Rac1 Mediates Type I Collagen-dependent MMP-2 Activation
ROLE IN CELL INVASION ACROSS COLLAGEN BARRIER*

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Yuzheng Zhuge and Jiahua Xu‡
From the Department of Dermatology, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794

Cell migration and proteolysis are two essential processes during tumor invasion and metastasis. Matrix metalloproteinase (MMP)-2 (type IV collagenase; gelatinase A), is implicated in tumor metastasis as well as in primary tumor growth. The Rho family of small GTPases regulates the dynamics of actin cytoskeleton associated with cell motility. In this report, we provide evidence that Rac1, one member of Rho-related small GTPases, is a mediator of MMP-2 activation in HT1080 fibrosarcoma cells cultured in three-dimensional collagen gel (3D-col) and that MMP-2 activation is required for Rac1-promoted cell invasion through collagen barrier. Stable expression of dominant negative (Rac1V12N17) and constitutively active Rac1 (Rac1V12), respectively, in HT1080 cells demonstrates that Rac1 promoted cell invasiveness across type I collagen and collagen-dependent MMP-2 activation. Active Rac1 is sufficient to induce MMP-2 activation in cells cultured in fibrin gel, an extracellular matrix component that does not support MMP-2 activation. The Rac1-dependent MMP-2 activation occurred in a cell-associated fashion and required MMP activities. Because the cell membrane-mediated MMP-2 activation requires MT1-MMP and low amount of issue inhibitor of matrix metalloproteinase-2 (TIMP-2), their expression was examined. Rac1 modulated MT1-MMP mRNA level and the accumulation of a 43-kDa form of MT1-MMP protein, in correlation with MMP-2 activation profile. However, TIMP-2 expression was independent of Rac1 activity. The coordinate modulation of MMP-2 activity and MT1-MMP expression/processing by Rac1 is consistent with cell collagenolytic activity. The C-terminal hemopexin-like domain of MMP-2, which interferes with the cell membrane activation of MMP-2, reduced Rac1-promoted cell invasiveness as monitored by collagen invasion assay. These results suggest that collagen-dependent MMP-2 activation and MT1-MMP expression/processing contribute to Rac-promoted tumor cell invasion through interstitial collagen barrier.

During metastasis, invasive cells must traverse tissue barriers comprised largely of type I collagen. This process depends on the ability of tumor cells to degrade the surrounding collagen matrix and then migrate through the matrix defects (1–3). The actin dynamics regulated by Rho family of small GTPases play a critical role in cell migration (4). The initiation of cell migration is characterized by actin polymerization at the leading edge and extension of a lamella in the direction of motion. Rac1, a member of the Rho family proteins that regulates the assembly of a meshwork of actin filaments at the cell periphery to produce lamellipodia (5), has been implicated in oncogenic transformation (6–8) and metastasis induction (9). Recent studies have demonstrated the direct role of Rac1 activity in cell invasiveness in type I collagen matrix (10, 11). Transfection of non-invasive mammalian epithelial cells T47D with active Rac1 induces cell invasion through type I collagen (10). Similarly, overexpression of adapter proteins p130Cas-associated substrate (CAS)/p-CrkII (Crk) induces Rac1-dependent COS-7 cell invasiveness in three-dimensional collagen (3D-col) culture (11). However, the proteolytic activities during Rac1-promoted cell invasion through type I collagen barriers remain undefined.

Increasing evidence has suggested that proteolytic activities at cell surface promote cell invasion (12). Matrix metalloproteinase (MMP)-2 (type IV collagenase; gelatinase A) is a cell surface-associated type I collagen-degrading MMP (13, 14). Overexpressed in different types of tumor (15), MMP-2 is involved in tumor metastasis, primary tumor growth, and angiogenesis (2, 16–19). Although considerable attention has been focused on the role of MMP-2 as a type IV collagenase in cell invasion across basement membrane, recent evidence has implicated MMP-2 in type I collagen remodeling by tumor cells (20) and the tubular organization of endothelial cells in 3D-col (21). In common with all MMPs, MMP-2 is synthesized and secreted as a latent precursor, requiring proteolytic removal of the propeptide for activation. The physiological mechanism that accounts for MMP-2 activation is under intense study. It is suggested that MMP-2 is activated in a membrane-associated mechanism after a two-step process that involves an initial cleavage of the zymogen followed by an autocatalytic conversion of the intermediate into a fully active enzyme (22–25). MT1-MMP, the best characterized member of MT-MMPs, is believed to carry out the initial cleavage of MMP-2 after the binding of MMP-2 proenzyme to an MT1-MMP-TIMP-2 complex at the cell surface (23, 26, 27). Interestingly, MT1-MMP becomes degraded to an inactive 43-kDa form during the MMP-2 activation (28–31). MT1-MMP is also overexpressed in several tumor tissues where activated MMP-2 is found (19). In

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‡ To whom correspondence should be addressed. Tel.: 631-444-2483; Fax: 631-444-3844; E-mail: jxu@mail.som.sunysb.edu.

The abbreviations used are: CAS, p130Cas-associated substrate protein; MMP-2, matrix metalloproteinase-2; MT1-MMP, membrane type 1-matrix metalloproteinase; MMP-9 type IV collagenase; MP-1, collagenase-1; MMP-13, collagenase-3; 3D-col, three-dimensional collagenase; ECM, extracellular matrix; Crk, c-CrkII; DMEM, Dulbecco’s modified Eagle’s medium; PA, plasminogen activator; TIMP-2, tissue inhibitor of matrix metalloproteinase-2; CTD, C-terminal hemopexin-like domain; CBD, fibronectin type-II-like modules; ConA, concanavalin A.
addition to its ability to activate MMP-2. MT1-MMP has intrinsic ECM degrading activity (32–36). Therefore MT1-MMP and MMP-2 activities at the cell surface provide a powerful combination for the localized ECM remodeling (33, 37).

To evaluate the hypothesis that MT1-MMP/MMP-2 proteolytic cascade might play a functional role in Rac1-induced tumor cell invasion through type I collagen-rich tissue barrier, we examined an invasive HT1080 fibrosarcoma cell line that showed elevated level of active MMP-2 during cell-fibrillar collagen interaction (38). In this report, we provide evidence that Rac1 is a mediator of collagen-stimulated MMP-2 activation and MT1-MMP expression/process, collagennolytic activity, and cell invasion through 3D-col. Furthermore, active MMP-2 contributes to Rac1-induced collagen invasive activity. Our findings suggest that Rac1 mediates MMP-2 activation and MT1-MMP expression/process during the encounter between invading tumor cells and type I collagen-rich stroma, thereby facilitating collagenolysis and cell invasion.

MATERIALS AND METHODS

Cell Culture and Plasminoids—HT1080 fibrosarcoma and HEP3 epidermoid carcinoma cell lines were maintained in DMEM containing 10% fetal bovine serum and heat-inactivated fetal bovine serum (HyClone) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and streptomycin (Life Technologies, Gaithersburg, MD). Stably transfected cell lines were maintained in medium that included 200 μg/ml G418 in addition to the above mentioned supplements. Plasminoids RSV-neo-fibronectinase, c-Myc-tagged Rac1V12N17, and Rac1V12 were kindly provided by Dr. Lorne Talchman, SUNY at Stony Brook, and Dr. Alan Hall, University College London, London, United Kingdom. Plasminoids containing cDNAs for MT1-MMP, MMP-2, TIMP-2, and 36B4 were purchased from ATCC.

Stable Transfection of HT1080 Cell Line—Subconfluent cell culture was co-transfected with one of testing plasminoids (Rac1V12N17, Rac1V12, and vector) and a plasmid construct containing the neomycin-resistant gene by the calcium phosphate precipitation technique as previously described (39). After transfection, individual colonies from Rac1V12N17- and Rac1V12-transfected cells were isolated after 2–3 weeks of 500 μg/ml G418 selection. As control, vector-transfected clones were pooled. All stably transfected cell lines were maintained in growth medium containing 200 μg/ml G418.

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Tetraspan Cells with Concanavalin A—Cells grown on tissue culture plates in growth medium (10% heat-inactivated fetal bovine serum/DMEM) were washed twice with serum-free medium before the addition of serum-free medium supplemented with 0.2% heat-inactivated lactoalbumin and 50 μg/ml concanavalin A (Sigma). Medium was then collected after 16–24 h for zymography.

Three-dimensional Collagen and Fibrin Cell Culture—Type I collagen fibrils were prepared according to a procedure previously described with some modification (40). Pepsin-solubilized bovine dermal collagen dissolved in 0.012 M HCl was 99.9% pure or fibrillogenic (Calbiochem-Novabiochem). Subsequently, cell-collagen or cell-fibrinogen suspension (5 × 104 cells/ml) was plated onto 24-well plates at 250 μl/well or 35-mm plastic dishes at 1.5 ml/dish. Cell-collagen cultures were incubated at 37 °C to form gel. The gelling of cell-fibrin cultures occurred in less than 5 min at room temperature after the addition of thrombin to a final concentration of 0.2 unit/ml. After both collagen and fibrin cell cultures formed gel, serum-free DMEM supplemented with 0.2% heat-inactivated lactoalbumin was added. Gels remained attached to the plastic dish for the duration of incubation.

Substrate Zymography—Conditioned medium was collected from cells cultured for 18–24 h in serum-free medium. To analyze the activity of cell-associated MMP-2, cells (7.5 × 105) in 35-mm plates were released from tissue culture plates by digestion with bacterial collagenase D (Roche Molecular Biochemicals, Indianapolis, IN) for 10 min or dispact (Becton Dickinson, Bedford, MA) for 5 min, respectively. Cells were washed gently with ice-cold phosphate-buffered saline 10–12 times and suspended in 150 μl of 1 × SDS sample buffer. Aliquots (total cell lysates) were immediately processed for enzymatic assay using zymography. Substrate zymography was performed as described previously with some modifications (41, 42). SDS-polyacrylamide (12% unless indicated in the figure legend) gels were co-polymerized with 1 mg/ml gelatin or 0.5 mg/ml type I collagen (Sigma). Samples (conditioned medium or total cell lysates) were resolved under nonreducing conditions. Gels were washed twice in 2.5% Triton X-100 for 1 h before and after incubation in 0.02% NaN3, pH 7.5, 5 mM CaCl2, and 0.02% Na3VO4 (gelatin substrate) or 100 mM Tris-HCl, pH 8.0, 5 mM CaCl2, 0.005% Brij-35, and 0.02% Na3VO4 (type I collagen substrate). At the end of the incubation, gels were stained with Coomassie Blue and destained.

Northern blot analysis—Total RNA was isolated from cells cultured in collagen or fibrin gel for 10 h and Northern analysis was performed as previously described (40). RNA was detected with α-32P-labeled cDNA probes for MT1-MMP and TIMP-2. Control probe was 36B4 cDNA.

Staining of Actin Cytoskeleton—Actin organization was visualized by staining with Texas Red-conjugated phalloidin (Molecular Probes, Eugene, OR). Cells in serum-free vitrogen solution were plated in 8-well treated chamber slides to form gel. After 24 h, cells in collagen gel were fixed in 4% paraformaldehyde, permeabilized with acetone at –20 °C, and stained with Texas Red-conjugated phalloidin for 30 min at room temperature. The images were captured using an epifluorescence microscope at the University Microscopy Imaging Center, SUNY at Stony Brook.

Northern Blot analysis—Total RNA was isolated from cells cultured in collagen or fibrin gel for 10 h and Northern analysis was performed as previously described (43). After detection by monoclonal antibodies against Rac1 (Upstate Biotechnology, Lake Placid, NY), MMP-2 (Chemicon, Temecula, CA), TIMP-2, and MT1-MMP (Calbiochem-Novabiochem), the blots were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

Collagen Fibril Dissolution—The capacity of cells to degrade type I collagen fibrils was assessed based on the modification of a procedure as described (35). Briefly, 300 μl of vitrogen solution at 1 mg/ml was added to 4-well plates in a 2.5× dilution of a 10 mg/ml vitrogen stock. The collagen fibril film was washed with several changes of distilled water and serum-free DMEM before a pellet of 4 × 105 cells in 25 μl of 10% fetal bovine serum/DMEM was dotted onto the center of each well. Cells were allowed to attach for 5 h, washed twice with serum-free DMEM, and then incubated for 3 days in serum-free DMEM supplemented with 0.2% lactoalbumin. Following the incubation, cells were removed with trypsin/EDTA and gelatinolytic staining in the wells was visualized by staining with Coomassie Blue.

For quantification of fibrillar type I collagen degradation, type I rat tail collagen (Becton Dickinson) was labeled with [3H]Acetic anhydride (Amersham Pharmacia Biotech) according to the protocols described (44, 45). 150 μl of [3H]-labeled type I collagen (0.5–1 × 106 cpm/mg protein) was allowed to polymerize in individual wells of a 48-well plate. Cells at 1.5 × 105/300 μl were added to each well and incubated at 37 °C. To follow the progressive degradation of collagen fibrils, aliquots (50 μl) of the medium were collected after 24 and 48 h. The soluble radioactivity was quantified in a liquid scintillation counter.

Expression and Preparation of MMP-2 Domains—A cDNA clone K-121 that contains the coding sequence for MMP-2 with a partially truncated propeptide domain (46) was used as a template for amplification with polymerase chain reaction. The regions corresponding to residues Gly17-Cys631 (C-terminal hemopexin-like domain; CTD) or Val391-Gln594 (fibronectin type-II-like modules; CBD) of MMP-2 proenzyme, respectively, were amplified. The resulting fragments were inserted into a pGEX-3X plasmid, respectively (Amersham Pharmacia Biotech, Uppsala, Sweden). The glutathione S-transferase fusion proteins were purified on Sepharose-coupled glutathione (Amersham Pharmacia Biotech) based on the manufacturer's instructions.

Collagen Invasion Assay—The assay was performed using the modification of procedures previously described (47). 50 μl of vitrogen solution at 1 mg/ml was applied to the upper compartment of each well in a 24-well Transwell plate (8-μm pore size; Costar) and allowed to gel at 37 °C. Cells at 5 × 104 in 200 μl of serum-free DMEM were added to the
upper chamber. Culture medium was added to the lower compartment. In indicated experiments, inhibitors or controls were added to both upper and lower chambers at the following final concentrations: 25 μM SC68180 (formerly SC44463, N-[3-(N-hydroxycarboanilido)-2-(2-methylpropyl)propanoyl]-O-methyl-L-tyrosine-N-ethylamide; a generous gift of Dr. W. C. Parks, Washington University, St. Louis), 100 μg/ml aprotinin (Sigma), 500 ng/ml recombinant TIMP-1 (Chemicon), 500 ng/ml recombinant TIMP-2 (Chemicon), 100 μg furin inhibitor Dec-Arg-Val-Lys-Arg-CHCl (Bachem Biochemicals), 500 ng/ml CTD (Gly417, Cys522, and 500 ng/ml CBD (Val191-Gln364). The invasion proceeded for 24 h at 37 °C. After incubation, the filters were fixed and stained with Diff-Quick staining kit (Fisher Scientific). The cells that reached the underside of the filter were counted. For each filter, the number of cells in 10 randomly chosen microscope fields was determined and averaged. Three invasion chambers were used per condition. The final values were the average of triplicates.

RESULTS

HT1080 Cells Stably Expressing Rac1 Mutants Exhibit Distinct Morphology and Invasive Properties in Three-dimensional Collagen Gel—A human fibrosarcoma cell line, HT1080, was transfected with dominant negative RacV12N17, constitutively active RacV12, or a vector control. Ectopic Rac1 expression was examined by Western analysis in G418-resistant clones. In a representative experiment, a monoclonal antibody against Rac1 detected both ectopic Rac1 tagged with c-Myc (upper band) and endogenous Rac1 (lower band) in lysates of cells expressing RacV12N17 (HN) and RacV12 (HV, but only endogenous Rac1 in vector-transfected cell extracts (HW) (Fig. 1A). Clones that express comparable levels of Rac1 were selected for further study.

Because the branching phenotype of carcinoma cells has been associated with their metastatic capacity (47), whether Rac1 mediates HT1080 cell invasion was assessed. RacV12 substantially increased, whereas RacV12N17 reduced, HT1080 cell invasion across 3D-col (Fig. 1C). To assess whether MMPs or plasminogen activator (PA)/plasmin system is responsible for this process, we monitored cell invasion in the presence of inhibitors for MMPs and PA/plasmin system, SC68180 and aprotinin, respectively. SC68180, formally known as SC44463, is a hydroxymate compound that has been shown to inhibit human keratinocyte migration on native type I collagen as a general MMP inhibitor (48). As shown in Fig. 1D, invasion of HW and HV cells through 3D-col was inhibited by SC68180. Interestingly, the active Rac1-induced cell invasion was reduced ~90% by the MMP inhibitor. In contrast, aprotinin did not impact on the collagen invasiveness. These data indicate that invasion of HT1080 cells across collagen depends on both Rac1 and MMP activities and that Rac1 requires active MMP activities to promote cell invasion.

MMP-2 Is Differentially Activated in HT1080 Cells Stably Expressing Rac1 Mutants Cultured in 3D-col—To investigate the Rac1-mediated MMP activity in HT1080 cells surrounded by collagen matrix, we monitored MMP production by gelatin zymography in these cell lines when cultured in 3D-col for 24 h (Fig. 2A). The conditioned medium of HEP3, an epidermoid carcinoma cell line that secretes MMP-1, MMP-2, and MMP-9 as detected by gelatin zymography (49), was used as a control (Fig. 2A, lane 1). Four major gelatinolytic bands with different intensities were detected in the serum-free medium of HW cell culture (Fig. 2A, lane 2). These bands corresponded to the inactive MMP-9 proenzyme (92 kDa) and three MMP-2 species, latent (L), intermediate (i), and active (A). Interstitial collagenase MMP-1 was undetectable in HT1080-derived cell lines in gelatin (Fig. 2A) as well as type I collagen zymography (Fig. 2B), a method that more sensitively detects MMP-1 than does MMP-2 (42). The distribution of three MMP-2 species was distinctly altered by the stable expression of RacV12N17 (HN) and RacV12 (HV). Active and latent MMP-2 became the major species in HV and HN cells, respectively (Fig. 2A, lanes 2–4), suggesting Rac1 specifically mediates MMP-2 proenzyme processing cells cultured in 3D-col. Active MMP-9 was not detected in this system; instead, latent MMP-9 at 92 kDa was detected at low level in both HW and HV cells, but nearly undetectable in HN cells (Fig. 2A).

Cells cultured in fibrin gel were used as control. Unlike 3D-col that induced MMP-2 processing, fibrin gel did not promote MMP-2 activation (Fig. 2A, compare lanes 2 and 5). Predominantly latent MMP-2, as well as low level of the intermediate form, were detected in HW and HN cells in fibrin gel (Fig. 2A, lanes 5 and 6). However, Rac1V12 induced MMP-2 activation by cells cultured in fibrin gel (Fig. 2A, compare lanes 5 and 7). Similar MMP-2 activation pattern was also observed using type I collagen zymography (Fig. 2B). Taken together, these results suggest that Rac1 is required for maximal activation of MMP-2 by 3D-col and that active Rac1 is sufficient to induce MMP-2 processing in the absence of collagen signal.

To determine the specificity of Rac1 in mediating collagen-induced MMP-2 activation, MMP-2 activating signals that are independent of Rac1 activity were sought. Canancalvan A (ConA) is known to activate MMP-2 (50). Stimulation of RacV12- or RacV12N17-transfected cells with ConA had similar effects on MMP-2 activation (Fig. 2C), suggesting that Rac1 is specifically involved in MMP-2 activation in response to type I collagen stimulation. Latent MMP-9 level remained similar in all three cell lines under this culture condition (Fig. 2C).

Rac1 Mediates Cell-associated Activation of MMP-2 by Type I Collagen—The activation of MMP-2 is thought to occur on cell membrane (51). To determine whether Rac1-dependent MMP-2 activation is associated with cells, cell-bound MMP-2 activity was assessed. Consistent with results from secreted MMP-2, the collagen-induced level of cell-associated active MMP-2 was greatly enhanced by RacV12, but reduced by RacV12N17 (Fig. 3A, lanes 4–6). Active MMP-2 was also detected in cell extracts prepared from RacV12-expressing cells grown in fibrin gel, a condition that did not stimulate MMP-2 activation (Fig. 3A, lanes 1–3). Western blotting of cell extracts detected both proenzyme and processed MMP-2 in response to collagen or RacV12 (Fig. 3B), confirming the zymographic results (Fig. 3A). Therefore, we conclude that Rac1 mediates cell-associated activation of MMP-2.

Rac1 Mediates MT1-MMP Expression and Processing—Cell-associated activation of MMP-2 requires MT-MMPs (51). Among several MT-MMPs identified to data, MT1-MMP is the best documented in its ability to activate MMP-2. To assess whether Rac1 mediates MMP-2 activation through MT1-MMP, we first determined whether MMP-2 activation by RacV12 requires MMP activities. The synthetic MMP inhibitor SC68180 abrogated MMP-2 activation (Fig. 4, lanes 2 and 5), whereas the inhibition of serine proteinases/plasmin system by aprotinin did not (Fig. 4, lanes 3 and 6), suggesting that MMP activities were responsible for the cell membrane activation of
MMP-2. The inhibitory effect of SC68180 on MMP-2 activation was observed in both cell and conditioned medium (Fig. 4).

Next, we assessed whether Rac1-mediated MMP-2 activation coincides with MT1-MMP expression. MT1-MMP mRNA was increased modestly by 3D-col (Fig. 5A, compare lanes 1 and 4). This induction was further enhanced by Rac1V12, but attenuated by Rac1V12N17 (Fig. 5, lanes 5 and 6). Rac1V12 had the ability to increase MT1-MMP mRNA in cells cultured in fibrin gel (Fig. 5A, lane 3). As shown in Fig. 5B, Western blotting detected two bands corresponding to 60 and 43 kDa, the two MT1-MMP species thought to represent the mature protein and its processing product, the N-terminal truncated protein, respectively (28, 52). Two unspecified bands of high molecular weight were also detected by monoclonal antibody against MT1-MMP, presumably representing an artifact of the antibody. Although the level of the 60-kDa form was enhanced by Rac1V12, HW and HN cells demonstrated comparable level of the 60-kDa MT1-MMP (Fig. 5B). Interestingly, the level of 43-kDa protein, the truncated MT1-MMP that was suggested to reflect the consumption of MT1-MMP in the activation of MMP-2 proenzyme (28), mirrored the MMP-2 activation pattern as observed earlier (Fig. 2A). As shown in Fig. 5B, both

FIG. 1. Stable expression of dominant negative and active Rac1 changes morphology, organization, and invasiveness of HT1080 fibrosarcoma cells in three-dimensional collagen matrix. A, HT1080 cells stably expressing vector only (HW), Myc-tagged Rac1V12N17 (HN), and Myc-tagged Rac1V12 (HV) were examined for expression of Rac1 protein. Lower band corresponds to endogenous Rac1 that migrates faster than tagged ectopic Rac1 (upper band). B, three HT1080-derived cell lines were cultured in 3D-col in serum-free medium for 1 day. Changes in cell morphology were visualized with epifluorescence microscope viewing of rhodamine phalloidin staining (a-c) and with phase-contrast microscope (d-f). a and d, HW cells; b and e, HN cells; c and f, HV cells. C, three HT1080-derived cell lines were added to the upper compartments of type I collagen invasion chamber. After 24 h cells that invaded to the underside of the filters were counted. Results shown are the mean of cell number in 10 randomly selected fields in a single representative experiment of six performed. D, the invasion of three HT1080-derived cell lines through 3D-col was examined in the presence of either 25 μM SC68180, a general inhibitor of MMP, or 100 μg/ml aprotinin, an inhibitor of PA/plasmin system. The number of invasive HW cells in the absence of inhibitors was set as 100%. The results are representative of four independent experiments.
collagen and Rac1V12 induced the level of 43-kDa MT1-MMP (lanes 3, 4, and 6). Therefore, Rac1 mediated both MT1-MMP expression/processing and the accumulation of the 43-kDa MT1-MMP processing product in correlation with MMP-2 activation.

Tissue inhibitor of matrix metalloproteinase-2 (TIMP-2), an effective inhibitor of MT1-MMP and membrane-associated MMP-2, is proposed to be a necessary component for MMP-2 activation at low concentration (23, 27). The role of Rac1 in TIMP-2 expression was also determined. Results from Northern and Western blotting indicated that TIMP-2 mRNA and protein levels were not obviously changed by either collagen or Rac1V12 (data not shown). An increase in the amount of MT1-MMP and little change in the level of TIMP-2 protein by colla-
gen or active Rac1 may imply a shift to increased cellular capability of activating MMP-2 proenzyme.

**Rac1 Mediates Type I Collagen Fibril Degradation**—Because both MMP-2 and MT1-MMP have the ability to degrade type I collagen (14, 33, 34), we wondered whether Rac1V12 might increase collagenolytic activity by HT1080 cells. To examine this possibility, fibrillar collagen degradation was assessed. Cells were seeded in a pellet on a reconstituted type I collagen fibril film and incubated in serum-free medium. Cells expressing vector control and Rac1V12 readily degraded the underlying collagen film to a different extent (Fig. 6A). Cells expressing Rac1V12N17, however, did not degrade as much the underlying collagen matrix. Interestingly, like 3D-col, the collagen fibril film also induced the processing of a fraction of MMP-2 proenzyme to active form in a Rac1-dependent manner (Fig. 6B). This process coincided with the modulation of collagenolytic activity.

To quantify the collagenolytic activity, cells were plated onto radioactive collagen fibril film. Released radioactivity indicated that collagenolytic activity in HW and HV cells was 1–5-fold higher than that in HN cells (Fig. 6C). Together these results demonstrated that Rac1 mediates collagenolytic activity in accordance with cell invasiveness (Fig. 1C), MMP-2 activation (Fig. 2A and Fig. 3), and MT1-MMP expression/processing (Fig. 5, A and B).

**Rac1-induced Cell Invasiveness in 3D-col Is Reduced by MMP-2 Inhibitors**—The C-terminal hemopexin domain (CTD) of MMP-2 has been extensively used to specifically inhibit cell-associated MMP-2 activation (22, 53, 54) and to study MMP-2 activity-dependent cellular functions (20, 36, 55). In contrast, the collagen-binding domain (CBD) of MMP-2 that contains fibronectin type II-like modules does not have inhibitory effect on MMP-2 activation as stimulated by ConA (54, 56). The recombinant CTD (Gly417-Cys631) and CBD (Val191-Gln364) were synthesized and tested for their ability to inhibit MMP-2 activation in HW and HV cells embedded in 3D-col (Fig. 7A). CTD inhibited MMP-2 activation in both cell lines in a concentration-dependent manner (Fig. 7A, lanes 1–6), whereas CBD did not have inhibitory effect (Fig. 7A, lanes 7 and 8). Therefore, CTD and CBD were used as a specific inhibitor of MMP-2 and a non-inhibitory control, respectively, in the collagen invasion assay.

To evaluate whether MMP-2 activity contributes to the invasive behavior of Rac1V12-expressing cells, we analyzed cell
transmigration across 3D-col in the presence of inhibitors of MMP-2 activation. Serum-free medium was taken from the upper chamber for analysis of proteolytic activities released by cells during invasion. CTD at 500 ng/ml completely inhibited Rac1V12-promoted MMP-2 activation during invasion (Fig. 7B, lane 5), whereas the control CBD at the same concentration did not impact on the process (lane 6), supporting the specificity of CTD inhibition of MMP-2 activation. Other positive and negative controls were also used. MMP-2 activation in active Rac1-expressing cells was not affected by TIMP-1 at 500 ng/ml (Fig. 7B, lane 2). In contrast, TIMP-2, a preferential inhibitor of MMP-2, suppressed MMP-2 activation at the same concentration (Fig. 7B, lane 3). A furin inhibitor, Dec-Arg-Val-Lys-Arg-CH₂Cl (58), is reported to reduce MT1-MMP expression/processing and collagenolytic activity. Therefore, our results support the notion that modulation of MMP-2 activation is at least one of mechanisms by which Rac1 mediates cell invasion through type I collagen matrix barrier.

**DISCUSSION**

The inhibition of MMP-2 activation by various inhibitors coincided with the reduced cell invasiveness (Fig. 7B). As much as 80% inhibition of cell invasion was observed in the presence of TIMP-2. The presence of CTD resulted in ~60% reduction in cell invasion. However, TIMP-1 and CBD, two controls that did not inhibit MMP-2 activation, did not affect Rac1-induced cell invasiveness. Therefore, our results support the notion that modulation of MMP-2 activation is at least one of mechanisms by which Rac1 mediates cell invasion through type I collagen matrix barrier.

The Rho group of small GTPases and MMPs are two protein families that play key roles in cell movement. Here we report that Rac1-regulated cellular MMP-2 activation by type I collagen and that MMP-2 activity is required for Rac1-induced cell invasion across type I collagen barrier. In correlation with MMP-2 activation, Rac1 also modulated collagen-induced MT1-MMP expression/processing and collagenolytic activity. This suggests that Rac1 mediates the collagen-dependent MT1-MMP/MMP-2 activity as one of mechanisms by which it induces cell invasion.

Cells expressing dominant negative Rac1 reduced MMP-2 activation by 3D-col, implying that collagen fibril-cell interaction initiates a proteolytic cascade to cleave MMP-2 proenzyme through Rac1. To determine that MMP-2 proenzyme processing coincided with Rac1 activation in cells cultured in 3D-col, we performed the pulling down assay that selectively detects the GTP-bound Rac1. However, Rac1 activity was unchanged in vector-expressing cells stimulated by type I collagen (data not shown). It has been recently proposed that the GTP-bound small GTPases stimulated by selective ECM signals may be difficult to detect by the pulling down assay because the activated forms probably appear very transiently and are rapidly down-regulated (60). An alternative approach was thus taken to confirm the role of active Rac1 in MMP-2 activation by 3D-col. The stable expression of active Rac1 enhanced 3D-col-induced MMP-2 activation, confirming the results showing the suppression of MMP-2 activation by dominant negative Rac1. Furthermore, Rac1V12 conferred cells with the ability to induce MMP-2 activation and MT1-MMP expression/processing when cells expressing active Rac1 were cultured in fibrin gel, a matrix that does not induce MMP-2 activation. Therefore, we conclude that active Rac1 is necessary and sufficient for collagen-induced MMP-2 activation.

Our finding is in line with the study showing that MMP-2 activation is induced by 3D-col via β₁ integrin (61). Cellular interaction with 3D-col has been shown to result in β₁ integrin aggregation (62). This mode of β₁ integrin modulation promotes MMP-2 activation/MT1-MMP processing (29) as well as phosphorylation of focal adhesion kinase (63). Activated focal adhesion kinase is linked to Rac1 by stimulating adaptor protein CAS (64), a molecule that promotes Rac1 activation after the complex formation with CrkII and DOCK180 (65–67). As expected from this chain of events (β₁ integrin aggregation → focal adhesion kinase → CAS/CrkII/DOCK180 → Rac1), the role of Rac1 in MMP-2 activation may reflect that of β₁ integrin aggregation. Indeed, we found that similar to β₁ integrin aggregation, active Rac1 was sufficient for induction of MMP-2 activation. Furthermore, a blocking antibody against β₁ integrin failed to inhibit Rac1V12-promoted MMP-2 activation in cells cultured in 3D-col, whereas β₁ integrin aggregation-induced MMP-2 activation was attenuated by the stable expression of Rac1V12N17 (data not shown).

MMP-2 activation is unique among secreted MMPs in that it occurs on cell membrane (51). Membrane-bound MT1-MMP is known to initiate MMP-2 activation (24, 26). It has been hy-
hypothesized that MT1-MMP and TIMP-2 form a “receptor” complex that binds MMP-2, resulting in the proteolysis of bound MMP-2 by an adjacent free MT1-MMP (23, 27, 68). This model predicts that the balance between TIMP-2 and MT1-MMP is of critical importance in determining the activation status of MMP-2. A variation in either TIMP-2 or MT1-MMP status could result in the modulation of MMP-2 proenzyme processing. Indeed, the culture of several cell types on or in 3D-coll induces MT1-MMP expression and, correspondingly, an increase in MMP-2 activation (21, 69). In line with these studies, our data showed that Rac1 induced cell-associated MMP-2 activation in correlation with altered level of MT1-MMP mRNA, but not TIMP-2 expression, implying that Rac1 activity facilitates a shift in balance toward increasing proteolytic activity of MMP-2. How might Rac1 mediate MT1-MMP expression that leads to MMP-2 activation? Active Rac1 has been previously reported to increase interstitial collagenase (MMP-1) expression in rabbit synovial fibroblasts through a series of events that include the induction of H_2O_2 production, NF-κB activation, and interleukin-1α secretion (70). Inflammatory cytokines including interleukin-1α and tumor necrosis factor-α can induce MT1-MMP expression in human endothelial cells (71). It is thus likely that in our cell system, Rac1 may regulate MT1-MMP expression and MMP-2 activation by modulating interleukin-1α production. However, the exact mechanism by which Rac1 mediates MT1-MMP expression and MMP-2 activation remains to be elucidated.

In addition to MT1-MMP expression, we also detected Rac1-mediated MT1-MMP processing as judged by Western analysis. MT1-MMP proteins were detected as two major bands, 60 and 43 kDa. The accumulation of the 43-kDa MT1-MMP mirrored the status of MMP-2 activation mediated by active or negative Rac1. Recently, increasing evidence has indicated that the generation of a 43-kDa truncated MT1-MMP directly correlates with MMP-2 activation by signals such as phorbol 12-myristate 13-acetate (28, 52, 72), ConA (29), fibronectin (31), and 3D-coll (30). In contrast, the level of 60-kDa MT1-MMP, most likely corresponding to the processed mature form of MT1-MMP (32), was not modulated by these stimulators. It is suggested that the 43-kDa form of MT1-MMP represents an inactive hypro-duct during the activation of MMP-2 proenzyme by active MT1-MMP, reflecting the consumption of MT1-MMP in the activation and release of MMP-2 (28). The spatial localization of MT1-MMP at invadopodia, a special membrane protrusion that is analogous to lamellipodia and makes contacts with the underlying ECM surface, is essential for its function in degrading ECM substrates (37). It is probable that MMP-2 activation by MT1-MMP also depends on the invadopodia localization (73). Therefore, the proper function of MT1-MMP may depend on both cellular expression and specific membrane localization. Rac1 may be involved in both processes. While it is conceptually unclear at this stage about the invadopodia structure in our three-dimensional tissue culture model, striking morphological differences among HW, HN, and HV cell lines were observed. We speculate that the Rac1-mediated spatial arrangement in 3D-coll may be a functional switch for MT1-MMP in our cell system. This could explain, at least partially, that as reflected by the 43-kDa form level, only a fraction of cellular MT1-MMP is accessible to MMP-2. This possibility will be the subject of future investigation.

A number of experimental approaches have been taken to determine the function of MMP-2 in cell motility. These approaches include the specific inhibition with neutralizing antibody against MMP-2 and the C-terminal hemopexin domain of MMP-2 (36, 74, 75) and the indirect inhibition with furin inhibitor and antisense oligonucleotides to prevent processing and synthesis of MT1-MMP, respectively (36, 59, 76). We assessed the functional role of MMP-2 in Rac1-induced cell invasion by inhibiting MMP-2 activity with reagents that target at the membrane activation of MMP-2 (the C-terminal hemopexin domain of MMP-2), MT1-MMP processing (furin inhibitor), activity of both MMP-2 and MT1-MMP (TIMP-2), and activity of general MMPs (SC68180). The inhibition of MMP-2 activation correlated with the reduction of Rac1-mediated cell invasiveness, suggesting that MMP-2 activity contributes to this process. It should be pointed out that MT1-MMP and MMP-2 may mediate cell invasion as components of a proteolytic activity cascade as well as two individual type I collagen-degrading MMPs. This notion is consistent with our finding that suppression of both MT1-MMP and MMP-2 activity by furin inhibitor or TIMP-2 more effectively reduced cell invasion than the inhibition of MMP-2 activation alone by its C-domain. However, it is also possible that these two inhibitors might target at a broader range of molecules involved in cellular invasion than the specific MMP-2 inhibitor C-domain does. While the function of MMP-2 is studied in the context of its activator MT1-MMP, the direct contribution of MT1-MMP in Rac1-mediated cell invasion may be studied in a cell system that does not express MMP-2. By using MMP-2-negative cell lines, Koshikawa et al. (36, 77) identified the MMP-2-independent function of MT1-MMP in cleaving laminin-5, a substrate of MMP-2. Whether native helical type I collagen can directly serve as a substrate of MMP-2 remains controversial (14, 78). Whereas Aimes and Quigley (14) have shown the evidence that suggests MMP-2 as an interstitial collagenase, Seltzer and Eisen (78) have indicated that the helix-relaxed, but not native helical, type I collagen is susceptible to digestion by purified MMP-2. In our cell systems, it is yet to be defined whether MMP-2 is sufficient to degrade native type I collagen or requires the helix relaxation by another MMP to initiate type I collagen cleavage process.

In summary, we have shown that Rac1 mediated collagen-induced MMP-2 activation and a shift in balance between MT1-MMP and TIMP-2 toward collagenolytic phenotype. Evidence was also presented that MMP activities, largely MT1-MMP and MMP-2, were necessary for Rac1-promoted cell invasion through 3D-coll. These findings suggest a mechanism by which the encounter between type I collagen and invading tumor cells could stimulate elevated level of MMP-2/TIMP-1-MMP activities, leading to increased collagenolysis. This reciprocal regulation between collagen and MT1-MMP/TIMP-2 is at least partially responsible for the Rac1-mediated cell invasion across collagen barrier. Therefore, the block of an activity chain, type I collagen → Rac1 → MT1-MMP → MMP-2, may impair the invasiveness of certain tumor cells in interstitial stroma.

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REFERENCES

1. MacDougall, J. R., and Matrisian, L. M. (1995) Cancer Metastasis Rev. 14, 351–362.
2. Stetler-Stevenson, W. G., Liotta, L. A., and Kleiner, D. E., Jr. (1993) FASEB J. 7, 1434–1444.
3. Morton, D. L., Essner, E. R., Kirkwood, J. M., and Parker, R. G. (1997) in Cancer Medicine (Holland, J. F., East, R. C., Morton, D. L., Frei, E., Kufe, D. W., and Weichselbaum, R. R., eds) pp. 2467–2499, Williams & Wilkins, Baltimore, MD.
4. Lauffenburger, D. A., and Horwitz, A. F. (1996) Cell 84, 359–369.
5. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) Cell 70, 401–410.
6. Qi, R. G., Chen, J., Kirn, D., McCormick, F., and Symons, M. (1995) Nature 374, 457–459.
7. Kholo-Sarfari, R., Solski, P. A., Clark, G. J., Kincir, M. S., and Der, C. J. (1995) Mol. Cell. Biol. 15, 6443–6453.
8. van Leeuwen, F. N., van der Kamen, R. A., Habets, G. G., and Collard, J. G. (1995) Oncogene 11, 2215–2221.
9. Habets, G. G., Scholtes, E. H., Zuidgeest, D., van der Kamen, R. A., Stam, J. C., Berns, A., and Collard, J. G. (1994) Cell 77, 537–549.
10. Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J., and Parise, L. V. (1997) *Curr. Opin. Cell Biol.* 10, 443–449.
11. Aimes, R. T., and Quigley, J. P. (1995) *J. Biol. Chem.* 270, 2891–2895.
12. Levy, A. T., Cioce, V., Sobel, M. E., Garbisa, S., Grigioni, W. F., Liotta, L. A., Itoh, T., Tanioka, M., Yoshida, H., Yoshioka, T., Nishimoto, H., and Itohara, S. (1999) *Cell* 98, 431–440.
13. Imren, S., Kohn, D. B., Shimada, H., Blavier, L., and DeClerck, Y. A. (1996) *J. Cell Biol.* 132, 1191–1201.
14. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G. (1991) *Cancer Res.* 51, 4033–4039.
15. Sato, H., and Seiki, M. (1994) *J. Cell Biol.* 125, 95–105.
16. Deryugina, E. I., Bourdon, M. A., Reisfeld, R. A., and Strongin, A. (1998) *J. Biol. Chem.* 273, 27119–27126.
17. Sato, H., and Seiki, M. (1994) *J. Biol. Chem.* 269, 25113–25119.
