Pro-collagenase-1 (Matrix Metalloproteinase-1) Binds the α2β1 Integrin upon Release from Keratinocytes Migrating on Type I Collagen*

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In injured skin, collagenase-1 (matrix metalloproteinase-1 (MMP-1)) is induced in migrating keratinocytes. This site-specific expression is regulated by binding of the α2β1 integrin with dermal type I collagen, and the catalytic activity of MMP-1 is required for keratinocyte migration. Because of this functional association among substrate/ligand, receptor, and proteinase, we assessed whether the integrin also directs the compartmentalization of MMP-1 to its matrix target. Indeed, pro-MMP-1 co-localized to sites of α2β1 contacts in migrating keratinocytes. Furthermore, pro-MMP-1 co-immunoprecipitated with α2β1 from keratinocytes, and α2β1, co-immunoprecipitated with pro-MMP-1. No other MMPs bound α2β1, and no other integrins interacted with MMP-1. Pro-MMP-1 also provided a substrate for α2β1-dependent adhesion of platelets. Complex formation on keratinocytes was most efficient on native type I collagen and reduced or ablated on denatured or cleaved collagen. Competition studies suggested that the interaction of pro-MMP-1 with α2β1 confines this proteinase to points of cell contact with collagen and that the ternary complex of integrin, enzyme, and substrate function together to drive and regulate keratinocyte migration.

Cells, either resting or activated, use a variety of surface receptors to sense the presence and location of specific molecules in the extracellular space. For example, integrin-ligand interactions tell cells which structural proteins they have encountered in the extracellular space, and in turn, these contacts activate signaling pathways involved in differentiation, proliferation, and gene expression, among other processes. During migration, cells need to proteolyze, to some extent, nearby extracellular matrix proteins, and hence, cell-matrix contacts can also instruct cells which proteinases are needed and where the enzyme should be targeted and released.

An example of cell-matrix-mediated spatial regulation of proteolysis is seen with collagenase-1 (MMP-1),* a matrix metalloproteinase, in human cutaneous wounds. In response to injury, collagenase-1 is induced in basal epidermal cells (keratinocytes) as the cells move off of the basement membrane and contact native type I collagen in the underlying dermis (1), and this inductive response is specifically controlled by the collagen-binding integrin α2β1 (2). As we demonstrated in various experiments, the catalytic activity of collagenase-1 is required and sufficient for keratinocyte migration on complex matrices containing type I collagen. For example, keratinocytes plated on mutant, collagenase-resistant type I collagen do not migrate, even in the presence of fibronectin and vitronectin; yet they express MMP-1 at levels equivalent to those released by cells on wild-type collagen (2). Keratinocyte migration is also completely inhibited by anti-collagenase-1 antibodies, which block the catalytic activity of the enzyme, and by anti-α2β1 blocking antibodies (2). Thus, MMP-1, collagen, and α2β1 function together in migrating keratinocyte during re-epithelialization of cutaneous wounds.

It is becoming increasingly clear that extracellular proteolysis is a cell-regulated process. After all, cells do not release proteases indiscriminately, especially enzymes like MMP-1 with such a defined substrate specificity. Rather they rely on precise interactions to accurately degrade, cleave, or process specific substrates in the pericellular space. Indeed, an emerging concept is that metalloproteinases, as for some serine and cysteine proteinases (3–6), are anchored to the cell membrane, thereby targeting their catalytic activity to specific substrates within the pericellular space. In recent years, specific cell-MMP interactions have been reported, such as the binding of gelatinase-A (MMP-2) to cell membranes (7) and to the integrin α5β1 (8), gelatinase-B (MMP-9) to CD44 (9), and matrilysin (MMP-7) to heparan sulfate proteoglycans (10). The membrane-type MMPs (MMP-14, -15, -16, -17, -24, and -25) are single-pass transmembrane proteins that are fixed and active at the cell surface and, in addition to acting as proteinases, may provide docking sites for other MMPs. Indeed, pro-MMP-2 also interacts with MMP-14 and TIMP-2 (tissue inhibitor of metalloproteinase 2) on the cell surface, and this trimeric complex is essential for activation of this gelatinase (11–14). It is likely that other MMPs are also attached to cells via specific interaction to membrane proteins, and determining these anchors will lead to identifying activation mechanisms and relevant substrates.

Because the α2β1 integrin regulates MMP-1 expression by binding the substrate of the enzyme, we assessed whether MMP-1 interacts with this collagen receptor on the surface of immunoprecipitation; IB, immunoblotting; BSA, bovine serum albumin; PAS, protein A-Sepharose.

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The abbreviations used are: MMP, matrix metalloproteinase; IP, immunoprecipitation; IB, immunoblotting; BSA, bovine serum albumin; PAS, protein A-Sepharose.
keratinocytes. We report here that pro-MMP-1 specifically binds the α5β1 integrin on keratinocytes migrating on type I collagen. Formation of the α5β1-pro-MMP-1 complex may provide a mechanism to increase the localized concentrations of enzyme, in turn facilitating the cleavage of type I collagen and keratinocyte migration across the dermal wound bed.

**EXPERIMENTAL PROCEDURES**

**Enzymes and Substrates**—Human pro-MMP-1 was purified by two-step chromatography from conditioned medium of phorbol-treated U937 cells as described (15). Matrilysin was purchased from Chemicon International, Temecula, CA. To isolate pro-MMP-1 from the cleavage products and metalloproteinases, autolysis of purified zymogens was induced with 1 mM 4-aminophenylmercuric acetate (Sigma) for 1 h at room temperature, and the two fragments were separated by size selection dialysis in 0.5 ml of 0.05 M Tris, 0.01 M CaCl2 using a 10,000 Molecular Weight Cut-Off Slide-a-Lyzer (Pierce). The purity and integrity of these fragments were verified by SDS-polyacrylamide gel electrophoresis. Cleaved collagen was prepared by incubating 500 μg of bovine type I monomeric collagen (Vitrogen; Collagen Corp., Palo Alto, CA) with 25 μg of 4-aminophenylmercuric acetate-activated MMP-1 overnight at room temperature. MMP-1 was removed by dialysis in phosphate-buffered saline for 4–6 h at 4 °C. Gelatin was made by heating type I collagen at 80 °C for 10 min.

**Antibodies**—Blocking antibody (6F1) to the α5 integrin subunit was provided by Dr. Barry Coller (Mount Sinai Medical Center, New York, NY). The following antibodies, which were purchased from Chemicon International, Inc., were used for immunoprecipitation (IP) and/or immunoblotting (IB) as indicated in parenthesis: MMP-1 antibodies, AB-806 (IP); AB-8114 (IB), and AB-8105 (IB); α5 integrin subunit antibody, MAB-1973 (IP/IB): α4 antibodies, MAB-1992 (IP) and AB-1936 (IP and immunostaining); α5 antibody, MAB-1992 (IP); α2 antibody, AB-1928 (IP); α2, antibody, MAB-1953 (IP); β1 antibodies, MAB-2252 (IP); and AB-1937 (IB). For immunostaining of human MMP-1, we used an antibody (IM35L) from Calbiochem (La Jolla, CA). Affinity-purified rabbit polyclonal antibodies against human MMP-1, MMP-3, and MMP-9 have been described (1, 16, 17).

**Exploits and Cell Culture**—Normal human adult skin was obtained from patients undergoing reductive mammaplasty or lateral abdominoplasty. Four-millimeter, full thickness punch biopsies were attached for paraffin embedding. Keratinocytes were isolated from human skin by trypsinization, and dehydrated through graded ethanol. Fixed cells were incubated in 4-aminophenylmercuric acetate-activated MMP-1 overnight at room temperature. The prepared keratinocyte suspension was allowed to adhere to type I collagen or gelatin-coated dishes for 24 h. The conditioned medium was collected, and the cells were washed three times with ice-cold phosphate-buffered saline and then lysed with 1 ml of 1.5% Triton X-100, 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, 2 mM phenylmethylsulfonyl fluoride, 20 μM L-α-tocopherol, and 12.5 μg/ml leupeptin. The cell lysates and conditioned media were precleared by incubating for 2 h at 4 °C in mTris buffer containing IPB-BSA (0.5% Triton, 200 mM NaCl, 10 mM MgCl2, 100 mg of BSA) containing protein A-Sepharose (PAS; Zymed Laboratories Inc., South San Francisco, CA). PAS was removed by centrifugation, and the preclearing procedure was repeated. Following the second centrifugation, the desired primary antibody was added to the supernatants, and the samples were incubated overnight at 4 °C with gentle movement. Antibody-antigen complexes were precipitated with PAS, washed twice with IPB-BSA-2 (0.4% Triton, 10 mM EDTA, 100 mg/ml BSA), and eluted from PAS by heating at 60 °C for 20 min in 60 μl of nondenaturing electrophoresis sample buffer. For cells treated with an anti-α5 blocking antibody (6F1 or MAB1950), complexes were formed with a rabbit polyclonal antibody (AB-806 for MMP-1 or AB-1936 for α5) and were precipitated from solution with Magna Bind Polyclonal Rabbit IgG and eluted with heat. The precipitates (MMP-1 or α5 or both) were pelleted, and the supernatants were removed.

**Immunoblotting**—Immunoprecipitates (30 μl; total volume, 60 μl) or samples of cell lysates or conditioned medium (500 μl; total volume, 1 ml) were resolved by denaturing electrophoresis through 10% (for metalloproteinases) or 12% (for integrins) polyacrylamide-SDS gels and transferred by semidry electrophoresis at 15 V for 20 min to nitrocellulose membranes (Hybond ECL; Amersham Pharmaacia Biotech) in 48 mM Tris, 39 mM glycine, 20% methanol, and 0.0375% SDS. Nonspecific binding sites were blocked by soaking membranes in 3% nonfat dry milk in Tris-buffered saline at 4 °C overnight. Blots were incubated with antisera against MMP-1 (AB-8114 or AB-8105), MMP-3, MMP-9, α5 (AB-1936), or β1 (AB-1937) polyclonal antisera in blocking buffer for 2 h and washed twice with sodium saline containing 0.1% Tween 20 for 20 min. Membranes were subsequently incubated with a 1:3000 dilution of a peroxido-conjugate donkey anti-rabbit IgG (Amersham Pharmacia Biotech) in blocking buffer for 1 h, washed twice, and developed with the Enhanced Chemiluminescence system (Amersham Pharmacia Biotech) in blocking buffer for 1 h, washed twice, and developed with the Enhanced Chemiluminescence system (Amersham Pharmacia Biotech) following the manufacturer’s instructions.

**Platelet Adhesion**—96-well microtiter plates were coated with native type I collagen, MMP-1, or matrilysin (MMP-7) as described above. Nonspecific protein binding sites were blocked by incubating with 0.5% BSA in Tris-buffered saline, pH 7.4, at room temperature for 2 h. The platelets were preadulted and washed as described (20), and 100 μl of platelet suspension were permitted to adhere to the substrate for 1 h at room temperature. Nonadherent platelets were removed by several washings. Adhesion was quantified by measuring the hexoseaminidase activity in the adherent platelets (21).

**Surface Labeling**—Proteins on the surface of keratinocytes were labeled using EZ-LinkTM Sulfo-NHS-LC-Biotinylation Kit (Pierce). Equal numbers of primary keratinocytes were plated, and 24 h later, cells were rinsed three times with serum-free medium and then incubated for 45 min in medium containing 1 mg/ml of Sulfo-NHS-LC-Biotin. The cells were then washed three times with medium to remove unbound biotin. Fresh medium was then added with various concentrations of MMP-1 or MMP-1 predomain for 4 h. Cell lysates were collected, and labeled surface proteins were precipitated using streptavidin beads and resolved by electrophoresis. Surface-associated MMP-1 was detected by immunoblotting. Alternatively, MMP-1 was directly immunoprecipitated from cell lysates and detected on gels using horse-radish peroxidase-linked streptavidin.

**RESULTS**

As is well established (22–25), the α5β1 integrin is localized circumferentially on basal keratinocytes in intact human skin (Fig. 1A). However, in response to injury, this integrin gathers along the basal surface of migrating keratinocytes at a wound edge (Fig. 1B). As we reported previously in studies of actual human wounds (1, 2), MMP-1 is selectively expressed by basal keratinocytes at the most forward edge of migration, and this same spatially confined pattern of expression was seen in ke-
Pro-collagenase-1 Binds the α2β1 Integrin

Keratinocytes that have migrated from the epidermal edge of skin biopsies placed in culture overnight and were then fixed and processed for paraffin embedding, A–D, biopsies (4 mm) of viable human skin were placed in culture overnight and were then fixed and processed for paraffin embedding. A, sections were stained for the α2 integrin subunit using alkaline phosphatase. In the center of the sample, where the skin is intact, signal for α2β1 was seen on the basal-lateral surface of basal keratinocytes (arrows). B, at the sample edge, keratinocytes had migrated down the exposed side of the biopsies along the dermis. At the forward edge of the epidermal front, intense staining for α2β1 was seen along the basal surfaces of keratinocytes (arrow). C and D, in situ hybridization revealed that MMP-1 mRNA is expressed by keratinocytes at the forward extent of and just behind the epidermal front (arrow), within the same population of cells showing marked redistribution of α2β1. Shown are paired bright field (C) and dark field (D) micrographs. E and F, primary human keratinocytes were isolated from normal adult skin, plated on slides coated with native type I collagen, and processed 6 h later for immunofluorescence using antibodies against MMP-1 and α2β1. A prominent signal for α2β1 (red) was seen along the periphery of spreading cells and at the more central close contacts, where co-localization with MMP-1 was evident (merged signal, yellow). Cell-associated fluorescence for MMP-1 (green) was also seen apart from α2β1. The two confocal images were taken at 0.5 (E) and 2.0 (F) μm above the substratum.

Pro-MMP-1 is bound to α2β1 on the surface of keratinocytes. Keratinocytes were plated on collagen-coated dishes, and 24 h later specific proteins were immunoprecipitated from cell lysates as described under “Experimental Procedures.” In the panels shown, the antibody used for precipitation is indicated at the top of each lane. The antibodies used for immunoblotting are indicated along the side of each panel. In A, purified pro-MMP-1 was included as a migration standard (Std) and, the migration of molecular mass standards is shown on the right (in kDa). In E, proteins were immunoprecipitated from membrane extracts of HaCaT keratinocytes. As described under “Experimental Procedures,” different antibodies were used if samples were immunoprecipitated and immunoblotted for the same antigen.

To assess this idea, we plated primary adult human keratinocytes on native type I collagen (hereupon called collagen). Reflecting the phenotype of basal cells involved in re-epithelialization in vivo, MMP-1 mRNA is expressed only in keratinocytes migrating from the colonies of proliferating and differen-
pro-MMP-1 binds αβ1 integrin: a novel observation.

We also used a platelet adhesion assay to assess direct binding of pro-MMP-1 to native, membrane-integrated αβ2 integrin (32). Platelets bound to dishes coated with collagen in the presence of Mn2+, and this interaction was inhibited with EDTA or 6F1 (Fig. 4), a blocking antibody that binds the I domain of αβ1 (33). Platelets also bound to pro-MMP-1 (Fig. 4), but they did not bind to matrilysin (MMP-7), another MMP expressed by migrating epithelial cells (34). Thus, by three distinct assays: co-immunoprecipitation, platelet adhesion, and solid phase binding to αβ2 integrin I domain (35), we demonstrated that pro-MMP-1 and active MMP-1 binds to the αβ2 integrin.

Increasing concentrations of 6F1 displaced pro-MMP-1 from the surface of keratinocytes (Fig. 5A), whereas a blocking antibody to αβ1 did not (Fig. 5B). For these studies, 6F1 was added 24 h post-plating to prevent cell detachment, and 2 h later, cells and media were harvested. Because 6F1 recognizes an epitope within the I domain, these results suggests that pro-MMP-1 interacts with this region of the αβ2 subunit. This conclusion was verified in various experiments reported in our accompanying paper (35).

The ability of pro-MMP-1 to interact with αβ2 was dependent on the nature of the collagen substratum. On gelatin, pro-MMP-1 did not co-immunoprecipitate with αβ2, even though appreciable levels of the enzyme were released into the medium (Fig. 5C). Gelatin did not influence the levels of αβ1 on the cell surface (Fig. 5C). In some experiments, plating on gelatin barred an interaction between MMP-1 and αβ1 (Fig. 5C), whereas in others, reduced levels of pro-MMP-1 were co-immunoprecipitated with αβ1 (Fig. 5D). Because each experiment was done with keratinocytes from skin of a different individual, these results may reflect person-to-person variability. Regardless, our data show that pro-MMP-1 interacts poorly, if at all, with αβ1 if keratinocytes are plated on denatured collagen. Similarly, reduced levels of pro-MMP-1 were co-immunoprecipitated with αβ1 from keratinocytes on enzymatically cleaved collagen (Fig. 5D).

A competition assay was used to begin to determine the site on pro-MMP-1 that interacts with αβ2. Keratinocyte surface proteins were labeled with biotin, and cultures were then incubated for 4 h with equimolar amounts of purified active MMP-1 or its pro-domain. Biotinylated surface proteins were precipitated using streptavidin beads, and membrane-associated pro-MMP-1 was detected by immunoblotting. Cell-bound pro-MMP-1 was progressively displaced with increasing con-
Pro-collagenase-1 Binds the $\alpha_2\beta_1$ Integrin

FIG. 5. Pro-MMP-1/integrin association is reduced by anti-$\alpha_2\beta_1$ antibodies and on keratinocytes grown on gelatin. A, keratinocytes were plated on type I collagen for 24 h and were treated for the last 2 h with increasing concentrations of 6F1, an anti-$\alpha_2\beta_1$ blocking antibody to an epitope within the I domain. The cell were washed of medium, the lysates were immunoprecipitated (IP) for MMP-1, and the resolved products were blotted (IB) for MMP-1 protein. In this experiment, both pro-MMP-1 and active MMP-1 were immunoprecipitated, but the association of both forms with the cell surface was reduced by 6F1. B, in a similar experiment with different primary keratinocytes, 6F1 again reduced the recovery of pro-MMP-1 from cell lysates, whereas a blocking antibody against $\alpha_2\beta_1$, another collagen-binding integrin, did not. C, keratinocytes were plated on heat-denatured collagen (gelatin) or on native fibrillar collagen with or without antibody 6F1 during the last 2 h. The cell layers were immunoprecipitated for $\alpha_2\beta_1$, and immunoblotted for or MMP-1 or $\alpha_2\beta_1$. Medium samples were immunoprecipitated and immunoblotted for MMP-1. D, keratinocytes were plated on gelatin, enzymatically cleaved collagen (Clvd Col), or native fibrillar collagen. The cell lysates were harvested, lysed, and immunoprecipitated for $\alpha_2\beta_1$ and immunoblotted for MMP-1. Recovery of integrin-associated pro-MMP-1 was reduced from keratinocytes on the denatured substrates. Different antibodies were used if samples were immunoprecipitated and immunoblotted for the same antigen.

Concentrations of active MMP-1, with >50% removed with 3.6 $\mu$M active MMP-1 (Fig. 6). Equal concentrations of the pro-domain had no effect on the recovery of cell-associated pro-MMP-1. We obtained similar results if pro-MMP-1 was immunoprecipitated from biotinylated cell lysates and detected on gels using horseradish peroxidase-linked streptavidin (data not shown). The efficiency of pro-MMP-1 displacement was possibly hampered by poor accessibility of the relatively large competitors to points of cell-substratum contacts. These findings suggest that pro-MMP-1 is anchored to the $\alpha_2\beta_1$ integrin via its catalytic, linker, or hemopexin domains, a conclusion that is more thoroughly refined in our accompanying paper (35).

DISCUSSION

Cell-matrix contacts provide an unambiguous signal informing cells which matrix protein they have encountered and, in turn, which proteinase is needed and where the enzyme should be delivered and released. During migration, cells attach to, extend over, and then release from a matrix substrate, and repeating these steps allows the cell to continue moving (36). Following injury to the epidermis, basal keratinocytes at the wound edge quickly move off of the basement membrane onto the underlying dermal matrix, which is rich in type I collagen. In addition to being a mechanism for attachment and spreading, the interaction of keratinocytes with dermal collagen provides a site-specific signal that, along with other processes, such as altered cell-cell contacts, initiates the epithelial response to wounding. This response is characterized, in part, by the prominent and invariable expression of pro-MMP-1 (37) and is regulated by the ligation of the $\alpha_2\beta_1$ integrin, which is constitutively expressed on keratinocytes (38), with dermal collagen (2).

Here, we report that pro-MMP-1 specifically binds the $\alpha_2\beta_1$ integrin on enzyme-expressing keratinocytes plated on native type I collagen. Co-localization of this proteinase with this collagen-binding integrin was seen by confocal immunofluorescence, and direct binding of pro-MMP-1 to the $\alpha_2\beta_1$ integrin was demonstrated by co-immunoprecipitation and platelet adhesion. In our accompanying paper, we demonstrate by solid phase assays that pro-MMP-1 interacts with the I domain of the $\alpha_2\beta_1$ integrin (35). The ternary complex of the $\alpha_2\beta_1$ integrin, type I collagen, and pro-MMP-1 would spatially confine proteolysis and selectively direct catalysis to points of cell-matrix contacts. There are several examples of cell-directed proteolysis, some of which are cited in the Introduction. As discussed by Owen and Campbell (39) and as demonstrated by their studies on serine proteinases in neutrophils (40, 41), anchoring enzymes to the cell surface provides both a mechanism to increase enzyme concentration at sites of proteolysis and a pericellular barrier to interference by natural proteinase inhibitors, which are abundant in tissue fluids. In addition to these functions, we propose that the $\alpha_2\beta_1$-pro-MMP-1 complex functions also as a molecular motor controlling and driving keratinocyte migration over a dermal collagen.

Because the formation of $\alpha_2\beta_1$-collagen contacts would precede the biosynthesis and secretion of pro-MMP-1, not all $\alpha_2\beta_1$-contacts would be complexed with enzyme. Indeed, our co-immunoprecipitation data demonstrate that pro-MMP-1 binds to a subset of the $\alpha_2\beta_1$ integrin receptors (Fig. 2D), and our confocal observations indicate that pro-MMP-1 binds to established $\alpha_2\beta_1$-collagen contacts (Fig. 1E). This spatial association suggests that MMP-1 functions to dissociate the integrin-collagen contacts, as suggested in our previous studies (2), rather than to break down matrix barriers that might be encountered at the forward extent of cell extension. The $\alpha_2\beta_1$ integrins bind native collagen with high affinity (42), and thus, clustering this...
Pro-collagenase-1 Binds the αβ1 Integrin

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We investigated the interaction of pro-collagenase-1 (procollagenase-1) with the αβ1 integrin and characterized its role in cell migration. Procollagenase-1 binds to the αβ1 integrin in a thiol-dependent manner and, in normal keratinocytes, induces cell motility. Procollagenase-1 is able to induce cell migration in the presence of its substrate, namely type I collagen, even in the absence of other growth factors. Procollagenase-1 binds to the αβ1 integrin in a thiol-dependent manner and, in normal keratinocytes, induces cell motility. Procollagenase-1 is able to induce cell migration in the presence of its substrate, namely type I collagen, even in the absence of other growth factors.

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Pro-collagenase-1 (Matrix Metalloproteinase-1) Binds the $\alpha_2\beta_1$ Integrin upon Release from Keratinocytes Migrating on Type I Collagen

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