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Communication

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

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Qiming Chen‡, Michael S. Kinch§¶, Tsung H. Lin‡, Keith Burridge§, R. L. Juliano‡‖

From the Departments of ‡Pharmacology and §Cell Biology and Anatomy, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599

Integrins can function as signal-transducing receptors capable of modulating cell growth and gene expression (Juliano, R. L., and Haskill, S. (1993) *J. Cell Biol.* 120, 577–585; Hynes, R. O. (1992) *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-D-lysine, a nonspecific adhesion-promoting polypeptide. Treatment of cells with 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 a sufficient stimulus 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 monocytes (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 FAK[1] activation, little is known of the biochemical basis of integrin-mediated signal transduction. By contrast, a wealth of recent studies have 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 are thus considered to be key molecules for the convergence of extracellular signals and their transmission into the nucleus (29, 30). Since early events in receptor tyrosine kinase-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). Cytochalasin D was from Aldrich. Calyculin A, myelin basic protein (MBP), laminin, type I collagen, and RGD peptide (GRGDSPK) were from Life Technologies, Inc. ^{125}I-protein A (low specific activity) was obtained from DuPont NEN. Protein G-Sepharose® 4 Fast Flow was purchased from Pharmacia Biotech Inc. [γ-^{32}P]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-D-lysine, or 100 μM GRGDSPK 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 μM cytochalasin D was included at this step). About 10^6 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

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‖ To whom correspondence should be addressed.

[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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for the times indicated in the figure legends. Following the incubations, the cells were washed twice with ice-cold PBS and then lysed in 250 µl of modified RIPA buffer consisting of 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 50 mM Hepes, pH 7.5, 1 mM Na_3VO_4, 50 mM NaF, 1 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 µg/ml anti-MAP kinase rabbit polyclonal antibody 691. Immune complexes were detected using ^{125}I-protein A (2 µCi/10 ml). The dried blots were exposed at −70 °C to 3M Medical Imaging Film.

Immune Complex Kinase Assay—MAP kinase activity was assayed according to Boulton and Cobb (31) with minor modification. Briefly, total cell lysates (200 µg/sample) were precleared with protein G-Sepharose, then incubated with 1 µg of anti-MAP kinase (956/837) rabbit polyclonal antibody 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-^{32}P]ATP, 50 µM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM benzamidine, 0.3 mg/ml MBP, and 25 mM 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 samples (8 µg of MBP for each) were electrophoresed on a 15% polyacrylamide gel. After staining with Coomassie Brilliant Blue, the gel was dried and exposed at −70 °C to 3M Medical Imaging Film overnight. Finally, the MBP bands were excised from the gel and counted by scintillation counting.

Immunolocalization of MAP Kinase—REF52 cells, grown to confluence, were deprived of sera for 48 h. The monolayers were then dissociated with trypsin/EDTA and incubated in suspension for at least 1 h at 37 °C. Following incubation of the cells on fibronectin-coated coverslips for 30–120 min at 37 °C, the samples were fixed for 5 min with 3.7% formaldehyde (Sigma) in PBS, permeabilized with 0.5% Triton X-100 (Sigma) in Tris-buffered saline, and stained with anti-MAP kinase antibody 691 and rhodamine-conjugated goat anti-mouse (Chemicon). The coverslips were washed in PBS, rinsed in deionized water, and mounted with Fluor-Save® (Calbiochem). Coverslips were viewed on a Zeiss Axiophot microscope. Fluorescence micrographs were taken on T-max 400 film (Eastman Kodak Co.).

RESULTS

To investigate the effect of cell adhesion to fibronectin on MAP kinase activity, Swiss 3T3 cells were kept in suspension (as controls) or plated on fibronectin-coated dishes and incubated for the times indicated in the legends. This was done in the absence of any exogenous mitogens. MAP kinase activation was initially examined by a mobility shift assay (32, 33). As shown in Fig. 1*a*, when cells adhered to fibronectin, there were distinct electrophoretic mobility shifts for both the 42- and 44-kDa forms of MAP kinases, with the degree of shifting reaching a maximum 10 min after the cells were plated on the substratum. Activation of MAP kinase enzymatic activity was confirmed by an immunocomplex kinase assay using MBP as a substrate. Consistent with the result of the mobility shift assay, MAP kinase activity was transiently increased in Swiss 3T3 cells plated on fibronectin. The maximal activity of MAP kinases was seen at 10 min and then the kinase activity declined (Fig. 1, *b* and *c*). When cells were kept in suspension, MAP kinase activity remained unchanged. The magnitude of adhesion-induced MAP kinase activation was similar to that caused by treatment of quiescent cells with growth factors such as epidermal growth factor (data not shown). The time course of MAP kinase activation approximated that of cell attachment. Thus, cell adhesion to fibronectin can promptly and effectively activate both the 42- and 44-kDa MAP kinases. We were concerned that the MAP kinase activation induced by cell adhesion to fibronectin might be due to soluble mitogens that were carried along as impurities in the fibronectin preparation. To obviate this possibility, we examined MAP kinase activation in cells adhering to substrata coated with synthetic peptides containing the RGD sequence, which is recognized by several members of the integrin family (2). As seen in Fig. 1*d*, adhesion to synthetic RGD peptides also caused a strong activation of MAP kinases, thus ruling out effects due to growth factor impurities. These observations also suggested that engagement of integrins is an important aspect of the adhesion-induced activation of MAP kinases.

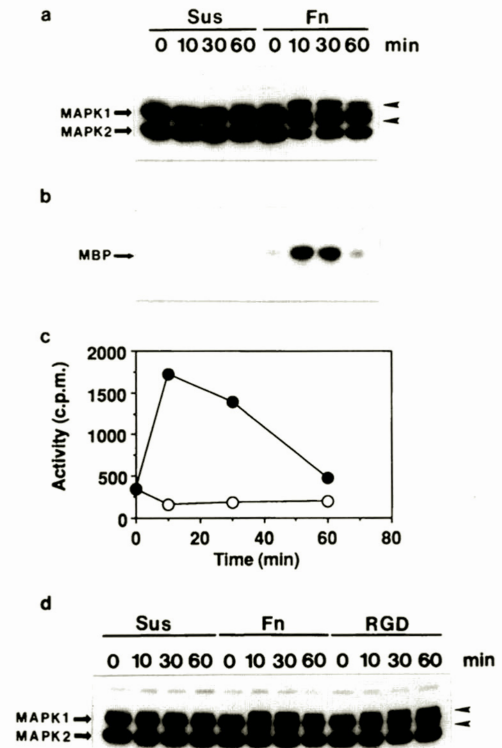

FIG. 1. **Adhesion to fibronectin or RGD peptide induces MAP kinase activation.** In *panels a–c*, Swiss 3T3 cells were grown to confluence in complete medium, dissociated with trypsin/EDTA, and then kept in suspension in serum-free medium (*Sus*) or plated on to a substratum coated with 10 µg/ml fibronectin (*Fn*) and incubated at 37 °C for 0, 10, 30, and 60 min. Following the incubations, total cell lysates were prepared and MAP kinase activity was examined by mobility shift assay and by immunocomplex kinase assay. (Throughout the legend and on the figure, the 44-kDa form of MAP kinase is designated MAPK1, while the 42-kDa form is designated MAPK2.) *Panel a* shows the mobility shift of both MAPK1 and MAPK2; the *arrowheads* indicate slower-migrating forms of MAPK1 and MAPK2 corresponding to the activated forms. In *panel b*, the activity of MAP kinases in immunoprecipitates was measured after addition of MBP as a substrate; phosphorylated MBP was separated by a 15% SDS gel and autoradiographed. The MBP bands were also excised and counted in a scintillation counter (*panel c*). The *filled circles* represent radiophosphate incorporated into MBP in cells plated on fibronectin, while the *open circles* represent incorporation in control cells. In *panel d*, confluent Swiss 3T3 cells were kept in suspension (*Sus*) or plated on substrata coated with 10 µg/ml fibronectin (*Fn*) or 100 µM synthetic GRGDSPK peptide (*RGD*) and incubated for varying times at 37 °C. After the incubations, MAP kinase activity was evaluated by the mobility shift assay as described above. The *arrowheads* indicate the activated forms of MAPK1 and MAPK2.

To test the generality of our observations, we examined adhesion-mediated effects on MAP kinases in other fibroblast lines. As seen in Fig. 2 (*left panel*), rat REF52 fibroblasts also displayed a marked activation of MAP kinases subsequent to cell adhesion to fibronectin. Mobility shift assays (*left panel, a*) showed that both the p42 and p44 forms of MAP kinase were activated in these cells. Activation was confirmed using immunocomplex kinase assays (*left panel, b* and *c*). In comparison to Swiss 3T3 cells, there was a higher basal level of MAP kinase

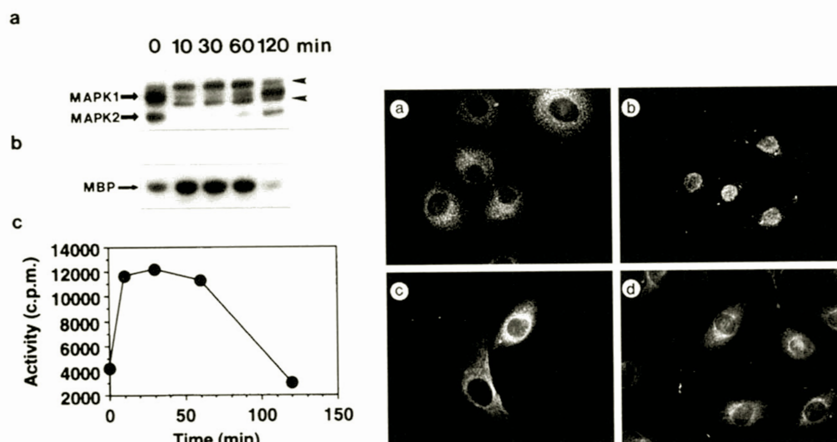

FIG. 2. **MAP kinase activation and immunolocalization in REF52 cells.** Rat fibroblasts (REF52), grown to confluence, were deprived of sera for 48 h. The monolayers were then dissociated with trypsin/EDTA and incubated in suspension for 1 h at 37 °C. The cells were plated on fibronectin-coated dishes for biochemical assay or on fibronectin-coated coverslips for microscopy. *Left panel*, MAP kinase activation. Following incubations on fibronectin-coated substrata for 0–120 min, total cell lysates were prepared and MAP kinase activity was examined by mobility shift assay (*a*) and by immunocomplex kinase assay (*b* and *c*), as described above. *Right panel*, immunolocalization. Following incubation of the cells on fibronectin-coated coverslips for 0–120 min at 37 °C, the samples were fixed and stained with anti-MAP kinase antibody and then rhodamine-conjugated secondary antibody. Stained coverslips were viewed and photographed on a Zeiss Axiophot fluorescence microscope. *a*, after 30 min of attachment to fibronectin; *b*, after 60 min of attachment to fibronectin; *c*, after 120 min of attachment to fibronectin; *d*, control cultures after incubation in serum-free medium.

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-D-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-D-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

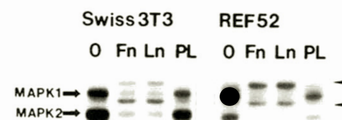

FIG. 3. **MAP kinase activation on fibronectin, laminin, and poly-D-lysine.** Swiss 3T3 or rat REF52 cells were plated in serum-free medium on dishes coated with 10 µg/ml fibronectin, laminin, or poly-D-lysine. After 10 min of incubation, the cells were lysed and MAP kinase activation determined by an electrophoretic mobility shift assay as described above. *Fn*, fibronectin; *Ln*, laminin; *PL*, poly-D-lysine.

plated on fibronectin with cytochalasin D, an inhibitor of actin filament formation (34), almost completely blocked the activation of MAP kinases (Fig. 4). 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-D-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 for adhesion-induced MAP kinase activation, as cytochalasin D treatment strongly inhibited this process. 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

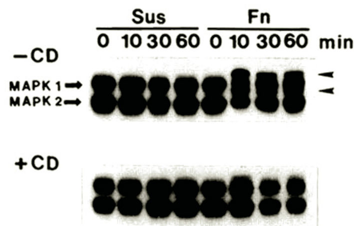

FIG. 4. **The effect of cytochalasin D on MAP kinase activation induced by cell adhesion to fibronectin.** Swiss 3T3 cells in serum-free medium pretreated with 10 μM cytochalasin D for 30 min (*+CD*) and control cells (*−CD*) were kept in suspension (*Sus*) or plated on fibronectin (*Fn*) and incubated at 37 °C for the indicated times (cytochalasin D was included in the medium for the treated group during the incubation). After the incubations, MAP kinase activity was evaluated by the mobility shift assay. Cytochalasin D treatment did not affect cell adhesion but did inhibit cell spreading.

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 substratum. 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 cytoplasm 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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