Platelet-derived Growth Factor and Fibronectin-stimulated Migration Are Differentially Regulated by the Rac and Extracellular Signal-regulated Kinase Pathways*

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Directed cell migration is essential for a variety of important biological processes ranging from development and angiogenesis to metastasis. Ras plays a pivotal role in the signaling cascade that governs chemotaxis of fibroblasts toward platelet-derived growth factor-BB (PDGF-BB). Ras activates multiple downstream pathways, which include the extracellular signal-regulated kinase (ERK), Rac, and Ral signaling cascades. We therefore investigated the role of the Rac and ERK pathways in cell migration. We showed that migration of fibroblasts toward PDGF-BB is inhibited by expression of dominant negative Asn-17 Rac1. Blocking of the ERK pathway by either expression of dominant negative Ala-218/Ala-222-mitogen-activated protein kinase kinase (A218/A222-MEK1) or by a MEK-specific inhibitor did not inhibit migration toward PDGF-BB. In contrast, migration toward soluble fibronectin was suppressed by inhibition of the ERK pathway but not by Asn-17 Rac1 expression. These results indicate that directed cell migration mediated by different receptor classes in response to different ligands differentially utilizes the Rac and ERK pathways and suggest that Rac might play a critical role in pathological processes such as angiogenesis and metastasis.

Directed cell migration or chemotaxis is a critical feature of several physiological and pathological processes, including development, wound healing, atherosclerosis, immunity, angiogenesis, and metastasis (1–4). Chemotaxis involves the sensing of a concentration gradient of chemoattractant, reorganization of the actin cytoskeleton, and subsequent movement toward the chemoattractant. Cytokine-induced reorganization of the actin cytoskeleton is mediated by members of the Rho family of GTP-binding proteins. Rho regulates stress fiber assembly, Rac controls lamellipodia formation, and Cdc42 directs the dynamics of filopodia (5–8).

We have shown previously that the GTP-binding protein Ras plays a central role in the signaling cascade that governs chemotaxis of fibroblasts toward PDGF-BB (9). However, the pathways downstream of Rac that control cell motility remain unknown. Ras activates several distinct effector pathways, including the ERK cascade, the pathway controlled by Rac, and the pathway initiated by RalGDS, the guanine nucleotide dissociation stimulator for RalA and -B, which are close relatives of Ras (10–17). Since Rac has been shown to control the formation of lamellipodia induced by Ras (5, 18), it is a likely candidate for regulating Ras-mediated directed migration. A possible role for Rac in directed migration is also suggested by observations showing that Ras- and growth factor-induced activation of Rac are mediated by phosphatidylidyinositol 3-kinase (17, 19), which in turn has been implicated in chemotaxis (20–22). In this study, therefore, we investigated the role of Rac in different types of cell migration: directed migration toward PDGF-BB and soluble fibronectin and random migration stimulated by lysophosphatidic acid (LPA).

EXPERIMENTAL PROCEDURES

Cell Lines—Derivatization, properties, and growth conditions of Asn-17 Rac1 (N17-Rac1)-expressing Rat1 fibroblasts have been previously described (12). ERK activation by 2.5 ng/ml PDGF-BB or 1 µg/ml LPA in these mutant Rac1-expressing lines is similar to that of control cells, indicating that the changes in cell migration stimulated by PDGF-BB and LPA are not a consequence of changes in expression level of the PDGF-β and LPA receptors.

For the establishment of A218/A222-MEK1-expressing lines, Glu-tagged full-length A218/A222-MEK1 (kindly provided by S. Macdonald and E. Porfiri, ONX) was subcloned as a BamHI–NcoI fragment into the expression plasmid pAUCT. This is a pBR-based vector that contains the neomycin resistance gene under the control of the thymidine kinase promoter, the Tet repressor-VP16 fusion cDNA under the control of the cytomegalovirus promoter, and the Tet operator driving the expression of interest (kindly provided by A. Pattaey, ONX). Rat 1 fibroblast lines expressing A218/A222-MEK1 or the pAUCT vector control were established after DNA transfection by selecting G418-resistant clones growing in the presence of tetracycline to maintain the expression of recombinant MEK1 mutant at low levels. Clones were maintained in high glucose (4.5 g/liter) Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units of penicillin, and 2 µg/ml streptomycin, 400 µg/ml G418, and 2 µg/ml tetracycline and kept at 37 °C and 5% CO2. Prior to experimental analysis, induction of recombinant Rac and MEK mutants was achieved by removing tetracycline for 24 h.

Expression levels of the Myc-tagged Rac mutants were obtained by immunoblotting using the 9E10 monoclonal antibody as in Ref. 23. Expression levels of the Glu-Glu-tagged MEK1 mutant were determined using an anti-Glu-Glu monoclonal antibody (24).

Kinase Assays—A218/A222-MEK1 expression was induced by removal of tetracycline for 24 h. Subsequently, cells were starved from serum for 18 h and induced with 10 nM epidermal growth factor for various periods of time. Cells were lysed in 20 mM Tris-HCl, pH 8.0, 137

LPA, lysophosphatidic acid; N17-Rac1, Asn-17 Rac1; ERK, extracellular signal-regulated kinase; A218/A222-MEK1, Ala-218/Ala-222-mitogen-activated protein kinase kinase.

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mM NaCl, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl$_2$, 1 mM sodium vanadate, 1 mM Pefabloc, 20 μM leupeptin, 10 μg/ml apropin, and 50 mM NaF. Cleared lysates were incubated for 2 h at 4 °C with anti-ERK1 E1.2 crude serum and protein A-Sepharose. The beads were washed twice with lysis buffer and once with kinase buffer (30 mM Tris-HCl, pH 8.0, 20 mM MgCl$_2$, and 2 mM MnCl$_2$). The kinase reaction was initiated by adding 30 μl of kinase buffer (10 μM cold ATP, 2.5 μCi of [γ-32P]ATP, and 7 μg of myelin basic protein) to the beads. After incubation for 30 min at 30 °C, reactions were stopped by adding sample buffer. Phosphorylated myelin basic protein was resolved on 14% SDS-PAGE and revealed by autoradiography.

Cell Migration Assays—Cell migration through collagen-coated filters was assayed as described previously (9). Cell binding to the collagen-coated filters of the various lines and control cells was determined at various times after incubation in the chemotaxis chamber. Nonadherent cells were washed off with phosphate-buffered saline, and adherent cells were counted under the microscope. Statistical analysis (two sample t test) was performed on the values for stimulated migration corrected for background migration (in the absence of stimulus).

Quantification of Growth Factor-induced Ruffling—Serum-starved cells were transferred to bicarbonate-free medium and mounted in a homemade observation chamber. Cell behavior was followed by means of video time lapse microscopy. Video frames were collected every 10 s with a Hamamatsu C2400 camera mounted on a Zeiss Axiovert 100TV inverted microscope provided with a × 40 Plan-NeoFluor NA 0.75 objective. Images were stored on a Panasonic TQ 2028F optical memory disk recorder. The number of ruffles per cell larger than 10 μm was determined at 2-min time intervals.

RESULTS

Rac Is Essential for Directed Migration toward PDGF-BB and Random Migration Stimulated by LPA—To investigate the function of Rac in cell migration, we employed Rat1 fibroblast lines expressing N17-Rac1 (12). We first characterized the migratory response of control Rat1 fibroblasts toward PDGF-BB, using a multwell Boyden chamber assay (25). This assay measures the movement of cells across a porous membrane in response to a concentration gradient of a chemoattractant and has been shown previously to provide quantitative measurement of lymphocyte, endothelial cell, and tumor cell motility in response to a variety of physiological effectors. Control vector-transfected cells showed optimal migration toward a gradient generated by concentrations of 2.5–5 ng/ml PDGF-BB in the bottom well. Checkerboard analysis in which the amount of attractant was varied in both the top and bottom wells of the Boyden chamber indicated that the Rat1 fibroblasts display predominantly directional motility (chemotaxis) toward a PDGF-BB gradient, with a minor component attributed to an increase in random motility (data not shown). Relative to vector control cells, the three independent clones of Rat1 fibroblasts expressing N17-Rac1 tested were strongly inhibited in their migration toward PDGF-BB ($p < 0.0005$), whereas their basal unstimulated motility was not altered (Fig. 1c). These results indicate that Rac is a key element in the signaling pathway involved in directional migration induced by PDGF-BB. Basal cell movement, in contrast, does not depend on Rac.

To further study the role of Rac in the regulation of cell motility, we investigated the response of the lines expressing mutant Rac proteins to LPA, a ligand that activates a G protein-coupled receptor (26). Checkerboard analysis demonstrated that LPA stimulates random, nondirectional migration (chemokinesis) in contrast to PDGF-BB, which stimulates directed cell migration. Here again, Rat1 fibroblasts expressing N17-Rac1 showed a strong reduction in LPA-stimulated migration ($p < 0.0005$) (Fig. 1d), indicating that Rac also plays an essential role in a signaling pathway utilized by LPA to control cell motility. Together with the previous data, these results also indicate that both random and directed stimulated cell motility are Rac-dependent and that motility signaling pathways triggered by tyrosine kinase and G protein-coupled receptors both employ Rac as a mediator.

To test whether the Rac pathway controls cell locomotion stimulated by other factors, we studied directed migration toward soluble fbronectin (FN), an integrin-mediated response. We previously showed that FN-stimulated migration is independent of Ras activity (9). At this moment it is not clear, however, whether migration toward soluble FN is chemotactic or haptotactic (migration guided by a gradient of increasing substrate adhesiveness).

Migration toward FN was not significantly inhibited by expression of N17-Rac (Fig. 1c). This indicates that, in contrast to motility stimulated by the growth factors PDGF-BB and LPA, Rac activity is not essential for migration toward fbronectin. These observations also further differentiate the motility pathway stimulated by fbronectin from those activated by the growth factors PDGF and LPA.

Inhibition of the ERK Pathway Impairs Directed Migration toward Soluble FN, but Not toward PDGF-BB—In addition to activating Rac, Ras also stimulates the ERK pathway, which is required for cell proliferation and transformation (11). The potential role of the ERK cascade in the regulation of cell motility is not yet clear, however. To study whether activation
of the ERK pathway is necessary for cell migration, we used Rat1 fibroblast lines expressing dominant-negative A218/A222-MEK1 (11) from a tetracycline-repressible promoter (27). Although these lines showed greatly diminished ERK activation in response to growth factors (up to 90% inhibition of ERK activation for the line shown in Fig. 2), migration toward PDGF-BB was unaltered (Fig. 2a). In addition, although LPA stimulates ERK activity in a Ras-dependent fashion (28), A218/A222-MEK1 expressing lines did not show any inhibition in LPA-stimulated motility (Fig. 2b). Thus, Rac controls PDGF- and LPA-stimulated motility independently of the ERK pathway.

In contrast to the above results, A218/A222-MEK1-expressing lines were significantly inhibited in migration toward soluble fibronectin (p < 0.0005) (Fig. 2c). The inhibition of fibronectin-stimulated migration was not caused by decreased adhesion of the A218/A222-MEK1-expressing lines to the filter separating the two chambers. Indeed, adhesion to the Boyden chamber collagen-coated filter of all of the cell lines used in this study (including the lines expressing the various Rac mutants) was indistinguishable from those of controls, and adhesion was independent of the presence of growth factors or soluble fibronectin in the bottom chamber (data not shown).

We further tested the role of the ERK pathway in cell migration using PD98059, a MEK-specific inhibitor (29), to block MEK activation. PD98059 at a concentration of 10 μM, which inhibits growth factor activation of ERK by 50–60% in Rat1 fibroblasts (30), significantly inhibited migration of vector control lines toward FN (p < 0.0005) but did not affect migration stimulated by either PDGF-BB or LPA (Fig. 3, a–c). These results confirm that activation of the ERK pathway is necessary for migration toward soluble fibronectin, indicating a novel function for the ERK pathway in integrin-mediated cell migration.

**Mechanism of Rac-mediated Migration**—The control of cell migration by Rac might be expected to involve the regulation of lamellipodial dynamics, which has been shown to be controlled by Rac (5). We therefore quantified PDGF-induced ruffling in Rat1 fibroblasts expressing N17-Rac1 and controls (Fig. 4). PDGF-induced ruffling was strongly inhibited by expression of N17-Rac1 in Rat1 fibroblasts, in agreement with results obtained in other cell types (5, 19). However, LPA at concentrations up to 1 μg/ml did not induce any ruffling response in Rat1 fibroblasts (data not shown), in line with earlier observations in Swiss 3T3 fibroblasts (6). These findings suggest that Rac may regulate cell motility independently of its role in lamellipodia formation.

**DISCUSSION**

Our data indicate that the Rac and ERK pathways mediate different types of migratory behavior, with the Rac pathway

![Fig. 2. A218/A222-MEK1 selectively inhibits directed migration toward soluble fibronectin. a, migration of A218/A222-MEK1-expressing Rat1 cells toward PDGF-BB (2.5 ng/ml). b, migration of A218/A222-MEK1-expressing Rat1 fibroblasts stimulated by LPA (1 μg/ml). c, directed migration of A218/A222-MEK1-expressing Rat1 fibroblasts toward soluble fibronectin (1 μg/ml). Other conditions were as in Fig. 1. d, upper panel, anti-Glu-Glu Western blots showing A218/A222-MEK1 expression levels; lower panel, 32P incorporation into myelin basic protein phosphorylated by immunoprecipitated ERK1 from cells treated with 10 μM epidermal growth factor for 10 min.

![Fig. 3. The MEK inhibitor PD98059 selectively inhibits directed migration toward soluble fibronectin. a, effect of 10 μM PD98059 on migration of Rat1 vector control cells toward PDGF-BB (2.5 ng/ml). b, effect of 10 μM PD98059 on migration of Rat1 vector control cells stimulated by 1 μg/ml LPA. c, effect of 10 μM PD98059 on migration of Rat1 vector control cells toward 1 μg/ml fibronectin. Other conditions were as in Fig. 1.](image-url)
controlling motility stimulated by PDGF-BB and LPA and the ERK pathway controlling motility stimulated by FN (Fig. 5). Because Ras is essential for both PDGF- and LPA-stimulated motility (9), our results also indicate that the Rac and ERK pathways can differentially mediate signals that emanate from Ras. This is consistent with results obtained with Ras effector loop mutants, which indicated that membrane ruffling and ERK activation are mediated by distinct Ras effectors (17, 18). The recent observation that Ras activation of Rac is mediated by phosphatidylinositol 3-kinase, is also in line with previous findings that chemotaxis toward PDGF is phosphatidylinositol 3-kinase-dependent (20–22). Interestingly, Ras, phosphatidylinositol 3-kinase, and Rac have also been shown to be necessary for hepatocyte growth factor/scatter factor-induced dispersion of Madin-Darby canine kidney cells (31–33), indicating that this signaling cascade may be utilized in a variety of motile responses and may play an important role in mammalian development (34).

Adhesion to fibronectin has been shown to activate ERK and may be responsible for shape-dependent cell proliferation (35–37). Our results indicate an additional role for the ERK pathway in fibronectin-stimulated migration. These results are consistent with a recent report, which shows that activation of the ERK pathway is necessary for haptotaxis stimulated by a collagen gradient (38). We have previously shown that migration toward soluble fibronectin is Ras-independent (9). Whether Ras plays a role in integrin-induced activation of ERK still remains to be resolved, however (39, 40).

The mechanism by which Rac controls migration remains to be elucidated. The inhibition of LPA-stimulated motility by N17-Rac1 suggests that the role of Rac in cell migration could be independent of its function in the control of lamellipodia. Although this may come as a surprise, it is consistent with recent studies on actin filament dynamics and ultrastructure in locomoting heart fibroblasts, which showed that the rate of cell locomotion correlates with the flow of actin filaments in the cell body and not with that in lamellipodia (41). Therefore, in addition to regulating the formation of lamellipodia, Rac may control more subtle cytoskeletal features, which still remain to be defined. Rac could also regulate cell migration by inducing the expression of matrix-degrading proteases, since AP1 and PEA3, two elements that are implicated in the control of matrix metalloprotease expression (42), are both activated by Rac (43, 44). A similar mechanism might be used by the ERK pathway to control cell migration toward fibronectin. Alternatively, the

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**FIG. 4.** PDGF-induced ruffling is inhibited in Rat1 fibroblasts expressing N17-Rac1. The number of ruffles per cell was determined at the maximum of the ruffling response caused by the indicated concentrations of PDGF-BB (see “Experimental Procedures”). Error bars indicate the S.E. of four fields of cells, comprising 7–10 cells each.

**FIG. 5.** The Rac and ERK pathways mediate different modes of migration. Rac regulates directed migration toward PDGF-BB and random migration stimulated by LPA. The downstream pathway utilized by Rac in the control of cell motility is likely to be distinct from the pathway involved in lamellipodia formation. Migration toward fibronectin depends on activation of the ERK pathway but is independent of Ras activation. The relationship between migration and transcription is still not understood.

ERK pathway could control migration toward FN via activation of myosin light chain kinase, as was shown to be the case for collagen-mediated haptotaxis (38).

The function of Rac in the regulation of directed cell migration is in line with the role of Tiam1, a Rac guanine nucleotide exchange factor, which facilitates cell invasion (45, 46). Furthermore, directed migration is a critical component in angiogenesis (4). Thus, the data presented in this paper suggest that Rac, in addition to its role in tumorigenicity (12, 13), may be an important signaling element in metastasis and angiogenesis.

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