Cell adhesion to the extracellular matrix triggers a cascade of intracellular biochemical signals regulated by the integrin family of receptors. Recent evidence suggests that integrin engagement may activate a mitogen-activated protein (MAP) kinase cascade that may cooperate with more clearly defined mitogenic signaling pathways to regulate cell proliferation, adhesion, and migration. Here we report that the adhesion-dependent activation of the MAP kinase Erk2 (extracellular signal-regulated kinase 2) occurs in serum-starved NIH3T3 cells, and that this activation of Erk2 is preceded by the activation of the small GTP-binding protein Ras in fibronectin-adherent cells. Inhibition of Ras signaling by expression of a dominant-inhibitory mutant of Ras (N17Ras) in NIH3T3 cells blocked adhesion-dependent activation of Erk2, although the focal adhesion kinase (FAK) was still activated in these cells. Furthermore, activation of this Ras-MAP kinase pathway activated cytosolic phospholipase A$_2$, leading to the release of arachidonic acid metabolites, and N17Ras also inhibited these events. However, N17Ras expression does not inhibit cell adhesion, spreading, or focal contact and stress fiber formation. These results suggest that, while integrin-dependent activation of this MAP kinase pathway is Ras-dependent, the integrin-dependent activation of FAK and several morphological events are Ras-independent. Thus, integrin-mediated signals involved in regulating cell morphology appear to diverge from those regulating MAP kinase activation at a level upstream of Ras activation.

Integrins are the major family of transmembrane receptors that mediate attachment to the extracellular matrix (ECM).$^1$ Engagement and clustering of integrins lead to the formation of structures, called focal adhesions, where integrins link to intracellular cytoskeletal complexes and bundles of actin filaments. These structures form a scaffolding for the association of signaling molecules that regulate signal transduction pathways leading to integrin-induced changes in cell behavior. Recently, much has been learned about the interactions between integrins and the ECM, although significantly less is known about the intracellular biochemical pathways that integrins regulate and the cellular functions that are thereby controlled (reviewed in Refs. 1-3).

One family of proteins whose role in integrin-mediated signaling has only recently begun to be examined is the Ras superfamily of small GTP-binding proteins. Ras, the canonical member of the superfamily, is activated in response to numerous soluble growth and serum factors. Subsequently, Ras activates a signaling pathway that includes the mitogen-activated protein (MAP) kinases Erk1 and Erk2 which eventually regulates transcription, translation, and the cytoskeleton (4). Erk1 and Erk2 are also activated when cells adhere to the ECM proteins fibronectin, laminin, collagen, and vitronectin (5-7), suggesting that integrin-matrix interactions activate a MAP kinase cascade that may be related to that activated by soluble growth factors. Adhesion to fibronectin also stimulates the tyrosine phosphorylation of the focal adhesion kinase (FAK) as well as its association with other signaling molecules (Src and Grb2/SOS) that may promote activation of the Ras signaling pathway (6). If so, then integrin-mediated activation of Ras would be expected downstream of FAK and upstream of Erk1/Erk2 activation.

Downstream targets for Erk1/Erk2 in the Ras-MAP kinase pathway include numerous potential substrates that fall into one of several categories (4): first, protein kinases such as p90$^rsk$ that are involved in protein translation; second, a group that includes nuclear proteins such as Elk1, a ternary complex factor involved in c-fos induction; finally, cytosolic phospholipase A$_2$ (cPLA$_2$), an enzyme that liberates arachidonic acid and its metabolites from glycerophospholipids. This final group is especially interesting in light of the observation that integrin clustering in HeLa cells induces the release of arachidonic acid metabolites and cell spreading, events that are inhibited by the PLA$_2$ inhibitor bromophenacyl bromide (8).

What role, if any, signaling molecules of the Ras-MAP kinase pathway play in each of these events remains to be examined, although a role for Ras in integrin-mediated signaling appears likely for two reasons. First, Ras can be found at sites of integrin clustering (9), and, second, treatment of Jurkat cells with integrin antibodies activates Ras (10). However, matrix-dependent activation of Ras has not been shown, and a role for Ras in integrin-dependent activation of MAP kinases remains to be established. Therefore, the purpose of this study was to examine signaling through the Ras-MAP kinase pathway and to determine whether or not this pathway plays a role in integrin-mediated cellular responses such as cell adhesion, spreading, and focal contact and stress fiber formation. We
found that Ras is rapidly and transiently activated in fibronectin-adherent NIH3T3 cells, and that this activation precedes the peak of Erk2 activation. To test if integrin-mediated Erk2 activation is Ras-dependent, we expressed a dominant-inhibitory Ras mutant (N17Ras) in NIH3T3 cells and found that it inhibits matrix-dependent activation of Erk2 and cPLA2 and arachidonic acid release, while integrin-dependent activation of FAK is not inhibited by N17Ras. Finally, although N17Ras appears to block the integrin-mediated Ras-MAP kinase pathway, it does not inhibit the formation of focal adhesions and stress fibers. These results suggest that, while integrin-mediated adhesion can activate the Ras-MAP kinase pathway, this pathway is not necessary for integrin-dependent focal contact and stress fiber formation.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies to Ha-Ras were purchased from Transduction Laboratories (for immunoblotting) and Santa Cruz Biotech (for GTP-loading). A pan-Erk antibody used for immunoblotting (which recognizes the major Erk in NIH3T3 cells, Erk2) was purchased from Transduction Laboratories, and the anti-phosphoerysine antibody (clone 4G10) was purchased from Upstate Biotechnology Inc. An agarose-conjugated anti-Erk2 antibody (Santa Cruz Biotech) was used for immunoprecipitation/kinase assays. The cPLA2 antibody was purchased from Santa Cruz Biotech. The anti-focal adhesion kinase (FAK) antibody (CK) was a gift from J. L. Guil (Cornell University). Reagents for immunofluorescence were obtained from Sigma (anti-vinculin antibody) and Molecular Probes (rhodamine phalloidin). Human plasma fibronectin was purchased from Collaborative Biomedicals and platelet-derived growth factor BB from Life Technologies, Inc. The dominant inhibitory Ras construct, pMMRasDN, was obtained from J. Brugge (ARIA Pharmaceuticals). A plasmid, pLENeo, containing the neomycin resistance gene, was provided by C. M. DiPersio (MIT). Dexamethasone (Pharmaceuticals). A plasmid, pLENneo, containing the neomycin resistance gene, was provided by C. M. DiPersio (MIT). Dexamethasone was purchased from Sigma. 32PO4, [3H]arachidonic acid for 18 h were washed, trypsinized, and replated on fibronectin- or polylysine-coated dishes as described above. After allowing the cells to adhere for 5 min at 37 °C, the dishes were rinsed once with prewarmed DMEM, and fresh DMEM was added to the dishes. The cells were returned to the incubator for 0, 5, 10, 15, or 35 additional minutes, and 50 μl of media were removed for scintillation counting. To ensure that equivalent amounts of radio labeled lipids were present in the parental NIH3T3 and N17Ras-expressing cells, equal numbers of cells were extracted with chloroform, and the radioactivity was determined by scintillation counting.

Immunofluorescence—Adherent cells were fixed, permeabilized, and stained as described previously (11).

RESULTS

Several studies have suggested a link between integrin-mediated signaling and the Ras-MAP kinase pathway. Specifically, adhesion of cells to ECM components such as fibronectin induces the activation, tyrosine phosphorylation, and nuclear translocation of MAP kinases through an integrin-dependent mechanism (5–7). However, a recent study has concluded that integrin engagement of the ECM is not sufficient to trigger the activation of Erk1 and Erk2 in serum-starved Swiss 3T3 cells (14). To examine the serum dependence of integrin-mediated MAP kinase activation, NIH3T3 cells were incubated in media containing 0, 0.5, or 0.1% serum for 18 h before replating in serum-free media on fibronectin-coated dishes for 0 to 40 min. MAP kinase activation was examined by mobility shift assay. As shown in Fig. 1A, we observed an upward gel mobility shift in Erk2 characteristic of its activation and phosphorylation on tyrosine and threonine residues. The shift was rapid, occurring as early as 5 min after plating the cells onto a matrix, and was maximal between 10 and 20 min. The activation of Erk2 was observed even in serum-starved (0%) cell lysates, although activation was somewhat enhanced when cells were maintained in even low levels of serum (0.5%), and higher concentrations of serum (10%) appeared to dampen integrin-mediated Erk2 activation.

The observed fibronectin-induced activation of MAP kinase suggested a potential role for activated Ras in integrin-mediated signal transduction (1). Therefore, we analyzed the activity state of Ras by quantitating the levels of GTP and GDP associated with Ras in lysates from suspended or fibronectin-adherent NIH3T3 cells (Fig. 1B). A rapid and transient activation of Ras was observed in cells plated onto fibronectin, with the peak of GTP associated with Ras occurring as early as measurable (5 min). Although the percentage of GTP associated with Ras in fibronectin-adherent cells was less than that observed in PDGF-stimulated cells, it was significantly greater than that in suspended or polylysine-adherent cells (Fig. 1B). This peak of Ras activation precedes both cell spreading (which can first be observed 15 min after the cells have been plated onto fibronectin) and the peak of Erk2 activation (see Fig. 1A), suggesting that Ras could regulate integrin-mediated Erk2 activation. However, this correlation does not ensure that Erk2...
activation is Ras-dependent.

To examine the regulation of fibronectin-induced MAP kinase activation by Ras, NIH3T3 cells were transfected with a dominant-inhibitory mutant of Ras (N17Ras). Expression of N17Ras in cells inhibits the activation of endogenous Ras, presumably by interfering with the exchange of GTP for GDP, and has been shown to block Ras-mediated growth factor signaling (15). Expression of N17Ras was controlled in these cells by the dexamethasone-inducible murine mammary tumor virus promoter. Parental NIH3T3 cells and two separate clones which expressed relatively low (DN1) and high (DN2) levels of N17Ras when stimulated with dexamethasone for 18 h (see Fig. 2A) were used in subsequent experiments. As shown in Fig. 2A, expression of N17Ras at relatively high levels (DN2) completely inhibited both PDGF- and matrix-dependent upward gel mobility shift characteristic of Erk2 activation. Furthermore, N17Ras inhibited the activation of Erk2 kinase activity (Fig. 2B). Expression of lower levels of N17Ras (DN1) delayed activation of Erk2 (Fig. 2A).

The ability of the dominant-inhibitory N17Ras to inhibit fibronectin-mediated activation of Erk2 places this MAP kinase downstream of Ras in integrin as well as growth factor signaling. In an attempt to delineate further the signaling pathway from integrins to Erk2, we examined whether the activation of FAK, a potential upstream mediator of integrin-dependent Ras and MAP kinase activation (6), is Ras-dependent. Expression of N17Ras did not inhibit the tyrosine phosphorylation of FAK in fibronectin-adherent cells (Fig. 2C). In fact, when expressed at the highest levels, N17Ras enhanced the tyrosine phosphorylation of FAK.

We next examined a potential downstream target for Erk2, cPLA2. Phosphorylation of cPLA2 by MAP kinase, which causes an electrophoretic mobility shift that correlates with an increase in enzymatic activity, is believed to account for the agonist-stimulated activation of cPLA2 (16). However, several recent studies suggest that MAP kinase-independent pathways exist for cPLA2 activation (17, 18). When cell lysates from NIH3T3 cells plated onto fibronectin were examined, we observed an upward gel mobility shift characteristic of cPLA2 activation (Fig. 3A), and this activation correlated with the release of arachidonic acid metabolites (Fig. 3B). These results are in agreement with studies in HeLa cells where integrin clustering is believed to induce integrin-dependent arachidonic acid release through activation of cPLA2 (8). Expression of the dominant-inhibitory N17Ras completely blocked the upward gel mobility shift indicative of cPLA2 activation and significantly inhibited the release of arachidonic acid metabolites (by 80% at the 20-min time point; see Fig. 3), indicating that Ras mediates integrin-dependent cPLA2 activation and arachidonic acid release in NIH3T3 cells.

Having established that integrin-mediated activation of both Erk2 and cPLA2 is Ras-dependent while FAK phosphorylation is not, we next addressed the question of how these signaling events might control morphological events such as cell spread-
growth factor-independent activation of Erk2 in NIH3T3 cells.

In this report, we have shown directly that integrin-dependent, Ras-dependent activation of MAP kinases suggested a link to the Ras signaling pathway described in this report. This observation that the adhesion-dependent activation of cPLA2-arachidonic acid pathway described in this report is not necessary for the formation of integrin-dependent morphological structures in NIH3T3 cells.

DISCUSSION

Although many cellular processes are known to be matrix-dependent, the contributions of signals transduced by integrins have only begun to be explored. The ability of integrins to activate MAP kinases suggested a link to the Ras signaling pathway (5–7). In this report, we have shown directly that adhesion of fibroblastic cells to a defined matrix can indeed activate Ras. This result is in agreement with another study in which treatment of lymphoid cells with integrin antibodies activated Ras (10) and suggests a potential pathway for integrin-dependent activation of MAP kinases. Using a dominant-inhibitory strategy, we have shown directly that integrin-dependent, growth factor-independent activation of Erk2 in NIH3T3 cells is Ras-dependent. Furthermore, cPLA2 appears to be a substrate for a Ras-activated kinase, presumably Erk2, and the release of arachidonic acid metabolites upon matrix adhesion is, in large part, Ras-dependent. However, in contrast with some previous suggestions, this pathway does not appear to regulate integrin-dependent morphological changes that occur when cells engage the ECM.

Why is integrin-dependent activation of the Ras-MAP kinase pathway important? First, it may play a role in regulating integrin-dependent gene expression. Adhesion of suspension-arrested cells induces the rapid expression of several genes including c-fos, a component of AP1 complexes (21). Activation of the Ras-MAP kinase cascade is known to induce MAP kinase phosphorylation of Elk1 and increase the transcriptional activation of c-fos. These events then regulate the activity of the AP-1 complex, thereby controlling cell proliferation (22). In this way, integrin-dependent activation of this pathway could regulate adhesion-dependent cell growth. Our results suggest that this integrin-dependent MAP kinase pathway converges with growth factor signaling at least at the level of Ras, perhaps through the formation of signaling complexes that integrate signals from integrins and growth factor receptors (23).

Second, Ras might be responsible for regulating cytoskeletal rearrangements involved in cell adhesion, spreading, and focal contact and stress fiber formation. Activated Ras can trigger a cascade which induces morphological changes such as membrane ruffling (19). However, Ras appears to do so through a MAP kinase-independent pathway (24). Furthermore, while there is considerable evidence that Ras can activate a signaling cascade (that includes the Rho family of small GTP-binding proteins) which regulates cytoskeletal dynamics (25), our observation that Ras inhibition does not prevent integrin-dependent cell adhesion, spreading, or focal contact and stress fiber formation in NIH3T3 cells suggests that a Ras-MAP kinase-independent pathway exists for these morphological events.

Ras has also been implicated in the growth factor-induced release of arachidonic acid (26, 27), and this is supported by our observation that the adhesion-dependent activation of cPLA2, and subsequent arachidonic acid release are Ras-dependent. However, our results suggest that this pathway is not essential for integrin-dependent adhesion or spreading of NIH3T3 cells on a fibronectin matrix. This observation is in contrast with previous studies in which arachidonic acid metabolites were reported to be essential for the spreading of HeLa cells on a collagen matrix (8, 28). The observed differences may be due to the different cell types, matrix proteins, and/or integrin receptors engaged in these studies; it is also possible that the reduced levels of arachidonic acid released by our cells expressing N17Ras may be sufficient to allow the cells to spread.

While our results indicate that actin rearrangements induced by engagement of integrins are Ras-independent, these events may nonetheless be mediated by Rho family members Rac and Rho which are essential for epidermal growth factor-
induced actin stress fiber formation (29). Both Rac and Rho stimulate actin polymerization through regulation of phosphatidylinositol metabolism, although the precise targets remain to be identified (30, 31). Hotchin and Hall (14) have recently shown that the formation of focal contacts requires an interaction of integrins with the ECM and a functionally active Rho which, in Swiss 3T3 cells, must be activated by serum factors. However, in NIH3T3 cells it appears either that serum starvation is not sufficient to turn off Rho or that Rho is activated upon engagement of integrins, or that integrin-dependent actin rearrangements are Rho-independent. Although it is presently unknown how actin rearrangements induced by integrin engagement and clustering may be mediated by Rho family members, future studies may aid in defining where integrin-dependent signaling pathways regulating cell morphology diverge from those regulating cell growth; we now know that they do so upstream of the Ras-MAP kinase pathway.

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Ras Activation Is Necessary for Integrin-mediated Activation of Extracellular Signal-regulated Kinase 2 and Cytosolic Phospholipase A2 but Not for Cytoskeletal Organization

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