Potentiation of Cell Migration by Adhesion-dependent Cooperative Signals from the GTPase Rac and Raf Kinase*

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The small GTPase Rac is thought to regulate cell movement by influencing actin cytoskeletal organization and membrane ruffling. However, cell migration also depends on the activation of mitogen-activated protein kinase (MAPK), which can regulate myosin motor function, an event critical for cell contraction. Evidence is provided that, during active cell adhesion to the extracellular matrix, Rac potentiates the MAPK pathway and influences cell migration by selectively synergizing with Raf kinase but not with Ras or MAPK kinase. In fact, the synergy between Rac and Raf kinase increases the chemotactic sensitivity of cells to epidermal growth factor by 1000-fold. Therefore, the role of Rac in cell migration not only depends on its ability to regulate actin cytoskeletal organization but also on its capacity to potentiate chemokine activation of MAPK in a manner that depends on active cell adhesion to the extracellular matrix.

Cell migration plays a critical role during development, wound repair, and inflammation as well as tumor cell dissemination (1, 2). These events are regulated by chemokines and adhesion proteins, which promote signaling events that influence cell migration by regulating actin organization and myosin motor function (3–6). The Rho family GTPases are known to affect the organization of the actin cytoskeleton in the migratory cell (7, 8). Activation of Rho leads to the formation of actin stress fibers and the assembly of focal adhesion (9), while Cdc42 is involved in the induction of filopodia (7, 10). The small GTPase Rac is known to promote actin cytoskeletal reorganization leading to membrane ruffling and cell migration (11, 12). Inactivation of Rac in Drosophila embryos leads to disruption of dorsal closure (13). In hematopoietic cells, Tiam1 (T-lymphoma invasion and metastasis gene 1), a known activator of Rac, has been shown to promote T cell invasion (14). In epithelial cells, active Rac and Cdc42 were shown to stimulate motile behavior of T47D mammary carcinoma cells, and this was associated with phosphorylation of 3-kinase activity (15). In some cases, Rho proteins can suppress the motile phenotype of cells. For example, Tiam1 and Rac inhibit hepatocyte growth factor-induced scattering of epithelial Madin Darby canine kidney cells by increasing E-cadherin-mediated cell-cell adhesion (16). It was suggested that the intracellular localization of the GTPase signaling complex changed, depending on which adhesive protein the cells were attached to, and this dictated whether it stimulated cell-cell adhesion or cell migration (17). Therefore, it appears that Rho GTPase activities and their influence on cell biology depend on the adhesive state of the cell.

Recent evidence indicates that the extracellular signal-regulated kinase (ERK) pathway contributes to cell migration in a manner independent of its ability to promote gene transcription or cell proliferation (18). Specifically, MAPK phosphorylates and thereby activates myosin light chain kinase, which in turn phosphorylates myosin light chain, leading to cell migration in the absence of chemokines (18). In this case, phosphorylation of myosin light chain by myosin light chain kinase promotes actin/myosin assembly and ATPase activity at the rear of migratory cells (6, 19, 20). However, MAPK may influence motility by decreasing integrin-mediated adhesion (21, 22). While Rac and MAPK appear to contribute to cell migration, it remains unclear how these molecules are regulated.

In this report, we investigated the role Rac plays in regulating the cell migration response. Evidence is provided that Rac not only promotes actin reorganization and membrane ruffling, but to our surprise, it increases the sensitivity of cells in response to EGF by 1000-fold. In this case, Rac is able to synergize with Raf kinase, leading to increased MAPK activity in a manner dependent on active cell adhesion. These results demonstrate that Rac GTPase activity regulates cell migration in two ways, by inducing actin reorganization and by influencing the MAPK pathway.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Rabbit polyclonal antibodies to MAPKs (ERK2 and c-Jun NH2-terminal kinase 1 (JNK1)), Ha-Ras, Raf1, MEK1, and EGFR were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-FLAG M2 monoclonal antibody was purchased from Sigma. Anti-EGFR and anti-phosphotyrosine (4G10) monoclonal antibodies were purchased from Santa Cruz Biotechnology and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively. Human recombinant EGF, platelet-derived growth factor-BB, and insulin were obtained from Genzyme (Cambridge, MA). Anti-active MAPK and PD 98059, a MEK inhibitor, were obtained from Promega (Madison, WI). A stock concentration (10 mM) of PD 98059 was prepared in Me2SO and frozen at –70 °C. Myelin basic protein (MBP) was from Upstate Biotechnology, Inc. GST-c-Jun(1–169) was obtained from Promega (Madison, WI). A stock concentration (10 mM) of PD 98059 was prepared in Me2SO and frozen at –70 °C. Myelin basic protein (MBP) was from Upstate Biotechnology, Inc. GST-c-Jun(1–169) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Rhodamine Phalloidin was from Molecular Probes, Inc. (Eugene, OR).

Cell Culture and Cell Transfection—COS-7 and NIH3T3 cells were...
from ATCC (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 1 mM glutamate.

For transient transfection experiments, 100-mm dishes of cells 60–80% confluent were pretreated by transfection using the Lipofectamine (Life Technologies, Inc.) method according to the protocol of the manufacturer. 1 μg of pCMV-ERK2 expression vector encoding constitutively active Raf1, Erk2, Raf1, MEK1, or inactive Rac1 was used in keeping total DNA at 5 μg/dish. After 36 h, COS-7 cells were placed in medium without serum for a further 18–24 h prior to harvesting and assaying kinase activities and migration, and NIH3T3 cells were made quiescent by maintaining them in Dulbecco's modified Eagle's medium containing 0.5% serum for 24 h. To perform transfection experiments, we achieved an 80% expression efficiency providing a 10–40-fold increase in the level of gene product of interest compared with endogenous protein levels as determined by Western blotting. 2 Cells used in migration assays, were cotransfected with 0.5 μg of a reporter construct encoding β-galactosidase (pCMV-β-galactosidase; Stratagene). 5-Bromo-4-chloro-3-indolyl-β-d-galactopyranoside was used as a β-galactosidase substrate to visualize the transfected cells. Cells analyzed by immunofluorescent staining were cotransfected with a green fluorescent protein (GFP) (pEGFP-C1, CLONTECH) as a cell transfection marker.

**Cell Adhesion and Migration Assay—**COS-7 cell migration assays were performed using Boyden chambers (6.5-mm diameter, 10-μm thickness, 8-μm pores, Transwell®, Costar, Cambridge, MA) containing polycarbonate membranes as described previously (23). The underside of the membrane of the upper chamber was coated with 10 μg/ml rat tail collagen type I (Upstate Biotechnology) or human fibronectin (Collaborative Biomedical Products) for 2 h at 37 °C, rinsed once with phosphate-buffered saline, and then placed into the lower chamber containing 500 μl of migration buffer (fibroblast basal medium with 0.5% bovine serum albumin, Clonetics). Cells were removed from culture dishes with Versene (0.526 mM EDTA in phosphate-buffered saline; Irvine Scientific, Santa Ana, CA), washed twice with migration buffer, and then resuspended in migration buffer (106 cells/ml). 100,000 cells were added to the top of each migration chamber and allowed to migrate for 5 h. In some cases, growth factors such as insulin or EGF were included in the lower chamber. Also, PD 98059 (25 μM) was used to preincubate cells for 30 min before they were detached, and the cells were allowed to migrate in the presence of the inhibitor. Nonmigratory cells on the upper membrane surface were removed with a cotton swab, and the migratory cells attached to the bottom of the membrane were stained with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside substrate. The number of 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside-positive migratory cells per membrane were counted with a Zeiss Axiovert 100 microscope (Zeiss, 63 objective).

**Immunoprecipitation and Immunoblotting—**Transfected COS-7 cells were rinsed twice with cold phosphate-buffered saline and then lysed in radiommune precipitation buffer (100 mM Tris, 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 1% aprotinin, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 mM EDTA, 1 mM sodium vanadate, and 50 mM NaF) for 30 min on ice. The lysates were clarified by centrifugation at 15,000 × g for 10 min at 4 °C, and protein concentration was determined using a Pierce Micro BCA protein assay kit (Pierce). EGFR from lysates containing 200 μg of total cellular protein were immunoprecipitated with protein G-bound anti-EGFR monoclonal antibody (Santa Cruz Biotechnology) for 2 h at 4 °C. Samples were subject to 8% SDS-PAGE and analyzed by immunoblots with anti-EGFR polyclonal antibody and anti-phosphotyrosine monoclonal antibody. Lysates containing 2–10 μg of total cellular protein were also immunoprecipitated with anti-ERK2 and anti-JNK1 polyclonal antibodies (Santa Cruz Biotechnology) for endogenous ERK2 and JNK1, respectively; anti-Ras, Raf, and MEK antibodies for their expressions; anti-FLAG M2 monoclonal antibody (Sigma) for Rac1 expression; and anti-ppERK polyclonal antibody (Promega) for active MAPK. Horseradish peroxidase-conjugated goat anti-mouse or rabbit secondary antibodies and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) were used to visualize the blotted proteins of interest.

**Protein Kinase Assays—**The ERK2 and JNK1 activities in cell lysates were determined by an in vitro immunocomplex kinase assay. The ability of ERK to phosphorylate MBP was determined according to Boulton and Cobb (24). Briefly, 500 μg of protein from cell lysates were precleared with protein A-Sepharose for 4 h in the cold and then incubated with protein A-Sepharose coupled with anti-ERK antibodies (4 mg/100 ml stock bead suspension; Pierce) overnight in the cold. Immunoprecipitates were rinsed three times with radioimmune precipitation buffer containing 0.1 M NaCl before incubation with 100 μl of reaction mixture containing 10 μCi of [32P]ATP, 10 mM MgCl2, 20 μM ATP, 1 mM dithiothreitol, 1 mM benzamidine, 0.04 mM mg/ml MBP, and 25 mM Hepes, pH 8.0, for 20 min at 30 °C. The reaction was stopped by adding 20 μl of boiling SDS sample buffer (6×). Phosphorylation of MBP was then analyzed by 15% SDS-PAGE, stained with Coomassie Blue, dried, and exposed to imaging film overnight. Endogenous JNK1 activity was determined similarly by immunocomplex kinase assays after using an anti-JNK1 polyclonal antibody to immunoprecipitate JNK from cell lysate, and its activity was measured using 2 μg of GST-c-Jun(-1–169) as substrate (25).

**Immunofluorescent Staining—**Transfected COS-7 cells grown on glass coverslips were serum-starved for 24 h and then treated with or without EGF (100 ng/ml) for 10 min. In some cases, cells were pretreated with PD 98059 (25 μM) for 30 min before EGF stimulation. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.2% Triton X-100 for 5 min, and stained with rhodamine-conjugated phallolidin (0.1 μg/ml) for 45 min. For all transfection cells, they were co-transfected with 0.5 μg of pEGFP as a reporter. Cell fluorescence was analyzed with a laser confocal microscope (model 1024; Bio-Rad) with a × 63 objective.

**RESULTS**

To examine the effects of endogenous Rac on cell migration, cells were transfected with Tiam1, a guanine nucleotide exchange factor specific for Rac that has been shown to promote T-lymphoma cell invasion into a fibroblast monolayer (14, 26). Cells expressing Tiam1 were allowed to migrate on a collagen-coated membrane in the absence of growth factors or chemokines. Tiam1-expressing cells showed a 3–4-fold increased haptotactic migration response when compared with mock-transfected control cells (Fig. 1A). This migration was blocked by expression of a mutationally inactive Rac (Rac1 N17) or by treatment of cells with an inhibitor of MEK (PD 98059) (27, 28). These data indicate that cell migration induced with Tiam1 is dependent on both Rac and MAPK. Importantly, while Tiam1 expression enhanced cell migration, it did not increase ERK activity in these cells as determined by an in vitro kinase assay using MBP as a substrate (data not shown). To further explore the roles of Rac and the MAPK pathway in migration, cells expressing active forms of Ras (H-Ras V12), Raf (Raf BXB), or MEK (MEK1EE) in the presence or absence of Rac1 N17 were allowed to migrate toward collagen. Active Ras, Raf, or MEK promoted haptotaxis, in each case, which was blocked by co-expression of Rac1 N17 (Fig. 1B).

Cells exposed to a chemokine gradient respond by moving toward the source of chemokine (29). To examine the role of Rac and MAPK in chemokine-directed cell migration, cells expressing Rac1 N17 or those exposed to PD 98059 were serum-starved and allowed to migrate in response to the chemotactic agents EGF or insulin. Both EGF and insulin induced cell migration that was completely blocked by expression of Rac1 N17 or exposure of cells to PD 98059 (Fig. 1C). In addition, expression of a mutationally inactive form of MEK (MEK1AA) (28, 30) completely blocked EGF-induced cell migration, confirming the results obtained with the MEK inhibitor PD 98059 (data not shown).

To investigate the general nature of these findings, we examined the role of Rac and MAPK in the migratory response of NIH3T3 fibroblasts to platelet-derived growth factor-BB or expression of Tiam1. As shown in Fig. 1D, like that observed for COS-7 cells, NIH3T3 cell migration induced by platelet-derived growth factor-BB or Tiam1 expression was blocked by expression of Rac1 N17 or treatment of cells with the MEK inhibitor PD 98059. Together, these results demonstrate that both che-
Directed cell migration during development, angiogenesis, and inflammation depends on both cell adhesion to the extracellular matrix and the ability of a cell to detect a broad chemokine gradient (31–34). To further evaluate the role of Rac in chemokine-dependent cell migration, cells were transfected with a mutationaly active Rac (Rac1 L61) and allowed to migrate on a collagen substrate in response to a broad concentration range of EGF. To our surprise, cells expressing active Rac showed a 1000-fold increased sensitivity to EGF compared with control cells. In this case, Rac potentiated cell migration toward 1 pg/ml EGF, while control cells required >1 ng/ml to achieve this level of migration. Importantly, active Rac did not increase the overall level of migration but, rather, enhanced the dose response to the growth factor (Fig. 3). COS-7 cell migration on collagen is mediated by \( \alpha_{6}\beta_{1} \) integrin (18). EGF-induced cell migration on collagen was abolished by preincubating cells with P4C10 monoclonal antibody (anti-\( \beta_{1} \) integrin) in the presence or absence of activated Rac (data not shown). Moreover, treatment of cells with EGF or expression of active Rac did not quantitatively or qualitatively influence the cells’ adhesiveness to a collagen substrate (data not shown). Together, these data demonstrate that Rac has the capacity to greatly potentiate the chemotactic effects of EGF, enabling cells to respond to a minute dose of growth factor.

To move, cells must continually make and break adhesion contacts (35). Therefore, we examined the role of Rac in EGF-dependent ERK activation in cells that were either actively engaged in adhesion to simulate the migratory process or those that were preattached and spread for several hours on a collagen matrix. The ability of EGF to activate ERK was enhanced by Rac only in cells undergoing active adhesion (Fig. 4A). Cells that had been preattached and spread on collagen for 4 h showed no such enhancement of ERK activity (Fig. 4A). Importantly, cells expressing Rac in the absence of EGF showed no enhanced MAPK activity or migration regardless of their adhesive state. Therefore, EGF appears to cooperate with Rac in an adhesion-dependent manner to potentiate MAPK activity as well as chemotactic cell migration. These findings provide a link between cell adhesion events and the coupling of Rac activity to the MAPK-dependent cell migration machinery.

To investigate the mechanism by which Rac influences EGF-dependent cell migration, lysates from cells exposed to varying concentrations of this chemokine in the presence or absence of Rac were examined for the level of EGF receptor (EGFR) phosphorylation. In this case, EGFR from cells expressing Rac1 L61 showed no increase in tyrosine phosphorylation in response to EGF over that seen with control cells (Fig. 4B), indicating that Rac impacts a downstream signaling event in the EGF-mediated cell migration response.

To begin to define the downstream mechanism(s) by which Rac influences the MAPK pathway and cell migration, cells were transfected with active Rac together with each component of the MAPK pathway. In this case, Rac was expressed in cells together with either a suboptimal dose (0.1 \( \mu \)g of DNA) or an optimal dose (1.0 \( \mu \)g of DNA) of active Ras, Raf, or MEK. In the absence of exogenously expressed Rac, the optimal dose, but not the suboptimal dose, of Ras, Raf, or MEK was sufficient to promote cell migration (Fig. 5). In addition, cells containing Rac1 L61, alone or in conjunction with the suboptimal dose of Ras or MEK, showed no migration over background. However, cells expressing Rac in conjunction with the suboptimal dose of Raf demonstrated a robust migration response (Fig. 5). These data demonstrate that Rac can potentiate cell migration in a cooperative or synergistic manner with Raf kinase.

The fact that chemokines promote MAPK activity and induce cell migration prompted us to assess whether the cell migration...
response induced by the synergy between Raf and Rac might be associated with increased MAPK activity in these cells. Rac1 L61 was expressed in the presence or absence of 0.1 or 1.0 μg of DNA encoding Raf BXB, and lysates from these cells were assessed for MAPK activity using myelin basic protein MBP as a substrate. MAPK activity could not be induced when cells were exposed to 0.1 μg of DNA (suboptimal dose) encoding Raf (Fig. 6, middle panel). However, expression of 0.1 μg of Raf DNA together with Rac was able to induce MAPK activity to the same level as that observed with optimal level (1.0 μg DNA) of Raf alone (Fig. 6, middle panel). While Rac synergized with Raf in promoting increased MAPK activity, no increase in Rac activation of endogenous JNK (36, 37) was observed, as measured by phosphorylation of c-Jun (Fig. 6, lower panel). Therefore, Rac and Raf function to preferentially enhance ERK activity.

To establish whether the migration response observed due to the cooperation of Rac and Raf requires MAPK activity, cells transfected with active Rac together with low dose (0.1 μg of DNA) active Raf were incubated in the presence or absence of PD 98059 and allowed to migrate toward a collagen substrate. This MEK inhibitor completely abolished cell migration induced with Rac/Raf (Fig. 7), indicating that this response requires MAPK activity. Therefore, we conclude that Rac and Raf can cooperate to activate MAPK, and this is required for cell migration.

DISCUSSION

Chemokines and adhesive components within the extracellular matrix initiate directed cell migration/invasion during development or in the adult organism, in response to injury or inflammatory events. Signals initiated by chemokine gradients and/or extracellular matrix proteins facilitate the conversion of cells from the stationary to the motile phenotype (38, 39). Cell migration depends on reorganization of the actin cytoskeleton and development of cell polarity enabling a cell to make new adhesive contacts at its leading edge and break existing contacts at its trailing edge. This is associated with actin reorganization and in some cases active membrane ruffling at the cells’ leading edge, an event mediated by the small GTPase Rac (9, 40), and contractile force at the rear of the cell, which can be regulated by actin/myosin motor function (6, 41). Recent evidence demonstrates that myosin motor function can be regulated, in part, by MAPK, which serves to phosphorylate and thereby activate myosin light chain kinase (18). In fact, mutationally inactive myosin light chain kinase or inhibitors of the MAPK pathway block myosin light chain phosphorylation and
in so doing disrupt haptotaxis or chemotaxis (18). Alternatively, increased ERK activity may induce motility by reducing the adhesivity of integrin contacts with the matrix (21, 22).

Evidence provided in this study indicates that Rac, commonly known to promote actin reorganization in motile cells, actually serves another primary function in the regulation of cell migration. During chemokine-induced migration activated Rac was able to increase the cells' sensitivity to EGF by 1000-fold. This potentiation occurred downstream of the EGF receptor, since the presence of active Rac did not change the tyrosine phosphorylation profile of this receptor in response to varying amounts of EGF. Rather, it was associated with Rac's ability to enhance the activation of MAPK induced by EGF. Importantly, this increased MAPK activity by Rac could only be observed in cells that were actively attaching to their substrate, since stably preattached cells did not show this effect. In fact, active adhesion and deadhesion are ongoing processes in migratory cells. Therefore, it is tempting to speculate that cell-associated proteases that can degrade extracellular matrix components and thereby release preattached cells from adhesive contacts may enhance chemotaxis, in part, by facilitating new adhesion events that we predict would enhance MAPK activity in a Rac-dependent manner. In addition to its ability to promote membrane ruffling, Rac can potentiate MAPK activity in response to EGF, an event critical for actin/myosin motor function (18, 42). We were able to separate these activities within the cell, since an inhibitor of MEK or a mutationally inactive form of this enzyme blocked cell migration without impacting the ability of Rac to promote membrane ruffling. In fact, immunofluorescent staining data presented shows that while...
observed that activation of endogenous Rac by the Rac guanine nucleotide exchange factor Tiam1 or chemokines such as EGF and insulin also promoted increased motility. Activation of endogenous Rac by Tiam1 leads to induction of motility, while exogenous expression of active Rac in the absence of EGF fails to enhance motility. This apparent discrepancy can be explained by several possibilities. For example, Tiam1 promotes the activity of endogenous Rac that leads to membrane ruffling at the cells’ leading edge, whereas overexpression of exogenous Rac L61 leads to membrane ruffling around the entire cell periphery (Fig. 2). In addition, expression of exogenous Rac in cells may compete, in part, for endogenous Rac in terms of intracellular localization. Alternatively, Tiam1 might activate or indirectly potentiate the activity of other signaling molecules in the cell. In any event, Tiam1-induced motility or that induced by chemokines was blocked by either dominant negative Rac or inhibition of MAPK. The fact that inhibition of MEK blocked migration yet did not influence Rac-dependent membrane ruffling demonstrates that MAPK influences motility without impacting Rac or membrane ruffling. In contrast, Rac activity not only leads to membrane ruffling, but, as shown here, it can potentiate MAPK activity in a cell expressing low levels of Raf kinase. This may explain how cells can be induced to migrate in vivo in response to relatively low levels of chemokine during the initial phases of angiogenesis or inflammation. Based on these findings, we propose that cell motility depends on two signaling pathways, one involving Ras/MAPK activity, leading to activation of actin/myosin motor function and/or release of adhesion contacts, and a second that involves Rac-dependent membrane ruffling. Our results also show that Rac, once activated, can ultimately influence the MAPK pathway, thereby enhancing the cell migratory response to chemotactic factors in an adhesion-dependent manner.

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