Matrix-dependent Tiam1/Rac Signaling in Epithelial Cells Promotes Either Cell–Cell Adhesion or Cell Migration and Is Regulated by Phosphatidylinositol 3-Kinase

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Abstract. We previously demonstrated that both Tiam1, an activator of Rac, and constitutively active V12Rac promote E-cadherin–mediated cell–cell adhesion in epithelial Madin Darby canine kidney (MDCK) cells. Moreover, Tiam1 and V12Rac inhibit invasion of Ras-transformed, fibroblastoid MDCK-f3 cells by restoring E-cadherin–mediated cell–cell adhesion. Here we show that the Tiam1/Rac-induced cellular response is dependent on the cell substrate. On fibronectin and laminin 1, Tiam1/Rac signaling inhibits migration of MDCK-f3 cells by restoring E-cadherin–mediated cell–cell adhesion. On different collagens, however, expression of Tiam1 and V12Rac promotes motile behavior, under conditions that prevent formation of E-cadherin adhesions. In nonmotile cells, Tiam1 is present in adherens junctions, whereas Tiam1 localizes to lamellae of migrating cells. The level of Rac activation by Tiam1, as determined by binding to a glutathione-S-transferase–PAK protein, is similar on fibronectin or collagen I, suggesting that rather the localization of the Tiam1/Rac signaling complex determines the substrate-dependent cellular responses. Rac activation by Tiam1 requires PI3-kinase activity. Moreover, Tiam1- but not V12Rac-induced migration as well as E-cadherin–mediated cell–cell adhesion are dependent on PI3-kinase, indicating that PI3-kinase acts upstream of Tiam1 and Rac.

Key words: cell migration • E-cadherin adhesion • PI3-kinase • Rac signaling • Tiam1

Cell migration is an essential process involved in development, wound healing, tumor invasion and metastasis. Migratory behavior of cells requires communication of individual cells with their environment, and is dependent upon the integration of diverse signals derived from the extracellular matrix and interactions with adjacent cells (Huttenlocher et al., 1995). The regulation of cytoskeletal changes associated with migratory behavior of cells has recently become focused on the family of the small Rho-like GTPases. Cdc42, Rac1, and RhoA regulate signal transduction pathways that mediate distinct cytoskeletal rearrangements required for cell migration. In quiescent fibroblasts, Rho activation is linked to the assembly of stress fibers and focal adhesions (Ridley and Hall, 1992). Activation of Cdc42 and Rac results in the formation of filopodia and lamellipodia, respectively (Ridley et al., 1992; Kozma et al., 1995; Nobes and Hall, 1995). The cytoskeletal changes induced by constitutively active V12Cdc42 and V12Rac are also associated with specific, integrin-based adhesion complexes and cell spreading (Nobes and Hall, 1995; Van Leeuwen et al., 1997; D'Souza-Schorey et al., 1998). In addition to these direct effects on the cytoskeleton, Rho-like proteins have been implicated in transcriptional activation (Vojtek and Cooper, 1995; Perona et al., 1997; Kheradmand et al., 1998), oncogenic transformation (Khosravi-Far et al., 1995; van Leeuwen et al., 1995), and metastasis formation (Habets et al., 1994). Similar to Ras proteins, the Rho-like GTPases are activated by the exchange of bound GDP for GTP, which is stimulated by guanine nucleotide exchange factors (GEFs).[1] GTPase-activating proteins stimulate the intrinsic GTPase activity and lead to inactivation of the GTPases (Van Aelst and D'Souza-Schorey, 1997).

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1. *Abbreviations used in this paper:* GEF, guanine nucleotide exchange factor; GST, glutathione-S-transferase; HGF, hepatocyte growth factor; o/n, overnight; PAK-CD, PAK-CRIB domain; PH domain, Pleckstrin homology domain; PI3-kinase, phosphatidylinositol 3-kinase; Tiam1, T-lymphoma invasion and metastasis gene 1.

Rho-like proteins have been shown to play an essential role in migration of hematopoietic cells. T-lymphoma invasion and metastasis gene 1 (Tiam1) was identified as an invasion-inducing gene in T-lymphoma cells (Habets et al., 1994) and functions as a specific activator or GEF for Rac1 (Michiels et al., 1995). V14Rho does not increase invasion of T-lymphoma cells into a fibroblast monolayer, but Rho activity is required for invasion. V12Cdc42 also induces invasion in T-lymphoma cells, but it remains to be established whether Cdc42 acts through activation of Rac or through common downstream signaling pathways (Stam et al., 1998). Similarly, locomotion of macrophages also requires Rac and Rho activity, whereas Cdc42 is involved in responding to a gradient of the chemokine colony stimulating factor-1 (Allen et al., 1998).

In epithelial cells, the effects of the small Rho-like GTPases seem contradictory, since they have been shown to be required for cell migration as well as for cell–cell adhesion. In epithelial keratinocytes, the activity of both Rho and Rac proteins is required for the assembly of specialized sites of cell–cell contacts, the adherens junctions (Braga et al., 1997; Takaishi et al., 1997). Furthermore, Tiam1 or V12Rac have been shown to inhibit hepatocyte growth factor (HGF)-induced cell scattering of epithelial Madin Darby canine kidney (MDCK) cells by increasing E-cadherin–mediated cell-cell adhesion (Hordijk et al., 1997). Moreover, Tiam1/Rac signaling inhibits motile and invasive behavior of Ras-transformed MDCK-f3 cells due to restoration of E-cadherin–mediated adhesions. Also Cdc42 has been implicated in the formation of adherens junctions, although activation of Rac by Cdc42 cannot be excluded (Kuroda et al., 1997). These data suggest that the activity of Rho-like GTPases is required to maintain the cytoskeletal architecture of a polarized epithelium. In contrast to these results are the findings that dominant-negative N17Rac inhibits the HGF-induced membrane ruffling and lamellipodia formation in MDCK cells, suggesting a role for Rac in cell motility (Ridley et al., 1995). Furthermore, V12Rac and V12Cdc42 stimulate motile behavior of T47D mammary carcinoma cells which requires phosphatidylinositol 3-kinase (PI3-kinase) activity (Keely et al., 1997). In colon carcinoma cells, integrin-mediated activation of PI3-kinase has been shown to stimulate Rac-dependent motile behavior (Shaw et al., 1997). Thus, in addition to a role for Rac in the establishment and maintenance of E-cadherin–mediated adhesions, Rac appears also to play an essential role in migratory responses of epithelial cells.

In this paper we have addressed these seemingly opposing effects of Rac in epithelial cells with respect to E-cadherin–mediated cell–cell adhesion and cell migration. Here we show that the different Rac-mediated cellular responses are dependent on the cell substrate. Rac activation inhibits migration of Ras-transformed MDCK-f3 cells by establishing E-cadherin–mediated cell–cell adhesion on fibronectin and laminin 1, but promotes motility on collagen substrates when E-cadherin–mediated adhesions are prevented. In nonmotile cells, Tiam1 is found in adherens junctions, whereas in migrating cells the protein localizes to lamellae and membrane ruffles. Since the Tiam1-mediated Rac activation on each substrate is similar, we suggest that the different intracellular localization of the Rac signaling complex determines whether Rac stimulates E-cadherin–mediated cell–cell adhesion or cell migration. Rac activation by Tiam1 requires PI3-kinase activity on all substrates. Furthermore, both the motility and cell–cell adhesion induced by Tiam1 but not by V12Rac depend on PI3-kinase activity. This indicates a role for PI3-kinase upstream of Tiam1 and Rac in cell migration as well as in the formation of E-cadherin–mediated cell–cell adhesion.

Materials and Methods

Antibodies and Adhesive Ligands

Antibodies against β-catenin, γ-catenin, α-catenin, p85, and Rac1 were obtained from Transduction Laboratories (Lexington, KY) and monoclonal 12CA5 antibody against the hemagglutinin-epitope tag from Boehringer Mannheim (Indianapolis, IN). Fibronectin, collagen type I, α-actinin antibody, and the monoclonal DECMA-1 antibody against E-cadherin were purchased from Sigma Chemical Co. (St. Louis, MO). Laminin type I and collagen type IV were obtained from Collaborative Biomedical Products (Bedford, MA).

Cells and Culture Conditions

MDCK and V12Ras-transformed MDCK-f3 cells (Behrens et al., 1989; Vleminckx et al., 1991) were cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL Life Technologies, Breda, The Netherlands) supplemented with 10% fetal calf serum (GIBCO-BRL Life Technologies). Stable cell lines expressing the hemagglutinin epitope-tagged C1199Tiam1 (encoding the 1,199 COOH-terminal amino acids of Tiam1), FLTiam1 (encoding full-length Tiam1), and the Myc epitope-tagged V12Rac construct were generated by retroviral transduction and selected with 0.8 mg/ml neomycin (Hordijk et al., 1997). MDCK-f3 cells expressing FLTiam1 were retrovirally transduced with control empty vector or p85 and Δp85 constructs, and subsequently selected on neomycin (0.8 mg/ml; GIBCO-BRL Life Technologies) and zeocin (0.2 mg/ml; Invitrogen, San Diego, CA). Recombinant HGF was added to a final concentration of 10 ng/ml as indicated. Different substrates (10 μg/ml or as indicated in the figure legend) were used to coat cell culture dishes overnight (o/n) as indicated. For experiments using soluble collagen (see Fig. 2), clusters of cells were allowed to attach on a fibronectin matrix for 3 h, before addition of 10 μg/ml soluble collagen I in phosphate-buffered saline containing 0.5% acetic acid. As control, phosphate-buffered saline containing 0.5% acetic acid lacking collagen was added.

Constructs and Retroviral Transductions

Hemagglutinin epitope-tagged FLTiam1, C1199Tiam1, and the Myc epitope-tagged V12Rac were cloned into a LZRS-IRES-Neo retroviral vector, a modified LZRS retroviral vector (Kinsella and Nolan, 1996), conferring neomycin resistance. Human Δp85α and p85α constructs were hemagglutinin epitope-tagged and analogue to constructs described by Dhand et al. (1994). A SalI/NotI fragment from pBK-HA-p85 or pBK-HA-Δp85, encoding a hemagglutinin epitope-tagged p85 or Δp85 subunit, was inserted between the XhoI and NotI sites of a LZRS-IRES-Zeo retroviral vector, conferring zeocin resistance. A detailed description of retroviral vectors will be described elsewhere (Michiels, F., manuscript in preparation). To produce retroviruses, Phoenix packaging cells (Kinsella and Nolan, 1996) were transfected with retroviral constructs encoding Tiam1, V12Rac, p85, and Δp85, as described previously (Michiels et al., 1995; Stam et al., 1998). In brief, 30,000 MDCK-f3 cells were transduced by culturing for 10 h in 2 ml of cell-free Phoenix supernatants in the presence of 4 μg/ml polybrene. Transduced cells were cultured for 48 h in fresh DME before transductants were selected in DME containing either neomycin (0.8 mg/ml) or a combination of neomycin (0.8 mg/ml) and zeocin (0.2 mg/ml). Expression of proteins in the pool of transduced cells was analyzed by Western blotting, as described previously (Michiels et al., 1997).

Immunofluorescence Studies

Cells grown on glass coverslips coated with fibronectin, laminin 1, or collagen I (10 μg/ml) were fixed in 3.7% formaldehyde in phosphate-buffered saline, containing 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Cells were permeabilized in 0.5% Triton X-100 for 3 min, blocked with 0.5% bovine serum

albumin in phosphate-buffered saline, and then incubated for 1 h with primary antibodies. Antibodies were visualized with FITC-labeled secondary antibodies (Zymed Labs, South San Francisco, CA). Cells were stained simultaneously with 2 U/ml TRITC-labeled phalloidin (Molecular Probes, Eugene, OR). C1199Tiam1 was stained with anti-DH (Habets et al., 1994) and E-cadherin with DECMA-1 antibody (Sigma Chemical Co.). Images were recorded with a Bio-Rad MRC-600 confocal laser scanning microscope (Hercules, CA).

Cell Surface Biotinylation

MDCK-f3 and C1199Tiam-expressing MDCK-f3 cells were seeded on tissue culture plastic and grown for 2 d to allow synthesis of their own matrix. All manipulations were performed at 4°C. In brief, the cells were incubated for 15 min in phosphate-buffered saline (supplemented with 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$) containing 500 μg/ml sulfo-NHS-biotin (Pierce Chemical Co., Rockford, IL), washed three times in phosphate-buffered saline containing 50 mM glycin, pH 7.4, lysed in RIPA buffer (25 mM Hepes, pH 8, 150 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 0.15% sodium dodecylsulfate, 10% glycerol, 1 mM EDTA, 3 mM $MgCl_2$, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin), centrifuged 30 min at 21,000 *g* and the supernatant was incubated with avidin-coated agarose beads (Sigma Chemical Co.) for 1 h. Immunoprecipitates of biotinylated surface proteins bound to avidin-agarose were washed three times in RIPA buffer and analyzed for E-cadherin (Decma monoclonal antibody, Sigma Chemical Co.) by Western blotting.

Cloning and Production of GST–PAK-CD Fusion Protein

The Rac activity assay is based on the Rap1 activity assay described by Franke et al. (1997). We used a glutathione-S-transferase (GST)–PAK-CD (PAK-CRIB domain) fusion protein, containing the Rac- and Cdc42-binding region from human PAK1B (GenBank/EMBL/DDBJ accession number AF071884). A fragment encoding amino acids 56–272 of PAK1B was generated by standard PCR using the oligos AGCTGGATCCATTTACCTGGAGAT and AGCTGAATTCATTTCTGGCTGTTGGATGTC, and then digested with BamHI/EcoRI and inserted between the BamH1 and EcoRI sites of pGEX2TK (Pharmacia Biotech, Piscataway, NJ) to yield GST–PAK-CD.

Escherichia coli BL21 cells transformed with the GST–PAK-CD construct were grown at 37°C to an absorbance of 0.3. Expression of recombinant protein was induced by addition of 0.1 mM isopropylthiogalactoside for 2 h. Cells were harvested, resuspended in lysis buffer (50 mM Tris-HCl, pH 8, 2 mM $MgCl_2$, 0.2 mM Na_2S_2O, 10% glycerol, 20% sucrose, 2 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin), and then sonicated. Cell lysates were centrifuged at 4°C for 20 min at 45,000 *g* and the supernatant was incubated with glutathione-coupled Sepharose 4B beads (Pharmacia Biotech) for 30 min at 4°C. Protein bound to the beads was washed three times in lysis buffer and the amount of bound fusion protein was estimated using Coomassie-stained SDS gels.

Rac Activity Assay

Cell culture dishes were coated o/n at 4°C with fibronectin, laminin 1, or collagen I (10 μg/ml). Cells were seeded in small clusters of 2–4 cells on the different matrices and analyzed after 4 h. The PI3-kinase inhibitor wortmannin was added as indicated at an initial concentration of 50 nM at 30 min after seeding. Because of the high instability of wortmannin, 3× fresh inhibitor (10 nM) was added every hour. 4 h after seeding, the cells were washed in ice-cold phosphate-buffered saline (containing 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$), incubated 5 min on ice in lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 1% NP-40, 10% glycerol, 100 mM NaCl, 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin), and then centrifuged for 5 min at 21,000 *g* at 4°C. Aliquots were taken from the supernatant to compare protein amounts. The supernatant was incubated with bacterially produced GST–PAK-CD fusion protein, bound to glutathione-coupled Sepharose beads at 4°C for 30 min. The beads and proteins bound to the fusion protein were washed three times in an excess of lysis buffer, eluted in Laemmli sample buffer (60 mM Tris, pH 6.8, 2% sodium dodecylsulfate, 10% glycerin, 0.1% bromphenol blue), and then analyzed for bound Rac1 molecules by Western blotting using a monoclonal mouse antibody against human Rac1 (Transduction Laboratories).

Migration Assays

Cell migration assays were performed using Transwell migration chambers (diameter 6.5 mm, pore size 8 μm; Costar Corp., Cambridge, MA) coated on both sides of the membrane with fibronectin, laminin 1, or collagen I (each 10 μg/ml) in phosphate-buffered saline o/n at 4°C. The coated filters were rinsed once with phosphate-buffered saline and placed into the lower chamber containing medium supplemented with 10 ng/ml recombinant HGF. Cells were added to the upper compartment of the Transwell chamber and allowed to migrate to the underside of the top chamber for 4–5 h. Cells coexpressing Tiam1 and p85 subunits of PI3-kinase were allowed to migrate for 3 h. Nonmigrated cells on the upper membrane were removed with a cotton swab, and migrated cells attached to the bottom surface of the membrane were fixed for 10 min in methanol, stained with Giemsa, and then counted.

Dissociation Assays

Dissociation assays were performed as described (Hordijk et al., 1997). The cells were seeded and allowed to grow for 2 d. 25 μM LY294002 was added for 4 h as indicated. The cells were scraped in cell culture medium and suspended by repeated pipetting (~20 times). The number of particles (cell clusters) was counted and divided by the number of total cells (Np/Nc).

Results

Reversion of the Ras Phenotype of MDCK-f3 Cells by Tiam1/Rac Is Substrate-dependent

We have shown that Tiam1, a GEF for Rac1, as well as constitutively active V12Rac revert the fibroblastoid phenotype of Ras-transformed MDCK-f3 cells to an epithelioid phenotype and inhibit migration and invasion of these cells, due to restoration of E-cadherin–mediated cell–cell adhesion (Hordijk et al., 1997). To investigate the effect of the cell substrate on these Rac-mediated responses, control and retrovirally transduced MDCK-f3 cells were seeded onto different matrices and analyzed for phenotypic reversion. Control MDCK-f3 cells retained a fibroblastoid phenotype on fibronectin, laminin 1, and collagen I substrates. The cells formed ruffles and lamellae and were unable to form adhesive E-cadherin–mediated cell–cell contacts (Fig. 1). Expression of C1199Tiam1 or constitutively active V12Rac reverted the fibroblastoid phenotype of MDCK-f3 cells to an epithelioid phenotype and inhibited the HGF-induced cell scattering on fibronectin or laminin 1, but not on a collagen I matrix (Fig. 1). V12Rac reverted the fibroblastoid phenotype less efficiently compared with C1199Tiam1, consistent with earlier findings (Hordijk et al., 1997). C1199Tiam1- or V12Rac-expressing cells acquired a polarized phenotype on collagen I and carried lamellae, a phenomenon usually associated with motile behavior (Fig. 1). This polarized phenotype was found in the presence (Fig. 1) and less pronounced in the absence of HGF. Tiam1 similarly inhibited the HGF-induced scattering of untransformed epithelial MDCK cells on fibronectin or laminin 1, but not on collagen I (data not shown), demonstrating that the substrate-dependent motility of Tiam1- or V12Rac-expressing MDCK-f3 cells was not caused by the presence of activated Ras in these cells. Apparently, Tiam1/Rac signaling promotes cell–cell adhesion on fibronectin or laminin 1, as found previously on tissue culture plastic (Hordijk et al., 1997), but permits motility on a collagen I matrix. We found no indication that MDCK-f3 cells produced motil-

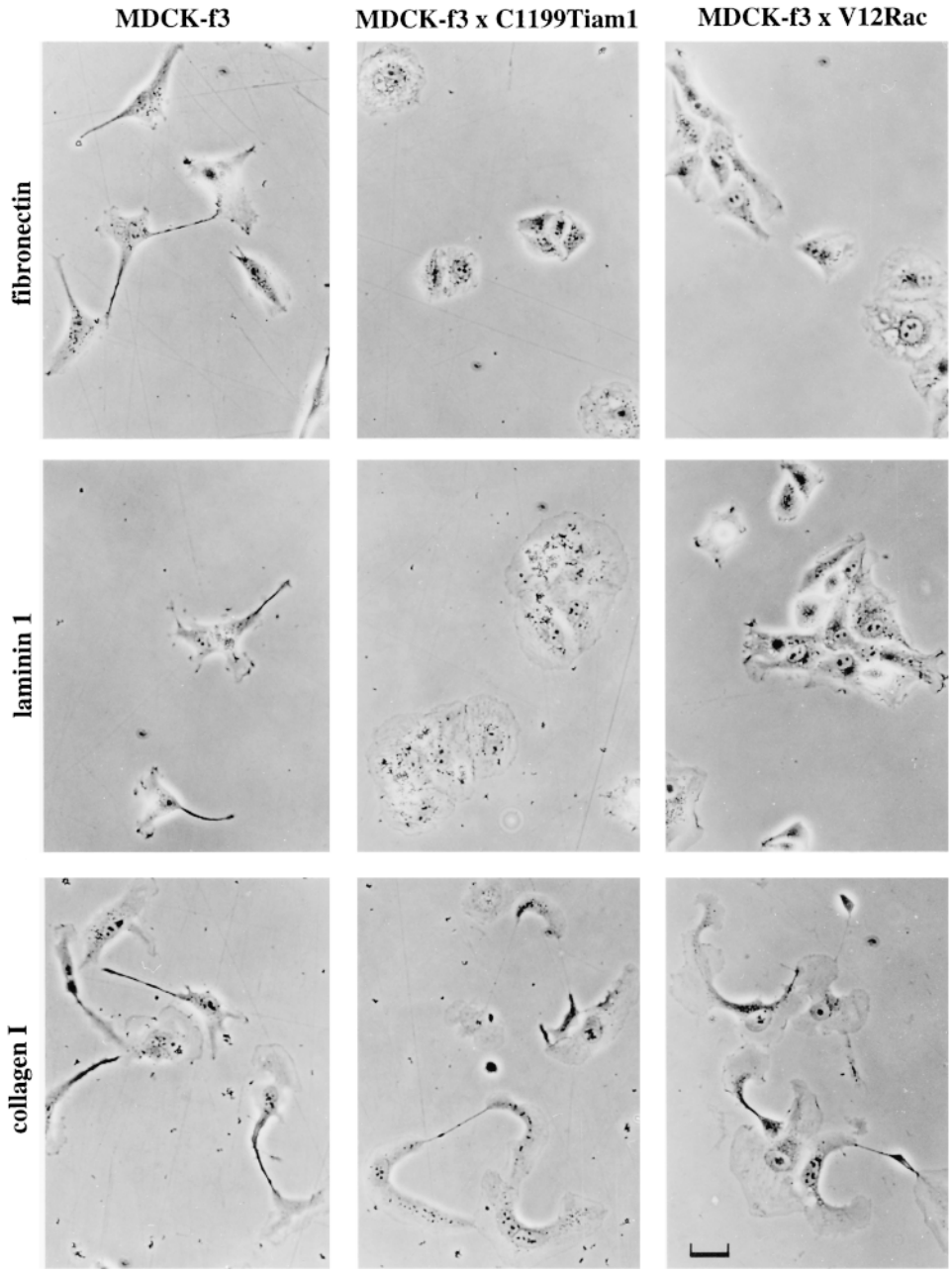

Figure 1. Reversion of the fibroblastoid phenotype of Ras-transformed MDCK-f3 cells to an epithelioid phenotype by C1199-Tiam1 or V12 Rac is dependent on the cell substrate. Control MDCK-f3 cells and C1199-Tiam1- or V12Rac-expressing MDCK-f3 cells were seeded in small clusters of 2–4 cells on fibronectin, laminin 1, or collagen I in the presence of HGF. Phase–contrast photographs were taken 10 h after seeding. Bar, 25 μm.

ity-inducing factors such as scatter factors or collagens, or that expression of C1199Tiam1 in MDCK-f3 cells inhibited the production of such factors (data not shown). Instead, control, C1199Tiam1- and V12Rac-expressing MDCK-f3 cells produced fibronectin in substantial amounts (data not shown), likely creating matrix conditions that caused the Tiam1/Rac-induced phenotypic reversion of MDCK-f3 cells grown on tissue culture plastic.

Collagen Induces a Migratory Phenotype in C1199Tiam1-expressing MDCK-f3 Cells

Adhesion of the C1199Tiam1-expressing MDCK-f3 cells to either fibronectin or collagen I substrates was comparable (data not shown), suggesting that specific signals are responsible for the collagen I-induced motility. To investigate this further, clusters of C1199Tiam1-expressing MDCK-f3 cells were seeded onto a matrix consisting of fibronectin and collagen I mixed in a ratio of 100:1 (Fig. 2 *B*). In contrast to a fibronectin matrix (Fig. 2 *A*), the colonies scattered on the mixed matrix as on collagen I and formed lamellae (Fig. 2 *B*), indicating that small amounts of collagen I are sufficient to induce motility. Moreover, colonies of C1199Tiam1-expressing cells already attached to fibronectin (Fig. 2 *A*) underwent an epithelial–mesenchymal transition upon addition of soluble collagen I (Fig. 2 *C*). These findings indicate that even small amounts of collagen I can induce cell scattering, even in the presence of a fibronectin matrix. To investigate the effects of other collagen types on colony morphology, clusters of C1199-

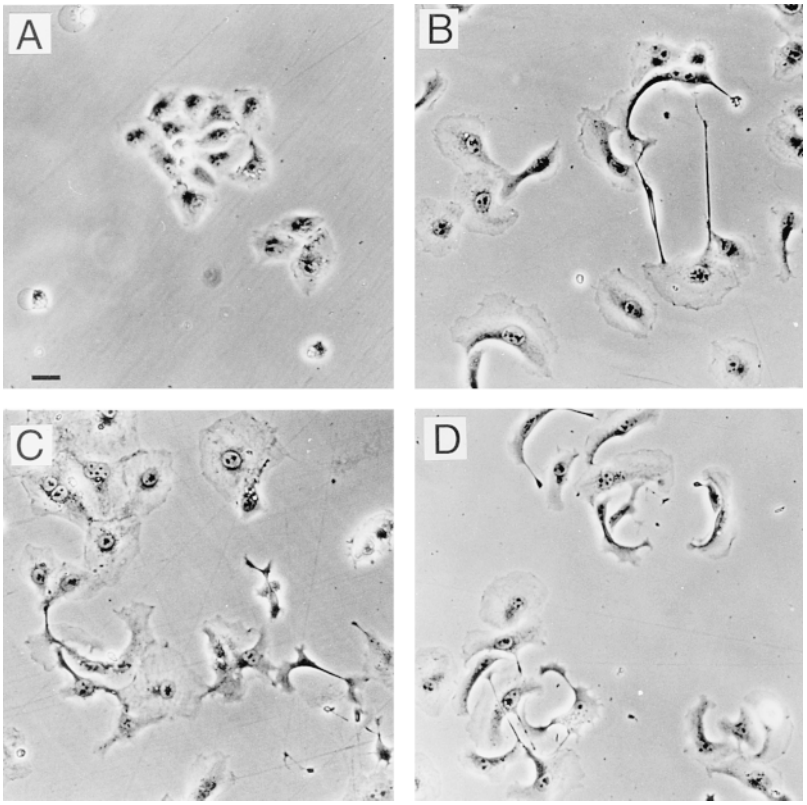

Figure 2. Morphological effects of matrix composition on C1199Tiam1-expressing MDCK-f3 cells. Small clusters of C1199Tiam1-expressing MDCK-f3 cells were seeded in the presence of HGF on (*A*) fibronectin (10 μg/ml), (*B*) a mixed matrix of fibronectin (10 μg/ml) and collagen I (0.1 μg/ml), (*C*) fibronectin (for 3 h), followed by addition of 10 μg/ml soluble collagen I, or (*D*) collagen IV (10 μg/ml). Phase–contrast photographs were taken 5 h after seeding. Bar, 25 μm.

Tiam1-expressing MDCK-f3 cells were seeded onto a collagen type IV matrix (Fig. 2 *D*). In the presence of HGF, the observed scatter response was comparable to that induced by collagen I (Fig. 2 *D*) but in the absence of HGF, collagen IV was less efficient than collagen I (data not shown). Denatured collagen did not induce cell scattering (data not shown), indicating that intact collagen is required to induce epithelial–mesenchymal transition. Fibrillar type I as well as mesh-forming type IV collagens seem to provide specific signals that interfere with the formation of E-cadherin–mediated adhesions and promote cell scattering of the C1199Tiam1-expressing MDCK-f3 cells.

The Intracellular Localization of Tiam1 Is Substrate-dependent

To address the matrix-dependent motility in relation to the intracellular localization of the C1199Tiam1 protein and E-cadherin–mediated adhesion, trypsinized C1199-Tiam1-expressing MDCK-f3 cells were seeded as small clusters of 2–4 cells in the presence of HGF onto different matrices and analyzed by immunocytochemistry (Fig. 3 *A*). On fibronectin or laminin 1, the cells formed small colonies and C1199Tiam1 was present at adherens junctions, where it colocalized with members of the cadherin complex including E-cadherin (Fig. 3, *B* and *C*), β-catenin, α-catenin, and α-actinin (data not shown). C1199Tiam1 was also present in HGF-induced membrane ruffles (Fig. 3, *A* and *C*). The Tiam1/Rac-induced restoration of E-cadherin–mediated adhesion on fibronectin and laminin 1 led to the accumulation of F-actin at sites of cell–cell contact (Fig. 3, *A* and *B*). This pronounced Rac effect has been described earlier in epithelial cells seeded on plastic (Braga et al., 1997; Hordijk et al., 1997; Takaishi et al., 1997). On a collagen I matrix, however, the C1199Tiam1-expressing MDCK-f3 cells established no E-cadherin–mediated adhesions and exhibited a motile phenotype. In these cells, C1199Tiam1 localized to membrane ruffles and lamellae, as well as in the cytoplasm (Fig. 3 *A*). We observed a diffuse E-cadherin staining in the cytoplasm of single, often polarized cells, as expected in the absence of E-cadherin adhesions (Fig. 3 *B*). Thus, the nature of the matrix affects the localization of Tiam1, directly or indirectly, and hence could regulate Rac activity either to increase E-cadherin–mediated adhesion or to promote motility.

Restoration of E-cadherin–mediated Adhesion by C1199Tiam1 Is Dependent on a Balance between Cell–Cell and Cell–Matrix Interactions

To analyze whether collagen I inhibits the formation of E-cadherin–mediated adhesions or promotes motility, we investigated the formation of cell–cell contacts by mimicking high cellular density conditions. Larger clusters (5–10 cells) of loosely attached, trypsinized C1199Tiam1-expressing MDCK-f3 cells were seeded onto a collagen I matrix. Under these conditions and in the absence of HGF, most cells were able to establish cell–cell contacts that were enriched in E-cadherin and F-actin. Some cells, however, at the outer edge of the colonies appeared to move away from the colony (Fig. 4, *arrowhead*). Addition of HGF increased the number of motile cells, synergizing with the collagen effect (Fig. 4). These data indicate that E-cad-

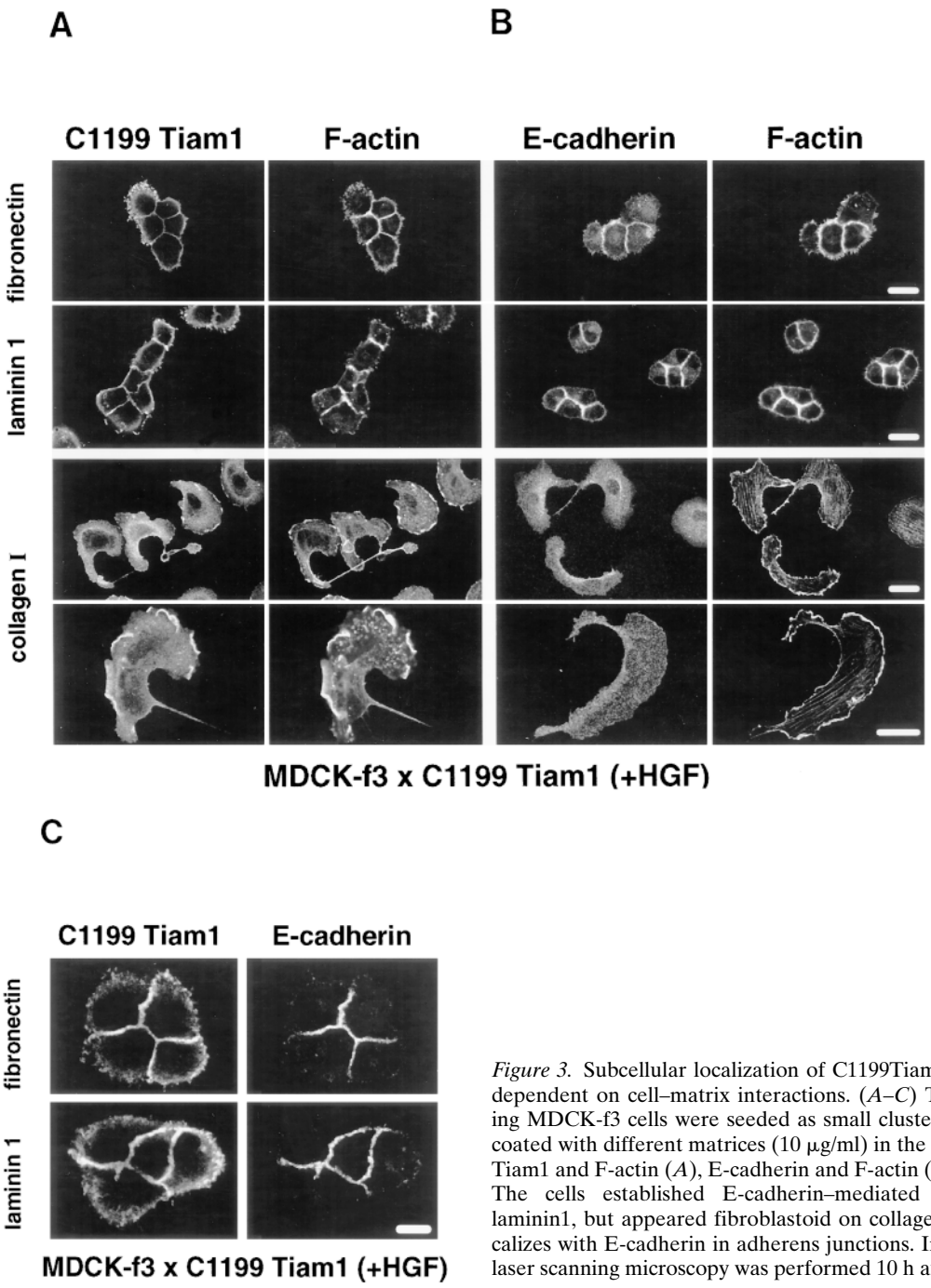

Figure 3. Subcellular localization of C1199Tiam1 expressed in MDCK-f3 cells is dependent on cell–matrix interactions. (*A–C*) Trypsinized C1199Tiam1-expressing MDCK-f3 cells were seeded as small clusters of 2–4 cells on glass coverslips coated with different matrices (10 μg/ml) in the presence of HGF, and stained for Tiam1 and F-actin (*A*), E-cadherin and F-actin (*B*), or Tiam1 and E-cadherin (*C*). The cells established E-cadherin–mediated adhesions on fibronectin and laminin1, but appeared fibroblastoid on collagen I. Note that C1199Tiam1 colocalizes with E-cadherin in adherens junctions. Immunofluorescence and confocal laser scanning microscopy was performed 10 h after seeding. Bar, 25 μm.

herin–mediated adhesions on collagen I are not prevented but are difficult to establish because of the motility-promoting effect of collagen I. Conditions that favor E-cadherin adhesions such as close contact between cells and the absence of HGF (Fig. 4), inhibit collagen-induced motility.

Tiam1-mediated Rac Activation Increases Cell-Surface E-cadherin

Biochemical analyses showed that the restoration of E-cadherin–mediated adhesions induced by Tiam1/Rac signaling in MDCK-f3 cells was not caused by an increase in the total pool of E-cadherin (Fig. 5 *A*). Also the expression of other components of the E-cadherin complex such as β-catenin, γ-catenin, and α-actinin were not changed in C1199Tiam1- or V12Rac-expressing cells, when compared with control cells (Fig. 5 *A*). By surface biotinylation we found that C1199Tiam1 induced a three- to fourfold increase in the amount of E-cadherin at the cell surface (Fig. 5 *B*, lane *2*), when compared with fibroblastoid MDCK-f3 cells (Fig. 5 *B*, lane *1*). This indicates that activation of Rac by Tiam1 stimulates the formation or stabilization of functional E-cadherin complexes at the cell surface leading to

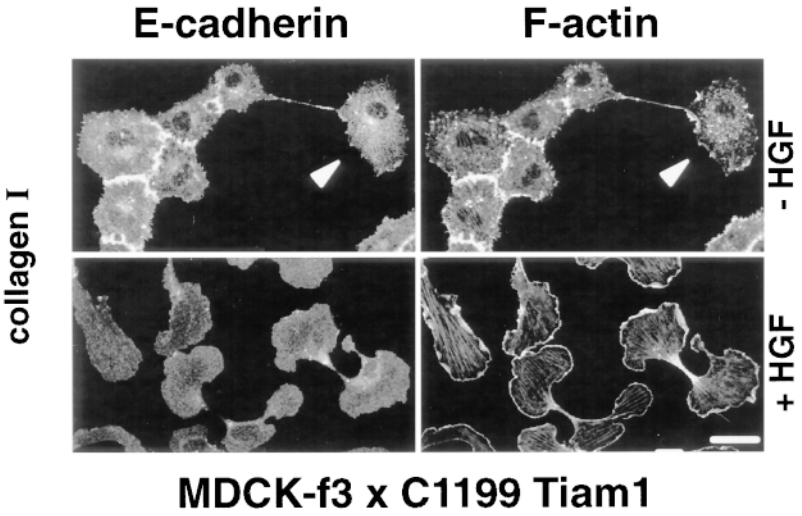

Figure 4. Collagen I-induced motility is dependent on a balance between cell–cell and cell–matrix interactions. C1199Tiam1-expressing MDCK-f3 cells were seeded as larger, loosely attached clusters of 5–10 cells on collagen I, to mimic conditions of high cellular density. In the absence of HGF, most cells formed E-cadherin–mediated adhesions, but some cells at the outer edge of the colony moved away from the colony (*arrowhead*). The presence of HGF increased motility, synergizing with the collagen effect. Immunofluorescence and confocal laser scanning microscopy was performed 10 h after seeding. Bar, 25 μm.

phenotypic reversion towards an epithelioid phenotype and inhibition of motile and invasive behavior.

Migration of C1199Tiam-expressing MDCK-f3 Cells Is Dependent on Substrate and Cell Density

The adhesion of C1199Tiam1-expressing MDCK-f3 cells to various substrates was comparable, suggesting that Tiam1/Rac-regulated cell–cell interactions played an important role in the substrate-dependent migratory responses. Therefore, the effect of the substrate on motile and invasive behavior was quantified using a transwell invasion assay. As shown in Fig. 6 *A*, expression of C1199Tiam1 decreased the infiltration of MDCK-f3 cells through filters coated with fibronectin or laminin 1 substrates ~10-fold when compared with control MDCK-f3 cells. On a collagen I matrix, C1199Tiam1-expressing cells showed a sevenfold increase in their migration rate as compared with the other substrates, but still displayed a reduced motility with respect to control cells (Fig. 6 *A*). We reasoned that the reduced migration rate might be due to E-cadherin–mediated cell–cell contacts which were favored by seeding the cells at high density (1.5×10^5 cells/well). Indeed, by adding HAV-peptides to block E-cadherin adhesion (Blaschuk et al., 1990), migration of C1199Tiam1-expressing MDCK-f3 cells on collagen I was enhanced with increasing amounts of E-cadherin–blocking peptide (Fig. 6 *B*). In contrast, addition of control peptide against N-cadherin had no effect on the migration rate of C1199Tiam1-expressing cells (Fig. 6 *B*). Moreover, at medium cell density (0.75×10^5 cells/well), migration of C1199Tiam1-expressing cells on a collagen I matrix was increased to levels comparable to those of control cells (Fig. 6 *C*), or even exceeded the migration of control cells at low cell density (0.15×10^5 cells/well) (Fig. 6 *D*). We conclude that motility triggered by collagen I is stimulated by Tiam1-mediated Rac activation, but only under conditions which prevent E-cadherin–mediated adhesions.

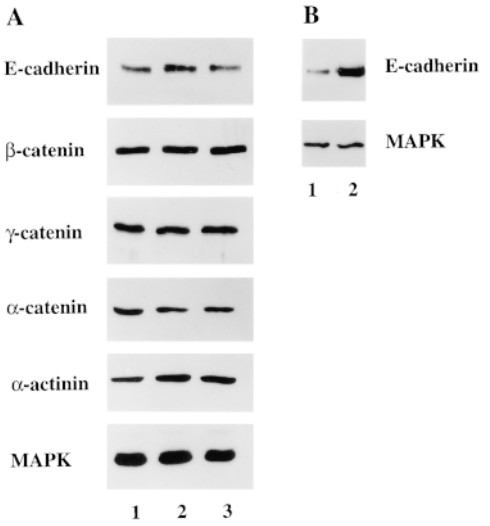

Figure 5. Expression of C1199Tiam1 in Ras-transformed MDCK-f3 cells increases the amount of functional E-cadherin molecules at the cell surface. (*A*) MDCK-f3 (lane *1*) and C1199Tiam- (lane *2*) or V12Rac-expressing MDCK-f3 cells (lane *3*) were grown for 2 d on plastic to allow production of their own (fibronectin) matrix. Expression of E-cadherin, β-catenin, γ-catenin, α-catenin, and α-actinin was compared in cell lysates by Western blot analysis. No differences in total amount of protein could be detected. (*B*) MDCK-f3 (lane *1*) and C1199Tiam-expressing MDCK-f3 cells (lane *2*) were grown as described in (*A*) and subjected to cell surface biotinylation. Biotinylated E-cadherin was immunoprecipitated with avidin-agarose and detected on Western blot using an antibody against E-cadherin. Equal amounts of protein were analyzed, as determined by probing the cell lysates with an antibody against mitogen-associated protein kinase. In cells expressing Tiam1, we found a threefold increase in E-cadherin present at the cell surface.

Tiam1-mediated Rac Activation on Fibronectin and Collagen I Is Similar

To investigate whether the substrate-dependent phenotypes were due to differences in the degree of Rac activation, we analyzed the level of Tiam1-mediated Rac activation in cells seeded on a fibronectin or collagen I substrate. For this, the GTPase binding domain (CRIB domain) of the kinase PAK, a downstream effector molecule for Rac and Cdc42 that specifically binds the activated, GTP-

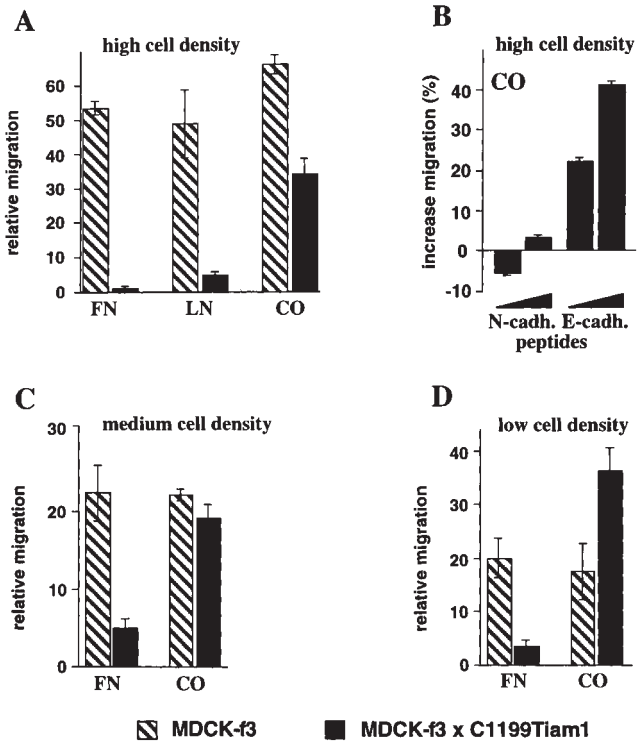

Figure 6. Tiam1 inhibits or promotes migration of MDCK-f3 cells in Transwell assays dependent on the substrate and cell density. Migration rates of (*A* and *B*) 1.5×10^5, (*C*) 0.75×10^5, or (*D*) 0.15×10^5 cells per well were determined towards HGF contained in the lower chamber of a Transwell. (*A*) Migration of C1199Tiam1-expressing MDCK-f3 cells is inhibited on fibronectin (*FN*) and laminin 1 (*LN*). The migration rate on collagen I (*CO*) is increased as compared with the other substrates, but is still inhibited when compared with control cells. (*B*) Addition of increasing amounts (400 and 600 μg/ml) of HAV-peptides against E-cadherin but not N-cadherin increases migration of C1199Tiam1-expressing cells on collagen I. (*C* and *D*) Migration rates determined with lower numbers of cells. On collagen I the motile behavior of C1199Tiam1-expressing MDCK-f3 cells, as compared with control MDCK-f3 cells, increases when the cell densities in the assay are reduced (compare *A*, *C*, and *D*). Each bar represents the mean ± SD of triplicate migration assays. One representative example of three independent experiments is shown.

loaded form of the GTPase (Manser et al., 1994), was fused to GST. Cell lysates derived from control or C1199Tiam1-expressing MDCK-f3 cells grown on different substrates were incubated with the GST–PAK fusion protein. Eluates of the fusion protein were subsequently analyzed for the presence of associated GTP–Rac by Western blotting. In control MDCK-f3 cells, seeded either on fibronectin or collagen I, we could hardly detect any endogenous active Rac protein (Fig. 7 *A*, lanes *1* and *2*). Expression of C1199Tiam1 induced strong activation of endogenous Rac on both fibronectin and collagen I (Fig. 7 *A*, lanes *3* and *4*), whereas the amounts of total Rac protein were comparable (Fig. 7 *A*, lanes *1–4*). The presence of HGF neither changed the level of active Rac in the control MDCK-f3 nor in the C1199Tiam1-expressing MDCK-f3 cells (Fig. 7 *A*, lanes *5–8*). Overexposure of the autoradiogram showed a low level of active Rac in MDCK-f3 cells which was independent of the presence of HGF (data not shown). Assaying Rac activities of extremely sparsely seeded cells on fibronectin or collagen I, in order to exclude any formation of E-cadherin–mediated adhesions, also resulted in similar Rac activities on both substrates (Fig. 7 *B*). The level of Tiam1-mediated Rac activation was thus not dependent on the cell substrate, suggesting that rather the different substrate-dependent intracellular localization of the Rac activity caused the opposite cellular effects on cell–cell adhesion and motility. This is supported by the localization of the Tiam1 protein (Fig. 3 *A*), either to adherens junctions or to lamellae, which suggests that specific localization of the Rac signaling complex is involved in promoting either cell–cell adhesion or cell migration.

Wild-type epithelial MDCKII cells showed a significant level of Rac activation in the absence of HGF (Fig. 7 *C*, lane *1*). Thus, the epithelial phenotype is characterized by a substantial Rac activity which is presumably required to maintain the epithelial layer (Braga et al., 1997; Takaishi et al., 1997; Tzuu-Shuh and Nelson, 1998). HGF induces cell motility of epithelial MDCKII cells, leading to fibroblastoid, scattered cells. Upon addition of HGF to MDCKII cells, we observed a small transient increase in Rac activation within the first 10 min of stimulation (Fig. 7 *C*, lane *2* and data not shown) which reached again the level of unstimulated cells after a few hours (Fig. 7 *C*, lane *3*). In this time period morphological changes in response to HGF became visible. Since HGF stimulation did not result in major changes of Rac activation, HGF might induce coupling of the observed Rac activity to different signaling complexes which are either involved in cell–cell adhesion or cell motility.

Tiam1-mediated Rac Activation Is Regulated by PI3-Kinase

Tiam1 requires membrane localization via its NH_2-terminal Pleckstrin homology (PH) domain to activate endogenous Rac (Michiels et al., 1997; Stam et al., 1997). Some PH domains have been shown to bind to lipid products generated PI3-kinase, as found for the isolated NH_2-terminal PH domain of Tiam1 (Rameh et al., 1997). We studied therefore, whether the Tiam1-mediated activation of Rac is dependent on PI3-kinase activity. C1199Tiam1-expressing MDCK-f3 cells were seeded on fibronectin or collagen I and activation of endogenous Rac was measured either in the absence or the presence of the PI3-kinase inhibitor wortmannin (Yano et al., 1993; Okada et al., 1994). Upon inhibition of PI3-kinase, the Tiam1-mediated Rac activation decreased on both the motility-suppressing fibronectin and the motility-promoting collagen I substrate (Fig. 7 *D*, lanes *1–4*). In contrast, the activity of the constitutively active V12Rac protein was not dependent on PI3-kinase (Fig. 7 *D*, lanes *5–8*). From these data we conclude that PI3-kinase activity is required for Tiam1-mediated activation of endogenous Rac.

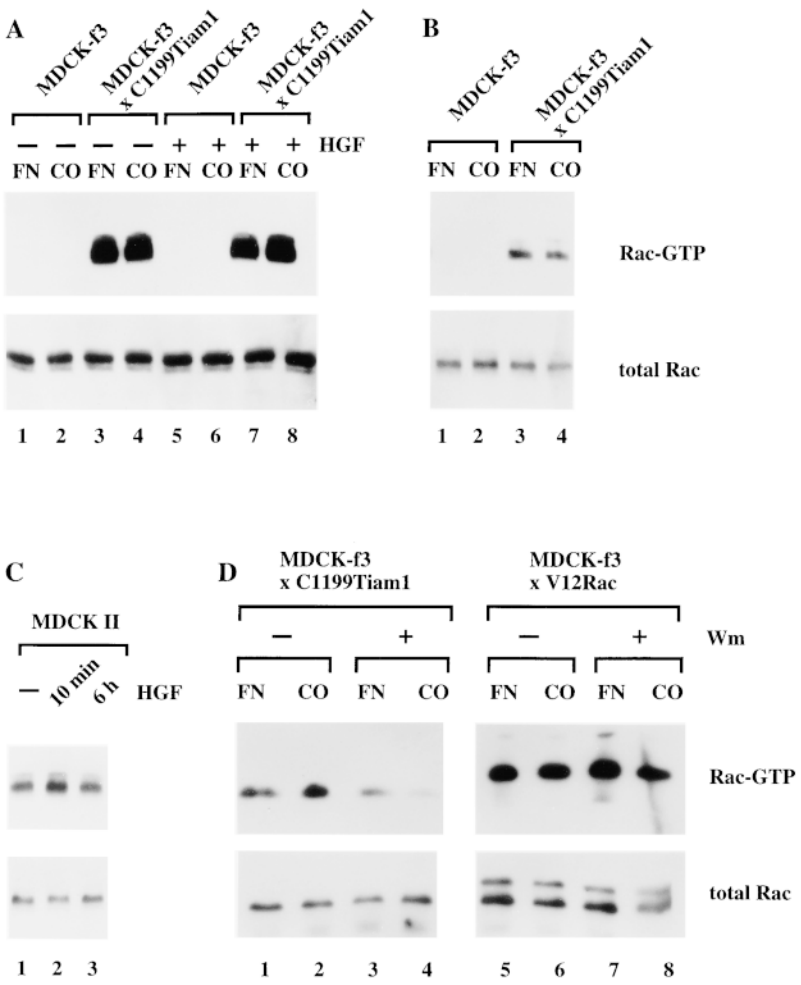

Figure 7. Tiam1-mediated Rac activation on fibronectin and collagen I does not differ and is dependent on PI3-kinase activity. (*A*) MDCK-f3 cells (lanes *1*, *2*, *5*, and *6*) or C1199Tiam1-expressing MDCK-f3 cells (lanes *3*, *4*, *7*, and *8*) were seeded in small clusters of 2–4 cells on a fibronectin (lanes *1*, *3*, *5*, and *7*) or collagen I (lanes *2*, *4*, *6*, and *8*) substrate in the absence (lanes *1–4*) or presence of HGF (lanes *5–8*). Cell lysates were incubated with GST–PAK-CD fusion protein and bound, active GTP–Rac molecules were analyzed by Western blotting. Cell lysates probed for total Rac are shown as a control. (*B*) To exclude effects of cell density on the Rac activity assay, MDCK-f3 cells (lanes *1* and *2*) or C1199Tiam1-expressing MDCK-f3 cells (lanes *3* and *4*) were seeded in small clusters (*2–4* cells) in a four- to fivefold lower density as in (*A*) on a fibronectin (lanes *1* and *3*) or collagen I (lanes *2* and *4*) substrate. Cell lysates were analyzed as described in *A*. (*C*) Rac activities of wild-type epithelial MDCKII cells in the absence (lane *1*) or presence of HGF for the indicated time (lanes *2* and *3*). Cell lysates were analyzed as described in *A*. (*D*) Rac activation in C1199Tiam1- (lanes *1–4*) or V12Rac-expressing MDCK-f3 cells (lanes *5–8*) in the absence (lanes *1*, *2*, *5*, and *6*) or the presence (lanes *3*, *4*, *7*, and *8*) of the PI3-kinase inhibitor wortmannin (50 nM). Cells were seeded on a fibronectin (lanes *1*, *3*, *5*, and *7*) or collagen I (lanes *2*, *4*, *6*, and *8*) substrate and cell lysates were analyzed as described in *A*. The Myc tag of V12Rac increases the apparent molecular weight of this protein and V12Rac exhibits therefore a slightly lower electrophoretic mobility than endogenous Rac. In cell lysates, Myc-tagged V12Rac as well as endogenous Rac are detected by the antibody against Rac (lanes *5–8*).

Both Tiam1-stimulated Cell Migration and E-cadherin–mediated Cell–Cell Adhesion Are Dependent on PI3-Kinase

Tiam1-mediated Rac activation was dependent on PI3-kinase activity both on a collagen I and fibronectin substrate (Fig. 7 *D*), suggesting that PI3-kinase is required for Tiam1/Rac-induced cell migration as well as E-cadherin–mediated cell–cell adhesion. To determine the role of PI3-kinase in Tiam1-induced migration, we compared migration rates of control and C1199Tiam1- or V12Rac-expressing MDCK-f3 cells on a collagen I substrate in the presence of the PI3-kinase inhibitor LY294002 at low cell density (Vlahos et al., 1994). The Tiam1- but not V12Rac-mediated migration was reduced three- to fourfold upon inhibition of PI3-kinase (Fig. 8 *A*), consistent with the effects of PI3-kinase inhibition on the Tiam1-mediated activation of Rac determined in the Rac activity assay (Fig. 7 *D*). Motility of the control Ras-transformed MDCK-f3 cells was also inhibited by LY294002 (Fig. 8 *A*), indicating that not only the Tiam1-stimulated migration but also the basal, Ras-mediated migration of these cells was dependent on PI3-kinase activity. Similar results were obtained with the structurally different PI3-kinase inhibitor wortmannin (data not shown). To substantiate these findings, we generated Tiam1-expressing MDCK-f3 cells that coexpress either the wild-type PI3-kinase subunit p85 or a dominant-negative mutant Δp85 that carries a deletion in the binding site for the p110 catalytic subunit of PI3-kinase (Dhand et al., 1994). Similarly to C1199Tiam1, FLTiam1 (encoding full-length Tiam1) activated endogenous Rac and promoted migration on a collagen I substrate. Expression of the dominant-negative subunit Δp85 reduced migration of MDCK-f3 cells expressing FLTiam1 by two- to threefold, whereas expression of the wild-type subunit p85 had no effect (Fig. 8 *B*). Consistent with the inhibitory effect of the dominant-negative Δp85 subunit on migration of FLTiam1-expressing cells, the migratory, polarized phenotype of FLTiam1-expressing MDCK-f3 cells on collagen I was prevented by dominant-negative Δp85 but not by the wild-type p85 subunit (Fig. 8 *C*). Adhesion and cell spreading on collagen I was not inhibited by expression of dominant-negative PI3-kinase, indicating that Δp85 does not interfere with adhesion or spreading of the cells, but rather specifically blocks migratory responses. Thus, both V12Ras- and Tiam1-induced migration but not V12Rac-induced migration was inhibited by PI3-kinase inhibitors and the dominant-negative Δp85 mutant of PI3-kinase. This indicates that PI3-kinase acts downstream of Ras and

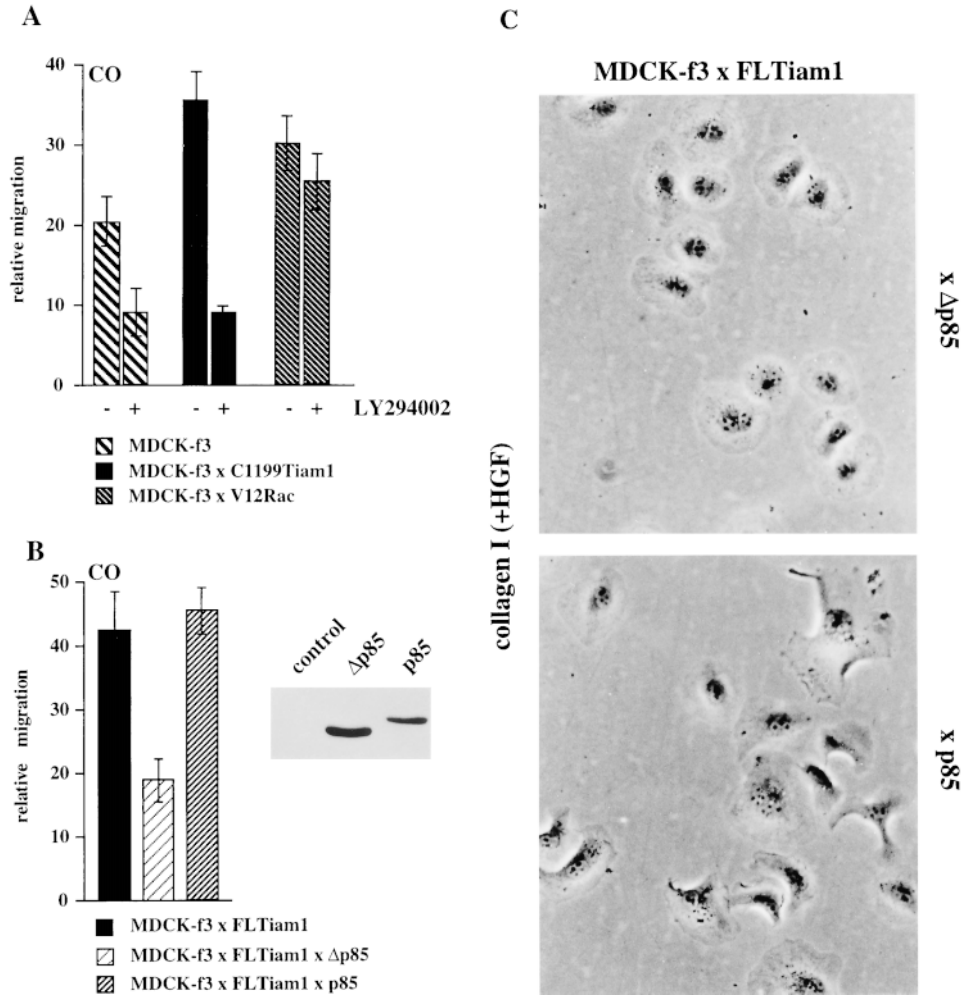

Figure 8. Tiam1- but not V12Rac-stimulated motility is dependent on PI3-kinase. (*A*) Migration rates of MDCK-f3 cells, C1199Tiam1-, or V12Rac-expressing cells were determined on collagen I towards HGF contained in the lower chamber of a Transwell (0.25×10^5 cells per well), either in the absence or presence of the PI3-kinase inhibitor LY294002 (15 μM). (*B*) Migration rates of FLTiam1-expressing MDCK-f3 cells (0.25×10^5 cells per well) transduced either with the control empty vector or with constructs encoding the PI3-kinase subunit wild-type p85 or dominant-negative Δp85. Both Δp85 and p85 subunits were expressed, as determined by immunoprecipitation (from 10×10^6 cells) with 12CA5 antibody against the hemagglutinin epitope of p85, followed by Western blot analysis of the precipitates with a monoclonal antibody against p85. In contrast to wild-type p85, dominant-negative Δp85 inhibited Tiam1-mediated migration twofold. Each bar represents the mean ± SD of triplicate migration assays. One representative example of three independent experiments is shown. (*C*) Coexpression of Δp85 but not p85 in FLTiam1-expressing MDCK-f3 cells inhibits the migratory, polarized phenotype of these cells on collagen I. Small clusters (2–4 cells) of trypsinized MDCK-f3 cells coexpressing FLTiam1 and either Δp85 or p85 were seeded in the presence of HGF onto collagen I and photographed after 4 h.

upstream of Tiam1 and Rac in signaling pathways required for migration.

Tiam1-mediated activation of Rac was dependent on PI3-kinase, suggesting that PI3-kinase might also play a role in the Tiam1-induced increase of E-cadherin–mediated cell–cell adhesion. To determine this, control and C1199Tiam1- or V12Rac-expressing MDCK-f3 cells were cultured for 2 d on plastic dishes to allow formation of their own (fibronectin) matrix. The degree of cell–cell adhesion was quantified using dissociation assays (Fig. 9, *A* and *B*), after treatment with the PI3-kinase inhibitor LY294002 for 4 h. Control MDCK-f3 cells were fibroblastoid on fibronectin and did not form E-cadherin–mediated adhesions (Fig. 1). The dissociation assay yielded virtually only single cells in the presence or absence of LY294002 (Fig. 9, *A* and *B*). In contrast, C1199Tiam1- and V12Rac-expressing cells reverted to an epithelioid phenotype on fibronectin by restoring E-cadherin–mediated adhesions (Figs. 1 and 3). Dissociation of these cells resulted in large cell aggregates, indicative for the formation of E-cadherin–mediated adhesions. Treatment of C1199Tiam1-expressing cells with LY294002 resulted in a significant decrease in size of the aggregates (Fig. 9, *A* and *B*), indicating that PI3-kinase is required for the formation and strength of E-cadherin–mediated adhesions. In contrast, the degree of adhesion between V12Rac-expressing cells, which formed smaller aggregates because V12Rac is less efficient in restoring E-cadherin–mediated adhesions than C1199Tiam1 (Fig. 1), was not significantly affected upon inhibition of PI3-kinase (Fig. 9, *A* and *B*). Similar results were obtained with the PI3-kinase inhibitor wortmannin (data not shown). Analysis of Rac activation in MDCK-f3 cells coexpressing FLTiam1 and Δp85 gave a decrease of Tiam1-mediated Rac activation by two- to threefold by Δp85 on a fibronectin as well as on a collagen I substrate (data not shown), consistent with the findings using the PI3-kinase inhibitors (Fig. 7 *D*). We conclude that Tiam1- but not V12Rac-induced E-cadherin–mediated cell–cell adhesion is dependent on PI3-kinase activity and that PI3-kinase acts upstream of Tiam1 and Rac in this process. In

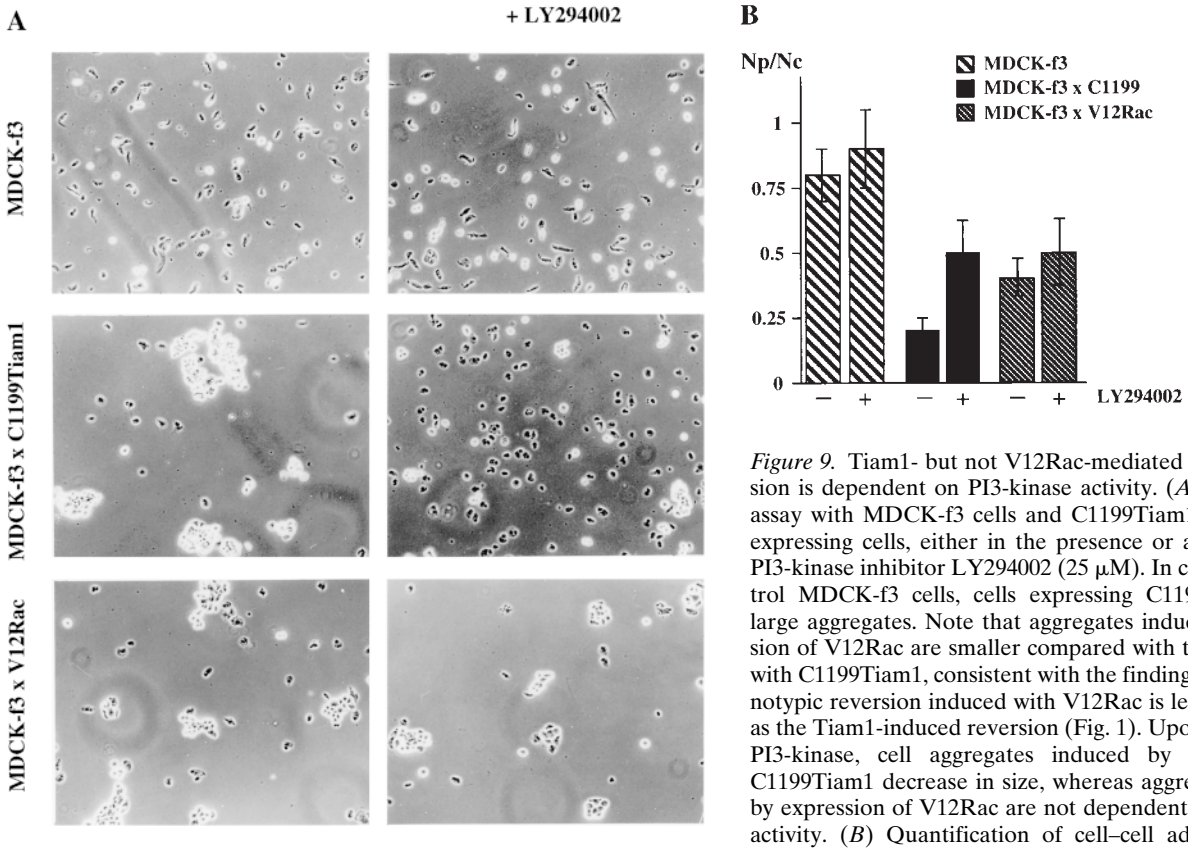

Figure 9. Tiam1- but not V12Rac-mediated cell–cell adhesion is dependent on PI3-kinase activity. (*A*) Dissociation assay with MDCK-f3 cells and C1199Tiam1- or V12Rac-expressing cells, either in the presence or absence of the PI3-kinase inhibitor LY294002 (25 μM). In contrast to control MDCK-f3 cells, cells expressing C1199Tiam1 form large aggregates. Note that aggregates induced by expression of V12Rac are smaller compared with those observed with C1199Tiam1, consistent with the findings that the phenotypic reversion induced with V12Rac is less pronounced as the Tiam1-induced reversion (Fig. 1). Upon inhibition of PI3-kinase, cell aggregates induced by expression of C1199Tiam1 decrease in size, whereas aggregates induced by expression of V12Rac are not dependent on PI3-kinase activity. (*B*) Quantification of cell–cell adhesion of the MDCK-f3 cell lines determined by dissociation assays and expressed as numbers of particles (cell clusters) per total number of cells (*Np/Nc*). Each bar represents the mean ± SD of triplicate dissociation assays. One representative example of four independent experiments is shown.

addition to the documented role of PI3-kinase in cell motility, PI3-kinase is also involved in the establishment of E-cadherin–mediated cell–cell adhesions.

Discussion

To become motile in processes such as wound healing and tumor invasion, epithelial cells undergo epithelial–mesenchymal transition accompanied by the disruption of E-cadherin–mediated cell–cell adhesions. Mutations in E-cadherin or members of the cadherin complex which link E-cadherin to the F-actin cytoskeleton, have been implicated in tumor formation and increased invasive and metastatic behavior of tumor cells (Birchmeier and Behrens, 1994). Furthermore, loss of E-cadherin–mediated adhesions is causal in the transition from adenomas to invasive carcinomas (Perl et al., 1998).

In the present paper we show that the Rho-like GTPase Rac1 in concert with the cell substrate plays a central role in the determination of the epithelial or mesenchymal phenotype of epithelial cells. On fibronectin and laminin 1, Tiam1/Rac signaling reverted the fibroblastoid phenotype of Ras-transformed MDCK-f3 cells and inhibited migration by restoring E-cadherin–mediated cell–cell adhesion. In contrast, on a collagen substrate, cells expressing Tiam1 or V12Rac exhibited a fibroblastoid phenotype and increased motile behavior. Tiam1 localized to adherens junctions in nonmotile epithelial cells on fibronectin and laminin 1 but was present in lamellae and membrane ruffles of fibroblastoid migratory cells on collagen. These different phenotypes were not due to Tiam1-dependent changes in Rac activity on the various substrates. This suggests that not Rac activation per se but rather the intracellular localization of the Rac signaling complex is responsible for the different phenotypes, as reflected by the substrate-dependent localization of the Tiam1 protein. Our data reconcile the reported opposing effects of Rac on motile behavior of epithelial cells. Rac activation has been shown to be required for and to promote E-cadherin–mediated cell–cell adhesion, leading to inhibition of invasion and migration (Hordijk et al., 1997; Braga et al., 1997; Takaishi et al., 1997). In contrast, other reports have demonstrated an essential role for Rac and PI3-kinase in enhanced migration of epithelial cells (Keely et al., 1997; Shaw et al., 1997). The roles of Tiam1/Rac signaling in migration of epithelial cells appear to be due to a balance between Rac-dependent, matrix-regulated effects on cell migration as well as E-cadherin–mediated cell–cell adhesion.

Fibroblastoid Ras-transformed MDCK-f3 cells contained a small amount of active Rac which was independent of both the substrate and HGF (Fig. 7 *A*). In contrast, epithelial wild-type MDCKII cells showed a substantial level of active Rac in the absence of HGF (Fig. 7 *C*). Apparently, V12Ras does not activate Rac in the fibroblas-

toid MDCK-f3 cell line but rather decreases the level of Rac activity by an as yet unexplained mechanism. As a consequence this may lead to loss of cell–cell adhesion and to Ras-mediated motile behavior. Reintroduction of Rac activity by Tiam1 or V12Rac restored cell–cell adhesion and suppressed the Ras-mediated motility in MDCK-f3 cells. The low Rac activity in the Ras-transformed MDCK-f3 cells indicates that in addition to Rac-mediated motility other pathways contribute to migration of these cells, as recently has been demonstrated (Potempa and Ridley, 1998).

We showed previously that the NH_2-terminal PH domain of Tiam1 is required for the proper localization of Tiam1 in membrane ruffles in fibroblasts and adherens junctions in epithelial cells (Hordijk et al., 1997; Michiels et al., 1997). The isolated NH_2-terminal PH domain binds with a 10-fold higher affinity to the PI3-kinase lipid product phosphatidylinositol (3,4,5)-trisphosphate in comparison to phosphatidylinositol (4,5)-bisphosphate or phosphatidylinositol (3,4)-bisphosphate (Rameh et al., 1997). Activation of Tiam1 most likely involves binding to lipid products of PI3-kinase, since Tiam1-mediated Rac activation is dependent on PI3-kinase activity (Fig. 7 *D*). Similar to Tiam1, activation of Vav, a GEF for Rac in hematopoietic cells, is dependent on PI3-kinase (Han et al., 1998). Lipid binding to the NH_2-terminal PH domain, may play an important role in localization of Tiam1 in specific signaling complexes at the plasma membrane, as has been shown for other signaling proteins containing PH domains (Kolanus et al., 1996).

Our findings implicate that PI3-kinase acts upstream of Tiam1/Rac in the signal transduction cascade, since Tiam1- but not V12Rac-induced cell–cell adhesion as well as cell migration was dependent on PI3-kinase activity. Similarly, Shaw et al. (1997) concluded that PI3-kinase acts upstream of Rac in PI3-kinase–induced motility of colon carcinoma cells. However, Keely et al. (1997) found that PI3-kinase is located downstream of Rac using T47D mammary carcinoma cells. This discrepancy may reflect cell type-dependent differences in the requirement of PI3-kinase for motility. The role of PI3-kinase in cell motility is well documented (Wennström et al., 1994; Royal and Park, 1995; Keely et al., 1997; Shaw et al., 1997). The PI3-kinase–dependent Rac activation by Tiam1, and the subsequent decrease in cell–cell adhesion upon inhibition of PI3-kinase activity, also suggests a role for PI3-kinase in the establishment of E-cadherin–mediated cell–cell adhesion. Tiam1-mediated Rac activation was decreased by dominant-negative Δp85 as well as by PI3-kinase inhibitors on a fibronectin as well as on a collagen I substrate, which argues that Tiam1 activity on both substrates is dependent on lipid products produced by PI3-kinase. The substrate-dependent formation of both lamellae and restoration of E-cadherin–mediated adhesion is accompanied by accumulation of F-actin at the respective sites (Fig. 3). This suggests that actin accumulation in lamellae as well as in adherens junctions is linked to PI3-kinase–induced, Tiam1-mediated Rac activation. The localization and/or composition of the Rac signaling complex appears to be matrix regulated and may, therefore, lead to opposite cellular responses.

We observed a transient small increase of active Rac after HGF-stimulation of epithelial wild-type MDCKII cells which reached levels of unstimulated cells after a few hours (Fig. 7 *C*). Thus, stimulation with HGF did not result in a major activation of Rac, suggesting that the HGF-induced switch from cell–cell adhesion to cell migration reflects coupling of active Rac to different signaling pathways. A possible mechanism for HGF-induced epithelial–mesenchymal transition of MDCKII cells could involve recruitment of the Tiam1/Rac-signaling complex from adherens junctions to specific membrane sites by localized, elevated levels of phosphatidylinositol (3,4,5)-trisphosphate, which increase upon HGF stimulation (Royal and Park, 1995). This might also explain the partial relocalization of Tiam1 from adherence junctions to membrane ruffles observed in MDCKII cells upon stimulation with HGF (Hordijk et al., 1997).

The adhesion to various substrates of C1199Tiam1-expressing MDCK-f3 cells was comparable, suggesting that Rac-induced motility on collagen is not necessarily coupled to the degree of adhesion and spreading. Small amounts of collagen were sufficient to induce cell scattering, even if the cells had already established cell–cell contacts on fibronectin (Fig. 2). Moreover, Tiam-expressing cells that coexpressed the dominant-negative PI3-kinase subunit Δp85 attached to and spread on collagen I, but were impaired in migration (Fig. 8). This suggests that specific, motility-inducing signals are derived from collagen which are independent from its adhesive function. Cells grown on fibronectin, laminin 1, or their own (fibronectin) substrate seemed to lack such signals and therefore established strong E-cadherin–mediated adhesions. The matrix-dependent effects of Tiam1/Rac signaling on migration of epithelial cells are consistent with the role for Tiam1/Rac in invasion of T-lymphoma cells (Habets et al., 1994; Michiels et al., 1995). Lymphoid cells do not establish strong homotypic interactions to prevent migration as found in epithelial cells.

Integrins have been shown to participate in cell migration. The $\alpha_2\beta_1$ integrin is required for collagen-induced motility of the bladder carcinoma cell line NBT-II (Tucker et al., 1990; Vallés et al., 1996) and also for V12Rac-induced motility of T47D cells on a collagen I matrix (Keely et al., 1997). In a colon carcinoma cell line, the $\alpha_6\beta_4$ integrin is essential for PI3-kinase–induced migration on a laminin 1 substrate, and mediates the formation and stabilization of actin-containing motility structures (Rabinovitz and Mercurio, 1997; Shaw et al., 1997). It thus appears that the expression of substrate-specific integrins may vary among carcinomas which may explain differences in substrate-dependent migratory responses of carcinoma cells. Epithelial–mesenchymal transition of epithelial cells could involve stimulation of collagen-specific integrins or the recently identified DDR receptors (Shrivastava et al., 1997; Vogel et al., 1997). This may lead to PI3-kinase activation at specific membrane locations, followed by disassembly of the Rac signaling complex at adherens junctions and assembly at lamellae. This complex presumably includes activators as well as downstream effector molecules, since V12Rac also induced substrate-dependent cellular responses, although less efficiently than Tiam1. Depending on the signals derived from the matrix, this signaling complex might either couple to proteins involved in the forma-

tion of adherens junctions or to molecules which stimulate cell migration. Tiam1 contains other sequences that contribute to localizing the protein at the membrane and mediate binding to large cytoskeletal protein complexes (Stam et al., 1997). Proteins with an adapter function may also be involved in the composition and targeting of the Rac signaling complex. Shc has recently been described to bind to the cytoplasmic domain of E-cadherin (Xu et al., 1997), to the cytoplasmic domain of β1 and β4 integrins (Mainiero et al., 1995; Wary et al., 1996), as well as to the collagen-activated DDR receptor (Vogel et al., 1997). Furthermore, it was shown that the matrix-induced formation and phosphorylation of a complex between p130CAS and the adaptor Crk is involved in the induction of Rac-mediated migration of carcinoma cells (Klemke et al., 1998). Similar to Tiam1, Crk and p130CAS localize to membrane ruffles in migratory cells (Klemke et al., 1998) and may thus be involved in specific intracellular localization of the Rac signaling complex.

Taken together, Rac activation in epithelial cells promotes E-cadherin–mediated cell–cell adhesion as well as cell migration. We conclude that the opposing data on the involvement of Rac signaling in migration of epithelial cells are due to matrix-regulated effects on E-cadherin–mediated adhesion and cell motility. The phenotype observed is determined by a balance between Rac-dependent cell-matrix and E-cadherin–mediated cell–cell interactions, which either promote or inhibit motility.

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