in the plasma membranes consistent with cell surface association (7–10). We have shown a surface localization of both gelatinases in carcinoma cells of breast tumors (11, 12) and epithelial cells of fibrocystic breast disease (12), and others have shown the localization of pro-MMP-9 in the tumor basement membrane zone of skin tumors (13) colocalizing with collagen IV. In recent years, much information has been gained on the cell surface association of pro-MMP-2. It has been shown that pro-MMP-2 binds to the cell surface via membrane type 1-MMP (MT1-MMP), a subclass of MMPs bound to plasma membranes (14, 15). Interestingly, the binding of pro-MMP-2 to MT1-MMP requires the participation of tissue inhibitor of metalloproteinase-2 (TIMP-2) (14). The trimer complex allows for pro-MMP-2 activation on the cell surface, possibly by another MT1-MMP molecule. A later study suggested that the cell surface association of pro-MMP-2 can also occur through the binding of the C-terminal domain of the enzyme to integrin αvβ3 (16). Thus, several mechanisms may play a role in the surface localization of pro-MMP-2.

Pro-MMP-9 is structurally similar to pro-MMP-2, with both enzymes containing three tandem copies of a 58-amino acid residue fibronectin type II-like module (17, 18), known as the gelatin-binding domain, which plays a role in binding to extracellular matrix components (19–21). Furthermore, both latent enzymes can bind to TIMP molecules in the C-terminal domain of the zymogen with pro-MMP-9 binding to TIMP-1 (17) and pro-MMP-2 to TIMP-2 (4, 5). Pro-MMP-9 also contains a 54-amino acid proline-rich extension of unknown function that is idase; EHS, Engelbreth-Holm-Swarm; ECL, enhanced chemiluminescence; mAb, monoclonal antibody; BVE, rat vascular endothelial.

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The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; MTI-MMP, membrane type 1-MMP; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PMSP, phenylmethylsulfonyl fluoride; HRP, horseradish peroxi-
similar to the α2(IV) chain of collagen V and in addition is a glycosylated enzyme (17). In contrast to pro-MMP-2, little is known about the interactions of pro-MMP-9 with the cell surface, yet pro-MMP-9 has been found to be present on the cell surface. Recent studies by Partridge et al. (22) showed that pro-MMP-9 is present in the plasma membrane and focal contacts of cultured endothelial cells. Pro-MMP-9 was also detected in the plasma membranes of human fibrosarcoma HT1080 cells (23), and the enzyme could be activated by a plasmin-dependent mechanism (24). We have recently shown that, upon induction with 12-O-tetradecanoylphorbol-13-acetate, pro-MMP-9 binds to the surface of human breast epithelial MCF10A cells (25). In this report, we further examined the binding of pro-MMP-9 to a variety of cell lines and demonstrated the existence of a single high affinity (Kd = 22 nm) binding site. Using immobilized pro-MMP-9, ligand blot analysis and co-immunoprecipitation experiments with surface biotinylated cells, we have identified the α2(IV) chain of collagen IV as the major pro-MMP-9-binding protein. These studies provide novel evidence on the interactions of pro-MMP-9 with α2(IV) that may play a role in the localization of zymogen at cell-matrix contacts and degradation of basement membranes.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant Gelatinases and TIMP-1**

Human recombinant pro-MMP-9, pro-MMP-2, and TIMP-1 were all expressed in HeLa cells using a recombinant vaccinia virus expression system and purified to homogeneity as described previously (26, 27). To obtain 35S-pro-MMP-9 and 35S-pro-MMP-2, expression of recombinant enzymes in infected HeLa cells was carried out in the presence of 15 μCi/ml [35S]methionine. The specific activities of [35S]-pro-MMP-9 and [35S]-pro-MMP-2 were 0.0159 μCi/μmol and 0.0106 μCi/μmol, respectively.

**Antibodies**

The monoclonal antibodies to human pro-MMP-9 (CA-209) and to pro-MMP-2 (CA-801 and CA-905) have been described previously (11, 12, 25, 28). An anti-pro-MMP-9 rabbit polyclonal antibody (pAB109) raised against a synthetic peptide (APRQRQGSTVLVTGDLGLRT) from the N-terminal domain of human pro-MMP-9 was a generous gift from Dr. Stetler-Stevenson (National Cancer Institute, National Institutes of Health, Bethesda, MD) (25). A polyclonal antibody against human TIMP-1 was raised in rabbits against human recombinant pro-MMP-2 (29). Mouse monoclonal antibodies (mAbs) against human TIMP-1 (IM 322) were purchased from Calbiochem (San Diego, CA). Chain-specific rat mAbs to human collagen IV were prepared as described previously (30). A mAb to the α2(IV) chain of human collagen IV (mAb 1910) was provided by Dr. Fred Miller (Karmanos Cancer Institute, Detroit, MI) and grown in DMEM supplemented with 10% fetal bovine serum (FBS) and RPMI 1640 medium supplemented with 10% FBS, respectively. Human HT1080 fibrosarcoma (CCL-121) cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM supplemented with 10% FBS. Rat vascular endothelial (RVE) cells derived from brain capillaries (32) and mouse lung microvascular endothelial (CD3) cells were obtained from Dr. Diptie (Department of Pathology, Wayne State University) and grown in DMEM supplemented with 10% FBS.

**Binding Assays—Cells (MCF10A, MDA-MB-231, PC3) grown to 80% confluence (3–5 days after seeding) in 12-well (35-mm) plates were infected with PBS or incubated with PBS containing 0.1% BSA. The cells were washed with 0.5 ml/well of 0.25% trypsin and plated in 12-well plates in 1 ml/tube of binding medium (25 mM Hepes (pH 7.5) with 0.5% BSA in DMEM). The medium was aspirated and concentrations of 1–40 nm) of 125I-pro-MMP-9 diluted in binding medium were added to each well (300 μl) in the presence or absence of 80-fold excess unlabeled pro-MMP-9. After a 45-min incubation at 4 °C, the medium was aspirated and the cells were washed four times with cold PBS containing 0.1% BSA. The cells were lysed with 0.5 ml/well of 0.5 n NaOH for determination of radioactive counts in a γ counter (Packard model 5650). The results were expressed as the mean of the values obtained from triplicate samples. The number of cells in each well was determined in quadruplicate wells.

Time-course experiments were similarly done, except that the concentration of 125I-pro-MMP-9 for each well was kept constant at 18 nm and the cells were harvested after various times at 4 °C. The nonspecific binding of 125I-pro-MMP-9 was determined in the presence of 80-fold excess unlabeled pro-MMP-9. Typically, specific binding represented an average of approximately 20–30% of total bound 125I-pro-MMP-9. The association rate constant (kon) of pro-MMP-9 was determined from the time-course experiment following logarithmic transformation of the data. Competition with specifically bound 125I-pro-MMP-9 to binding of 125I-pro-MMP-9 to the cell surface follows a second-order binding isotherm. Thus, the kon is determined from the slope of a line plotted as ln(LReq/(LReq – LR)) versus time as described previously (33), where LReq is the quantity of 125I-pro-MMP-9 bound at 120 min and LR is the quantity of 125I-pro-MMP-9 bound at the times 0, 2, 15, and 30 min. The slope of this line was determined by linear regression analysis using Microsoft Excel™, and the error represents the standard deviation of the slope. The equilibrium binding constant (Kd) and the number of binding sites per cell were determined by nonlinear curve-fitting analysis using the GraphPad Prism™ software version 2.0 and by Scatchard analysis. The slope and the intercept from the Scatchard analysis were determined by linear regression analysis using Microsoft Excel™. For HT1080 and RVE cells, binding was performed with 125I-pro-MMP-9 (3.6 nm) in the presence and absence of 80-fold excess unlabeled pro-MMP-9 for 45 min at 4 °C and the amount of specific ligand bound (fmol/cells) was determined. Competition of 125I-pro-MMP-9 binding with pro-MMP-2 was carried out in a ligand binding assay as described above, except that 80-fold excess unlabeled pro-MMP-2 was used instead of unlabeled pro-MMP-9.

**Cellular Distribution of Bound 125I-Pro-MMP-9**

MCF10A cells were incubated with cold binding medium and then incubated with 18 nm/ well of 125I-pro-MMP-9 in triplicate wells for 45 min at 4 °C. The medium was aspirated, and the cells were washed four times with cold binding medium. Each well then received 0.5 ml of prewarmed (37 °C) binding medium, and the plates were incubated at 37 °C for various times. At each time point, the plates were removed and rinsed with PBS, followed by the addition of 0.5 ml of PBS containing 0.5% Pronase E (Sigma) in PBS. The cells were incubated at 4 °C for 30 min, and the monolayer was dislodged by gentle pipetting and transferred to a microcentrifuge tube. The samples were centrifuged for 5 min at 2000 × g, and the supernatant (cell surface-bound fraction) was transferred to a new tube. The pellet (internalized fraction) was washed once with PBS and then resuspended in 0.5 ml of PBS. The radioactivity of the three fractions, in triplicate, was measured in a γ counter. Internalization studies in the presence of TIMP-1 were performed similarly, except that the cells were incubated with 125I-pro-MMP-9 and TIMP-1 complex that was previously formed by incubating 125I-pro-MMP-9 with TIMP-1 for 30 min at 22 °C.

**Competition of 125I-Pro-MMP-9 Binding with Purified α2(IV)**

125I-Pro-MMP-9 (3.6 nm) was incubated with 1.5 at 4 °C with 2-fold molar excess of affinity-purified α2(IV) at a final concentration of 7 nm in the presence of 5 μM EDTA. Binding assays with MCF10A cells were conducted as described above.

**Coupling of Pro-MMP-9 to Affi-Gel 10**

One milligram of recombinant pro-MMP-9 was coupled to Affi-Gel 10 via its cysteine residue using the sulfo-NHS-LC-biotin reagent (Pierce). The reagent-coupled pro-MMP-9 was dialyzed against PBS, pH 7.4, containing 0.5% BSA and 0.02% (v/v) Tween 20. The solution was sterilized by filtration through a 0.22-μm filter and was stored at −20 °C. The biotinylated pro-MMP-9 was bound to a 1 ml Sepharose G-25 (fine) column equilibrated with collagenase buffer. The specific activity of 125I-pro-MMP-9 and 125I-TIMP-1 was determined after trichloroacetic acid precipitation and quantitation by densitometry of the proteins in Coomasie Blue-stained SDS-polyacrylamide gels relative to a standard curve of unlabeled purified proteins. Typically, the specific activities of 125I-TIMP-1 and 125I-pro-MMP-9 were 0.049 μCi/μmol and 0.322 μCi/μmol, respectively. No detectable autocatalytic/degradation forms of MMP-9 were observed in the iodinated enzyme as determined by both gelatin-zymography and autoradiography.

**Cells**

Human immortalized breast epithelial MCF10A cells (31) were grown as described previously (25). MDA-MB-231 breast cancer cells and PCs prostate cancer cells were grown by Dr. Fred Miller (Karmanos Cancer Institute, Detroit, MI) and grown in DMEM supplemented with 10% fetal bovine serum (FBS) and RPMI 1640 medium supplemented with 10% FBS, respectively. Human HT1080 fibrosarcoma (CCL-121) cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM supplemented with 10% FBS. Rat vascular endothelial (RVE) cells derived from brain capillaries (32) and mouse lung microvascular endothelial (CD3) cells were obtained from Dr. Diptie (Department of Pathology, Wayne State University) and grown in DMEM supplemented with 10% FBS.
nont pro-MMP-9 in 50 mM Hepes (pH 7.5), 5 mM CaCl₂, 150 mM NaCl, and 0.02% Brij-35 was allowed to bind to 1 ml of Affi-Gel 10 (Bio-Rad) for 5 h at 4 °C with rotation in the presence of 60 mM CaCl₂. After coupling, the matrix (Affi-Gel 10-pro-MMP-9) received a 150-μl volume of 1 M ethanolamine (pH 8) and incubated with rotation for 1 h at 4 °C. The matrix was allowed to settle, and the supernatant was centrifuged to SDS-PAGE to determine the amount of uncoupled pro-MMP-9. The Affi-Gel 10-pro-MMP-9 matrix was washed four times with collagenase buffer. The immobilized pro-MMP-9 maintained its capability to bind TIMP-1, as determined by binding of 125I-TIMP-1 compared with solution TIMP-1, as determined by binding of 125I-TIMP-1 compared with solution.

Affinity Purification of the 190-kDa Protein—MCF10A cells were lysed with 0.8 ml/150-mm plate ice-cold lysis buffer (25 mM Tris (pH 7.5), 100 mM NaCl, 1% Nonidet P-40, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 5 mM benzamidine, and 1 mM PMSF). After a centrifugation (20 min, 14,000 rpm), the supernatant was collected and incubated (4 °C) with Affi-Gel 10-pro-MMP-9 batchwise overnight, poured into a column (Polyprey™, Bio-Rad), and the flow-through fraction collected. The matrix was washed with 20 ml of 25 mM Tris (pH 7.5), 150 mM NaCl, 5 mM CaCl₂, 20% Me₂SO, 2 mM PMSF, 5 mM benzamidine, 10 μg/ml leupeptin, and 10 μg/ml aprotinin (wash 1), followed by 10 ml of the same buffer as described above but containing 150 mM NaCl (wash 2).

The 190-kDa protein was eluted from the column with 4.5 ml of 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM CaCl₂, 20% Me₂SO, 2 mM PMSF, 5 mM leupeptin, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM benzamidine, and 1 mM PMSF. After a centrifugation (20 min, 14,000 rpm), the supernatant was collected and incubated (4 °C) with Affi-Gel 10-pro-MMP-9 batchwise overnight, poured into a column (Polyprey™, Bio-Rad), and the flow-through fraction collected. The matrix was washed with 20 ml of 25 mM Tris (pH 7.5), 500 mM NaCl, 0.1% Nonidet P-40, 2 mM PMSF, 5 mM benzamidine, 10 μg/ml leupeptin, and 10 μg/ml aprotinin (wash 1), followed by 10 ml of the same buffer as described above but containing 150 mM NaCl (wash 2).

The elution of pro-MMP-9, pro-MMP-2, and active MMP-9 was determined (6-fold) with a Centriprep-10 (Amicon). The medium was analyzed by silver-stained SDS-PAGE (34) and ligand blot as described below. The flow-through, wash 1, wash 2, and eluate fractions were analyzed by SDS-PAGE to determine the amount of uncoupled pro-MMP-9. The same procedure was used with silver-stained SDS-PAGE (34) and ligand blot as described below. The protein concentrations of each column fraction were determined by the BCA protein assay (Pierce) reagent. The same procedure was used with lysates of surface biotinylated cells, except that 0.5–1 ml of lysates were incubated with 50 μl of Affi-Gel 10-pro-MMP-9 or uncoupled Affi-Gel 10 matrix. After binding, the matrix was washed with 1 ml of cold HNTG buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 10% glycerol) followed by one wash in the same buffer containing 500 mM NaCl and three washes in HNTG buffer. After a brief centrifugation, the bound proteins were eluted with 40 μl of collagenase buffer with 10% Me₂SO and subjected to SDS-PAGE, blotting, and detection by streptavidin-HRP or ligand blot as described below.

Microsequencing—The affinity-purified 190-kDa protein was subjected to 7% SDS-PAGE under reducing conditions, followed by staining with 0.25% Coomassie Brilliant Blue. The band containing the 190-kDa protein was excised from the gel, and the pieces were washed three times (15 min each) with Millipore water. The gel slices were washed three times (15 min each) in 50% acetonitrile, HPLC grade, and then lyophilized to dry and stored at −80 °C. The dried material was reconstituted in 30 μl of a lysis buffer containing HNTG buffer and 10% acetonitrile, HPLC grade, and then extruded for 30 s at 100 psi. The MCF10A cells were grown in the presence of daily additions of ascorbate (75 μg/ml) and the conditioned medium was obtained as described.

Cell Surface Biotinylation—Surface proteins were biotinylated with sulfo-NHS-biotin (Pierce) as described previously (25). The biotinylated cells were lysed with 2 ml/dish of ice-cold lysis buffer, the lysates incubated for 1 h on ice, and the supernatant collected after a 15-min centrifugation (13,000 × g) at 4 °C. The supernatants were analyzed immediately for the presence of pro-MMP-9-binding proteins by pro-MMP-9 immunoprecipitation or immunoprecipitation with pro-MMP-9 and detection with streptavidin-HRP as described below.

Preparation of Confluent Medium—Confluent cultures of MCF10A cells were incubated with serum-free DMEM/F-12 (15 ml/150-mm dish) for 24 h at 37 °C. The medium was collected, centrifuged, and concentrated (6-fold) with a Centriprep-10 (Amicon). The medium was analyzed by immunoblot analysis as described above. In some experiments, cells were grown in the presence of daily additions of ascorbate (75 μg/ml) and the conditioned medium was obtained as described.

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RESULTS

Characteristics of 125I-Pro-MMP-9 Binding to MCF10A Cells—Previously, we showed that exposure of MCF10A cells to 12-O-tetradecanoylphorbol-13-acetate results in the secretion of pro-MMP-9 that associates with the cell surface (25). To measure the binding of pro-MMP-9, we carried out binding...
assays of radioiodinated enzyme to untreated MCF10A cells to avoid interference with the endogenously produced pro-MMP-9 (25). Fig. 1A shows time-dependent binding of $^{125}$I-pro-MMP-9 to MCF10A cells at 4 °C. Logarithmic transformation of the data revealed a $k_m$ value of 6.94 ± 0.67 × 10^4 M$^{-1}$ s$^{-1}$ (Fig. 1A, inset). Incubation of the cells with increasing concentrations (1–40 nM) of $^{125}$I-pro-MMP-9 in the presence of 80-fold excess of unlabeled enzyme demonstrated a specific saturable binding (Fig. 1B). Scatchard analysis revealed a $K_d$ value of 2.16 ± 0.16 × 10^{-8} M and 1.3 × 10^6 sites/cell (Fig. 1B, inset). Similar values were obtained after nonlinear curve-fitting analysis of the data ($K_d = 2.49 ± 0.27 × 10^{-8}$ M and 1.4 × 10^5 sites/cell). We found no evidence of cell surface activation of $^{125}$I-pro-MMP-9, as determined by SDS-PAGE followed by autoradiography or by gelatin zymography (data not shown).

Ligand binding assays were also performed with prostate carcinoma PC3 cells ($K_d = 2.62 ± 0.56 × 10^{-8}$ M and 5.5 × 10^4 sites/cell) and breast carcinoma MDA-MB-231 cells ($K_d = 2.13 ± 0.23 × 10^{-8}$ M and 1.08 ± 0.1 × 10^5 sites/cell). In addition, similar $k_m$ values ($-6.7-8.1 × 10^{-4}$ M$^{-1}$ s$^{-1}$) were obtained with both cell lines. We also measured the specific binding of $^{125}$I-pro-MMP-9 to HT1080 and RVE cells and obtained the following values: HT1080 cells, 7 fmol/10^5 cells; RVE cells, 12.4 fmol/10^5 cells.

Binding specificity for pro-MMP-9 was determined in MCF10A cells using pro-MMP-2 as a competitor as described under “Experimental Procedures.” These studies showed that pro-MMP-2 caused a 27% decrease in specific $^{125}$I-pro-MMP-9 binding with 3.3 fmol/1.3 × 10^5 cells in the presence of unlabeled pro-MMP-2 compared with 4.5 fmol/1.3 × 10^5 cells with pro-MMP-9 alone. Thus, pro-MMP-2 slightly competes with pro-MMP-9 for the same binding site on MCF10A cells.

Cellular Distribution of $^{125}$I-Pro-MMP-9 and a $^{125}$I-Pro-MMP-9-TIMP-1 Complex After Binding to MCF10A Cells—We investigated the fate of pro-MMP-9 after binding using ligand internalization studies as described under “Experimental Procedures.” Fig. 2A shows that the amount of $^{125}$I-pro-MMP-9 associated with the cell pellet (internalized ligand) reached a maximum of 21% of the total ligand bound after 10 min of incubation at 37 °C. Later time points showed little or no change (<5%) in the radioactivity of the pellet fraction. Concurrently, after a 10-min incubation, ~80% of the total cell-associated radioactivity was recovered by Pronase E digestion (representing cell surface-bound enzyme). This fraction exhibited a decline in radioactivity consistent with dissociation of cell surface-bound ligand and its appearance in the supernatant fraction (Fig. 2A). SDS-PAGE analysis demonstrated that the radioiodinated enzyme in the supernatant fraction remained in the latent form at all times examined. In contrast, the internalized enzyme was degraded to low (>20 kDa) molecular mass fragments (data not shown). Thus, binding of pro-MMP-9 to MCF10A cells is not followed by significant ligand internalization.

Binding studies with urokinase plasminogen activator demonstrated that binding of this activator to its receptor in the presence of plasminogen activator inhibitor-1 results in rapid internalization of the enzyme/inhibitor complex (36). To determine if a similar process could occur with pro-MMP-9, we examined the cellular partitioning of a $^{125}$I-pro-MMP-9-TIMP-1 complex after binding to MCF10A cells as described above. These experiments showed no significant differences in the cellular distribution of the free (Fig. 2A) versus that of the complex (Fig. 2B) ligand after incubation at 37 °C for up to 2 h. Furthermore, the association of pro-MMP-9 with TIMP-1 did not alter the rate of ligand dissociation into the supernatant fraction as >90% of the radioactivity in this fraction was tri-chloroacetic acid-precipitable. Additionally, autoradiograms of the samples after SDS-PAGE showed no differences in cellular distribution between free and complexed zymogen (data not shown).

Identification of a 190-kDa Cell Surface Protein That Binding to MCF10A Cells—The above binding data were consistent with the existence of a single pro-MMP-9-binding component on the cell surface. To identify the putative pro-MMP-9-binding protein, we carried out an affinity purification using immobilized pro-MMP-9 (Affi-Gel 10-pro-MMP-9). Lysates of surface-biotinylated MCF10A cells were incubated with Affi-Gel 10-pro-MMP-9 matrix or uncoupled Affi-Gel 10 and the bound proteins were eluted with 10% Me2SO. The eluted proteins were then detected by either streptavidin-HRP or ligand blot analysis as described under “Experimental Procedures.” Fig. 3 shows that MCF10A cells express a major 190-kDa protein that specifically and consistently binds to the Affi-Gel 10-pro-MMP-9 matrix (Fig. 3, A, lane 2; and B, lanes 2, 4, and 5) but not to the
uncoupled matrix (Fig. 3, A and B, lanes 1 and 3). Several minor biotinylated proteins (~70–90 kDa) were found to bind, albeit inconsistently, to the pro-MMP-9 affinity matrix (Fig. 3A, lane 2). The 190-kDa protein was detected by streptavidin-HRP (Fig. 3A, lane 2) consistent with biotinylation and consequently, cell surface localization. The streptavidin detection was specific since blots of samples derived from lysates of non-biotinylated cells and incubated with Affi-Gel 10-pro-MMP-9 matrix were negative (Fig. 3A, lane 4). The presence of the 190-kDa protein in the non-biotinylated cells was confirmed by ligand blot analysis (Fig. 3B, lane 4). Electrophoretic migration of the 190-kDa protein was similar under nonreducing conditions (Fig. 3B, lane 5) or reducing conditions (Fig. 3A, lanes 2 and 4). In contrast, under nonreducing conditions, pro-MMP-9 exhibited the presence of monomer and dimer forms (Fig. 3B, lane 6), as expected (37).

To determine whether the 190-kDa protein forms a precipitable complex with pro-MMP-9, lysates of MCF10A cells were incubated with exogenous pro-MMP-9 and subjected to a co-immunoprecipitation experiment with anti-MMP-9 antibodies as described under “Experimental Procedures.” The immunoprecipitates were analyzed by ligand blot analysis. This experiment showed that the 190-kDa protein co-precipitates with pro-MMP-9 (Fig. 3C). In the absence of enzyme, the 190-kDa protein was not detected, demonstrating the specificity of the interaction. Taken together, these results demonstrate that MCF10A cells express a 190-kDa cell surface protein that specifically binds to pro-MMP-9.

Affinity Purification of the 190-kDa Protein and Hydrodynamic Studies—The 190-kDa protein was purified from lysates of MCF10A cells using the Affi-Gel 10-pro-MMP-9 matrix as described under “Experimental Procedures.” Fig. 4 shows the analysis of the column fractions in a silver-stained SDS-PAGE (Fig. 4A) and by ligand blot analysis (Fig. 4B). The 190-kDa protein was detected in the 20% Me₂SO eluate (Fig. 4, A and B, lane 2). Analysis of the protein content in each fraction indicated a ~3300-fold purification. The affinity-purified 190-kDa protein was subjected to hydrodynamic studies for determination of the sedimentation coefficient and Stokes radius to determine the native molecular mass. As shown in Fig. 5A, the 190-kDa protein was detected in six fractions derived from the gradient as shown by ligand blot analysis (Fig. 5A, inset). A sedimentation coefficient of 7.9 S was calculated from two determinations using known protein standards. The Stokes radius of the 190-kDa protein was determined by gel filtration using protein standards (Fig. 5B) and was calculated to be 52.6 Å. By combining the sedimentation and Stokes radius (38), a native molecular mass of 192,000 was calculated. These data and the electrophoretic mobility of the 190-kDa protein on SDS-PAGE (Fig. 3) indicate that this protein is monomeric.

The 190-kDa Pro-MMP-9-binding Protein Is the α2(IV) Chain of Collagen IV—The affinity-purified 190-kDa protein was submitted for microsequencing as described under “Experimental Procedures.” Analyses of three HPLC-purified peptides...
obtained after tryptic digestion revealed the following amino acid sequences: GVS/GFPGAD/GIP/GHPQGG/PRG, DGYQ/GPD/GPRG, and KIAQPGTVGFGQ, which correspond to residues 109–128, 325–335, and 1396–1408, respectively, of the human α2(IV) chain of collagen IV (39). Immunoblot analysis of the 190-kDa protein demonstrated reactivity with three different mAbs (H22, H25, and H21) against the human α2(IV) chain (Fig. 6A). Furthermore, co-immunoprecipitation of the 190-kDa protein from an MCF10A cell lysate with exogenous pro-MMP-9 using an anti-MMP-9 antibody followed by immunoblot analysis with H22 mAb further demonstrated that the coprecipitated 190-kDa protein is the α2(IV) chain (Fig. 6B).

**Specificity of the Binding of 125I-Pro-MMP-9 to α2(IV)**——To evaluate the role of α2(IV) in the surface binding of pro-MMP-9, a binding assay was carried out with 125I-pro-MMP-9 that was preincubated with affinity-purified α2(IV), as described under “Experimental Procedures.” The results showed a 78% reduction in specific binding, from 4.5 to 1.0 fmol of pro-MMP-9 bound/1.3 × 105 cells, when 125I-pro-MMP-9 (3.6 nm) was incubated with 2-fold molar excess (~7 nm) of α2(IV) before the binding assay. Since α2(IV) is a substrate of MMP-9 (shown below), MCF10A cells were incubated (45 min, 37 °C) with MMP-9 (1.2 pmol/well) to remove surface-associated α2(IV). This treatment resulted in an 80% reduction in 125I-pro-MMP-9-specific binding (from 6.4 to 1.4 fmol/1.3 × 105 cells). In contrast, when the cells were treated with MMP-9 in the presence of TIMP-1, a significant recovery of 125I-pro-MMP-9-specific binding (from 1.4 to 4.4 fmol/1.3 × 105 cells) was observed, suggesting that TIMP-1 prevented the degradation of the surface-associated α2(IV). Collectively, these experiments suggest that the cell surface binding of pro-MMP-9 is mediated by α2(IV).

**Expression of α2(IV) in Cultured Cells and Binding to Pro-MMP-9**——We examined the expression of α2(IV) in cell extracts and on the surface of MCF10A, MDA-MB-231, HT1080, RVE, and CD3 cells by surface biotinylation, immunoblots and ligand blot analyses. Fig. 7 shows that all the cells examined express α2(IV) that specifically bound to the pro-MMP-9-affinity matrix (Fig. 7, A–D) or co-precipitated with pro-MMP-9 (Fig. 7E). Surface biotinylation followed by Affi-Gel 10-pro-MMP-9 purification demonstrated that α2(IV) is readily detected on the surface of MCF10A (Fig. 7B, lane 1), HT1080 (Fig. 7B, lane 3) and RVE (Fig. 7D) cells but could not be detected on the surface of MDA-MB-231 cells (Fig. 7B, lane 2). However, in the latter, α2(IV) was identified in the cell lysate (Fig. 7A, lane 2) consistent with the ligand binding data demonstrating a reduced number of pro-MMP-9 binding sites/cell in the MDA-MB-231 cells. Expression of α2(IV) was also detected in mouse endothelial CD3 cells by co-immunoprecipitation with pro-MMP-9 and detection by ligand blot analysis (Fig. 7E, panel 5). Thus, these studies demonstrate that monomeric α2(IV) is expressed by a variety of cells and can be detected on the cell surface.

**Binding of Pro-MMP-9 to Collagen IV**——The identification of α2(IV) and its binding to pro-MMP-9 raised the question about interactions of this enzyme with triple-helical collagen IV. Since the most abundant collagen IV is a heterotrimeric protein composed of two α1(IV) and one α2(IV) chains (40), we examined whether MCF10A cells produce trimeric collagen IV and whether it binds to pro-MMP-9. Concentrated serum-free conditioned medium of MCF10A cells was subjected to SDS-PAGE under nonreducing conditions, followed by immunoblot analysis using various antibodies to collagen IV. As shown in Fig. 8, mAbs to either the α2(IV) (Fig. 8, lane 1, mAb 1910) or α3(IV) (Fig. 8, lane 2, H11) chains recognized a protein of >450 kDa, likely to represent trimeric collagen IV. In contrast, H22 antibodies against α2(IV) did not react with trimeric collagen IV (Fig. 8, lane 3) suggesting that its epitope is not exposed under nonreducing conditions. A protein of ~160 kDa was also detected, although to a lower extent, with both H11 and H22 antibodies. The nature of this protein is unknown. It should be noted that, under these conditions, monomeric α2(IV) chain
could not be detected in the media of MCF10A cells by immunoblot and ligand blot analysis. Furthermore, growth of MCF10A cells in the presence of daily added ascorbate (75 μg/ml) did not alter the localization and amounts of the α2(IV) chain (data not shown).

The ability of pro-MMP-9 to bind collagen IV was tested by a co-immunoprecipitation experiment after the addition of pro-MMP-9 to the media of MCF10A cells. As control, pro-MMP-9 was also added to a cell lysate to co-precipitate α2(IV). These studies demonstrated that, although the cell-associated α2(IV) coprecipitated with pro-MMP-9 (Fig. 9A, lane 2), the secreted trimeric collagen IV did not (Fig. 9A, lane 1). Additionally, the collagen IV in the conditioned medium did not bind to the pro-MMP-9-affinity matrix (data not shown). The interaction of pro-MMP-9 with native collagen IV was further investigated using purified EHS collagen IV and the pro-MMP-9-affinity matrix as described under “Experimental Procedures.” After the affinity step, the bound and unbound fractions were analyzed by ligand blot analysis under reducing conditions for the presence of mouse collagen IV chains. Fig. 9B shows that mouse collagen IV did not bind to the affinity matrix (Fig. 9B, lane 2). However, the α1(IV) and α2(IV) chains were readily detected in the unbound fraction (Fig. 9C, lane 2). It should be noted that, under the conditions of the ligand blot analysis, the mouse α1(IV) chain was detected. Taken together, these results suggest that pro-MMP-9 binds to monomeric α2(IV) with an affinity greater than that to triple-helical collagen IV.

**Domain Analysis of Pro-MMP-9-α2(IV) Interactions**—To define the pro-MMP-9 domains that interact with α2(IV), we examined the binding of a pro-MMP-9-TIMP-1 complex since TIMP-1 is known to bind to the C-terminal domain of pro-MMP-9 (4, 37, 41). In addition, we questioned whether the active form of MMP-9, lacking the N-terminal domain, binds α2(IV). A lysate of MCF10A cells was incubated with either a pro-MMP-9-TIMP-1 complex or with MMP-9. The proteins were then immunoprecipitated with either anti-MMP-9 or anti-TIMP-1 antibodies and the immunoprecipitates were resolved by ligand blot analysis. As shown in Fig. 10, α2(IV) coprecipitated with pro-MMP-9, as expected (Fig. 10A, lane 1). In contrast, TIMP-1 alone did not bind to α2(IV) (Fig. 10A, lane 2). In the presence of a pro-MMP-9-TIMP-1 complex, α2(IV) was co-immunoprecipitated by either anti-TIMP-1 (Fig. 10A, lane 4) or anti-MMP-9 antibodies (Fig. 10A, lane 3). Thus, the pro-MMP-9-TIMP-1 complex interacts with α2(IV). Development of the same blot with anti-TIMP-1 antibodies (Fig. 10B, lanes 5–8) showed TIMP-1 in the samples containing inhibitor (Fig. 10B, lanes 6–8). When MMP-9 was added to MCF10A lysates, α2(IV) could not be detected in the immunoprecipitate (Fig. 10C, lane 9) or in the supernatant fraction of the immunoprecipitates suggesting that it was probably degraded by MMP-9. However, α2(IV) coprecipitated with the active enzyme (Fig. 10C, lane 10) when the experiment was carried out in the presence of 10 mM EDTA. Thus, neither the N-terminal nor the C-terminal domain (at least the region of TIMP-1 interaction) of pro-MMP-9 appears to be critical for binding to α2(IV). However, we have consistently found that a pro-MMP-9-α2(IV) complex failed to co-precipitate with gelatin-agarose matrix (data not shown), suggesting a role for the gelatin-binding domain in the interaction of pro-MMP-9 with α2(IV).

**Affinity of Pro-MMP-9 and Pro-MMP-2 for α2(IV)**—Since pro-MMP-9 bears a high degree of sequence similarity with pro-MMP-2 and both possess a gelatin-binding domain (41), we determined the ability of pro-MMP-2 to compete for the binding of α2(IV) to pro-MMP-9. The α2(IV) was co-immunoprecipitated with pro-MMP-9 and anti-MMP-9 antibodies in the absence or presence of pro-MMP-2 (equivolum or 5-fold molar excess, relative to pro-MMP-9) and in the presence of EDTA to prevent degradation of α2(IV) by any trace of MMP-2. The immunoprecipitates were resolved by ligand blot analysis. As shown in Fig. 11, α2(IV) co-precipitates only in the presence of pro-MMP-9 (Fig. 11A, lanes 2–4). No significant differences in the amounts of α2(IV) co-precipitating with pro-MMP-9 were detected in the presence of equal molar amounts (Fig. 11A, lane 3) or excess (5-fold) molar amounts of pro-MMP-2 (Fig. 11A, lane 4), suggesting that under these conditions only pro-MMP-9 bound to α2(IV). In agreement with these results, α2(IV) incubated with pro-MMP-2 alone did not form a co-precipitable complex as

![Figure 6](image6.png)  
**Fig. 6. Immunoblot analysis of the 190-kDa protein with anti-α2(IV) antibodies.** A, affinity-purified α2(IV) (50 ng/lane) was subjected to 4–12% SDS-PAGE under reducing conditions followed by immunoblot analysis with three different mAbs, H22, H25 and H21, to the human α2(IV) chain and detection by ECL. B, a lysate (1 ml) of MCF10A cells prepared as described under “Experimental Procedures” was incubated (1 h, 4°C) with (+) or without (−) pro-MMP-9 (0.5 μg/ml) and then immunoprecipitated with anti-MMP-9 antibodies and protein G-Sepharose beads. The immunoprecipitates were resolved by 4–12% SDS-PAGE under reducing conditions, followed by immunoblot analysis with H22 antibodies and detection by ECL. IP, immunoprecipitation; IB, immunoblot.

![Figure 7](image7.png)  
**Fig. 7. Expression of α2(IV) in cultured cells.** MCF10A (lane 1), MDA-MB-231 (lane 2), HT1080 (lane 3), RVE (panel 4), and CD3 (panel 5) cells were examined for α2(IV) expression in lysates of non-biotinylated (A, C, and E) or surface-biotinylated cells (B and D). Samples were incubated with either Affi-Gel 10-pro-MMP-9 (+) or Affi-Gel 10 alone (−) (A–D) or subjected to a co-immunoprecipitation with pro-MMP-9 (+) and anti-pro-MMP-9 antibodies (+) (E). Detection of α2(IV) was achieved by immunoblot with anti-α2(IV) antibodies (A and C) or streptavidin-HRP (B and D), or by ligand blot analyses with pro-MMP-9 as a probe (E). Asterisks indicate the position of α2(IV). Arrow shows pro-MMP-9 detected in the ligand blot.
determined using anti-MMP-2 antibodies and ligand blot analysis (Fig. 11A, lane 6). Consistently, pro-MMP-2 was detected in the immunoprecipitates when the same blot was developed with anti-MMP-2 antibodies (Fig. 11B, lane 8).

To obtain a quantitative measurement of the relative affinities of pro-MMP-9 and pro-MMP-2 for the α2(IV) chain, we examined the ability of α2(IV) to form a complex with either pro-MMP-2 or pro-MMP-9 by co-chromatography and the dissociation constants (K_d) were determined as described under “Experimental Procedures.” Fig. 12 shows that both pro-MMP-9 and pro-MMP-2 bind α2(IV) as a function of proenzyme concentration. Under these conditions, maximal binding was observed at concentrations of enzymes of ~150 nM for pro-MMP-9 and >750 nM for pro-MMP-2. From these data, the K_d values of α2(IV) for pro-MMP-9 and pro-MMP-2 were calculated to be approximately 45 nM and ≥350 nM, respectively.

**DISCUSSION**

Previously, we have shown the surface association of pro-MMP-9 in 12-O-tetradecanoylphorbol-13-acetate-treated MCF10A cells (25). Other studies have described the presence of pro-MMP-9 on plasma membranes of HT1080 cells (23) and in focal contacts of endothelial cells (22). Here, we have characterized the binding parameters of pro-MMP-9 and demonstrated that the proenzyme binds with high affinity (K_d ∼20–30 nM) to the surface of a variety of cell types. In MCF10A cells, internalization studies demonstrated that binding of pro-MMP-9, free or in complex with TIMP-1, is not followed by ligand internalization. Furthermore, under the experimental conditions, surface binding of pro-MMP-9 does not result in zymogen activation, possibly due to the lack of activators in the cell culture system. However, recent studies demonstrated that surface-associated pro-MMP-9 can be activated by a plasmin-dependent mechanism (23, 24). Taken together, these findings suggest that the surface association of pro-MMP-9 is not directly associated with activation and/or internalization but plays a role in confinement of proenzyme in areas of cell-matrix contacts, where it could become the target for potential pro-MMP-9-activating proteinases.

We identified a 190-kDa cell surface protein in various cell lines that specifically bound to a pro-MMP-9-affinity matrix

**FIG. 8.** Expression of collagen IV in the media of MCF10A cells. Aliquots (20 μl) of concentrated serum-free conditioned media of MCF10A cells were subjected to 4–12% SDS-PAGE under nonreducing conditions followed by transfer to a nitrocellulose membrane. Collagen IV was probed with mAbs against either α2(IV) (mAb 1910 (lane 1) and H22 (lane 3)) or α1(IV) (H11 (lane 2)), and detection was performed using ECL as described under “Experimental Procedures.” The arrow demonstrates the position of the trimeric collagen IV of ~450 kDa.

**FIG. 9.** Binding of pro-MMP-9 to collagen IV. A, concentrated serum-free conditioned medium (lane 1) or a lysate (lane 2) of MCF10A cells were incubated (1 h, 4 °C) with pro-MMP-9 (0.5 μg/ml) and then immunoprecipitated with anti-MMP-9 antibodies and protein G- Sepharose beads. The immunoprecipitates were resolved by 4–12% SDS-PAGE under reducing conditions followed by ligand blot analysis. B and C, EHS collagen IV (10 μg/ml) was incubated (overnight, 4 °C) with the Affi-Gel 10-pro-MMP-9 matrix (30 μl) in lysis buffer (0.5 ml, final volume). After incubation, the supernatant was collected and the beads were processed as described under “Experimental Procedures.” The proteins in the bound (B) and the unbound (C) fractions were resolved by 4–12% SDS-PAGE under reducing conditions followed by ligand blot analysis. B, lane 2, bound fraction; C, lane 2, unbound fraction. Asterisks show the α2(IV) chain. Open arrows in C indicate the position of the α1(IV) and α2(IV) chains of mouse EHS collagen IV. Lane 1 in B and C shows the affinity-purified α2(IV), as a control.

**FIG. 10.** Binding of a pro-MMP-9-TIMP-1 complex and MMP-9 to α2(IV). Lysates (0.5 ml) of MCF10A cells were incubated (1 h, 4 °C) with either pro-MMP-9 (0.5 μg/ml) (lanes 1 and 5), TIMP-1 (0.33 μg/ml) (lanes 2 and 6), a pro-MMP-9-TIMP-1 complex (lanes 3, 4, 7, and 8) or with MMP-9 (0.5 μg/ml) (lanes 9 and 10) in the absence (lanes 1–9) or presence (lane 10) of 5 mM EDTA. The samples were immunoprecipitated with mAbs to either pro-MMP-9 (CA-209) (lanes 1, 3, 5, 7, 9, and 10) or TIMP-1 (1M 322) (lanes 2, 4, 6, and 8). The immunoprecipitates were resolved by 4–12% SDS-PAGE under reducing conditions and the proteins detected by ligand blot analysis (A and C). The membrane in A was subsequently developed by immunoblot analysis (B) using a rabbit anti-TIMP-1 polyclonal antibody. The proteins of approximately 55 and 20 kDa are the heavy and light chains of the IgG molecule, respectively.

**FIG. 11.** Co-immunoprecipitation of pro-MMP-9 with α2(IV) and competition with pro-MMP-2. Affinity-purified α2(IV) (~0.5 μg) was incubated (1 h, 4 °C) without (lanes 1, 5, and 7) or with 5 nM pro-MMP-9 (lanes 2–4) or with 5 nM pro-MMP-2 (lanes 6 and 8) in the presence of 10 mM EDTA. The samples in lanes 3 and 4 also received 5 nM (1:1 pro-MMP-9/pro-MMP-2 molar ratio) and 25 nM pro-MMP-2 (1:5 pro-MMP-9/pro-MMP-2 molar ratio), respectively. The samples were immunoprecipitated with either anti-pro-MMP-9 (CA-209) (lanes 1–4) or anti-MMP-2 (CA-801 and CA-805) (lanes 5–8) antibodies as described under “Experimental Procedures.” A, the immunoprecipitates were resolved by 4–12% SDS-PAGE under reducing conditions followed by ligand blot analysis. B, the samples in lanes 5 and 6 from A were examined by immunoblot analysis using a polyclonal antibody to pro-MMP-2. The protein of approximately 55 kDa is the heavy chain of the IgG molecule.
and formed a co-precipitable complex with pro-MMP-9. Microsequencing of the affinity-purified protein revealed a complete sequence homology to the α2(IV) chain of human collagen IV (39). Additionally, immunoblot analysis with chain-specific antibodies (30) corroborated that the 190-kDa protein is α2(IV). Several observations indicate that the cell surface association of pro-MMP-9 is mediated by α2(IV). (i) Preincubation of pro-MMP-9 with affinity-purified α2(IV) significantly inhibited ligand binding. (ii) Pretreatment of MCF10A cells with MMP-9 reduced by 80% the binding of proenzyme, likely due to the degradation of surface-associated α2(IV) as observed in experiments with soluble α2(IV) and MMP-9. Accordingly, treatment of cells with MMP-9 and TIMP-1 resulted in the recovery of the majority of ligand binding; (iii) Binding of pro-MMP-9 to the cells exhibited an affinity (Kd = 22 nM) that was in close agreement with the affinity (Kd = 45 nM) of the enzyme for purified α2(IV). In addition, there was a good correlation between the number of pro-MMP-9 binding sites determined in the binding assays and the level of expression of surface-associated α2(IV). For example, the breast cancer MDA-MB-231 cells that exhibited 10-fold less binding sites than MCF10A cells showed undetectable levels of surface-associated α2(IV). The reason for the low levels of α2(IV) on the surface of these malignant cells is unknown but may be related to their inability to retain α2(IV) on the cell surface since α2(IV) was detected intracellularly. Thus, in vitro, the surface association of pro-MMP-9 would depend on the ability of each cell type to express and retain α2(IV) on the cell surface. Whether pro-MMP-9 and pro-MMP-2 bind also to monomeric α1(IV) is yet unknown and remains to be determined. However, the fact that only a single polypeptide, α2(IV), was specifically and consistently co-precipitated with pro-MMP-9 or bound to immobilized proenzyme suggests a unique and preferential interaction between pro-MMP-9 and α2(IV).

Co-immunoprecipitation experiments using a pro-MMP-9-TIMP-1 complex or MMP-9 demonstrated that neither the C-terminal nor the N-terminal domains of pro-MMP-9 appeared to be critical for interactions with α2(IV). However, we could not precipitate the α2(IV)/pro-MMP-9 complex with gelatin-agarose beads, suggesting a role for the gelatin-binding domain. In this regard, it was interesting to observe that pro-MMP-2, when compared with pro-MMP-9, exhibited a lower affinity for α2(IV) as determined by the co-immunoprecipitation and gel filtration experiments. Since pro-MMP-2 contains a similar gelatin binding domain (41), this suggests that the interactions of α2(IV) with pro-MMP-9 are also influenced by sites/domains other than the gelatin binding domain and/or by the three-dimensional conformation of pro-MMP-9. It would be of interest to determine whether the 54-amino acid proline-rich α2(IV)-like extension that is uniquely present in pro-MMP-9 (17, 41), plays any role in the binding of the enzyme to α2(IV). In the cell binding assays, pro-MMP-2 slightly competed with pro-MMP-9 binding, suggesting that surface-associated α2(IV) is also available for pro-MMP-2 binding. However, given the lower affinity of pro-MMP-2 for α2(IV) and the existence of alternate high affinity surface binding sites for pro-MMP-2 (14), this enzyme would be expected to bind to the cell surface via a different mechanism (14, 16).

The binding of gelatinases to collagen IV has been examined in previous studies (21, 42); however, the sites of interaction were not defined. The results presented here suggest that a major high affinity binding site for pro-MMP-9 in collagen IV must reside within the α2(IV) chain. However, our data also indicate that neither triple-helical collagen IV secreted by MCF10A cells nor EHS collagen IV bound to pro-MMP-9 under conditions of α2(IV) binding. In agreement with these results, we have recently observed a weak affinity (Kd = 2.15 μM) of pro-MMP-9 for EHS collagen IV as determined by surface plasmon resonance. Thus, whereas monomeric α2(IV) forms a tight complex with pro-MMP-9 (Kd, nM), trimeric collagen IV binds pro-MMP-9 with very low affinity (Kd, μM). This is also consistent with the studies of Steffensen et al. (21), who showed a weaker affinity of a recombinant gelatin-binding domain of pro-MMP-2 for trimeric collagen IV compared with that for denatured collagen IV. This has led to the suggestion that binding sites for the gelatin-binding domain in denatured collagen IV may be masked in triple-helical collagen IV (21). Therefore, it is conceivable that the binding of pro-MMP-9 to α2(IV) is mediated by sites that are cryptic in collagen IV and that are exposed after partial denaturation and/or degradation of the collagen IV molecule. If so, these sites would have to be conserved after partial proteolysis to allow binding of pro-MMP-9. Such a scenario would probably involve a partial degradation of the collagen IV network by a protease(s) other than the gelatinases as the catalytic efficiency of MMP-2 and MMP-9 against native collagen IV has been reported to be limited (43–45). After collagen IV degradation, secretion of pro-MMP-9 by pro-MMP-9 producing-cells in close association with the basement membrane would then facilitate binding of pro-MMP-9 to α2(IV). After activation of the α2(IV)-bound pro-MMP-9, the enzyme would then contribute to the complete degradation of the collagen IV network consistent with its ability to degrade denatured collagens (41).

Another possible scenario for the results observed here may involve binding of pro-MMP-9 to a surface-associated α2(IV). Although the origin and binding mechanism of α2(IV) chains to the cell surface remain to be elucidated, our in vitro studies with a variety of cell lines (epithelial, endothelial and fibrosarcoma) clearly show that, although some α2(IV) chains are assembled with α1(IV) into trimeric collagen IV molecules, a portion of single α2(IV) chains are secreted and deposited on the cell surface. The surface-associated α2(IV) chains appear to be stable as determined by surface biotinylation and pulse-chase analysis. Indeed, we have found that α2(IV) chains can be detected in the cellular compartment of both MCF10A and HT1080 cells and in the supernatant of the latter even after a

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4 M. Olson and R. Fridman, unpublished results.
10-h chase period without any evidence of processing and/or degradation. These findings are in contrast with the general truth that active proteinases are present in the experimental system, as they can hydrolyze non-triple-helical collagen chains as shown here with α2(IV) and MMP-9. Thus, the integrity and eventually the detection of α(IV) chains would depend on the presence of active proteinases and/or inhibitors in each particular culture system and tissue. Detection of monomeric α(IV) chains may also depend on the extraction procedures and/or on the antibodies used since some antibodies may only recognize α(IV) chains in triple-helical conformation. Here we have used immobilized pro-MMP-9 and chain-specific mAbs raised against synthetic peptides, which independently allowed for the detection of native and stable α2(IV) chains on the cell surface. Although the processing, localization and stability of monomeric α2(IV) warrants further in vitro and in vivo studies, it is tempting to speculate that surface-associated α2(IV) may anchor pro-MMP-9 on the cell surface after autocrine or paracrine secretion. After activation by pro-MMP-9-activating enzymes, MMP-9 would then play a role in localized surface proteolysis. Since the binding of pro-MMP-9 to the α2(IV) chain is of high affinity and involves the zymogen form, it will be important in future studies to address the activation of pro-MMP-9 and its interactions with TIMP-1 in the context of pro-MMP-9 bound to α2(IV).

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