c-Raf-mediated Inhibition of Epidermal Growth Factor-stimulated Cell Migration*

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Epidermal growth factor stimulates migration of a number of cell types, yet the signaling pathways that regulate epidermal growth factor-stimulated migration are poorly defined. In this report, we employ a transient transfection migration assay to assess the role of components of the Ras-mitogen-activated protein (MAP) kinase signaling pathway in epidermal growth factor-stimulated chemotaxis of rat embryo fibroblasts. Expression of dominant negative Ras blocks epidermal growth factor-mediated chemotaxis, while constitutively active Ras has no effect on chemokinesis or chemotaxis. PD98059 and U0126, inhibitors of MAP kinase kinase (MEK) activity, decreased epidermal growth factor-stimulated migration, while kinase-defective MEK1, an inhibitor of MAP kinase activation, enhanced migration. To understand the paradoxical effects of these molecules on epidermal growth factor-induced migration, we examined the role of c-Raf on migration. Expression of either wild type c-Raf or the catalytic domain of c-Raf effectively inhibited epidermal growth factor-stimulated cell migration. We suggest that, whereas Ras activity is necessary to promote epidermal growth factor-stimulated migration, sustained activation of c-Raf may be important in down-regulating migratory signaling pathways triggered by epidermal growth factor receptor activation. Further, activation of c-Raf upon inhibition of the MEK-MAP kinase pathway may contribute to the inhibition of cell migration observed with pharmacological MEK inhibitors.

Regulated cell migration is physiologically important for embryonic development, wound healing, and immunological responses associated with inflammation. Moreover, unregulated migration is a central feature of some pathological states, most notably tumor metastasis. While it is clear that regulating cell migration is important for homeostasis of an organism, the signal transduction events that regulate motility are poorly defined.

Cells can be stimulated to move toward soluble factors (chemotaxis) including growth factors, such as epidermal growth factor (EGF),1 or substrate-bound components (haptotaxis), typically ECM. EGF binding to its cell surface receptor elicits signal transduction pathways that culminate in migration (1–3). It has been reported that EGF-stimulated migration requires receptor tyrosine kinase activity and autophosphorylation induced upon EGF binding (2–4). Phosphotyrosine residues on the activated EGFR bind Src homology 2 domains of PLCγ, Grb2, and Shc in vivo (5–8). Cells treated with the pharmacological PLC inhibitor, U73122, or expressing a mutant EGFR incapable of activating PLCγ fail to migrate to EGF indicating that PLCγ activity is necessary for migration in response to EGF (3).

Binding of Grb2 and/or Shc to the activated EGFR stimulates the Ras → Raf → MEK → ERK1/ERK2 (MAP kinase) signal transduction pathway (4). Several groups have investigated the role of this pathway in cell motility. In particular, Ras activity appears to be indispensable for cell motility in a number of systems (9–16). Indeed, overexpression of dominant interfering Ras proteins inhibits chemokinesis, PDGF-stimulated migration (13), and wound repair (15). However, expression of dominant negative Ras is reported to have no effect on haptotactic migration toward fibronectin (13).

The role of MAP kinase in mediating steps necessary for migration is somewhat less clear. Haptotactic migration of FG pancreatic carcinoma cells or Rat1 fibroblasts to collagen or fibronectin, respectively, can be blocked by the MEK-specific inhibitor, PD98059 (17, 18). Such migration can be stimulated by ectopically expressed constitutively active MEK, suggesting a regulatory role for MAP kinase in haptotaxis (17, 18). PDGF-stimulated chemotaxis of Rat1 fibroblasts was unaffected by PD98059 treatment, implying that MAP kinase is not necessary for PDGF-mediated migration (18). Recent reports have indicated that EGF-stimulated MAP kinase activation is not sufficient to promote migration of mouse NR6 cells expressing the EGFR (3). However, treatment of these cells with PD98059 inhibits EGF-stimulated migration, suggesting that MAP kinase is necessary for migration to occur (19).

In this study, we employed a transient transfection migration assay to assess the role of components of the Ras-Raf-MEK-MAP kinase signaling pathway in EGF-stimulated REF52 cell migration. As reported previously, treatment of cells with PD98059 inhibited EGF-stimulated cell migration. However, additional experiments showed that overexpression of catalytically inactive MEK stimulated EGF-induced migration, whereas the overexpression of activated MEK failed to stimulate cell migration. Catalytically inactive MEK binds to and titrates c-Raf away from endogenous substrates while the pharmacological MEK inhibitors increase c-Raf activity. These observations prompted examination of the role of c-Raf in the

nase; ERK, extracellular signal-regulated kinase; PDGF, platelet-de- rived growth factor; RRF, rat embryo fibroblasts; BSA, bovine serum albumin; PBS, phosphate-buffered serum; PE3K, phosphoinositide 3-ki- nase; GFP, green fluorescent protein; DMEM, Dulbecco’s modified Ea- gle’s medium; HA, hemagglutinin.

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¶ The abbreviations used are: EGF, epidermal growth factor; ECM, extracellular matrix; EGFR, EGF receptor; PLCγ, phospholipase Cγ; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase ki-
regulation of EGF induced cell migration. We show that overexpression of either wild type c-Raf or activated c-Raf consistently inhibited EGF-induced cell migration of REF52 cells. We conclude that, whereas Ras activity is necessary to promote EGF-stimulated migration, the sustained activation of c-Raf in REF52 cells may be important in down-regulating the migratory signaling pathways triggered by EGF receptor activation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—REF52 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 10 μg/ml penicillin (Life Technologies, Inc.), and 0.25 μg/ml streptomycin (Life Technologies, Inc.). EGF (Sigma) was reconstituted in DMEM containing 0.1% fatty acid-free BSA (Sigma). PDGF-BB (Amersham Pharmacia Biotech) was reconstituted in 4 mM HCl, 0.1% BSA. PD90859 (Biomol) and U0126 (Promega), MEK inhibitors, were reconstituted in Me2SO and used at a final concentration of 50 μM. U73122 (Biomol), a PLC inhibitor, and the inactive congener U73433 (Biomol) were reconstituted in chloroform and stored at −20 °C. Aliquots were dried under a nitrogen stream, resuspended in DMEM containing 1% fatty acid-free BSA, and used immediately at a final concentration of 1 μM. Wortmannin (Sigma), herbinycin (Calbiochem), and genistein (Life Technologies, Inc.) were reconstituted in Me2SO and used at final concentrations of 100 nM, 875 nM, and 100 μg/ml respectively.

**Plasmids**—All constructs in this study used a cytomegavirus promoter to drive expression of a peptide-epitope-tagged protein. HA-tagged A17Ras, N17Ras, and L61Ras (20, 21) were kind gifts of R. Jove (University of South Florida, Tampa, FL). The wild type c-Raf, activated c-Raf (22W) (22), and the empty vector pcDNA3-FLAG. Extravated MEK1 (S218/222D), and kinase-defective MEK1 (K97A) subcloned into pCMVHA have been described previously (23). FLAG-tagged ERK2 was constructed by digesting murine c-Raf with EcoRI and subcloning into the same sites in pcDNA3-FLAG. Extraneous pBluescript sequence between the HindIII site and the erk2 start site was removed using the Transformer mutagenesis kit (CLONTech) and an oligonucleotide spanning the FLAG peptide tag expressed on each ectopically expressed protein (12CA5 for HA-tagged Ras and MEK, M2 (Sigma) for FLAG-tagged c-Raf). For activity assays, 0.5–1.0 × 10⁶ cells, seeded on 100-mm dishes, were transfected with 9.9 μg of DNA in a 4:1 ratio of Superfect (Qiagen). Twenty-four to 48 h later, the cells were stimulated, harvested, and used for protein kinase assays described previously (23).

**Transfection Assays**—For migration assays, cells (0.5–1.0 × 10⁶/100-mm dish) were transfected with 10 μg of DNA in a 4:1 ratio of expression plasmid to GFP (which identifies positively transfected cells) using 60 μl of Superfet (Qiagen). Between 24 and 48 h after the addition of DNA, the transfection efficiency was determined by calculating the ratio of GFP-positive cells to total cells (counted in four random (magnification, ×320) fields using a Zeiss Axiostar 135TV inverted fluorescence microscope). Migration of transfected cells was assessed using the Boyden chamber assay described below.

For activity assays, 0.5–1.0 × 10⁶ cells, seeded on 100-mm dishes, were transfected with 9.9 μg of mutant MEK1 DNA and 0.1 μg of FLAG-ERK2 or 9.9 μg of mutant c-Raf DNA and 0.1 μg of wild type MEK1 DNA. Twenty-four to 48 h later, the cells were stimulated, harvested, and used for protein kinase assays described previously (23).

**Immunofluorescence**—Co-expression of individual constructs together with GFP was confirmed by transfecting 2.5–5 × 10⁶ cells plated on coverslips in 35-mm dishes with 2 μg of total DNA in a 4:1 ratio of test construct to GFP using 10 μl of Superfet (Qiagen). Twenty-four to 48 h later, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Cells transfected with individual test constructs and GFP were stained with monoclonal antibodies to the peptide tag expressed on each ectopically expressed protein (12CA5 for HA-tagged Ras and MEK, M2 (Sigma) for FLAG-tagged c-Raf) at a final concentration of 1.5–5 μg/ml. Texas red-conjugated goat anti-mouse IgG was used as a secondary antibody at a final concentration of 1.5–2 μg/ml. The number of cells expressing both green (GFP) and red (c-Raf or MEK) protein was determined by counting four random 400× magnification fields using a Leitz DMR fluorescence microscope. Co-expression frequency of each test construct and GFP was always greater than 80%.

**Cell Migration and Adhesion Assays**—Cell migration was assessed using modified Boyden chambers (tissue culture-treated, 6.5 mm diameter, 10 μm thickness, 8 μm pores; BioCoat, Becton Dickinson). For growth factor-mediated chemotaxis, the indicated concentrations of EGF or 0.7 ng/ml PDGF-BB were placed in the lower chamber in DMEM in the absence of serum (DMEM/0). To assess fibronectin stimulated haptotaxis, the underside of the membrane was coated with 1.5 μg/ml fibronectin suspended in PBS in the lower chamber and PBS was placed in the upper chamber. After incubating the chambers overnight at 4 °C, the fibronectin solutions were removed and DMEM/0 was added to the lower chamber. For adhesion assays, 2.5 × 10⁶ cells were plated on either uncoated 24-well plates or plates coated with 1.5 μg/ml fibronectin overnight at 4 °C and allowed to adhere for 6 h at 37 °C. The cells were washed twice in PBS, fixed with 4% paraformaldehyde at room temperature for 20 min, and washed twice with PBS. For inhibitor studies, cells were pretreated with each inhibitor for 15 min (16 h for herbinycin) prior to harvest. The inhibitor remained in both chambers of each well for the duration of the migration assay.

Migration assays were performed by suspending 1 × 10⁶ REF52 cells in DMEM/0 in the upper chamber and allowing the cells to migrate for 6 h at 37 °C. Nonmigrating cells on the upper side of the membrane were removed with a cotton swab. Cells that had migrated to the underside of the membrane were washed twice in PBS, fixed with 4% paraformaldehyde at room temperature for 20 min, and washed twice with PBS. Migration or adhesion was assessed by counting four 320× fields of cells using a Zeiss Axiostar 135TV inverted fluorescence microscope. Nontransfected cells were stained with crystal violet prior to counting to aid in visualization. For experiments using transfected cells, the number of fluorescent and total cells that migrated (observed by phase microscopy without counter staining) were determined and used to calculate the percentage of fluorescent cells that migrated. This percentage was normalized to the transfection efficiency for each construct to obtain the “relative migratory index.” The data presented represent the mean ± standard deviation. Each experimental group was analyzed using single-factor analysis of variance. If the global F test for differences among any one of the groups was significant at the 0.05 level, a planned test was performed (24) to determine which transfected group(s) differed from vector controls using Student’s tests assuming unequal variance. Statistical significance was defined as p < 0.05.

**RESULTS**

EGF Stimulates Migration of Fibroblasts—EGF has been reported to stimulate migration of many normal and tumorigenic cells (1), including NR6 fibroblasts engineered to express exogenous EGFFR (2, 3). To identify signals that regulate EGF-stimulated migration of the normal rat embryo fibroblasts, REF52, we determined the optimal concentration of EGF required to stimulate migration of these cells. Cells were allowed to migrate for 6 h at 37 °C in response to increasing concentrations of EGF placed in the lower well of a Boyden chamber (Fig. 1). The maximal, 2-fold increase in migration was induced with 3–10 ng/ml EGF (Fig. 1). Higher, mitogenic concentrations of EGF (25 ng/ml; Fig. 1) failed to stimulate migration significantly above background levels, consistent with reports of PDGF-stimulated migration (24).

EGF-stimulated NR6 migration requires EGF tyrosine phosphorylation and kinase activity (2) as well as PLCγ activity (3). We assessed the requirement for these activities, as well as PI3K activity, in EGF-stimulated migration of REF52 using pharmacological inhibitors. Treatment of REF52 cells with the tyrosine kinase inhibitors genistein or herbinycin completely inhibited chemokinesis and EGF-mediated chemotaxis (data not shown), thereby supporting the previous report of a requirement for tyrosine phosphorylation (2). Treatment of REF52 cells with 1 μM U73122, a PLC inhibitor, blocked EGF-stimulated migration (Table I) while having no effect on chemokinesis (data not shown). The inactive congener U73433, had no effect on cell migration. Finally, cells treated with PI3K inhibitors wortmannin or LY294002, failed to inhibit EGF-stimulated migration (Table I) at concentrations that blocked PDGF-stimulated migration (data not shown). These experiments support the previously reported requirement for PLC 

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2 S. Eblen, unpublished data.
activation in EGF-stimulated cell migration and interestingly fail to provide evidence for a significant role of PI3K.

**Overexpression of Dominant Acting Ras Affects EGF-stimulated Migration**—Because EGF-stimulated migration requires receptor activation and tyrosine phosphorylation (Ref. 2 and Table I) as well as receptor-mediated PLCγ activation (Ref. 3 and Table I), we sought to determine which components of the EGF receptor signaling pathway regulated migration. Previous reports have implicated Ras as a regulator of cell migration (9–16). Thus, we employed a transient transfection migration assay (see “Experimental Procedures”) to assess the effects of mutant Ras expression on EGF-stimulated migration. REF52 cells were transfected with mutant Ras constructs; the transfected cells were harvested 24–48 h later, and cell migration to 10 ng/ml EGF was measured as described under “Experimental Procedures.” Both EGF-mediated chemotaxis and chemokinesis of REF52 cells expressing dominant negative Ras (A17Ras or N17Ras) were reduced compared with cells expressing an empty vector control (pKH; Fig. 2, A and B). In contrast, expression of constitutively active Ras had no effect on chemokinesis or EGF-stimulated motility (Fig. 2, A and B). Overexpression of A17Ras or N17Ras had no significant effect on fibronectin-stimulated migration (Fig. 2C), indicating that dominant negative Ras likely blocks migration by affecting EGFR-regulated signaling rather than direct inhibition of components of the migration machinery. Dominant negative Ras expression also had no effect on cell adhesion (Fig. 2D). Western analysis indicated that Ras expression in REF52 cells was equivalent among the mutant Ras constructs (data not shown). In parallel experiments, expression of N17Ras and V12Ras in REF52 cells effectively blocked PDGF-stimulated migration in agreement with previous reports (data not shown; Ref. 13). Together, these data show that a Ras-dependent signal is required for EGF-mediated migration.

**Differential Effects of MEK1 Inhibitors on EGF-stimulated Migration**—To identify Ras effectors that may mediate chemotaxis in response to EGF, we examined molecular components of the Ras-Raf-MEK-MAP kinase pathway. Initially, we tested the effects of PD98059, an inhibitor of MEK activation (25), on EGF-stimulated migration of REF52 cells. Cells pretreated for 15 min with 50 μM PD98059 or MeSO were stimulated to migrate to 10 ng/ml EGF in the continued presence or absence of inhibitor. PD98059 decreased EGF-stimulated cell migration to levels comparable with chemokinesis (Table I). U0126, an inhibitor of MEK activation and MEK catalytic activity (26), inhibited EGF-stimulated migration as well as chemokinesis (Table I; data not shown). These data are consistent with a role for MEK in EGF-stimulated migration.

To further explore the requirement for MEK1 activation in migration, expression constructs encoding kinase-defective MEK1, activated MEK1 or the empty vector control (pCHA) were co-transfected into REF52 cells with GEP-expressing plasmids and EGF-stimulated migration of transfected cells was assessed (Fig. 3). Surprisingly, kinase-defective MEK1 expression enhanced EGF-stimulated migration 2-fold while having no effect on chemokinesis (Fig. 3, A and B). In contrast, activated MEK1 had no significant effect on chemokinesis or chemotaxis (Fig. 3, A and B), and neither of the mutant MEK1 constructs affected adhesion to fibronectin (Fig. 3C). The enzymatic activities of kinase-defective and activated MEK1 constructs were confirmed by measuring the activity of co-expressed FLAG-ERK2 (Fig. 3D). As anticipated, kinase-defective MEK1 inhibited EGF-stimulated ERK2 activity approximately 50% while activated MEK1 increased ERK2 activity approximately 4–5-fold in unstimulated cells. These data indicate that MEK-dependent activation of MAP kinase is not sufficient for EGF-stimulated migration of REF52 cells. Since kinase-defective MEK titrates c-Raf away from endogenous substrates, the paradoxical effects of the pharmacological MEK inhibitors and kinase-defective MEK on cell migration indicated the possible involvement of c-Raf in both the regulation of MEK/MAP kinase activation and cell migration.

**c-Raf Expression Inhibits EGF-stimulated Migration**—Recently, it has been shown that PD98059, in addition to blocking ERK activity, increases c-Raf activity in Swiss 3T3 cells treated with a number of stimuli (27). Thus, we tested the effects of PD98059 and a second MEK inhibitor, U0126, on c-Raf activity in REF52 cells. Cells were pretreated with 50 μM PD98059, 50 μM U0126, or MeSO for 15 min and stimulated with 10 ng/ml EGF or vehicle for 1 h in the continued presence or absence of either MEK inhibitor. To quantitate c-Raf activity, immunoprecipitated, endogenous c-Raf was tested for its ability to phosphorylate kinase-defective MEK in vitro (Fig. 4). PD98059 had no effect on basal c-Raf activity in unstimulated cells; U0126 marginally increased basal c-Raf activity (Fig. 4A). However, PD98059 or U0126 treatment enhanced EGF-stimulated c-Raf activity approximately 2–3- or 5–6-fold, respec-
relatively, to the level observed from EGF treatment alone (Fig. 4A). EGF-stimulated MAP kinase phosphorylation was decreased by PD98059 treatment and completely blocked by U0126 treatment (Fig. 4B). Basal MAP kinase phosphorylation was blocked by both PD98059 and U0126, as evidenced by the failure to detect binding of a phospho-ERK antibody and as the lack of mobility shift of ERK2 on ERK2 immunoblots (Fig. 4B). Thus, these results indicate that PD98059 or U0126 inhibition of MEK can lead to an increase in c-Raf activity presumably by blocking MAP kinase-dependent negative feedback inhibition of c-Raf (28–30).

To determine if increased c-Raf activity was sufficient to inhibit EGF-induced cell migration, expression constructs encoding wild type c-Raf, the catalytic domain of c-Raf (22W), or the empty vector control (pC) were transfected together with GFP-expressing plasmids into REF52 cells and the effects on cell motility were assessed (Fig. 5). Expression of individual c-Raf constructs had no significant effect on chemokinesis (Fig. 5A). However, both wild type c-Raf and 22W inhibited EGF-stimulated migration approximately 30–50% (Fig. 5B). The ability of 22W or wild type c-Raf to inhibit migration was not due to an effect on adhesion since cells expressing these constructs adhered to fibronectin as well as cells expressing the negative vector controls (Fig. 5C).

The activity of each c-Raf construct was assessed by co-transfecting empty vector, wild type c-Raf, or truncated c-Raf together with HA-tagged wild type MEK1 into REF52 cells. Immunoprecipitated MEK1 activity was assayed by virtue of its ability to phosphorylate ERK2 in vitro (Fig. 5D). As shown in Fig. 5D, wild type c-Raf and 22W increased c-Raf activity approximately 4–6-fold in unstimulated cells, relative to the empty vector control. EGF treatment increases c-Raf activity approximately 7–10-fold in cells expressing wild type c-Raf or 22W compared with EGF-stimulated cells expressing the empty vector control (Fig. 5D). Taken together, these data indicate that increased c-Raf activity correlates with the inhibition of EGF-stimulated migration. These results also suggest the possibility that treatment of cells with PD98059 or U0126
may indirectly lead to the activation of c-Raf and inhibition of migration through an alternative c-Raf-dependent pathway.

**DISCUSSION**

In this report, we provide evidence that EGF-stimulated migration of REF52 cells is differentially regulated by Ras and its downstream effector, c-Raf. Blocking Ras activity by transient overexpression of dominant negative Ras constructs (A17Ras or N17Ras) in a population of REF52 cells substantially inhibits chemokinesis and EGF-stimulated chemotaxis while failing to significantly inhibit haptotactic migration to fibronectin. However, transient expression of activated variants of Ha-Ras (V12Ras or L61Ras) fail to stimulate chemokinesis, chemotaxis, or haptotaxis. These results confirm previously reported studies carried out in other cell types, showing that Ras is necessary but not sufficient to promote cell migration to many growth factors (12–14). We also present the first evidence for c-Raf being a regulator of the cell migration signaling pathway. Treatment of REF52 cells with the MEK inhibitor PD98059 or U0126 inhibited migration of REF52 cells toward EGF. Treatment of REF52 cells with PD98059 or U0126 also resulted in a detectable enhancement of c-Raf kinase activity in response to EGF. Expression of wild type c-Raf or activated c-Raf, however, significantly inhibited EGF-induced REF52 cell migration. These studies provide evidence
that the activation state of c-Raf may be an important sensor in regulating the cell migration signaling pathway.

Several downstream effectors of Ras, including MAP kinase, have been suggested as potential regulators of cell motility including chemotaxis and haptotaxis (3, 14, 17, 18). Inhibition of MAP kinase activation with the MEK-specific inhibitor, PD98059 (25, 27), is reported to significantly reduce fibronectin-stimulated migration of Rat1 cells (18), collagen-stimulated migration of FG carcinoma cells (17), and EGF-stimulated migration of NR6 or human diploid fibroblast cell lines (19). Indeed, we find that both PD98059 and the MEK inhibitor, U0126, inhibit EGF-stimulated migration of REF52 cells (Table I), consistent with MAP kinase playing a role in the regulation of EGF-stimulated migration. Interestingly, U0126 appears to be a more effective inhibitor of cell migration (Table I), inhibiting both EGF-induced migration and chemokinesis. This may reflect the fact that U0126 is a significantly more potent inhibitor of MAP kinase (Ref. 26 and Fig. 4B) and would suggest that even chemokinesis requires some basal level of MAP kinase activity. Alternatively, U0126 may be acting on another pathway important for chemokinesis. Treatment of cells with PD98059 or U0126 also led to an elevation of c-Raf activity. Serum-depleted REF52 cells were pretreated with 50 or 10 ng/ml EGF stimulation for 1 h with or without PD98059 or U0126 treatment. c-Raf activity was determined by measuring the relative level of MEK1 phosphorylation following incubation of immunoprecipitated c-Raf with kinase-defective MEK1 substrate (KD MEK1) in vitro as described under “Experimental Procedures.” Levels of c-Raf in each immunoprecipitate were determined by Western analysis using a c-Raf-specific monoclonal antibody. B, ERK phosphorylation, detected by Western analysis of whole cell lysates using a phosphospecific ERK monoclonal antibody (phospho-ERK), was used as a measure of ERK activation. Serum-depleted REF52 cells were pretreated with 50 μM PD98059 or 50 μM U0126 for 15 min, followed by stimulation with vehicle or 10 ng/ml EGF for 1 h in the continuous presence of 50 μM PD98059 or 50 μM U0126. Western analysis of ERK2 (ERK2:IB) using monoclonal antibody B3B9 confirmed equivalent levels of ERK in each lane. The data shown are representative of at least three independent experiments.

FIG. 4. PD98059 or U0126 stimulates c-Raf activity. A, endogenous c-Raf was immunoprecipitated from REF52 cells following vehicle or 10 ng/ml EGF stimulation for 1 h with or without PD98059 or U0126 treatment. c-Raf activity was determined by measuring the relative level of MEK1 phosphorylation following incubation of immunoprecipitated c-Raf with kinase-defective MEK1 substrate (KD MEK1) in vitro as described under “Experimental Procedures.” Levels of c-Raf in each immunoprecipitate were determined by Western analysis using a c-Raf-specific monoclonal antibody. B, ERK phosphorylation, detected by Western analysis of whole cell lysates using a phosphospecific ERK monoclonal antibody (phospho-ERK), was used as a measure of ERK activation. Serum-depleted REF52 cells were pretreated with 50 μM PD98059 or 50 μM U0126 for 15 min, followed by stimulation with vehicle or 10 ng/ml EGF for 1 h in the continuous presence of 50 μM PD98059 or 50 μM U0126. Western analysis of ERK2 (ERK2:IB) using monoclonal antibody B3B9 confirmed equivalent levels of ERK in each lane. The data shown are representative of at least three independent experiments.

studies have demonstrated that activated c-Raf is a negative regulator of the cellular migratory response. The mechanism(s) by which c-Raf might negatively regulate cell migration is presently unknown. The ability of c-Raf overexpression to inhibit EGF-stimulated migration suggests that c-Raf may down-regulate EGF-dependent signals that promote migration, including signals to or from Ras and PLCγ. Indeed, negative feedback mechanisms have been described that lead to Ras desensitization in response to growth factor stimulation (32–39). In some cases, uncoupling Ras activation from growth factor receptor signaling involves MEK- or MAP kinase-dependent phosphorylation of SOS, and correlates with the dissociation of SOS from the growth factor receptor complex either by release from Grb2 (32–35) or from Shc as a Grb2/SOS complex (36–38). While c-Raf overexpression results in increased MEK activity (Fig. 5), it is unlikely that MEK-dependent desensitization of Ras, mediated by SOS phosphorylation, regulates EGF-stimulated migration since active MEK overexpression has no effect on EGF-stimulated migration (Fig. 3). In addition, hormone-induced activation of ΔRaf1:ER (an estrogen-regulated version of activated c-Raf) has been reported to increase MEK, MAP kinase activity and SOS phosphorylation in NIH3T3 cells, yet cells containing activated c-Raf retain the ability of EGF to stimulate Ras activity (39). However, prolonged increases in c-Raf activity did result in decreased EGFR tyrosine phosphorylation (39), suggesting that proteins that interact directly with the EGFR, including PLCγ, may not be activated to the same level in cells overexpressing active c-Raf. Therefore, increased c-Raf activity may indirectly alter PLCγ-mediated migratory events following EGF stimulation.

An alternative possibility is that c-Raf targets cellular components of the migration machinery, perhaps components of the adhesion-signaling pathway, in response to EGF-stimulated signaling events. Expression of an activated form of c-Raf, Raf-BXB, has been reported to suppress integrin activation, suggesting that c-Raf may inhibit engagement of activated integrins with ECM proteins (40). In addition, cells overexpressing active c-Raf appeared to spread more poorly compared with controls (40). The c-Raf-dependent inhibition of integrin binding of ECM proteins may well correlate with the inability of the cell to reorganize existing focal adhesions, resulting in an inhibition of cell migration. Indeed, EGF-stimulated migration of NR6 cells overexpressing the EGFR has been correlated with the loss of focal adhesions following EGF treatment (19). Treatment of these cells with PD98059 both inhibits migration and reduces the extent of focal adhesion loss in response to EGF, leading the authors to suggest that MAP kinase regulates EGF-initiated migration by promoting focal adhesion disassembly and remodeling. However, since PD98059 also en-
hances growth factor stimulated c-Raf activity (27, Fig. 4), PD98059 could inhibit focal adhesion disassembly by enhancing c-Raf activity. While we found no alteration in the ability of cells overexpressing c-Raf to adhere to fibronectin (Fig. 5), more subtle changes in adhesion complexes or adhesion-based signaling may be compromised by c-Raf overexpression.

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FIG. 5. c-Raf expression inhibits EGF-stimulated migration. REF52 cells expressing pC, empty vector control, wild type c-Raf (Wt c-Raf), or the catalytic domain of c-Raf (22W) were stimulated to migrate to vehicle (A) or 10 ng/ml EGF (B) or allowed to adhere to 1.5 μg/ml fibronectin (C). The data represent the mean relative migratory index or mean relative adhesion (see “Experimental Procedures”) ± standard deviation for seven to nine independent experiments. *, p values < 0.05 determined as described under “Experimental Procedures.” EGF stimulated 3.5 ± 1.5-fold increase in migration. D, the activity of each c-Raf construct was determined by its ability to activate co-expressed epitope-tagged MEK1. Immunoprecipitated MEK1 activity was measured as the ability to phosphorylate ERK2 in vitro (ERK2P). The fold increase in MEK1 activity of each construct ± EGF stimulation relative to unstimulated pC is indicated. Relative levels of immunoprecipitated MEK1 were detected using an HA-specific antibody (MEK1:IB). 

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