Rac Affects Invasion of Human Renal Cell Carcinomas by Up-regulating Tissue Inhibitor of Metalloproteinases (TIMP)-1 and TIMP-2 Expression*

Received for publication, June 1, 2001, and in revised form, September 6, 2001
Published, JBC Papers in Press, September 10, 2001, DOI 10.1074/jbc.M105049200

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Rho-like GTPases, including Cdc42, Rac1, and RhoA, regulate distinct actin cytoskeleton changes required for adhesion, migration, and invasion of cells. Tiam1 specifically activates Rac, and earlier studies have demonstrated that Tiam1-Rac signaling affects migration and invasion in a cell type- and cell substrate-specific manner. In the present study, we examined the role of Tiam1-Rac signaling in migration and invasion of human renal cell carcinomas. Stable overexpression of Tiam1 or constitutively active V12-Rac1 in a human renal cell carcinoma cell line (clearCa-28) strongly inhibited cell migration by promoting E-cadherin-mediated cell-cell adhesion. Blocking E-cadherin-mediated adhesion by E-cadherin-specific HAV peptides allowed cells to migrate, but was not sufficient to antagonize Tiam1- and V12-Rac1-induced inhibition of Matrigel invasion, suggesting that Rac may influence invasion also through other mechanisms. Indeed, Tiam1-mediated Rac activation induced transcriptional up-regulation of tissue inhibitor of metalloproteinases-1 (TIMP-1) and post-transcriptional up-regulation of TIMP-2, whereas secretion and activity levels of their counterparts, matrix metalloproteinase-9 and matrix metalloproteinase-2, respectively, were not affected. Application of recombiant TIMP-1 and TIMP-2 proteins significantly inhibited invasion of mock-transfected clearCa-28 cells, supporting a role of TIMPs in Rac-mediated inhibition of invasion. To our knowledge, this is the first evidence that increased Rac signaling may inhibit invasion of epithelial tumor cells by up-regulation of TIMP-1 and TIMP-2.

Tumor invasion is a complex biological process, during which tumor cells detach from the primary tumor and infiltrate the surrounding tissue. This process requires loss of cell contacts between tumor cells, active cell migration, adhesion to the extracellular matrix (ECM) and proteolytic degradation of the ECM (1, 2). On the molecular level, a number of different molecules, including cadherins, integrins, proteases, and growth factors, have been implicated in the regulation of tumor cell invasiveness (2).

Family members of the Rho-like GTPases, including Cdc42, Rac1, and RhoA, mediate distinct actin cytoskeleton changes required for cell adhesion, migration, and invasion (3–5). Their effects both on migration and invasion of epithelial cells seem to be at least in part cell type- and cell substrate-specific. Sustained activation of Rac by overexpression of the Rac-specific activator, Tiam1, or overexpression of constitutively active V12-Rac1 induces invasion and metastasis of murine T lymphoma cells (6, 7). In contrast, activation of Rac promotes E-cadherin-mediated cell-cell adhesion and, therefore, inhibits migration and invasion of epithelial cells (8–11). However, in other studies, Rac promotes migration and invasion of epithelial cells, as shown for T47D, colon epithelial, and Madin-Darby canine kidney (MDCK) cells (12–15). These seemingly opposing effects of Rac on both migration and invasion of epithelial cells were reconciled at least in part by the observations that the effects of Rac depend on the cell-substrate used, whether or not the formation of E-cadherin-mediated cell-cell adhesions is prevented, and the cell type studied (14, 16). For instance, in human keratinocytes it has recently been demonstrated that, although Rac is required for the formation of E-cadherin-dependent cell-cell adhesions, activation of Rac may also lead to the disruption of these adhesions in a time- and concentration-dependent manner (17).

In recent studies on mesenchymal cells, Rac has also been implicated in the regulation of distinct matrix metalloproteinases (MMPs) (18, 19). MMPs represent a family of proteases, which are subclassified according to their substrate preferences and structural properties (for review, see Ref. 20). Of all MMPs, MMP-2 (gelatinase-A, 72-kDa type IV collagenase) and MMP-9 (gelatinase-B, 92-kDa type IV collagenase) in particular are known to play a pivotal role in tumor invasion by proteolytic degradation of major basement membrane components such as type IV collagen, laminin, and nidogen (20–22). Like most MMPs, MMP-2 and MMP-9 are secreted as latent pro-enzymes (pro-MMPs), which are extracellularly activated through proteolytic cleavage by other MMPs or other enzyme classes (20, 21). Activities of MMPs are counterbalanced by specific tissue inhibitors of metalloproteinases (TIMPs), four different isoforms of which have been identified so far (23–25).

Although all TIMPs inhibit MMP-2 and MMP-9 as well as other MMP activities by forming 1:1 molar stoichiometric complexes with active enzymes, the association of TIMPs with other MMP activities by forming 1:1 molar stoichiometric complexes with active enzymes, the association of TIMPs with proteolytic degradation of major basement membrane components such as type IV collagen, laminin, and nidogen (20–22). Like most MMPs, MMP-2 and MMP-9 are secreted as latent pro-enzymes (pro-MMPs), which are extracellularly activated through proteolytic cleavage by other MMPs or other enzyme classes (20, 21). Activities of MMPs are counterbalanced by specific tissue inhibitors of metalloproteinases (TIMPs), four different isoforms of which have been identified so far (23–25). Although all TIMPs inhibit MMP-2 and MMP-9 as well as other MMP activities by forming 1:1 molar stoichiometric complexes with active enzymes, the association of TIMPs with pro-MMPs, which prevents subsequent activation of these pro-enzymes, is more specific; TIMP-1 associates exclusively with pro-MMP-9, TIMP-2 and TIMP-4 only with pro-MMP-2.
In contrast, TIMP-3 interacts with both pro-enzymes (23, 26, 27). Imbalances between pro-MMPs/MMPs and TIMPs are thought to influence the invasive phenotype of cells, and consequently overexpression of any of the TIMP isoforms or application of recombinant TIMP proteins were found to inhibit invasion in different tumor models (23, 28, 29). To our best knowledge, a role for Rac in regulating TIMP expression has not been reported yet.

Here we have analyzed the role of Rac signaling in invasion and migration of human renal cell carcinoma (RCC). We show that activation of Rac promotes E-cadherin-mediated cell-cell adhesion and inhibits cell dissociation in RCC cells. Activation of Rac also inhibits the more complex process of Matrigel invasion. Invasiveness could not be restored by blocking E-cadherin-mediated cell-cell adhesion, suggesting the existence of an additional, E-cadherin-independent mechanism through which activated Rac may inhibit invasion. In search for this mechanism, we found selective up-regulation of TIMP-1 and TIMP-2 by Tiam1 and Rac. The functional relevance of this up-regulation was substantiated by demonstrating that TIMP-1 and TIMP-2 inhibit Matrigel invasion of mock-transfected control cells.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Stable Gene Transfection, and Cell Culture Conditions—**The human clear cell RCC cell line clearCa-28 is an androgenic Tiam1 (30) and was chosen for transfection experiments and studies on Rac-mediated invasion. ClearCa-28 was kindly provided by Dr. Gerharz (Heinrich-Heine-University, Duesseldorf, Germany) and was stably transfected by retroviral transduction with either empty vector (pLZRS), an active mutant of Tiam1 (C1199-Tiam1) (9) or Myc-epitope-tagged, constitutively active V12-Rac1 as described (9). In the present study, C1199-Tiam1-31, the effects of C1199-Tiam1 and V12-Rac1 observed in clearCa-28 cells (see “Results”) were verified in a human colon carcinoma cell line, which was stably transfected as described above. This cell line was established in our laboratory from a histologically confirmed adenocarcinoma of the colon and designated as DusCol-1B. A detailed characterization of this cell line will be described elsewhere.2 Stably transfected cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS) (both Sigma) and antibiotics. G418 (Sigma) was used as a selection marker for the presence of pLZRS in a concentration of 500 μg/ml. HepG2 cells, which were obtained from the German Collection of Cell Cultures (DSMZ, Braunschweig, Germany) and were maintained in RPMI 1640 medium supplemented with 10% FCS (both Sigma) and antibiotics. Cell lines were incubated at 37 °C in an atmosphere of 5% CO2.

**Matrigel Invasiveness**—In vitro invasiveness was determined by means of the Matrigel invasion assay, which was essentially performed as recently described (32). In this invasion assay, tumor cells have to overcome a reconstituted basement membrane by a sequential process of proteolytic degradation of the substrate and active migration. Costar transwells (pore size 8 μm) were coated with Matrigel (Becton Dickinson), dried at 37 °C in an atmosphere of 5% CO2, and reconstituted with serum-free medium. The homogeneity of the coating was checked by protein stain. A single cell suspension of 50,000 tumor cells in serum-free medium was inoculated to the upper chamber, after having added conditioned serum-free medium to the lower chamber. Tumor cell invasiveness was determined by counting all tumor cells in five randomly selected counting areas at the lower surface of the filter after an incubation period of 24 h. Cells on the upper surface were wiped away, filters were fixed in methanol, and stained with hematoxylin and eosin. Invasion of mock-transfected clearCa-28 cells was also analyzed in the absence or presence of recombinant TIMP-1 and TIMP-2 proteins (Chemicon) as indicated. Invasion of both mock-transfected cells and C1199-Tiam1-expressing cells was also investigated in the presence of E-cadherin-specific inhibitory HAV peptides (500 μg/ml) (14, 33). Each assay was performed in triplicate and was repeated twice. Data were statistically analyzed by the nonparametric Mann-Whitney U test.

**Cell Migration Assay**—Cell migration was determined as tumor cell spreading from three-dimensional tumor cell spheroids as described recently (32). Spheroids were produced by incubating tumor cells on a grary shaker and were used because they recapitulate the in vivo situation where cell-cell and cell-substrate interactions are involved in cell migration (1). Each spheroid (6 mm diameter), each 0.4 mm in diameter, were selected and singly transferred to a 24-well plate. Subsequently, tumor cells were incubated for 48 h, and the capability of tumor cells to leave the spheroid by active locomotion was determined by measuring the longest and shortest diameter of the outgrown field vertical to each other after 48 h. Migration was determined on laminin (Sigma), as laminin is a major component of basement membranes and was used in a similar experiment for cell spreading previously. A single out-growth covered by tumor cells (A) was ellipse- or circle-like in shape, its size was calculated according to the following formula: A = (longest diameter × shortest diameter)/4 × π. The data were statistically analyzed by the nonparametric Mann-Whitney-U test.

**Cell-Cell Adhesion/Growth Patterns**—To investigate cell-cell adhesion and growth patterns, single cell suspensions of stably transfected cell lines were seeded in either normal cell culture medium or medium supplemented with human E-cadherin- or N-cadherin-specific inhibitory HAV peptides (500 μg/ml) (14, 33).

**Immunoblotting**—For detection of Tiam1 or Myc epitope-tagged V12-Rac1 expression, cells were lysed in a buffer containing 150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, 20 μM aprotinin, 20 μM leupeptin, and 200 μM phenylmethylsulfonyl fluoride (32). For detection of E-cadherin, α-catenin, β-catenin, or γ-catenin, cells were lysed in radioimmunoprecipitation assay buffer containing 50 mM NaCl, 25 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1% β-mercaptoethanol, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride (34). Secretion levels of TIMPs were determined in the same concentrated serum-free medium as used for zymography (see below). Protein concentrations were determined by the Bradford method, and equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis. Expression/secretion levels of the respective proteins were determined by commercially available TIMP-1- and TIMP-2-specific immunoassay kits (Chemicon), according to the manufacturer’s instructions.

**Quantitative TIMP-1 and TIMP-2 Immunoassays**—Cells were incubated for 24 h in serum-free medium. Supernatants were collected and calibrated with the number of cells in culture, was concentrated 15-fold by ultrafiltration (Centricon 10, Amicon), mixed with Laemmli sample buffer, and subjected to electrophoresis on a 10% SDS-PAGE gel containing 0.1% β-mercaptoethanol. Proteins were transferred to a nitrocellulose membrane, which was probed with the anti-TIMP antibodies. Bound antibodies were visualized by chemiluminescence (ECL, Roche Molecular Biochemicals) was used.

**Zymography**—Amounts and activities of secreted MMP-2 and MMP-9, also referred to as 72- and 92-kDa gelatinases, respectively, were determined by gelatin zymography as described (32). Briefly, cells were cultivated in serum-free medium for 24 h. The supernatant, calibrated with the numbers of cells in culture, was concentrated 15-fold by ultrafiltration (Centricon 10, Amicon), mixed with Laemmli sample buffer without reducing agents, and incubated at 37 °C for 30 min. Samples were subjected to nonreducing SDS-polyacrylamide gel electrophoresis with 0.2% gelatin co-polymerized in a 10% polyacrylamide gel. After electrophoresis, gels were washed in 2.5% Triton X-100; incubated in 50 mM Tris, pH 7.5, 20 mM NaCl, 5 mM CaCl2 for 24 h, and stained with Coomassie Blue R. Because we were interested in the proactivity of MMP-2 and MMP-9 and the fact that conversion of latent proenzymes of MMP-2 and MMP-9 to the active enzymes results in the removal of a 10-kDa amino-terminal domain, each protease can be determined by gelatin zymography, as indicated by clear bands of gelatinolytic activity of the respective molecular weights (22). Amounts and activities of cell membrane-associated MMP-2 and MMP-9 were determined as described by Cao et al. (35).
Northern Blot Analysis—Northern blots for TIMP-1 and TIMP-2 were performed as described previously (36). Probes were obtained from purified inserts of the following plasmids, which were kindly provided by Dr. P. Brown (British Biotech Pharmaceuticals Ltd., Oxford, United Kingdom): human entire TIMP-1 cDNA in pGEM-4Z (insert: EcoRI/EcoRI), human entire TIMP-2 cDNA in pGEM-4Z (insert: HindIII/EcoRI).

Chloramphenicol Acetyltransferase (CAT) Reporter Gene Assays—To investigate the effects of Tiam1 and Rac on TIMP-1 promoter activation, HepG2 cells were cotransfected with a −102/+95 human TIMP-1 promoter CAT-reporter construct (37), kindly provided by Dr. I. M. Clark (University of East Anglia, Norwich, United Kingdom), and FL-Tiam1, C1199-Tiam1, C580-Tiam1, wild-type (WT)-Rac1, V12-Rac1, or the empty expression vector (pMT2SM). C1199-Tiam1 was also cotransfected with Myc epitope-tagged dominant negative N17-Rac1 (in pMT2SM) in addition to the promoter construct to verify that Tiam1-induced effects were mediated by Rac. Cloning procedures of the constructs used have been described previously (7, 31, 38).

Cells were seeded at 1.6–2 × 10^5 cells/well in 24-well plates, allowed to settle overnight, and transiently transfected using FuGENE (Roche) according to the manufacturer’s instructions (3 μl of FuGENE/μg DNA, and 100 ng of DNA/construct and well). Prior to application of the respective FuGENE-DNA solutions, FCS-containing RPMI medium was substituted by FCS-free RPMI medium, supplemented with 0.1% bovine serum albumin. 48 h after transfection, cells were washed twice in cold phosphate-buffered saline, lysed using 40–50 μl/well “reporter gene lysis buffer” (Roche), and subsequently frozen at −80 °C until use. CAT reporter gene assays were then performed using the CAT-enzyme-linked immunosorbent assay kit (Roche) with 10–20 μl of the lysate. CAT levels were normalized to protein levels determined with the BCA method (Pierce). For all tested combinations, cells were transfected at least in triplicate wells and at least two independent experiments were performed.

RESULTS

Tiam1-Rac Signaling Influences Migration and Invasion—To investigate the role of Tiam1-Rac signaling in migration and invasion of human RCC, clearCa-28 cells were stably transfected by retroviral transduction with either empty vector (mock), C1199-Tiam1 or constitutively active V12-Rac1, and pools of stably expressing cells were used for further investigations (Fig. 1A). Stable overexpression of C1199-Tiam1 or V12-Rac1 in clearCa-28 cells resulted in a strong and significant inhibition of cell migration on laminin (p < 0.001), when compared with mock-transfected tumor cells (Fig. 1B), consistent with the finding that Tiam1 activates Rac. Similar effects as observed for three-dimensional tumor cell spheroids were seen, when migration was analyzed in the scratch assay (data not shown). Moreover, C1199-Tiam1 and V12-Rac1 also significantly inhibited the more complex process of Matrigel invasion (p = 0.006 and p = 0.018, respectively; Fig. 1C), which also requires proteolytic degradation of the extracellular matrix. Given the established role of Rac in promoting E-cadherin-mediated cell-cell adhesion (8–11), these results suggest that the observed inhibitory effects of C1199-Tiam1 and V12-Rac1 on invasion may result at least partly from Rac-mediated inhibition of cell migration, because of up-regulation of E-cadherin-based cell-cell adhesion. The inhibitory effects of C1199-Tiam1 and V12-Rac1 on migration and invasion cannot simply be explained by effects on proliferation, because both C1199-Tiam1- and V12-Rac1-transfected cells exhibited even a higher proliferation activity than mock-transfected control cells (data not shown).

**Effects of Tiam1-Rac Signaling on Homotypic Cell-Cell Adhesion and Consequences for Invasion**—C1199-Tiam1 and V12-Rac1 also affected cell-cell adhesion and in vitro growth patterns. ClearCa-28 cells, stably transfected with empty vector, exhibited a spindle-shaped phenotype and a dissociated growth pattern as was seen in the nontransfected parental cell line (Fig. 2). In contrast, expression of C1199-Tiam1 or V12-Rac1 induced a polygonal epithelial phenotype and a growth pattern in solid tumor islands, indicative for increased cell-cell adhesion. The effects of V12-Rac1 were less pronounced than those of C1199-Tiam1, which is in accordance with previous observations in epithelial cells (9). To explore whether C1199-Tiam1- and V12-Rac1-induced cell-cell adhesion was mediated by E-cadherin, as was reported from Ras-transformed MDCK cells (9), growth patterns were analyzed in the presence of either human E-cadherin- or human N-cadherin-specific inhibitory HAV peptides. Incubation with a human E-cadherin-specific inhibitory HAV peptide prevented the formation of stable cell-cell adhesion in C1199-Tiam1-expressing cells and induced a spindle-shaped phenotype as well as a dissociated growth pattern, whereas no effects were seen upon exposure to a human N-cadherin-specific blocking peptide (Fig. 3). Similar results were obtained when V12-Rac1-expressing cells were used (data not shown). From these results we conclude that activation of Rac inhibited a dissociated growth pattern as well as migration of human RCC cells by promoting E-cadherin-mediated cell-cell adhesion.

To investigate whether Tiam1/Rac-induced E-cadherin-mediated cell-cell adhesions are caused by up-regulation of E-
cal, or \( \gamma \)-catenin, expression levels of either of these proteins were determined by immunoblotting. However, no significant effects were observed upon transfection of C1199-Tiam1- and V12-Rac1-expressing cells exhibited strongly increased cell-cell adhesions, as evidenced by a growth pattern in solid tumor islands. *Bars*, 30 \( \mu m \).

**Fig. 2.** Cell-cell adhesion and growth patterns in human clearCa-28 cells. Parental cells and mock-transfected cells exhibited a spindle-shaped phenotype and a dissociated growth pattern, whereas C1199-Tiam1- and V12-Rac1-expressing cells exhibited strongly increased cell-cell adhesions, as evidenced by a growth pattern in solid tumor islands. *Bars*, 30 \( \mu m \).

**Fig. 3.** Effects of cadherin-specific inhibitory HAV peptides on cell-cell adhesion of C1199-Tiam1-expressing cells. There were no effects of N-cadherin-specific HAV peptides, and almost complete reversion of C1199-Tiam1-induced cell-cell adhesion and induction of a dissociated growth pattern by HAV peptides specific for E-cadherin. Similar effects were observed when V12-Rac1-expressing cells were used. *Bars*, 40 \( \mu m \).

**Fig. 4.** Protein expression of E-cadherin, \( \alpha \), \( \beta \), and \( \gamma \)-catenin in human clearCa-28 cells transfected with empty vector (mock), C1199-Tiam1, or V12-Rac1, as determined by immunoblotting from total cell lysates. Results are representative for three independent experiments.

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was analyzed in the absence and presence of human E-cadherin-specific inhibitory HAV peptides, respectively (Fig. 5). Surprisingly, E-cadherin-specific HAV peptides (500 \( \mu g/ml \)) had no effect on the inhibitory function of C1199-Tiam1 on Matrigel invasion, whereas these peptides slightly increased Matrigel invasion of mock-transfected control cells and influenced cell-cell adhesion as well as cell migration. This indicates that promotion of E-cadherin-mediated cell-cell adhesion was not the only reason for the inhibitory effect of C1199-Tiam1 in the Matrigel invasion assay, and suggested an additional molecular mechanism, through which activation of Rac may inhibit Matrigel invasion.

Tiam1-Rac Signaling Affects Invasion by Influencing the Expression/Secretion of TIMPs—Matrigel invasion requires proteolytic degradation of the extracellular matrix, and imbalances between MMPs and TIMPs are thought to play a pivotal role in tumor invasion and metastasis (22). Therefore, we investigated whether Tiam1-Rac signaling also affects the ex-
pression of proteases and protease inhibitors. Supernatants of our stably transfected cell lines were analyzed for secreted TIMP-1, TIMP-2, TIMP-3, and TIMP-4 proteins, all of which have been shown to inhibit invasion in different tumor models (23, 26, 27). By immunoblotting, TIMP-1 and TIMP-2 secretion levels were found to be markedly higher for cell lines transfected with C1199-Tiam1 or V12-Rac1 than for mock-transfected control cells (Fig. 6A). In contrast, TIMP-3 was absent in supernatants of all cell lines investigated, and TIMP-4 secretion levels did not significantly differ between the different cell lines. Similar results were obtained when TIMP protein expression was analyzed from total cell lysates (data not shown). Moreover, quantification of TIMP-1 and TIMP-2 concentrations in supernatants of mock- and C1199-Tiam1-transfected cells by means of TIMP-1- and TIMP-2-specific immunoassays confirmed the effects observed by immunoblotting (Fig. 6B).

Because imbalances between TIMPs and MMPs rather than distinct individual secretion levels are considered to determine the invasive phenotype (22, 23), the cell lines were also analyzed for the secretion of active and latent MMP-2 and MMP-9, which are major targets of TIMP-2 and TIMP-1, respectively. By gelatin zymography, all cell lines proved to secrete MMP-2 and MMP-9 exclusively as latent pro-enzymes, and no differences in secretion levels were observed between the different cell lines (Fig. 6A). Other MMPs with known gelatinolytic activities such as MMP-1, MMP-3, MMP-7, MMP-8, MMP-10, MMP-13, MT1-MMP, MT2-MMP, and MT3-MMP (20, 21, 40) were not secreted in detectable amounts. Moreover, similar results as observed for secreted MMPs were obtained, when cell membrane-associated levels and activities of MMPs were analyzed by gelatin zymography (data not shown). Because TIMP-1 and TIMP-2 have been shown to specifically prevent activation of pro-MMP-9 and pro-MMP-2, respectively, these results suggest that activation of Rac induces imbalances between these pro-MMPs and their specific inhibitors by selective up-regulation of TIMP-1 and TIMP-2.

To exclude that these effects are restricted to clearCa-28 renal cell carcinoma cells, we also investigated the effects of C1199-Tiam1 and V12-Rac1 in a human colon carcinoma cell line (DusCol-1B). Again, stable overexpression of C1199-Tiam1 and V12-Rac1, respectively, resulted in a significant inhibition of Matrigel invasion (p < 0.05) as well as increased secretion of TIMP-1 and TIMP-2 proteins, whereas MMP secretion levels, as determined by zymography, were not affected (Fig. 7, A–C). These results exclude a cell line-specific effect and suggest that selective up-regulation of TIMP-1 and TIMP-2 by Rac activation may be a more general mechanism.

To substantiate the functional relevance of Tiam1/Rac-induced up-regulation of TIMP-1 and TIMP-2, invasion of mock-transfected clearCa-28 cells was determined in the presence and absence of recombinant TIMP proteins. Application of recombinant TIMP-1 and TIMP-2 proteins, respectively, resulted in a dose-dependent inhibition of Matrigel invasion with significant inhibitory effects at concentrations of 0.1 μg/ml (or higher) of either protein (p < 0.05, Fig. 8). These concentrations...
were lower than the concentration of endogenous TIMP-2 and ~5-fold the concentration of endogenous TIMP-1 in supernatants of C1199-Tiam1 and V12-Rac1 as determined by immunoblotting with Tiam1- and Myc epitope-specific antibodies, respectively. The respective bands are indicated by asterisks (*). B, significant inhibitory effects ($p < 0.05$) of C1199-Tiam1 and V12-Rac1 on invasion, as determined by the Matrigel invasion assay. C, secretion levels of MMP-2 and MMP-9 were determined in concentrated serum-free supernatants by gelatin zymography. MMP-2 was exclusively secreted as latent proenzyme (pro-MMP-2), whereas MMP-9 was absent. Secretion levels of TIMPs were determined by immunoblotting from the same concentrated serum-free supernatants as used for zymography.

In the present study, we have shown that sustained activation of Rac strongly inhibits cell migration, cell dissociation, and the more complex process of Matrigel invasion of human RCC in vitro. Moreover, we found, that in addition to promoting E-cadherin-mediated cell-cell adhesion, selective up-regulation of TIMP-1 and TIMP-2 is another mechanism through which Rac may inhibit invasion of epithelial cells. Rac-induced up-regulation of TIMP-1 occurred predominantly on the transcriptional level and was mediated by Rac. Moreover, the fact that TIMP-1 promoter activation by Tiam1 and Rac was observed in different cell lines also suggests that this effect is not restricted to RCC tumor cells, but may also hold true for other cell types.

**DISCUSSION**

In the present study, we have shown that sustained activation of Rac strongly inhibits cell migration, cell dissociation, and the more complex process of Matrigel invasion of human RCC in vitro. Moreover, we found, that in addition to promoting E-cadherin-mediated cell-cell adhesion, selective up-regulation of TIMP-1 and TIMP-2 is another mechanism through which Rac may inhibit invasion of epithelial cells. Rac-induced up-regulation of TIMP-1 occurred predominantly on the transcriptional level, whereas up-regulation of TIMP-2 occurred on the post-transcriptional level.

In epithelial MDCK cells and keratinocytes, Rac promotes the formation of E-cadherin-mediated cell-cell adhesion (8–11) and consequently inhibits cell migration on laminin 1 and fibronectin (9, 14). In contrast, a recent study on keratinocytes suggests that, in a time- and concentration-dependent manner, active Rac may also disrupt cadherin-dependent cell-cell adhesions and promote a dissociated growth pattern (17). In human RCC, we found that sustained activation of Rac promoted E-cadherin-mediated cell-cell adhesion. This effect did not result from up-regulation of E-cadherin or $\alpha$-, $\beta$-, or $\gamma$-catenin, because expression levels of either of these proteins were not affected by C1199-Tiam1 or V12-Rac1. This effect is most likely based on Rac-induced increase in actin polymerization at sites of cell-cell contacts, which promotes the formation of functional cadherin-based cell-cell adhesions (9, 11).

Aside from an inhibitory effect on cell migration, activation of Rac also inhibited the more complex process of Matrigel invasion, for which degradation of the extracellular matrix is required. The inhibitory effect of C1199-Tiam1 on Matrigel invasion of RCC cells was hardly blocked by E-cadherin-specific HAV peptides, suggesting that the inhibitory effect of Tiam1 on invasion could not simply be explained by the Rac effect on E-cadherin-based adhesions. Apparently, blocking of E-cadherin-mediated cell-cell adhesion was not sufficient to induce Matrigel invasion, indicating that Rac may inhibit Matrigel invasion also through other mechanisms. Indeed, we found that sustained activation of Rac induced selective and marked up-regulation of TIMP-1 and TIMP-2 proteins, whereas neither the amounts nor the activities of secreted and membrane-associated MMP-2 and MMP-9 were affected. Other MMPs with known gelatinolytic activities were not secreted in detectable amounts. Thus, Rac signaling induced imbalances be-
tween pro-MMP-2/TIMP-2 and pro-MMP-9/TIMP-1, resulting in a relative dominance of both inhibitors. We found a similar selective up-regulation of TIMP-1 and TIMP-2 without effects on MMP expression also upon Rac activation in human colon carcinoma cells, indicating that this effect of Rac may also hold true for other epithelial cells. In contrast to the well known inhibitory function of TIMP-2 on pro-MMP-2 activation, it has also been reported that low concentrations of TIMP-2 may facilitate activation of pro-MMP-2 by membrane type-1 MMP (41). In clearCa-28 tumor cells, however, Tiam1/Rac-induced up-regulation of TIMP-2 did not result in activation of pro-MMP-2 as verified by zymographic analysis of membrane-associated amounts and activities of MMP-2 (data not shown).

The functional relevance of Rac-induced up-regulation of TIMP-1 and TIMP-2 in invasion was further substantiated by demonstrating that recombinant TIMP-1 and TIMP-2 proteins significantly inhibited invasion of control cells. Recombinant TIMP-2 significantly inhibited Matrigel invasion of control cells even at a concentration that was lower than that of endogenous TIMP-2 in supernatants of C1199-Tiam1-expressing cells. For recombinant TIMP-1, significant inhibitory effects on Matrigel invasion were observed at concentrations that were at least 5-fold higher than the concentration of endogenous TIMP-1 in supernatants of C1199-Tiam1-expressing cells. This, however, does not exclude a role of TIMP-1 in Tiam1/Rac-induced inhibition of Matrigel invasion, but it may result from different biological activities of recombinant and endogenously produced TIMP-1 proteins. Given the well established role of TIMPs as inhibitors of invasion and the fact that Tiam1/Rac-induced up-regulation of TIMP-1 and TIMP-2 was found in RCC and colon carcinoma cells, we conclude that selective up-regulation of TIMP-1 and TIMP-2 by Rac activation may be an important and general mechanism in the regulation of tumor invasiveness.

Recent investigations on mesenchymal cells also suggested a role for Rac in integrin-induced MMP-1 expression (18) and collagen I-induced MMP-2 activation (19). Increased expression or activation of distinct MMPs in response to integrin-mediated cell-substrate adhesion has also been reported by others, but the role of Rac was not analyzed in these studies (42, 43). Nevertheless, it is remarkable that these effects were partly substrate-specific and depended on the expression of distinct integrin receptors. In RCC cells, secretion and activity levels of MMP-2 were not affected by Rac (Fig. 6A), and MMP-1 was neither expressed nor secreted by any of the cell lines used (data not shown). These differences may result from differences between the experimental systems, as we used epithelial rather than mesenchymal cells.

In the present study, we also found that up-regulation of TIMP-1 and TIMP-2 by Rac is mediated by different mechanisms. By Northern blotting and CAT-reporter gene assays, we could demonstrate that Tiam1-induced up-regulation of TIMP-1 occurred predominantly on the transcriptional level. This effect was only seen with Tiam1 mutants, known to acti-
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Effects of Rac on the degree of E-cadherin-mediated cell-cell adhesion, the selective up-regulation of TIMP-1 and TIMP-2 may be another mechanism through which Rac signaling is able to inhibit tumor invasion in epithelial cells.

Acknowledgments—We thank A. Florange-Heinrichs and S. Slwik for excellent technical assistance, M. Ringler and M. Bellack for preparation of some of the figures, M. Heydthausen for statistical evaluations, and S. Nockemann for critically reading the manuscript.

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A Role of Rac Signaling in TIMP Expression
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J. Biol. Chem. 2001, 276:41889-41897.
doi: 10.1074/jbc.M105049200 originally published online September 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105049200

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