Binding and Localization of $M_r$ 72,000 Matrix Metalloproteinase at Cell Surface Invadopodia

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ABSTRACT

Degradation (turnover) of collagenous matrix occurs on the surface of specialized membrane extensions termed "invadopodia," which are sites of cell invasion into the extracellular matrix. Here we show the localization of the $M_r$ 72,000 type IV collagenase of the matrix metalloproteinase family at invadopodia. When added exogenously, latent $M_r$ 72,000 collagenase binds to invadopodia of chicken embryo fibroblasts transformed by Rous sarcoma virus, whereupon the bound collagenase loses its propeptide. The collagenase binds to a component contained within the detergent extract of transformed cells, and increased levels of the active $M_r$ 62,000 form of the collagenase are seen here. Such an association is not detected in the detergent extract derived from normal cells. Using a recently developed cell fractionation procedure to collect cell surfaces enriched in invadopodia, we show that the $M_r$ 72,000 collagenase associates with the invadopodial fraction and active forms of the enzyme become immobilized on the collagenous surface. Thus, invadopodia direct intense localized degradation of the extracellular matrix by concentrating active membrane-associated collagenases at sites of cellular invasion.

INTRODUCTION

For nearly 30 years since the discovery of collagenases (1), biologists have inferred that tissue remodeling requires controlled degradation of connective tissue macromolecules, which is essential for morphogenesis during development, bone repair, wound healing, angiogenesis, neutrophil migration, and tumor invasion (2-4). The degradation process is often confined to localized sites at the cell surface that are in contact with the ECM (5-7). However, members of the MMP family, such as the $M_r$ 72,000 type IV collagenase (8-11), are secreted in zymogen form and are bound to their natural inhibitors, the TIMPs (12-17). Physiological activation is required to achieve matrix degradation; this might occur after binding of proenzymes or proenzyme-TIMP complexes to the plasma membrane. Several morphological and biochemical observations have suggested that activation takes place after binding of proenzymes or their complexes with TIMPs to the plasma membrane (6, 18-20). There is more recent evidence for this hypothesis. The $M_r$ 72,000 MMP can be activated by incubation with isolated fibroblast membranes, and the resulting activity is blocked by TIMP-2 (21). The COOH-terminal domain of collagenase and stromelysin is essential for membrane activation and modulates interactions with TIMPs but is not required for catalysis (22, 23). Emonard et al. (24) have found that the breast cancer cell lines MDA-MB-231 and MCF-7 contain a cell surface, high affinity receptor for the $M_r$ 72,000 MMP. Brown et al. (25) have shown that detergent extracts from 12-O-tetradecanoylphorbol-13-acetate-stimulated HT-1080 human fibrosarcoma and concanavalin A-treated WI-38 human fibroblast cell lysates are able to process exogenously added $M_r$ 72,000 MMP in a calcium-dependent manner.

In a separate study using a rapid procedure of isolating membrane-bound proteases (26), we have found that highly invasive human melanoma cell lines, including LOX and RRPML7951, contain activated $M_r$ 72,000 MMP in association with the cell membrane that contacts the ECM, whereas the latent form is present in the conditioned medium and cytosol. Other studies have identified an mAb CP1 that recognizes a unique plasma membrane structure called invadopodia in RSV-CEF that has associated with it a complex of active proteases essential for the invasion of transformed cells through the ECM. These transformed chicken embryonic fibroblasts have been shown to be invasive in vitro while the nontransformed cells are noninvasive (19). Recently, it has been shown that the RSV-CEF produce 3-5 times higher levels of the "70 kDa" MMP than CEF as well as an activated $M_r$ 62,000 form produced exclusively by the RSV-CEF, and some of this MMP is complexed with a $M_r$ 22,000 avian form of TIMP-2 (21). Here we address the specific question whether invadopodia are sites of cell surface binding and activation of the $M_r$ 72,000 collagenase.

MATERIALS AND METHODS

Cell Culture. Primary cultures of CEF were prepared from 9-day-old White Leghorn embryos (Truslow Farms Incorporated, Chestertown, MD) and infected with a wild-type Schmidt-Ruppin strain or ts68 temperature-sensitive strain of RSV. Cells were cultured in Dulbecco's modified Eagle's medium containing 4500 mg/liter of glucose, 4% fetal calf serum, 1% chicken serum, 10% tryptose phosphate broth, 1% (v/v) penicillin-streptomycin, and 2 mM glutamine (Flow Laboratories, Inc., Rockville, MD). Cells were passaged every 2-3 days using trypsin and EDTA (GIBCO, Grand Island, NY), and all experiments were performed on third or fourth passage cultures of CEF and RSV-CEF.

Localization of Active Proteases on the Cell Surface. CEF and RSV-CEF were seeded on rhodamine-labeled type I collagen gelatin films prepared as described (5, 19) with the modification that the coverslips were coated with 2% gelatin, 2% sucrose, and 0.02% NaCl in PBS, and fixed for 30 min at 4°C with 2% glutaraldehyde in PBS. The cells were incubated on these films in serum-containing medium for 24 h to visualize local degradation of the type I collagen-gelatin film by transformed cells. The metalloproteinase inhibitor CI-CH$_2$-(C-O)-(OH-N)-DL-Phe-L-Ala-L-Ala-NH$_2$ (NP-20, Enzyme System Products, Livermore, CA), 0.1 mM, was added to the 6-h incubation period. Cells were fixed, permeabilized, and labeled with rat mAb CP1 that recognized invadopodia, followed by fluorescein-goat anti-rat antibody (Rockland, Inc.). Specimens were photographed with a Planapo 63/1.4 objective on a Zeiss Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) by epifluorescence microscopy for rhodamine and fluorescein labelling or differential interference contrast microscopy for morphology.

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3 The abbreviations used are: CEF, chicken embryo fibroblasts; ECM, extracellular matrix; MMP, matrix-metalloproteinase; RSV-CEF, Rous sarcoma virus-transformed chicken embryo fibroblasts; TBS, Tris-HCl buffered saline; TIMP-2, tissue inhibitor of metalloproteinase-2; TBS++, TBS containing 2 mM calcium and 2 mM magnesium; RSV, Rous sarcoma virus; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.
Localization of Exogenously Added M, 72,000 MMP on the Cell Surface. CEF or RSV-CEF were cultured overnight on coverslips in serum-free medium. Both latent M, 72,000 MMP-TIMP-2 complex derived from conditioned medium of human melanoma A2058 cells (50 μg/ml) or a recombiant form of the M, 72,000 MMP, purified from a recombinant vaccinia virus mammalian cell expression system (50 μg/ml) (27), were isolated from conditioned medium by gelatin-columns followed by dimethyl sulfoxide elution (28, 29) and were biotinylated (Pierce, Rockford, IL; according to the instructions provided by the manufacturer). The recombiant enzyme was almost entirely latent, although some trace amounts of the M, 68,000 intermediate form were present. It was able to digest gelatin and collagen type IV after p-aminophenylmercuric acid treatment, it bound TIMP-2 resulting in an 86% decrease in activity compared to the activated enzyme and was recognized by Ab45 and H1 antibodies (27). Both of these biotinylated enzymes were found to have the same activity as the nonbiotinylated forms when examined by gelatin zymography (data not shown). Immunostaining of the endogenous enzyme in association with the invadopodia further substantiates that the biotinylated forms behave in the same way as the endogenous enzyme (Fig. 3). These biotinylated enzymes were then added to the CEF or RSV-CEF cultures. The cells were incubated for 30 min at 4°C or 4°C. The cells were fixed, immunolabeled with rat anti-invadopodia mAb CP1, and then secondarily stained with rhodamine conjugated goat anti-rat antibody, as well as fluorescein-conjugated goat anti-biotin, to detect MMP or MMP-TIMP-2 complex. Binding of biotinylated fibronectin or bovine serum albumin to the surface of cell layers was used as a control for specific binding.

Association of M, 72,000 MMP Activity with RSV-CEF Detergent Extracts. To prepare enriched chicken M, 72,000 MMP, confluent CEF or RSV-CEF cell layers were cultured in serum-free medium with or without 20 μg/ml cycloheximide for 1 h. Cells were fixed and doubly labeled with rat mAb CP1 and either rabbit polyclonal antibody Ab45 (directed against native M, 72,000 human type IV collagenase) or H1 (directed against the propeptide sequence of the enzyme that is cleaved off during activation of the enzyme, thus recognizing only the inactive form) (28, 30). Ab45 (Fig. 4B) and H1 antibodies (data not shown) cross-reacted with the chicken enzyme found in 20-fold concentrated serum-free medium conditioned by CEF and RSV-CEF. The Ab45 and H1 antibodies recognized the chicken enzyme inside CEF and RSV-CEF cells (Fig. 3), as well as that on ECM fibrils around CEF cells in a pattern similar to that seen when observing the exogenously added biotinylated MMP to normal cell cultures (Fig. 2).

RESULTS

Identification of Active Proteases Associated with Invadopodia. CEF or RSV-CEF were cultured at 41°C in serum-containing medium until confluent on cross-linked gelatin matrix films in tissue culture dishes. Plasma membranes adhering to the substratum were isolated by shearing the cell body away from the membranes in contact with the cross-linked gelatin films as recently described (26), with the modification that the buffer used for washing and then shearing the cells was TBS++. Fractions of contact membranes and cell bodies were extracted with either 1.5% Triton X-114 in TBS++ for partitioning into detergent and aqueous phases (Fig. 5B, Lanes CD', CA', CD, and CA), or 1% SDS buffer (Fig. 5B, Lane TT). These fractions were analyzed for their proteolytic activity by gelatin zymography (32). For immunofluorescent light microscopy of cell contact plasma membranes, membranes remaining on the gelatin films were fixed in 3% paraformaldehyde plus 0.1% Triton X-100 in PBS for 10 min. The films were immunolabeled for invadopodia using rat mAb CP1, followed by fluorescein-conjugated goat anti-rat secondary antibody.

Fig. 1. Collagen-degrading activity on cell surface invadopodia of transformed cells. a and b, localization of the CPI antigen at sites of collagen degradation. Arrowheads, invadopodia that were labeled intensively for CPI and that correspond to sites of type I collagen-degradation. c and d, inhibition of collagen degradation by RSV-CEF with 0.1 mM of the metalloproteinase inhibitor NP-20. Bar, 10 μm.
Fig. 2. Binding of exogenously added M, 72,000 MMP to the plasma membrane at invadopodia. a and b, binding of M, 72,000 MMP-TIMP-2 complex to the surface of normal cells. CP1 invadopodial marker is low in normal cells (a) and MMP-TIMP-2 associates with the extracellular matrix being assembled by the cells (b). In c and d, binding of M, 72,000 MMP-TIMP-2 complex to ventral surface invadopodia of transformed cells. MMP-TIMP-2 complex (arrow in d) is seen to colocalize with the CP1 invadopodia staining (arrow in c). e and f, binding of a recombinant M, 72,000 MMP to the surface of normal cells. In normal cells there is no invadopodial CP1 labeling (e) and the recombinant MMP is seen to be associated with the matrix (f). g and h, binding of exogenous recombinant M, 72,000 MMP to ventral surface invadopodia of transformed cells. The transformed cell demonstrates invadopodial staining on the ventral surface (arrow in g) and the exogenous recombinant M, 72,000 MMP is seen to be associated with the invadopodia (arrow in h). Bar, 25 μm.

tially, two major problems were encountered that hindered visualization of cell surface associated MMPs on invadopodia. The intense labeling of the intracellular MMP pool together with the presence of secreted MMP that was immobilized on the substratum made it difficult to identify cell surface MMPs, although some CP1 invadopodial staining was observed to colocalize with the anti-M, 72,000 MMP staining (Fig. 3, a and b, arrowhead). To eliminate the contribution of the intracellular pool of CP1 and MMP labeling to the image, cells were preincubated with cycloheximide (20 μg/ml) for 1 h, a treatment known to clear intracellular pools of protein (Fig. 3, c and d). Following this treatment, the invadopodial CP1 staining clearly colocalizes with the anti-M, 72,000 MMP staining in the focal plane of the ventral cell surface (Fig. 3, c and d, arrowheads). By this method, we demonstrate that a chicken enzyme related to the human M, 72,000 MMP colocalizes with the CP1 antigen. However, although the MMP complex bound at the invadopodia was recognized by the antibodies to native M, 72,000 MMP (antibody Ab45) (Fig. 3, b and d), it was not...
antibody and the HI antibody (data not shown) both recognize the MT.

The MMP activity does not bind directly to the detergent phase of Triton-X 114 extracts from CEF and RSV-CEF, prepared as described above, do not contain any M, 72,000 MMP (Fig. 4B, Lanes 3 and 5, respectively). After incubating the detergent phase of CEF with concentrated CEF medium supplemented with 2 mM calcium and magnesium then repartitioning, no MMP is seen in association with the detergent phase (Fig. 4B, Lane 4), confirming the result of gelatin zymography described above (Fig. 4A). However, M, 72,000 as well detected by the antibodies against the propeptide sequence of latent M, 72,000 MMP (H1 antibody) (Fig. 3f) or TIMP-2 (data not shown), suggesting that active MMP is bound to the invadopodia.

A biochemical approach was used to further examine the association of exogenous M, 72,000 MMP with membrane components found in the detergent phase of Triton-X 114 extracts from CEF and RSV-CEF. The serum-free medium conditioned by RSV-CEF is enriched with the M, 72,000 MMP and it was used as the source of the enzyme.

The medium was incubated with the detergent phase components of cell extracts: alone (Fig. 4A, Lanes 1 and 2); in the presence of 5 mM EDTA (Lanes 3 and 4); and 2 mM calcium and 2 mM magnesium (Lanes 5 and 6); these mixtures were then repartitioned to yield the detergent phases and aqueous phases as indicated above, respectively. From all combinations, we observed that the M, 72,000 and M, 62,000 MMP activities from the RSV-CEF-conditioned medium associated only with the detergent phase of the RSV-CEF in the presence of 2 mM calcium and magnesium cations (Fig. 4A, Lane 5). After incubation with the conditioned medium, no MMP activity could be detected in the detergent phases of membrane extracts of CEF (data not shown) and RSV-CEF alone or when supplemented with EDTA (Fig. 4A, Lanes 1 and 3, respectively). In addition, MMP activity was recovered in the aqueous phase of these incubation mixtures (Fig. 4A, Lanes 2, 4, and 6). The MMP activity does not bind directly to the detergent phase of the Triton-X-114 detergent solution alone (data not shown).

The above observation that the M, 72,000 MMP associates with membrane components of transformed cells and may then be activated is further substantiated by immunoblot analysis (Fig. 4B). The Ab45 antibody and the HI antibody (data not shown) both recognize the M, 72,000 MMP present in serum-free medium conditioned by CEF (Fig. 3B, Lane 1) and RSV-CEF (Fig. 4B, Lane 2). The detergent phase of Triton-X 114 extracts from CEF and RSV-CEF, prepared as described above, do not contain any M, 72,000 MMP (Fig. 4B, Lanes 3 and 5, respectively). After incubating the detergent phase of CEF with concentrated CEF medium supplemented with 2 mM calcium and magnesium then repartitioning, no MMP is seen in association with the detergent phase (Fig. 4B, Lane 4), confirming the result of gelatin zymography described above (Fig. 4A). However, M, 72,000 as well

Fig. 3. Indirect immunofluorescent localization of endogenous active M, 72,000 MMP in invadopodia. In a and b, both MMP and CPI antigens were observed in transformed cells. Extensive intracellular antigens obscure their colocalization, although some colocalization of CPI staining and M, 72,000 MMP staining is seen (arrowhead). In c and d, after a 1-h preincubation of transformed cells with 20 µL/ml cycloheximide, precise colocalization of membrane M, 72,000 MMP staining (polyclonal antibody Ab45) (arrowhead in c) with invadopodia (arrowhead in e) is observed. In e and f, colocalization of the inactive form of the enzyme using polyclonal antibody H1 (f) which binds the propeptide sequence of the enzyme, with invadopodial membranes (arrowhead in e) is not observed. Bar, 25 µm.

Fig. 4. Binding of exogenously added M, 72,000 MMP to detergent extracts isolated from CEF and RSV-CEF. A, gelatin zymography of the gelatinase activities that were associated with the detergent phase of mixtures of the RSV-CEF-conditioned medium and the Triton-X 114 extracts of RSV-CEF (Lanes 1, 3, and 5) and that remained in the aqueous phase of the medium-cell extract mixture (Lanes 2, 4, and 6). Lane 1: gelatinase activities associated with the detergent phase of the cell extracts incubated with the conditioned medium. Lane 2, gelatinase activities present in the aqueous phase from the incubation shown in Lane 1. Lane 3, gelatinase activities associated with the detergent phase of the cell extracts incubated with RSV-CEF-conditioned medium in the presence of 5 mM EDTA. Lane 4, gelatinase activities present in the aqueous phase from the incubation shown in Lane 3. Lane 5, gelatinase activities associated with the detergent phase of the cell extracts incubated with the conditioned medium containing 2 mM calcium and 2 mM magnesium. Lane 6, gelatinase activities present in the aqueous phase from the incubation shown in Lane 5. Note that exogenously added M, 72,000 and M, 62,000 MMP present in the conditioned medium associates only with the detergent phase of the transformed cells in the presence of excess calcium and magnesium cations (Lane 5, arrowhead), but the MMP does not associate with the detergent phase of the medium-cell extract mixture under other conditions (Lanes 1 and 3). The zymogram represents one set of experiments out of seven repeated sets; 7.5% acrylamide SDS-gel was used and molecular weights were determined by comparison with M, 72,000 and M, 62,000 MMP from HT1080-conditioned medium (data not shown). B, immunoblot analysis of exogenously added M, 72,000 MMP from CEF cultures and its association with RSV-CEF detergent extracts. In Lanes 1 and 2, the M, 72,000 MMP present in the medium conditioned by CEF and RSV-CEF, respectively, are recognized by the rabbit polyclonal antibody Ab45. In Lanes 3 and 5, no antigen is detected in the detergent phase of CEF and RSV-CEF Triton-X 114 extracts, respectively. In Lane 4, after incubating the detergent phase of CEF extracts with the conditioned medium supplemented with 2 mM calcium and magnesium then repartitioning, no antigen is associated with the CEF detergent phase. In Lane 5, M, 62,000 and M, 72,000 polypeptides are found in association with the detergent phase of RSV-CEF extract after incubating the extract with the conditioned medium supplemented with 2 mM calcium and 2 mM magnesium then repartitioning. Note the specific appearance of the M, 62,000 MMP polypeptide only in the detergent phase of the RSV-CEF extract (Lane 6). The immunoblot represents one set of experiments out of four repeated sets; 6.5% acrylamide SDS-gel was used and molecular weights were determined by comparison with M, 72,000 and M, 62,000 MMP from HT1080-conditioned medium (data not shown).
as the activated Mr 62,000 MMP (11) are seen in association with the RSV-CEF Triton-X 114 detergent phase after incubating the RSV-CEF detergent phase with concentrated CEF medium supplemented with 2 mM calcium and magnesium then repartitioning (Fig. 4B, Lane 6). The presence of the Mr 62,000 MMP in association with the RSV-CEF detergent phase may represent an activation of the exogenously added MMP, since the CEF-conditioned medium contains very little if any Mr 62,000 MMP.

To characterize the endogenous gelatinase activity associated with invadopodia-rich cell membranes, we prepared a ventral membrane fraction from RSV-CEF that were invading a cross-linked gelatin film. This membrane fraction, called the contact membranes (Fig. 5A), can be visualized by differential interference contrast microscopy and mAb CP1 immunofluorescence (Fig. 5A, differential interference contrast and CP1, respectively). The isolated contact membranes contain highly enriched clusters of the invadopodial antigen (Fig. 5A). Solubilization of the contact membranes with SDS-polyacrylamide gel electrophoresis sample buffer exhibits various gelatinase activities (Fig. 5, Lane 77") including the latent Mr 72,000 intermediate and CP1, respectively. The isolated contact membranes contain highly enriched clusters of the invadopodial antigen (Fig. 5A). Solubilization of the contact membranes with SDS-polyacrylamide gel electrophoresis sample buffer exhibits various gelatinase activities (Fig. 5A).

This degradation occurs when the cells were cultured in medium containing serum or 5% plasminogen-depleted fetal calf serum and in the presence of 20 mM e-aminocaproic acid (32). Since serum contains various protease inhibitors, the invadopodia associated proteases may resist the action of serum-borne protease inhibitors such as TIMP-2, perhaps by forming tight contacts with the ECM surrounding invadopodia. However, smaller synthetic inhibitors may be able to enter this space. Thus, MMPs may bind to invadopodial membranes, become activated, and escape inhibition by secreted inhibitors allowing substrate degradation at the invadopodia-ECM interface.

This report shows the binding of both latent recombinant Mr 72,000 MMP and the MMP-TIMP-2 complex to the plasma membrane at invadopodia. Furthermore, the antibody that recognizes the active form of Mr 72,000 MMP labeled the invadopodia, whereas the antibody against the propeptide sequence did not, suggesting that activated MMPs are present at these sites. The Mr 72,000 MMP associates with components from the detergent extract of RSV-CEF when supplemented with calcium and magnesium, and increased levels of Mr 62,000 MMP are present. However, MMP does not associate with the detergent extract of CEF. In isolated invadopodia-rich fractions, activated Mr 72,000 MMP from the invadopodia membrane may become immobilized on its underlying gelatin film. These data suggest a key proteolytic mechanism in which the cell uses invadopodia-associated active MMP to degrade surrounding tissues at focal cellular invasion.

Previously, we showed that RSV-transformed cells can locally degrade a fibronectin substratum at cell contact sites (5). This localized degradation is sensitive to small synthetic metalloproteinase inhibitors, such as NP-20, whereas invadopodial organization is not (35). This degradation occurs when the cells were cultured in medium containing serum or 5% plasminogen-depleted fetal calf serum and in the presence of 20 mM e-aminocaproic acid (32). Since serum contains various protease inhibitors, the invadopodia associated proteases may resist the action of serum-borne protease inhibitors such as TIMP-2, perhaps by forming tight contacts with the ECM surrounding invadopodia. However, smaller synthetic inhibitors may be able to enter this space. Thus, MMPs may bind to invadopodial membranes, become activated, and escape inhibition by secreted inhibitors allowing substrate degradation at the invadopodia-ECM interface.

This report shows the binding of both latent recombinant Mr 72,000 MMP and the MMP-inhibitor complex to the plasma membrane at invadopodia and both free and complexed enzyme bind equally well to the invadopodia (Fig. 2). The enzyme associates with ECM fibrils and not membranes of normal CEF (Fig. 2) and does not associate with membrane extracts of normal CEF. However, it associates with the membrane extract of malignantly transformed cells and forms a lower molecular weight form (Fig. 4), suggesting that the putative binding component is differentially expressed upon malignant transformation. This observation also suggests that the TIMP-2 molecule itself does not bind to the putative binding component and does not obscure any sites on the MMP that play a role in membrane binding. The fact that the exogenously added Mr 72,000 MMP binds to the invadopodia in the presence of endogenous enzyme suggests a dynamic interaction between the enzyme and its cell surface receptor.
Immunofluorescent studies indicate that the invadopodia-associated M, 72,000 MMP loses the immunoreactive propeptide (Fig. 3) and TIMP-2 (data not shown). Chen et al. (11) have observed that RSV-CEF produce enhanced levels of M, 72,000 MMP compared to normal CEF and postulate that the inhibition by TIMP-2 may be overcome as a result of this MMP overproduction. This is consistent with our finding that no TIMP-2 is localized at the invadopodia.

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