

## Distinct Mechanisms Mediate the Initial and Sustained Phases of Integrin-mediated Activation of the Raf/MEK/Mitogen-activated Protein Kinase Cascade\*

(Received for publication, April 9, 1998, and in revised form, July 24, 1998)

Alan K. Howe‡ and Rudy L. Juliano§

From the Department of Pharmacology and The Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina 27599-7365

**Integrin-mediated adhesion to the extracellular matrix activates the canonical mitogen-activated protein kinase cascade, although the exact mechanism is not fully resolved. We show that integrin-mediated activation of Raf-1, an upstream regulator of mitogen-activated protein kinase, occurs in two phases. Efficient early activation of Raf required Raf-Ras interaction but was not affected by protein kinase C (PKC) inhibitors, while a lower, sustained level of activity was independent of Raf-Ras interaction but was reduced by PKC inhibitors. The combination of PKC inhibition and lack of Ras binding completely blocked integrin-mediated Raf activity. The activity of a membrane-bound Raf mutant that is deficient in Ras binding (Raf-R89L-CAAX) was also regulated by adhesion. Raf-R89L-CAAX activity was low in nonadherent cells, was rapidly stimulated to wild-type levels by cell adhesion, and remained at nearly maximal levels longer than wild-type activity. The activation of wild-type and mutant Raf proteins was ablated by cytochalasin D, demonstrating that cytoskeletal organization is required for activation of Raf, even when targeted to the membrane. These data suggest distinct initial and sustained phases of integrin-mediated Raf activation that require Raf membrane localization and possibly PKC activity, respectively, and that integrin-mediated adhesion may regulate a cytoskeleton-associated factor(s) responsible for Raf activation.**

It has been known for some time that a cell's interaction with the extracellular matrix (ECM)<sup>1</sup> can have profound effects on cell division, differentiation, and survival. Interactions between cells and the ECM are mediated predominantly by heterodimeric transmembrane proteins known as integrins (1, 2). It has become increasingly clear that integrins not only medi-

ate the physical attachment of cells to ECM but also generate a variety of signals that communicate the status of this attachment to the interior of the cell (3–5). These signals can regulate a variety of cellular functions, including cell morphology and motility, apoptosis, differentiation, and cell growth and division (3, 6).

Among the signals generated by integrin-mediated cell adhesion is activation of the canonical MAPK cascade (7–12). The mechanism of activation of this cascade in response to mitogens such as peptide growth factors has been delineated in great detail (13). It involves dimerization and autophosphorylation of the growth factor receptor tyrosine kinase (RTK), recruitment of an adaptor protein-guanine nucleotide exchange factor complex (e.g. GRB2-mSOS), and GTP loading and activation of small GTPase Ras (14). Ras binds the serine/threonine kinase Raf (15) and recruits it to the membrane, where it is activated by a complex and as yet unresolved mechanism (16). Once activated, Raf phosphorylates and activates MEK (MAPK/extracellular signal-regulated kinase), which in turn phosphorylates and activates MAPK.

While integrin-mediated activation of the MAPK cascade has been reported by several groups, the mechanism(s) through which this occurs is currently somewhat controversial. The events immediately upstream of MAPK seem clear; both Raf-1 and MEK are activated by integrin-mediated adhesion (7, 17), and MEK activity is required for integrin-mediated MAPK activation (7). However, the events upstream of Raf are less clear. On one hand, integrin-mediated Raf activation may occur through a pathway that is essentially a recapitulation of the canonical growth factor-activated pathway. Thus, integrin engagement induces autophosphorylation of the focal adhesion kinase pp125<sup>FAK</sup>, which may serve as a surrogate RTK domain and point of purchase for adaptor protein exchange factor complexes (10, 18). In this model, integrin-mediated activation of the Raf/MEK/MAPK cascade is dependent on both pp125<sup>FAK</sup> and Ras. Indeed, several groups have demonstrated that expression of dominant negative Ras (Ras<sup>N17</sup>) can inhibit integrin-mediated MAPK activation (17–22). However, our laboratory and others have demonstrated that integrin-mediated MAPK activation can occur independently of pp125<sup>FAK</sup> (21, 23). Furthermore, work from our laboratory has provided evidence for a Ras-independent component of integrin-mediated MAPK activation (7). Specifically, a level of expression of Ras<sup>N17</sup> sufficient to block signaling from a constitutively active mSOS exchange factor was unable to inhibit integrin-mediated activation of MEK (7). Also, expression of an amino-terminal fragment of Raf-1 (amino acids 23–284) containing the Ras binding site (24) blocked the activation of the MAPK pathway by oncogenic Ras (Ras<sup>V12</sup>) but not by integrin-mediated cell adhesion (7).

In this report, we focus on the mechanism of activation of

\* This work was supported in part by National Institutes of Health (NIH) Grant GM26165 (to R. L. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ A Lineberger Comprehensive Cancer Center Postdoctoral Fellow (National Cancer Institute, NIH, Training Grant CA09156-23).

§ To whom correspondence should be addressed. Dept. of Pharmacology, University of North Carolina at Chapel Hill School of Medicine, Mary Ellen Jones Bldg. #920, CB# 7365, Chapel Hill, NC 27599-7365. Tel.: 919-966-4383; Fax: 919-966-5640; E-mail: arjay@med.unc.edu.

<sup>1</sup> The abbreviations used are: ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; RTK, receptor tyrosine kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; Fn, fibronectin; PBS, phosphate-buffered saline; TPA, 12-O-tetradecanoylphorbol-13-acetate; BIM, bisindolylmaleimide; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; cytoD, cytochalasin D; WT, wild type.

Raf-1 by integrin-mediated cell adhesion. Our results show that integrin-mediated activation of Raf-1 seems to comprise two phases: an early phase that is enhanced or optimized by membrane localization and is independent of protein kinase C (PKC) activity and a later, sustained phase that is independent of Ras-Raf association but is blocked by inhibitors of PKC.

#### EXPERIMENTAL PROCEDURES

**Cell Culture, Plasmids, and Transfections**—NIH3T3 fibroblasts were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Plasmids encoding various myc-tagged Raf-1 alleles (pEFHm-Raf-WT, -Raf-R89L, -Raf-R89L-CAAX, and -Raf-K375M) were a kind gift from Drs. Richard Marais and Christopher Marshall (25). A plasmid encoding oncogenic Ras (pZIP-Ras<sup>V12</sup>) was originally obtained from Dr. Channing Der. For transfections, cells were seeded at  $3 \times 10^5$  cells/well in six-well (35-mm diameter) plates (Falcon Corp.) or  $1 \times 10^6$  cells/60-mm dish and incubated overnight at 37 °C. Complexes containing 8  $\mu$ l of LipofectAMINE (Life Technologies, Inc.) and 2  $\mu$ g of plasmid DNA (unless otherwise noted) were formed and applied to cells according to the manufacturer's instructions. For transfection of cells in 60-mm dishes, 25  $\mu$ l of LipofectAMINE and 5  $\mu$ g of plasmid were used. Cells were grown to confluence (usually 24–36 h after transfection), refed with serum-free DMEM, and harvested for specific applications as described below.

**Adhesion of Cells to Fibronectin-coated Plates**—Plastic Petri dishes (Corning) were coated in an overnight incubation at room temperature with 25  $\mu$ g/ml human fibronectin (Fn; Collaborative Biochemicals) or 5  $\mu$ g/ml poly-L-lysine (Sigma) in phosphate-buffered saline (PBS) (2 ml/60-mm dish; 5 ml/100-mm dish). Plates were washed once with PBS and once with sterile, deionized water before use. Serum-starved cells were trypsinized, resuspended in DMEM containing 2% bovine serum albumin (lipid- and globulin-free; Sigma), and 2 mg/ml soybean trypsin inhibitor (Life Technologies), collected by brief centrifugation, and washed once with DMEM, 2% BSA. Cells were incubated in suspension in DMEM, 2% BSA at 37 °C with gentle rotation for 1 h and then transferred to coated plates for the indicated times or kept in suspension for an equivalent time. Cells were then harvested for the appropriate analysis as described in the figure legends and below. In some instances, rather than being harvested by trypsinization, serum-starved cells were stimulated with TPA (100 nM, 10 min) as controls. Note that cells were replated onto Fn-coated plates at a density no greater than 30% confluence to minimize cell-cell contact.

**Pharmacological Reagents**—PKC activity was inhibited using either calphostin C or bisindolylmaleimide (BIM) (LC Laboratories). Calphostin C was activated by exposure to room light for 30 min at room temperature before use. Cells were incubated in serum-free DMEM containing the indicated amounts of the inhibitors or in 0.1% (v/v) Me<sub>2</sub>SO as a control, for either 12–16 h (long term treatment) or 1 h (short term treatment) before harvesting. For long term treatments, inhibitors or Me<sub>2</sub>SO were present in all media (except for trypsin) throughout a given experiment. For short term treatments, inhibitor or Me<sub>2</sub>SO was added to the cells at the beginning of their incubation in suspension. To prevent actin filament assembly, cytochalasin D was added at a concentration of 2  $\mu$ M to cells in suspension 45 min before replating.

**Preparation of Cell Extracts and Western Blotting**—Cells were washed twice with ice-cold PBS and then lysed in an appropriate volume of radioimmunoprecipitation assay buffer (25 mM Tris, pH 7.4, 137 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol) or Triton lysis buffer (25 mM Tris, pH 8.0, 137 mM NaCl, 1.0% Triton X-100, 10% glycerol) containing protease and phosphatase inhibitors (PMSF/aprotinin/pepstatin and sodium orthovanadate/sodium fluoride, respectively). Dishes were scraped, and lysates were transferred to microcentrifuge tubes, which were then vortexed and incubated on ice for 10 min. Insoluble debris was removed by centrifugation at 14,000 rpm for 10 min at 4 °C, and the clarified lysates were used for determination of protein concentration and subsequent steps. For Western blot analysis, 10–20  $\mu$ g of cell extract or one-tenth the total volume of an immunoprecipitation complex were mixed with the appropriate volume of 4 $\times$  Laemmli sample buffer, boiled for 5 min, separated by SDS-PAGE (7.5% acrylamide for Raf, 10% for MEK and MAPK, 12% for Ras), and transferred to nitrocellulose. Membranes were blocked in PBS containing 5% (w/v) nonfat dry milk and 0.1% (v/v) Tween 20 and incubated with primary antibodies against Raf-1 (sc-133 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or R19120 (Transduction Laboratories); 1:1000), MEK1 and -2 (M17030 (Transduction Laboratories), 1:1000), Ras (Y13–259 (Calbiochem), 1:250), or MAPK (sc-154 (Santa

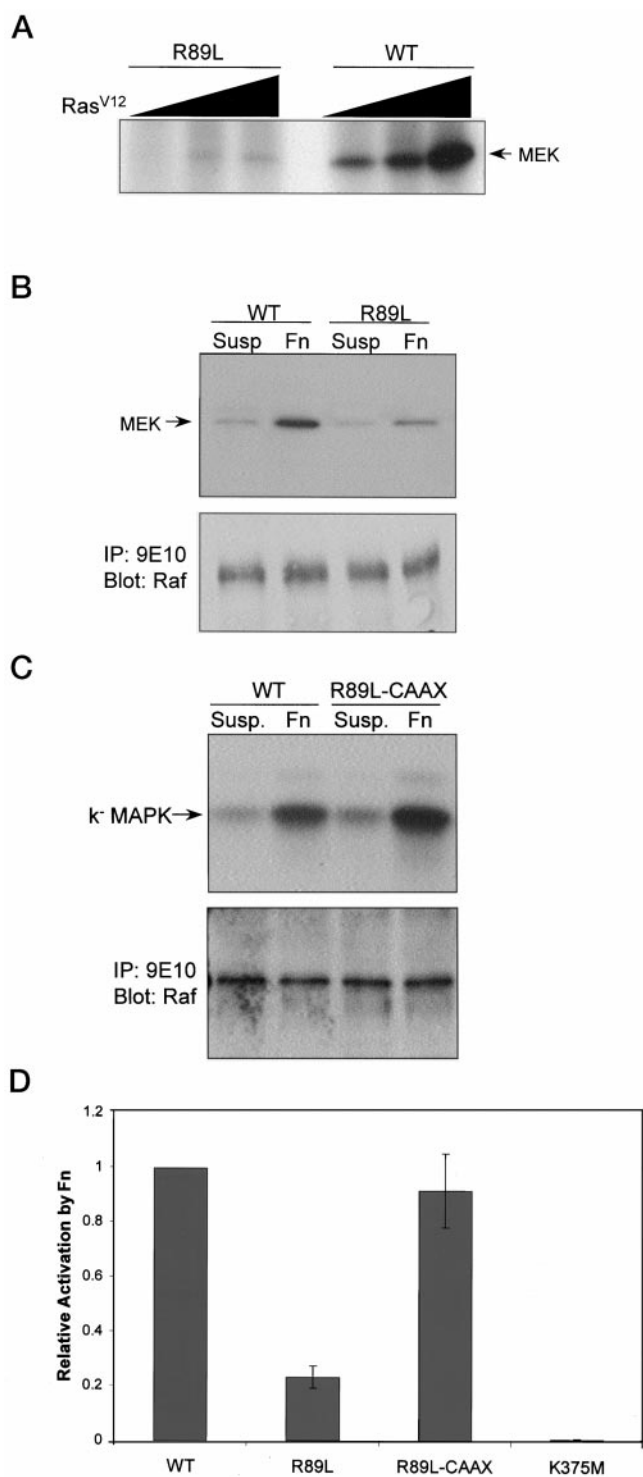
Cruz Biotechnology), 1:1000) for 1 h at room temperature or overnight at 4 °C. After extensive washing, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse (1:2000) or goat anti-rabbit (1:5000) Santa Cruz Biotechnology) and washed again, and immunoreactive bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Immunoprecipitations and Kinase Assays**—Ras-Raf co-immunoprecipitations were performed essentially as described (26), using 2.5–3.0 mg of cellular protein for each experimental point. For kinase assays, cell extracts (200–800  $\mu$ g) were incubated with one of the antibodies described above (or 9E10 (Berkeley Antibody Company) for myc-tagged Raf proteins), using 1  $\mu$ g of antibody for every 100  $\mu$ g of cellular protein, for 2 h at 4 °C, and then with 30  $\mu$ l of protein A/G-agarose for an additional 30 min. MAPK immunocomplexes were washed twice with radioimmunoprecipitation assay buffer; once with 100 mM Tris, pH 8.6, 500 mM LiCl; and once with kinase buffer (25 mM HEPES 7.4, 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, 1 mM dithiothreitol). MEK immunocomplexes were washed three times with Triton lysis buffer and once in kinase buffer. myc-tagged Raf immunocomplexes were washed three times with Triton lysis buffer and twice with Raf kinase buffer (25 mM HEPES 7.4, 10 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM dithiothreitol, 5  $\mu$ M ATP). Immunocomplexes were then incubated in 40  $\mu$ l of kinase buffer containing 20  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol; NEN Life Science Products) and the appropriate substrate (5  $\mu$ g of myelin basic protein for MAPK assays, 2  $\mu$ g of kinase-dead MAPK for MEK assays, or 0.5  $\mu$ g of kinase-dead MEK for Raf assays) for 30 min at room temperature. In some instances, Raf activity was assayed in a linked assay containing 0.1  $\mu$ g of inactive MEK plus 1  $\mu$ g of kinase-dead MAPK. Reactions were terminated by the addition of 40  $\mu$ l of 2 $\times$  Laemmli sample buffer and boiling for 2–5 min. Reaction products were separated by SDS-PAGE and visualized from the stained, dried gel either by autoradiography or by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA). Quantitation of substrate phosphorylation was performed using ImageQuant software (Molecular Dynamics).

#### RESULTS

**Integrin-mediated Adhesion Activates a Mutant of Raf Deficient in Ras Binding**—Although Ras plays an important role in the activation of Raf by growth factors, its role in integrin-mediated signaling is a point of contention. It is firmly established that Ras and Raf form a stable complex and that this direct interaction is required for Ras-mediated Raf activation (15, 16). However, preliminary co-immunoprecipitation experiments failed to demonstrate a significant increase in Raf-Ras association upon integrin-mediated adhesion.<sup>2</sup> To more directly address the question of whether interaction with Ras is required for integrin-mediated activation of Raf-1, cells were transiently transfected with plasmids encoding either wild-type Raf (Raf-WT) or a mutant allele containing an arginine to leucine substitution at position 89, within the Ras binding domain (Raf-R89L) (25). This single point mutation eliminates Raf interaction with Ras and renders Raf refractory to activation by Ras (27). The inability of Ras to activate the R89L mutant was confirmed by co-transfection of Raf plasmids with increasing amounts of a plasmid encoding the activated, oncogenic Ras allele Ras<sup>V12</sup> (Fig. 1A). Immunoprecipitation of the transfected, epitope-tagged kinase showed that the wild-type Raf was robustly activated by oncogenic Ras, while Raf-R89L, as expected, was not significantly activated by Ras. The activity of these Raf alleles in response to integrin-mediated adhesion was then measured (Fig. 1B). We found that adhesion to fibronectin could activate both the WT and R89L forms of Raf, although the level of activation of the R89L mutant was lower than that of Raf-WT (Fig. 1, B and D). To confirm that the level of activation seen for Raf-R89L was indeed significant, the activity of a catalytically inactive mutant of Raf (K375M) was determined in nonadherent and Fn-attached cells and used as a measure of zero activation (Fig. 1D). The results of several ( $n \geq 5$ ) experiments were averaged (Fig. 1D), which showed

<sup>2</sup> A. Howe and R. Juliano, unpublished observations.



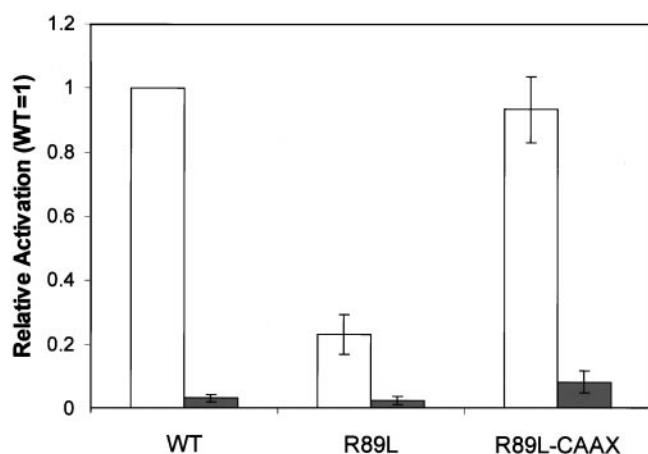
**FIG. 1. Integrin-mediated activation of wild-type and mutant Raf proteins.** NIH3T3 cells were transiently transfected with plasmids encoding epitope-tagged Raf-WT, a point mutant deficient in Ras-binding (R89L), a membrane-associated form of this mutant (R89L-CAAX), or a catalytically inactive mutant (K375M). *A*, cells were co-transfected with plasmids encoding WT or R89L Raf and increasing amounts (1, 3, and 9  $\mu$ g) of a plasmid encoding oncogenic Ras (Ras<sup>V12</sup>). After reaching confluence, cells were serum-starved overnight and harvested, and the transfected Raf proteins were immunoprecipitated with an antibody (9E10) against the myc epitope tag. The activity of the immunoprecipitated proteins was assayed as described under "Experimental Procedures," using kinase-dead MEK as a substrate. *B* and *C*, cells transfected with the indicated Raf constructs were grown to confluence, serum-starved, cultured in suspension, and either replated onto Fn-coated dishes (*Fn*) or maintained in suspension (*Susp.*) for 15 min. Tagged proteins were immunoprecipitated using the 9E10 anti-myc tag antibody and analyzed by either *in vitro* kinase assay (*top parts*

that, after 15 min of adhesion, the activation of Raf-R89L was significant but 4–5-fold lower than the level of activation of wild-type Raf. It should be noted that activation of Raf clearly involves specific adhesion to Fn, mediated primarily by integrins, because cells adhering to the nonspecific cationic polymer poly-L-lysine do not activate Raf (data not shown). In summary, these experiments demonstrate that significant activation of Raf by integrin-mediated adhesion can occur in the absence of direct Ras-Raf interaction but that this interaction clearly enhances integrin-mediated Raf activation.

**Integrin-mediated Adhesion Regulates Raf-R89L-CAAX Activity**—It is well established that localization of Raf-1 to the plasma membrane is important for its activation (28) and that one function of Ras is to mediate this localization (25, 29). However, the nature of the membrane-associated machinery responsible for Raf activation remains unclear (16). To examine the importance of membrane localization in integrin-mediated Raf activation and to determine if the function of Ras could be supplanted by targeting Raf to the membrane, we used a version of the R89L mutant that carries at its C-terminus a 20-amino acid sequence known as a CAAX box (Raf-R89L-CAAX). This sequence directs prenylation and membrane localization of Raf and, in adherent cells, results in a high constitutive level of Raf-R89L-CAAX activity, despite its inability to bind Ras (25, 28). Cells were transiently transfected with Raf-WT or Raf-R89L-CAAX, serum-starved, harvested, and either kept in suspension or plated on fibronectin for 15 min and analyzed by an immunoprecipitation kinase assay (Fig. 1C). Interestingly, Raf-R89L-CAAX activity, which is normally high in attached cells, was low in cells in suspension but increased upon cellular attachment to fibronectin, and the level of this activation was similar to that for Raf-WT (Fig. 1, C and D). Subcellular fractionation, membrane isolation, and Western blot analysis indicated that the low level of activity of Raf-R89L-CAAX in non-adherent cells was not due to a loss or reduction of membrane localization of the protein during suspension culture conditions (data not shown). These data suggest that integrin-mediated cell adhesion can efficiently activate Raf-1 when Raf is able to localize to the cell membrane, as with Raf-WT or Raf-R89L-CAAX, while significant, but reduced, activation can occur even when membrane localization is compromised, as with Raf-R89L. Furthermore, the lack of Raf-R89L-CAAX activity in nonadherent cells suggests that cell adhesion may regulate the membrane-associated machinery responsible for Raf activation after its translocation to the membrane.

**Integrin-mediated Activation of Wild-type and Mutant Raf Alleles Requires Actin Cytoskeletal Organization**—The regulation by adhesion of Raf-R89L-CAAX suggests that membrane-proximal regulators of Raf activity are sensitive to integrin function. One of the most important functions of integrins is to anchor actin filaments to the membrane at points of contact with the ECM, thereby supporting the assembly of stress fibers and organization of the actin cytoskeleton. To examine whether cytoskeleton assembly is important for integrin-mediated Raf activation, we treated cells with cytochalasin D (cytoD), a widely used and well characterized inhibitor of actin filament formation. Previous reports have established that disruption of the actin cytoskeleton, through treatment with cytoD or expression of dominant negative Rho, inhibits integrin-mediated MAPK

of *B* and *C*) or by Western blotting with anti-Raf antibody (*bottom parts*). *D*, relative integrin-mediated activation of Raf proteins. The fold activation (*i.e.* the ratio of activity after plating to the activity in suspension) was determined for WT and mutant Raf proteins. For each experiment, these values were normalized to the WT value, which was given an arbitrary unit of 1. The error bars indicate the S.D. ( $n = 5, 7, 6,$  and  $3$  for WT, R89L, R89L-CAAX, and K375M, respectively).

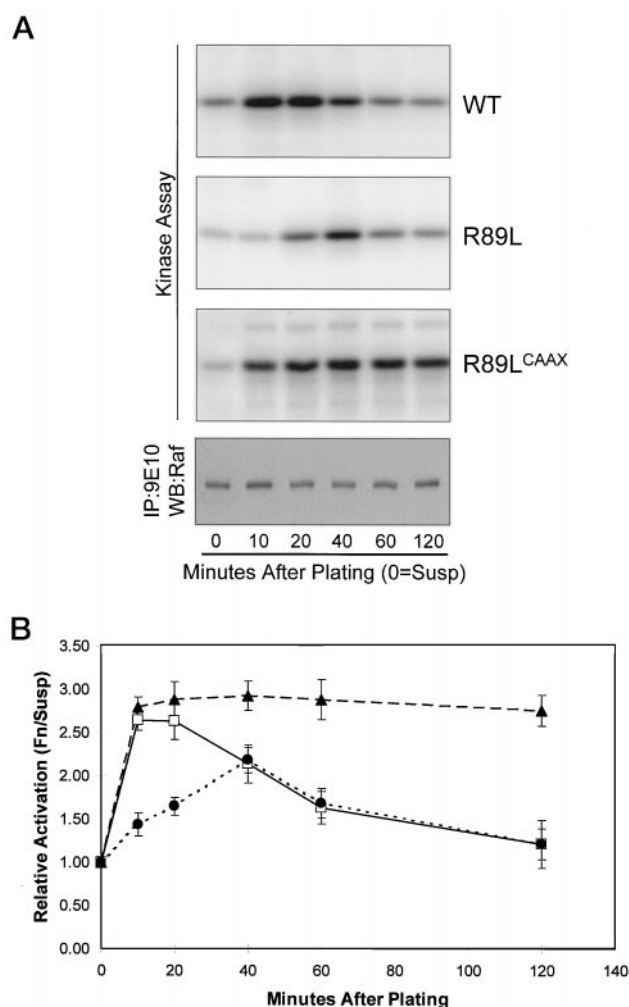


**FIG. 2. Disruption of actin cytoskeleton assembly blocks integrin-mediated activation of WT and mutant Raf proteins.** Cells were transfected with the indicated plasmids and cultured as described for Fig. 1. Me<sub>2</sub>SO (0.02% (v/v); white bars) or 2  $\mu$ M cytochalasin D (gray bars) was added to cells while in suspension, 45 min prior to replating. Cells were replated onto Fn-coated dishes or maintained in suspension for an additional 15 min. In each case, the cells were fully adherent; however, the cytochalasin D-treated cells were noticeably less spread than the control cells. The activity of the epitope-tagged kinases was assayed by an immunoprecipitation kinase assay using the 9E10 antibody, and the fold activation by adhesion to Fn was calculated. Values were normalized to the fold activation of Raf-WT in control (Me<sub>2</sub>SO)-treated cells. The error bars indicate S.D. ( $n = 3$  for all points).

activation (8, 9, 11, 30). In agreement with this, cytoD inhibited integrin-mediated activation of Raf-WT, as well as Raf-R89L and, interestingly, Raf-R89L-CAAX (Fig. 2). This demonstrates that cytoskeletal organization is required for integrin-mediated Raf activation, even for a membrane-targeted form of Raf.

**Time Course of Integrin-mediated Activation of Wild-type and Mutant Raf Alleles**—Compared with the response to most soluble mitogens, activation of the MAPK cascade by integrin-mediated adhesion is of lower magnitude and longer duration (3). In NIH3T3 cells, integrin-mediated MAPK activity peaks 10–15 min after adhesion and returns to basal levels by 2 h (7, 8). Important aspects of the relative activation of Raf-WT and the R89L and R89L-CAAX mutants may not be fully realized by a single time point. Therefore, we determined the time course of integrin-mediated activity of Raf-WT, Raf-R89L, and Raf-R89L-CAAX. As described previously for MAPK, integrin-mediated Raf-WT activity peaked at 10–20 min after adhesion to fibronectin and slowly returned to near basal levels by 2 h (Fig. 3). As described above in Fig. 1, the activation of Raf-R89L at early time points after attachment (*i.e.* 10–15 min) is significantly lower than Raf-WT. However, the level of activity of Raf-R89L throughout the later time points (*i.e.* 40–120 min) is almost indistinguishable from WT levels (Fig. 3). Specifically, Raf-R89L exhibits the same level of activity as Raf-WT at 40 min after cellular adhesion to fibronectin, and the rate at which this activity returns to basal level (*i.e.* the level present in nonadherent cells) parallels the rate of decay of WT activity. It is important to note that, for these experiments, transfections were performed on cells in six-well dishes, and upon trypsinization, all cells from a given plate were pooled, replated on Fn, and then harvested at different times. Using a pooled cell population for the time course experiments eliminates variability in levels of Raf expression between time points, a point that was confirmed by Western blot analysis of the immunoprecipitated Raf isoforms (Fig. 3A, bottom panel).

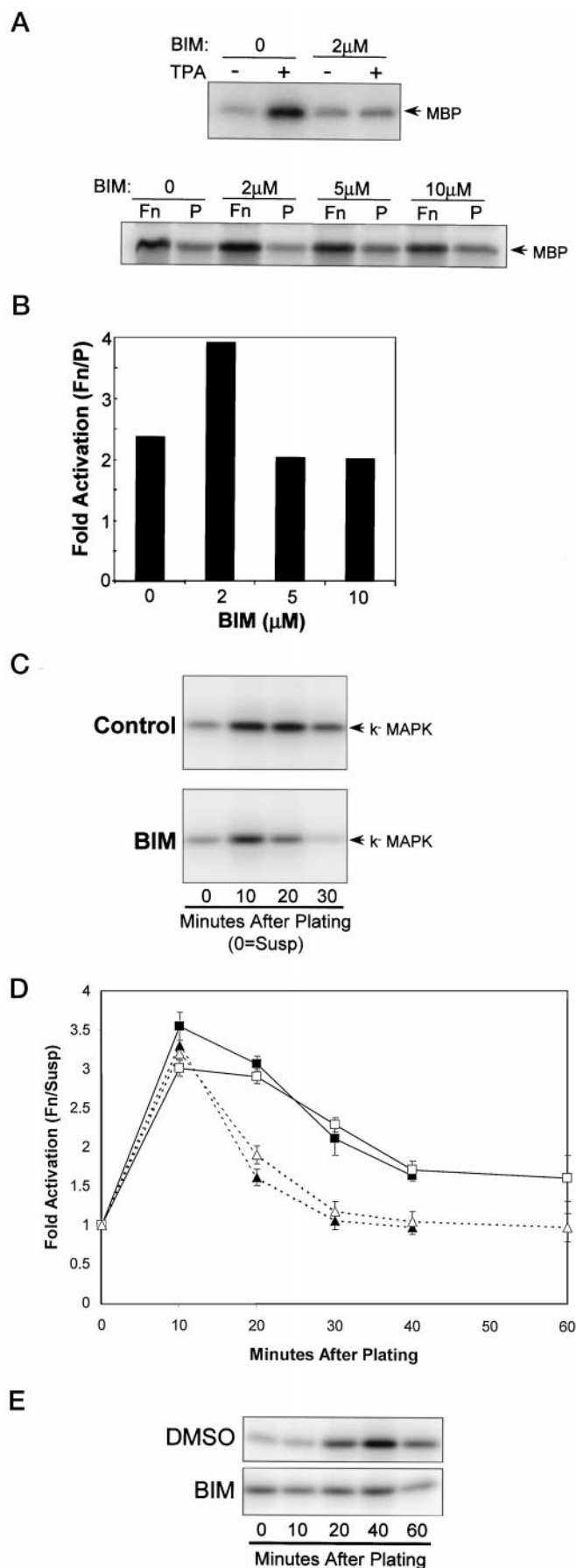
In contrast to the allele containing only the R89L point mutation, the membrane-targeted Raf-R89L-CAAX was activated as efficiently as Raf-WT at early time points after cell adhesion (Fig. 1, C and D; Fig. 2). However, unlike both



**FIG. 3. Time course of integrin-mediated activation of WT, R89L, and R89L-CAAX Raf.** A, cells were transfected with the indicated plasmids, cultured briefly in suspension, and plated onto Fn-coated dishes as described for Fig. 1. Cells were harvested at the indicated times, and Raf activity was determined by an immunoprecipitation kinase assay. For each transfection, a portion (1/6) of each immunoprecipitate was removed prior to kinase assay and analyzed by SDS-PAGE and Western blotting with anti-Raf antibody. A typical example, demonstrating equal amounts of immunoprecipitated Raf-WT, is shown (bottom part). B, average fold activation and S.D. values from four independent transfections (Raf-WT (open squares), Raf-R89L (filled circles), and Raf-R89L-CAAX (filled triangles)).

Raf-WT and the R89L point mutant, the activity of the R89L-CAAX mutant did not return to basal levels by 2 h but rather persisted at greater than 90% of maximum levels for the length of the experiment. Although a detailed, extended time course of adhesion-mediated Raf-R89L-CAAX activity has not been done, preliminary experiments suggest that the activity returns to base line within 8 h after adhesion (data not shown). These results demonstrate that constitutive localization of Raf to the cell membrane allows for a significantly prolonged activation of Raf upon cellular adhesion to fibronectin.

**Inhibitors of PKC Activity Block the Sustained Phase but Not the Initial Phase of Integrin-mediated Activation of the MAPK Cascade**—The results above suggested that integrin-mediated adhesion might regulate factors involved in the activation of membrane-localized Raf. In an attempt to determine what these factors might be, we began to investigate the importance of known positive regulators of Raf activity. Members of the PKC family of kinases are well known for their ability to activate MAPK through Raf (31–35). To evaluate whether PKC



**FIG. 4. Inhibition of PKC affects the sustained but not the initial phase of integrin-mediated activation of the Raf/MAPK pathway.** *A*, confluent NIH3T3 cells were incubated overnight in se-

activity was required for integrin-mediated activation of the Raf/MEK/MAPK cascade, we assayed integrin-mediated MAPK activity in the absence and presence of the PKC inhibitor BIM. Cells were treated with the indicated concentrations of BIM overnight, treated with phorbol ester for 10 min or replated onto fibronectin- or poly-L-lysine-coated plates for 15 min, and then harvested for MAPK immunoprecipitation kinase assay. While the activation of MAPK elicited by 100 nM TPA, a PKC agonist, was almost completely inhibited by 2  $\mu$ M BIM (Fig. 4*A*, *top*), adhesion-mediated MAPK activation was still apparent in the presence of up to 10  $\mu$ M BIM (Fig. 4, *A* (*bottom*) and *B*). A similar pattern was seen using another PKC inhibitor, calphostin C (data not shown), and similar results were obtained for integrin-mediated activation of both MEK (data not shown) and Raf (see below). Although the level of MAPK activity induced by adhesion to Fn decreased slightly with increasing amounts of BIM, the background level, *i.e.* the activity in nonadherent cells, also decreased. Therefore, the -fold activation, *i.e.* the ratio of activity in Fn-adherent and -nonadherent cells, was not significantly different in the absence or presence of BIM. Thus, at early time points, the inhibition of PKC does not affect the ability of integrin-mediated adhesion to activate the MAPK pathway.

To determine whether the sensitivity to PKC inhibitors changed over the course of integrin-mediated activation of the MAPK cascade, the time course of Raf activity in the absence or presence of BIM was assayed. Cells were incubated with BIM either overnight or for 1 h before replating. Under both treatment conditions, both treated and untreated cells exhibited peak integrin-mediated Raf activity between 10 and 15 min after plating (Fig. 4, *C* and *D*). However, in cells treated with BIM, integrin-mediated Raf activity dropped from peak to basal levels significantly faster than in untreated cells. Specifically, integrin-mediated Raf activity returned to base line between 30 and 40 min after plating in the presence of inhibitor, while in the absence of inhibitor, the level of Raf activity within this time period was still at 75–80% of peak activity, and took longer than 60 min to return to base line. These data suggest that PKC activity is not required for the early or initial phase of integrin-mediated activation of the MAPK cascade, but it does

rum-free DMEM containing 0.1% (*v/v*) Me<sub>2</sub>SO (0) or the indicated amounts of BIM and then either stimulated with 100 nM TPA (*top*) or cultured briefly in suspension and then replated onto dishes coated with poly-L-lysine (*P*) or Fn (*bottom*). Cells were harvested 10 min after TPA stimulation or 15 min after plating, and MAPK activity was measured by an immunoprecipitation kinase assay using myelin basic protein (*MBP*) as a substrate. *B*, graphic representation of densitometric values obtained from *A* (*bottom*). *C*, cells were treated with 0.1% (*v/v*) Me<sub>2</sub>SO (*Control*) or 2.5  $\mu$ M BIM and cultured as described. Cells were harvested at the indicated times, and Raf activity was measured by immunoprecipitation and a linked kinase assay utilizing recombinant wild-type MEK, kinase-dead MAPK (*k<sup>-</sup> MAPK*), and [ $\gamma$ -<sup>32</sup>P]ATP. *D*, confluent cells were treated overnight with 0.1% (*v/v*) Me<sub>2</sub>SO (*filled squares*) or 2.5  $\mu$ M bisindolylmaleimide (*filled triangles*) before trypsinization, suspension culture, and re-plating on Fn-coated plates. Alternatively, cells were treated for 1 h, while in suspension, with Me<sub>2</sub>SO (*open squares*) or BIM (*open triangles*) at the same concentrations as above. Cells were extracted at the indicated times, and Raf activity was assayed by an immunoprecipitation kinase assay using kinase-dead MAPK as a terminal phosphoacceptor. Reaction products were separated by SDS-PAGE, visualized by autoradiography, and analyzed on a Molecular Dynamics PhosphorImager. The average fold activations and S.D. values from three independent experiments are shown. *E*, cells were transfected with plasmid encoding Raf-R89L, grown to confluence, serum-starved overnight, and then incubated in suspension for 1 h in the presence of 0.1% (*v/v*) Me<sub>2</sub>SO or 2.5  $\mu$ M bisindolylmaleimide as indicated. Cells were plated onto Fn-coated dishes and harvested at the indicated times for analysis of Raf activity by linked kinase assay with kinase-dead MAPK described above. The results shown are representative of three separate transfection experiments.

contribute to prolonged or sustained activity. This conclusion must be tempered by the consideration that, although we used well known PKC inhibitors, it remains possible that these drugs affect another enzymatic activity that is involved in the process under study. Future pursuit of this issue will entail identification of the PKC isoform(s) putatively involved in Raf regulation.

If integrin-mediated activation of Raf does indeed comprise an initial membrane localization phase and a later, sustained phase governed by PKC, then compromising membrane localization of Raf while inhibiting PKC activity should conspire to completely abrogate integrin-mediated Raf activation. To test this hypothesis, we treated Raf-R89L-transfected cells with either solvent or PKC inhibitor and then assayed the activity of the tagged kinase at various time points after adhesion to Fn (Fig. 4E). As demonstrated previously in Fig. 3, Raf-R89L activity, in the absence of PKC inhibitor, is slightly increased early after attachment and then peaks and persists at later time points. After treatment with BIM, however, attachment to Fn fails to stimulate Raf-R89L activity above the level in non-adherent cells over the entire course of the experiment.

#### DISCUSSION

Activation of the MAPK cascade in response to integrin-mediated cell adhesion has been described by a number of laboratories and in a number of cell types (for a review, see Ref. 3). Understanding the pathways that connect integrins and Raf is fundamental to understanding the activation of the MAPK pathway by adhesion. However, since the exact mechanism of Raf activation in response to mitogens remains unresolved, the mechanism of integrin-mediated Raf activation is likely to also be somewhat elusive.

In an attempt to further define this mechanism, several groups have defined certain factors as either requisite or dispensable for integrin-mediated activation of the MAPK cascade, often with conflicting results. For example, there is good experimental evidence that suggests integrin-mediated MAPK activation involves pp125<sup>FAK</sup> (18), Ras (18, 19), and phosphatidylinositol 3-kinase (17), but there is also substantial evidence for mechanisms that are independent of pp125<sup>FAK</sup> (21, 23), Ras (7), and phosphatidylinositol 3-kinase (22). In many of the studies in which inhibitors of various activities were used, the effects of these inhibitors were measured at a single time point (usually corresponding to the peak of activity) or at a narrow range of time points that did not encompass the full period of MAPK activation. The results of the current study demonstrate that initial and sustained phases of integrin-mediated activation of the MAPK cascade are governed by distinct biochemical mechanisms. Specifically, the initial phase of activation, *i.e.* the relatively high level of activity observed early after adhesion to fibronectin, is not affected by PKC inhibitors but requires efficient translocation of Raf-1 to the plasma membrane. This translocation can be provided by interaction with Ras or by constitutive localization by CAAX-directed prenylation. In contrast, the sustained phase of activation, *i.e.* the submaximal activity that persists for tens of minutes up to hours, is independent of Ras-Raf association, is blocked by PKC inhibitors, and is lengthened by constitutive membrane localization of Raf.

The profile of Raf-R89L activation implicates Ras-Raf interaction as important for the initial phase but not necessarily the later sustained phase of integrin-mediated Raf activation. At first glance, the current results are seemingly at odds with a previous report from our laboratory in which inhibition of Ras function by expression of dominant negative Ras or disruption of Ras-effector interactions by expression of the amino terminus of Raf did not affect integrin-mediated MEK activation (7). The time course analysis of the current study provides a relatively straightforward explanation for this apparent discrep-

ancy. As shown in Fig. 3, the adhesion-induced activities of Raf-WT and Raf-R89L are equivalent at 30–40 min after plating. In our previous study, measurements to determine Ras independence were also made at 30 min after plating. Thus, it is reasonable to expect that partial inhibition of Ras by expression of a dominant negative would have only a slight effect on integrin-mediated activation of the MAPK pathway at this time point. Indeed, our current results suggest that the effect of inhibiting Ras might vary along the time course of integrin-mediated activation of the MAPK pathway. An important caveat is that in the current studies, only direct Raf-Ras interaction was blocked; the function of endogenous Ras was not perturbed. This contrasts with other studies (18, 19) using dominant negative alleles where presumably all effectors downstream of Ras were blocked. These effectors may influence integrin-mediated Raf and subsequent MAPK activation in indirect ways. In this manner, a high level of expression of a Ras dominant negative may affect integrin-mediated activation of the Raf/MEK/MAPK pathway by both direct (*e.g.* disruption of Ras-Raf interaction) and indirect (*e.g.* inhibition of other Ras effectors) means. In light of the current findings, it appears clear that Ras does contribute to the initial phase of activation of the MAPK pathway by cell adhesion. However, it is also clear that other factors, such as PKC and cytoskeletal elements (see below), influence the process at later times.

There is now substantial evidence that suggests an involvement of integrin- or cytoskeleton-associated structures in Raf activation. It has been established that activation of Raf by soluble mitogens results in its translocation to the plasma membrane and that this translocation is indeed important for Raf activation (25, 28, 36, 37). Our laboratory has previously shown that integrin-mediated adhesion is required for activation of Raf by mitogens (38). Furthermore, activated Raf exists in a large multiprotein complex that is associated with the plasma membrane and/or cytoskeleton (37). Although these observations concern activation of Raf by soluble mitogenic factors, it has now been established that integrin-mediated cell adhesion to insoluble matrix proteins also activates Raf (Refs. 17 and 39; this study) and can recruit Raf into integrin-associated complexes (12). In the current study, we show that integrin-mediated activation of wild-type and mutant Raf proteins was inhibited by treatment of the cells with cytoD, demonstrating that organization of the actin cytoskeleton is important for adhesion-mediated Raf activation. This supports earlier observations made by several groups that cytoD blocks integrin-mediated MAPK activation (8, 9, 11, 12, 40). Disruption of actin filament assembly may block a membrane-proximal signaling event that is downstream of integrin engagement and cytoskeleton organization but is required for Raf activation. The exact factors that mediate activation of membrane-bound Raf have yet to be identified, but candidates include tyrosine kinases (*e.g.* Src, Lck) (25, 41–43), various PKC isoforms (*e.g.*  $\alpha$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$ ) (33, 35, 44), and phospholipids (*e.g.* phosphatidic acid and phosphatidylserine) (45, 46). Alternatively, disruption of actin filament assembly may preclude the formation of a higher order cellular structure to which Raf is recruited for activation. This latter scenario is particularly intriguing, considering the inhibition by cytoD of R89L-CAAX-Raf, which permanently resides in the membrane. However, these hypotheses are certainly not mutually exclusive.

The efficient initial activation of Raf-R89L-CAAX demonstrates that the role of Ras-Raf interaction in integrin-mediated Raf activation is to target Raf to the membrane. The regulation by adhesion of Raf-R89L-CAAX is an intriguing observation for a number of reasons. As mentioned before, activation of Raf involves membrane translocation and deposi-

tion into a putatively cytoskeleton-associated cellular compartment. The low level of activity of Raf-R89L-CAAX in suspension implies that the membrane-associated Raf-activating factors are not functional in nonadherent cells but become functional upon cell adhesion. This suggests that integrin-mediated cell adhesion regulates the unknown, membrane-associated machinery responsible for activating Raf after its localization to the membrane, either by activating a component(s) of the machinery or by allowing efficient localization of these components.

The activity of Raf-R89L-CAAX is down-regulated far more slowly than Raf-WT, enduring at nearly maximum levels for at least 2 h. This demonstrates an important difference in the regulation of Raf targeted to the membrane by protein prenylation *versus* Raf targeted by more "natural" means, *i.e.* through interaction with Ras. One possible explanation is that the specific locations within the membrane to which Raf is targeted by Ras and by CAAX-directed prenylation may be different, and the Raf-activating factor(s) present in each may be quantitatively or qualitatively different. Another possibility is that inactivation of Raf after adhesion is the result of "diffusion" of Raf away from membrane-bound activating factors rather than an inactivation of the factors themselves. Thus, Raf-R89L-CAAX, which is unable to leave the membrane, stays active longer than Raf-WT.

Our data would suggest that PKC is not part of the machinery responsible for the initial, acute activation of Raf but that it may be involved in the subsequent maintenance of Raf activity. Raf is directly phosphorylated and activated by PKC (31–33), and PKC activity can be stimulated by integrin-mediated cell adhesion (47). Many reports have demonstrated that PKC-mediated activation of Raf can occur independently of Ras (33–35, 48, 49) and therefore independently of Ras-mediated membrane localization. This suggests that either PKC can activate Raf in the absence of membrane translocation or that there exists a mechanism for translocation that does not involve Ras. In this regard, it is interesting to note that Raf and PKC have been shown to form a stable complex *in vivo* (34, 50), and this may serve to recruit Raf to the plasma membrane. Interestingly, there is increasing evidence that demonstrates direct regulation of PKC activity by aggregated syndecan-4 (51, 52). Syndecan-4 is a transmembrane heparan sulfate proteoglycan that acts with integrins as a co-receptor for ECM ligands and plays a critical role in the formation of focal adhesions and actin stress fibers (53). It would be intriguing to investigate whether integrins and syndecans coordinately regulate different phases of the signaling pathways initiated by adhesion to Fn.

In summary, integrin-mediated activation of the Raf/MEK/ MAPK cascade appears to comprise an initial PKC-independent phase that is optimized by translocation of Raf to the plasma membrane, followed by a sustained phase that is blocked by PKC inhibitors. We have also shown that the activity of a constitutively membrane-associated mutant of Raf can be regulated by integrin-mediated adhesion. Since activated Raf exists as part of a membrane- and cytoskeleton-associated complex, this regulation suggests that integrins may play an important role in controlling the activation or localization of membrane-associated factor(s) responsible for Raf activation. In addition to its role in direct adhesion-mediated signaling, adhesion-dependent regulation of the Raf activation machinery may also be involved in the adhesion-dependent activation of the MAPK cascade by growth factors (38, 52). Future studies will be directed toward defining in more detail the adhesion-dependent regulation of Raf proteins targeted to the membrane, examining adhesion-induced changes in Raf subcellular

localization, and determining the integrin-regulated membrane components or structures that influence Raf activity.

## REFERENCES

- Hynes, R. O. (1992) *Cell* **69**, 11–25
- Gille, J., and Swerlick, R. A. (1996) *Ann. N. Y. Acad. Sci.* **797**, 93–106
- Aplin, A. E., Howe, A. K., Alahari, S. K., and Juliano, R. L. (1998) *Pharmacol. Rev.* **50**, 197–263
- Clark, E. A., and Brugge, J. S. (1995) *Science* **268**, 233–239
- Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) *Annu. Rev. Cell Dev. Biol.* **11**, 549–599
- Howe, A., Aplin, A. E., Alahari, S., and Juliano, R. (1998) *Curr. Opin. Cell Biol.* **10**, 220–231
- Chen, Q., Lin, T. H., Der, C. J., and Juliano, R. L. (1996) *J. Biol. Chem.* **271**, 18122–18127
- Chen, Q., Kinch, M. S., Lin, T. H., Burridge, K., and Juliano, R. L. (1994) *J. Biol. Chem.* **269**, 26602–26605
- Morino, N., Mimura, T., Hamasaki, K., Tobe, K., Ueki, K., Kikuchi, K., Takehara, K., Kadowaki, T., Yazaki, Y., and Nojima, Y. (1995) *J. Biol. Chem.* **270**, 269–273
- Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) *Nature* **372**, 786–791
- Zhu, X., and Assoian, R. K. (1995) *Mol. Biol. Cell* **6**, 273–282
- Miyamoto, S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K., and Yamada, K. M. (1995) *J. Cell Biol.* **131**, 791–805
- Seeger, R., and Krebs, E. G. (1995) *FASEB J.* **9**, 726–735
- Downward, J. (1996) *Cancer Surv.* **27**, 87–100
- Marshall, M. (1995) *Mol. Reprod. Dev.* **42**, 493–499
- Cutler, R. E., and Morrison, D. K. (1997) *Curr. Opin. Cell Biol.* **9**, 174–179
- King, W. G., Mattaliano, M. D., Chan, T. O., Tschlis, P. N., and Brugge, J. S. (1997) *Mol. Cell Biol.* **17**, 4406–4418
- Schlaepfer, D. D., and Hunter, T. (1997) *J. Biol. Chem.* **272**, 13189–13195
- Clark, E. A., and Hynes, R. O. (1996) *J. Biol. Chem.* **271**, 14814–14818
- Mainiero, F., Murgia, C., Wary, K. K., Curatola, A. M., Pepe, A., Blumberg, M., Westwick, J. K., Der, C. J., and Giancotti, F. G. (1997) *EMBO J.* **16**, 2365–2375
- Wary, K. K., Mainiero, F., Isakoff, S. J., Marcantonio, E. E., and Giancotti, F. G. (1996) *Cell* **87**, 733–743
- Wei, J., Shaw, L. M., and Mercurio, A. M. (1998) *J. Biol. Chem.* **273**, 5903–5907
- Lin, T. H., Aplin, A. E., Shen, Y., Chen, Q., Schaller, M., Romer, L., Aukhil, I., and Juliano, R. L. (1997) *J. Cell Biol.* **136**, 1385–1395
- Brтва, T. R., Drugan, J. K., Ghosh, S., Terrell, R. S., Campbell-Burk, S., Bell, R. M., and Der, C. J. (1995) *J. Biol. Chem.* **270**, 9809–9812
- Marais, R., Light, Y., Paterson, H. F., and Marshall, C. J. (1995) *EMBO J.* **14**, 3136–3145
- Finney, R., and Herrera, D. (1995) *Methods Enzymol.* **255**, 310–323
- Dent, P., Reardon, D. B., Morrison, D. K., and Sturgill, T. W. (1995) *Mol. Cell Biol.* **15**, 4125–4135
- Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M., and Hancock, J. F. (1994) *Science* **264**, 1463–1467
- Leever, S. J., Paterson, H. F., and Marshall, C. J. (1994) *Nature* **369**, 411–414
- Renshaw, M. W., Toksoz, D., and Schwartz, M. A. (1996) *J. Biol. Chem.* **271**, 21691–21694
- Carroll, M. P., and May, W. S. (1994) *J. Biol. Chem.* **269**, 1249–1256
- Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U. R. (1993) *Nature* **364**, 249–252
- Cai, H., Smola, U., Wixler, V., Eisenmann-Tappe, I., Diaz-Meco, M. T., Moscat, J., Rapp, U. R., and Cooper, G. M. (1997) *Mol. Cell Biol.* **17**, 732–741
- Cacace, A. M., Ueffing, M., Philipp, A., Han, E. K., Kolch, W., and Weinstein, I. B. (1996) *Oncogene* **13**, 2517–2526
- Ueda, Y., Hirai, S., Osada, S., Suzuki, A., Mizuno, K., and Ohno, S. (1996) *J. Biol. Chem.* **271**, 23512–23519
- Dent, P., and Sturgill, T. W. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 9544–9548
- Wartmann, M., and Davis, R. J. (1994) *J. Biol. Chem.* **269**, 6695–6701
- Lin, T. H., Chen, Q., Howe, A., and Juliano, R. L. (1997) *J. Biol. Chem.* **272**, 8849–8852
- Chen, Q., Lin, T. H., Der, C. J., and Juliano, R. L. (1996) *J. Biol. Chem.* **271**, 18122–18127
- Takahashi, M., and Berk, B. C. (1996) *J. Clin. Invest.* **98**, 2623–2631
- Morrison, D. K., Kaplan, D. R., Escobedo, J. A., Rapp, U. R., Roberts, T. M., and Williams, L. T. (1989) *Cell* **58**, 649–657
- Fabian, J. R., Daar, I. O., and Morrison, D. K. (1993) *Mol. Cell Biol.* **13**, 7170–7179
- Stokoe, D., and McCormick, F. (1997) *EMBO J.* **16**, 2384–2396
- van Dijk, M., Muriana, F. J., van Der Hoeven, P. C., de Widt, J., Schaap, D., Moolenaar, W. H., and van Blitterswijk, W. J. (1997) *Biochem. J.* **323**, 693–699
- Ghosh, S., Strum, J. C., Sciorra, V. A., Daniel, L., and Bell, R. M. (1996) *J. Biol. Chem.* **271**, 8472–8480
- Ghosh, S., and Bell, R. M. (1997) *Biochem. Soc. Trans.* **25**, 561–565
- Vuori, K., and Ruoslahti, E. (1993) *J. Biol. Chem.* **268**(29), 21459–21462
- Arai, H., and Escobedo, J. A. (1996) *Mol. Pharmacol.* **50**, 522–528
- Zou, Y., Komuro, I., Yamazaki, T., Aikawa, R., Kudoh, S., Shiojima, I., Hiroi, Y., Mizuno, T., and Yazaki, Y. (1996) *J. Biol. Chem.* **271**, 33592–33597
- van Dijk, M. C., Hilkemann, H., and van Blitterswijk, W. J. (1997) *Biochem. J.* **325**, 303–307
- Oh, E. S., Woods, A., Lim, S. T., Theibert, A. W., and Couchman, J. R. (1998) *J. Biol. Chem.* **273**, 10624–10629
- Oh, E. S., Woods, A., and Couchman, J. R. (1997) *J. Biol. Chem.* **272**, 8133–8136
- Couchman, J. R., and Woods, A. (1996) *J. Cell. Biochem.* **61**, 578–584
- Renshaw, M. W., Ren, X.-D., and Schwartz, M. A. (1997) *EMBO J.* **16**, 5592–5599