Integrin-mediated Cell Adhesion Activates Mitogen-activated Protein Kinases*

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Integrins can function as signal-transducing receptors capable of modulating cell growth and gene expression (Juliano, R. L., and Haskill, S. (1983) J. Cell Biol. 120, 577–585; Hynes, R. O. (1987) Cell 69, 11–25). An early event in integrin signaling in fibroblasts and other cells involves activation of pp125*FAK*, a cytoplasmic tyrosine kinase (Hanks, S. K., Calalb, M. B., Harper, M. C., and Patel, S. K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8487–8491; Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Parsons, J. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5192–5196). Here we report a novel aspect of integrin-mediated signal transduction. We demonstrate that adhesion of cells to substrata coated with extracellular matrix proteins, or with a synthetic peptide containing the RGD sequence, can cause activation of mitogen-activated protein (MAP) kinases in 3T3 or REF52 fibroblasts. Activation of MAP kinases seems to depend on integrin engagement rather than simply on cell attachment. Thus, MAP kinases are activated when cells adhere to substrata coated with the integrin ligands fibronectin or laminin, but not when cells adhere to poly-L-lysine, a nonspecific adhesion-promoting polypeptide. Treatment of cells with cytoskeleton-modifying agents such as cytochalasin D, an inhibitor of actin microfilament assembly, almost completely blocks adhesion-induced MAP kinase activation, indicating a critical role for the cytoskeleton. In REF52 cells, we have observed that activation of MAP kinases is accompanied by redistribution of the protein to the nucleus, suggesting that the activated kinases may impinge on factors regulating gene expression. Thus, integrin-mediated cell adhesion seems to cause activation and nuclear translocation of MAP kinases. This may have important implications for the regulation of cell growth and differentiation by the extracellular matrix.

Recent evidence suggests that integrins function not only as adhesive proteins but also as receptors capable of transducing biochemical signals to the interior of the cell (1, 2). Integrins have been implicated in the regulation of gene expression in fibroblasts and monococytes (5–7) and in the control of tumor cell growth (8–12). In fibroblasts and certain other cell types, ligation of integrins results in the activation and autophosphorylation of pp125*FAK*, a cytoplasmic tyrosine kinase (3, 4, 13–17). Aside from FAK1 activation, little is known of the biochemical basis of integrin-mediated signal transduction. By contrast, a wealth of recent studies has elucidated a consensus signaling pathway for peptide ligands that activate receptor tyrosine kinases. Ligand-receptor interactions cause receptor tyrosine kinase dimerization and autophosphorylation, engagement of SH2/SH3 domain adaptor proteins, accumulation of Ras-GTP, and activation of a kinase cascade comprising Raf-1, MAP kinase kinases, and MAP kinases (18–21). The MAP kinase family of serine/threonine kinases appears to be common to signaling pathways initiated by a wide range of growth and differentiation factors (22–24). Substrates for MAP kinases include transcription factors (25, 26) and other kinases (27). Translocation of MAP kinases from the cytosol to the nucleus can be triggered by serum and growth factors (28). MAP kinases thus considered to be key molecules for the convergence of extracellular signals and their transduction into the nucleus (29, 30). Since early events in receptor tyrosine integrin-mediated signaling and integrin-mediated signaling both involve activation of tyrosine kinases, we hypothesized that the two signal transduction pathways might share other common elements as well. Here we show that attachment of Swiss 3T3 or REF52 cells to a fibronectin substratum leads to MAP kinase activation and translocation from the cytosol to the nucleus. We also demonstrate that actin filament assembly plays an important role in this adhesion-mediated signaling event.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Swiss 3T3 cells and Dulbecco’s modified Eagle’s medium (DMEM) were supplied by the Comprehensive Cancer Center (University of North Carolina, Chapel Hill, NC). Anti-MAP kinase antibodies 956/837 and 691 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human plasma fibronectin was from Collaborative Research (Bedford, MA). Cytoskeletal D was from Aldrich. Calyculin A, myelin basic protein (MBP), laminin, type I collagen, and RGD peptide (GRGDSP) were from Life Technologies, Inc. (125I-protein A [low specific activity] was obtained from DuPont NEN. Protein G-Sepharose4 Fast Flow was purchased from Pharmacia Biotech Inc. [γ-32P]ATP (4500 Ci/mmol) was from ICN Biomedicals (Costa Mesa, CA). All other reagents were acquired from Sigma or Fisher.

**Preparation of Adhesive Ligand-coated Dishes**—Substrata were prepared by allowing a 10 μg/ml solution of fibronectin, laminin, type I collagen, poly-1-lysine, or 100 μg GRGDSP peptide to adsorb to 60-mm tissue culture dishes (Falcon 3002) at room temperature overnight, followed by blocking with 2% bovine serum albumin (BSA) for 2 h at room temperature. The dishes were rinsed twice with phosphate-buffered saline (PBS) prior to use.

**Cell Culture, Adhesion to Substrata, and Preparation of Total Cell Lysates**—Murine Swiss 3T3 or rat REF52 fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Confluent cells were dissociated from culture flasks with 0.05% trypsin, 0.53 mM EDTA. The suspended cells were washed once in DMEM containing 10% serum, then washed three times in DMEM supplemented with 2% BSA. Cell suspensions were incubated in DMEM, 2% BSA at 37 °C for 30 min on a rotator (in some experiments, 10 μg cytoskeleton D was included at this step). About 0.1 g cells were applied to dishes coated with adhesive ligands (some cells were kept in suspension on a rotator as controls) and incubated at 37 °C.

1 The abbreviations used are: FAK, focal adhesion kinase; MAP, mitogen-activated protein; DMEM, Dulbecco’s modified Eagle’s medium; MBP, myelin basic protein; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

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was determined using the Pierce bicinchonic acid assay.

the cells were washed twice with ice-cold PBS and then lysed in 250 mM p-nitrophenyl phosphate, 20 nM calyculin A, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 5 mM benzamidine. Protein was determined using the Pierce bicinchonic acid assay.

MAP Kinase Mobility Shift—Total cell lysate (40 µg) was electrophoresed on a 15% polyacrylamide gel, blotted onto nitrocellulose, and incubated with 0.1 µCi/ml anti-MAP kinase rabbit polyclonal antibody 691. Immune complexes were detected using 125I-protein A incubated with 0.1 pg/ml anti-MAP kinase rabbit polyclonal antibody 691. Total cell lysates (200 µg/sample) were precleared with protein G-Sepharose for 1 h on ice followed by adding BSA-blocked protein G-Sepharose for 2 h on ice. Immunoprecipitated proteins were recovered by centrifugation in a microcentrifuge for 5 min. The immunoprecipitates were washed two times with 0.25 M Tris, pH 7.6, and once with 0.1 M NaCl and 50 mM Hepes, pH 8.0. The immunoprecipitated MAP kinases were incubated in 100 µl of a mixture containing 0.5–1 µCi of [γ-32P]ATP, 10 mM MgCl2, 1 mM dithiothreitol, 1 mM benzamidine, 0.3 mg/ml MBP, and 25 µg Hepes, pH 8.0, at 30 °C for 20 min. The reactions were stopped by removing the supernatants from the pelleted immunocomplexes and boiling with SDS sample buffer. The MBP bands were excised from the gel and counted by autodigraph.
activity in REF52 cells in suspension, but the kinase activation subsequent to adhesion was quite strong and more persistent in these cells. We have also observed adhesion-induced MAP kinase activation in NIH 3T3 cells and in WI38, a human fibroblast line (data not shown). Thus, the effect seems to occur in a number of fibroblast lines.

The REF52 cells proved to be a favorable system for examining the intra-cellular localization of MAP kinases by immunofluorescence microscopy. Swiss 3T3 cells were less suited for this purpose since they remained fairly rounded during the period of maximal MAP kinase activation and round cells are difficult for microscopy. By contrast, the REF52 fibroblasts spread well on fibronectin and had a more prolonged time course of MAP kinase activation. As seen in Fig. 2 (right panel), MAP kinases were largely distributed within the REF52 cell cytoplasm in a perinuclear concentration after 30 min of contact with a fibronectin substratum (right panel, a). However, after 1 h on fibronectin, the MAP kinases in many REF52 cells were detected within the nucleus (right panel, b). This nuclear localization was transient and MAP kinases were present in the cytoplasm of most cells after 2 h (right panel, c), similar to the situation in control cells (right panel, d). Thus, translocation to the nucleus followed the initial activation of MAP kinases, while the period of nuclear localization overlapped with the period of maximal kinase activity.

To confirm that integrin ligation, rather than merely cell attachment, was required for activation of MAP kinases, we examined this process when cells were allowed to adhere to substrata coated with different macromolecules. We used extracellular matrix proteins, including fibronectin and laminin, that are clearly specific ligands for integrins (2), as well as poly-o-lysine, a positively charged, nonspecific, adhesion-promoting polypeptide. As seen in Fig. 3, attachment of either Swiss 3T3 fibroblasts or REF52 fibroblasts to fibronectin or laminin produced a distinct activation of MAP kinases, whereas attachment to poly-o-lysine failed to activate MAP kinases. Since the cells were >95% attached on both specific and nonspecific substrata, this indicates that integrin ligation is important and that cell adhesion alone does not result in significant activation of MAP kinases.

We sought to determine factors that might influence the activation of MAP kinases by cell adhesion. Treatment of cells plated on fibronectin with cytochalasin D, an inhibitor of actin filament formation (34), almost completely blocked the activation of MAP kinases (Fig. 3). The inhibition of MAP kinases by cytochalasin D could be due to effects on actin filament assembly in localized regions of the cell or due to the inhibition of overall cell spreading. The latter explanation seems unlikely since the activation of MAP kinases in 3T3 cells occurred within 10 min of plating on fibronectin, at which time the cells were largely rounded and had barely begun to spread.

DISCUSSION

We have demonstrated that adhesion of mouse or rat fibroblasts to substrata coated with fibronectin, laminin, or RGD-containing peptides can cause a strong and prompt activation of MAP kinases. We have also observed nuclear translocation of MAP kinases in rat fibroblasts subsequent to adhesion to fibronectin. Integrin engagement seems to be critical, since adhesion of fibroblasts to substrata coated with the nonspecific adhesion promoting polypeptide poly-o-lysine did not cause significant MAP kinase activation. These observations indicate that integrin signal transduction pathways share at least one common element with the better known signaling pathway triggered by soluble mitogens, namely activation of MAP kinases. Some degree of actin filament organization seems to be critical, since adhesion of fibroblasts to substrata coated with the nonspecific adhesion promoting polypeptide poly-o-lysine did not cause significant MAP kinase activation. The effect of cytochalasin D seems to be exerted at the level of localized actin filament assembly, since activation of MAP kinases in 3T3 cells does not require extensive cell spreading.

Integrin-mediated cell adhesion causes both activation of MAP kinases and tyrosine phosphorylation of FAK (3, 4); thus, it seems possible that FAK might be an upstream component in...
the pathway leading to MAP kinase activation. Although our current data do not directly address the potential relationship between FAK and MAP kinases, several observations are consistent with this possibility. Both FAK activation and MAP kinase activation occur rapidly as cells attach to a fibronectin substrate. Although the activation of MAP kinase is transient while the tyrosine phosphorylation of FAK is persistent (14–17), this may be due to induction of phosphatases that regulate MAP kinases (35). The observation that cytochalasin D treatment blocks both MAP kinase activation and integrin-mediated activation of FAK (15, 36) shows that both events depend on cytoskeletal organization, suggesting that they may be related. Furthermore, preliminary data (not shown) indicate that tyrosine kinase inhibitors known to affect FAK can partially block integrin-mediated MAP kinase activation. Thus, adhesion-induced activation of FAK and of MAP kinases display several similarities, suggesting, although clearly not proving, that they may be part of the same signaling pathway.

MAP kinases are thought to play a key role in conveying signals from the cytosol to the nucleus (29, 30). Thus, the finding that integrin-mediated cell adhesion causes activation and nuclear translocation of MAP kinases may be very germane to other observations showing that ligation of integrins can trigger gene expression in several cell types (1) and that anchorage to a substratum plays a key role in cell cycle progression (37). It seems likely that integrin-mediated signaling paths impinging on MAP kinases may make a major contribution to the control of cell growth and differentiation.

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