Integrin-mediated Activation of Mitogen-activated Protein (MAP) or Extracellular Signal-related Kinase Kinase (MEK) and Kinase Is Independent of Ras*

Qiming Chen, Tsung H. Lin, Channing J. Der, and R. L. Juliano‡

From the Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599

The integrins are a family of cell surface receptors that mediate adhesive interactions with the extracellular matrix and also generate signals that influence cell growth and differentiation. Ligation and clustering of integrins causes activation and autophosphorylation of focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase, and results in the transient activation of p42 and p44 mitogen-activated protein (MAP) kinases. Initial evidence has suggested that the integrin signaling pathway may share common elements with the canonical Ras signal transduction cascade activated by peptide mitogens such as epidermal growth factor (EGF). In this report we demonstrate that Raf-1 and MAP or extracellular signal-related kinase kinase (MEK), key cytoplasmic kinases of the Ras cascade, are activated subsequent to integrin-mediated adhesion of mouse NIH 3T3 fibroblasts. We also show that MAP kinase is downstream of MEK in the integrin signaling pathway. However, in contrast to the receptor tyrosine kinase signaling cascade, integrin-mediated signal transduction seems to be largely independent of Ras. Dominant negative inhibitors of Ras-dependent signaling failed to block integrin-mediated activation of MEK. In addition, while treatment with the peptide mitogen EGF clearly increased GTP-loading of Ras, little effect was observed in response to integrin-dependent cell adhesion. Thus, integrin-mediated activation of MEK and MAP kinase in 3T3 cells occurs primarily by a mechanism that is distinct from the Ras signal transduction cascade.

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‡To whom correspondence should be addressed. Tel.: 919-966-4383; Fax: 919-966-5640.

The abbreviations used are: FAK, focal adhesion kinase; MAP, mitogen-activated protein; MAPKK, MAP kinase kinase; MAPKKK, MAP kinase kinase kinase; BSA, crystalline, lipid-free, bovine serum albumin; EGF, epidermal growth factor; Fn, fibronectin; DMEM, Dulbecco’s minimal essential medium; K- MAPK, kinase-dead MAP kinase; MEK, MAP or extracellular signal-related kinase kinase.
bicinchonic acid assay.

Transfections—In some cases, 3T3 cells transfected with a vector expressing the chicken β1 integrin subunit (32) were used. Transfections were performed with LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Cells expressing chicken β1 subunit were selected using magnetic beads (DYNAL) coated with the monoclonal anti-chicken β1 integrin antibody W1B10 (32). The selected cells were expanded in medium containing 250 μg/ml G418 (Life Technologies, Inc.). Cells were further selected twice using MicroCell® ector flasks (Applied Immune Sciences) coated with W1B10, and expanded prior to use. Transient transfections of expression constructs encoding an epitope tagged–MEK (EE-MEK) (33), co-transfection with the Raf 23–284 construct (34), with a membrane-targeted, constitutively activated Sos-1 construct (35), with empty control vectors, or with activated or dominant-negative Ras constructs in the pZI vector (36), were also done using the LipofectAMINE procedure.

Purification of Recombinant Histidine-tagged Wild Type MEK and Kinase-defective MAP kinases—Histidine-tagged wt MEK and kinase-defective MAP kinase were prepared according to Gardner et al. (37) with modification. Briefly, bacterial cell lysates were clarified by centrifugation at 10,000 × g for 15 min at 4 °C, the clear supernatant was incubated with 0.5 ml of Ni²⁺-nitrilotriacetic acid-agarose beads (Qiagen, Chatsworth, CA) in the presence of 25 mM imidazole and 500 mM NaCl, on a rotator for 1 h at 4 °C. The beads then were washed three times with 1 ml of lysis buffer containing 25 mM imidazole and 500 mM NaCl. The histidine-tagged kinase was purified by eluting 3 times with 1 ml of 200 mM imidazole followed by a linear 0–500 mM NaCl gradient in a buffer comprised of 25 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol, 0.01% Nonidet P-40, 0.1% β-mercaptoethanol, and 1 mM benzamidine. Protein concentration was determined using the Coomassie dye-binding assay (Bio-Rad).

Immune Complex Kinase Assays—Cell lysates were preclarified with 30 μl of protein G-Sepharose beads for 15 min at 4 °C. Raf-1, MEK, EE-tagged MEK (33), or MAP kinase were immunoprecipitated by incubation of the lysates at 4 °C for 1 h with appropriate antibodies: 0.5 μg of anti-Raf-1 polyclonal antibody (C-12, Santa Cruz Biotechnology, Santa Cruz, CA) for Raf-1; 2 μg of anti-MEK1 and -2 (M17030, Transduction Laboratories, Lexington, KY) for MEK1 and MEK2; 5 μg of anti-polyEE monoclonal antibody (Onyx Pharmaceuticals, Richmond, CA) for EE-tagged MEK1; 1 μg of anti-ERK1 polyclonal antibody (K-23, Santa Cruz Biotechnology) for p42 and p44 MAP kinases. This was followed by incubation of the immunocomplexes with 30 μl of protein G-Sepharose beads for another 1 h at 4 °C. The beads then were washed with cold RIPA buffer once and a cold washing buffer (0.25 M Tris-HCl, pH 7.5, 0.1 mM NaCl) twice. Changes in MEK, EE-MEK, and MAP kinase activity were monitored by resuspension of immunoprecipitates in kinase assay buffer comprised of 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 25 μM ATP, 5 μCi of [γ-^32P]ATP, and 2 μg of kinase-defective MAP kinase (for MEK and EE-MEK) or 10 μg of myelin basic protein (MBP) (for MAP kinase) and incubating them at room temperature for 30 min. Raf-1 kinase activity was measured by incubating the resuspended immunocomplexes in 30 μl of kinase assay mixture containing 13.3 mM Tris-HCl, pH 7.5, 13.3 mM MgCl₂, 13.3 mM dithiothreitol, 32.5 μM ATP, 5 μCi of [γ-^32P]ATP, and 0.5 μg of wt MEK at room temperature for 15 min followed by adding 10 μl of 0.2 mg/ml kinase-defective MAP kinase and incubating another 15 min at room temperature. Reactions were stopped by adding 20 μl of 3 × SDS sample buffer and boiling for 2 min. The samples were electrophoresed on SDS-polyacrylamide gels. The phosphorylated substrate bands were quantitated either by excising the bands and scintillation counting or by phosphorimaging.

Western Blot and MAP Kinase Mobility Shift Assay—EE-MEK was immunoprecipitated with anti-polyEE antibody as described above and blotted by anti-MEK1 and -2 (0.1 μg/ml). FAK was immunoprecipitated with 4 μg of anti-FAK monoclonal antibody (F15020, Transduction Laboratories), and EGF receptor by 5 μl of an antisera recognizing EGF receptor cytoplasmic domain from Dr. S. Earp (University of North Carolina, Chapel Hill, NC). Tyrosine-phosphorylated FAK and EGF receptor were detected by Western blotting with an anti-phosphotyrosine antibody (145-095, Transduction Laboratories). Loading of FAK was evaluated by Western blotting with an anti-FAK polyclonal antibody as described (14). In some cases, MAP kinase activity was ascertained by a mobility shift assay as described previously (28).

RESULTS

This investigation was designed to compare integrin-mediated signaling with the better known signal transduction pathway triggered through receptor tyrosine kinases and to evaluate the role of Ras in the integrin pathway. Integrin-dependent adhesion of suspended NIH 3T3 cells to a fibronectin-coated substrate led to rapid and robust tyrosine phosphorylation of pp125^FAK, but not of the 185-kDa EGF receptor (Fig. 1, A and C). Conversely, treatment of adherent 3T3 cells with EGF caused activation and autophosphorylation of the EGF receptor, but only slightly enhanced the high basal level of FAK tyrosine phosphorylation typical of attached cells (Fig. 1, B and D). Treatment of suspension cells with EGF strongly activated the EGF receptor, but had no effect on FAK (Fig. 1, B and D). The overall patterns of tyrosine phosphorylation in lysates of cells stimulated by integrin ligation or by peptide growth factors also showed marked differences between the two situa-
Thus, as expected, cell adhesion and growth factor stimulation triggered distinct initial tyrosine phosphorylation events, with EGF primarily activating its cognate receptor and integrin-mediated cell adhesion primarily activating FAK.

The cytoplasmic serine/threonine kinases Raf-1 (a MAP-KKK) and MAP kinase, as well as the dual-function kinase MEK (a MAPKK), were each strongly activated by either integrin-mediated attachment of suspended NIH 3T3 cells to fibronectin or by treatment of adherent cells with EGF (Fig. 2A). The increase in kinase activities over basal levels was usually somewhat higher for maximally effective (100 ng/ml) concentrations of EGF than for coating concentrations of fibronectin (25 μg/ml) that were maximally effective in promoting adhesion. Both the 42-kDa and 44-kDa forms of MAP kinase were activated by either EGF stimulation or integrin-mediated adhesion (28).

The kinetics of integrin activation of Raf, MEK, and MAP kinase were essentially the same, with maximal activation within 10 min of the beginning of the adhesion process and then a gradual decline over a period of 1 to 2 h. This is similar to previously reported kinetics for adhesion-induced MAP kinase activation in Swiss 3T3 cells (28).

To confirm that the observed effect on MAP kinase was specifically due to integrins, we examined kinase activity in 3T3 cells transfected with a chicken β1 integrin subunit and adhering to surfaces coated with an anti-chicken β1 monoclonal antibody or to surfaces coated with an antibody to CD44, an abundant non-integrin cell surface protein (39); adhesion to fibronectin was used as a positive control. As seen in Fig. 2C, specific adhesion mediated by the β1 integrin, but not by CD44,
FIG. 5. Dominant-negative constructs that block Ras signaling do not affect integrin-mediated MEK activation. In A, NIH 3T3 cells were transiently co-transfected using LipofectAMINE with 0.4 μg of pCMV EE-MEK and 0.4, 0.8, or 1.6 μg of pCGNraf 23-284 (total amount DNA was supplemented with pCGN empty vector to 2 μg for each 35-mm tissue culture dish). Transfected cells were placed in suspension and then allowed to attach to a fibronectin-coated substratum as described in the legend of Fig. 1. EE-MEK was immunoprecipitated with an anti-polyEE monoclonal antibody, and EE-MEK activity was monitored by its ability to phosphorylate K-MAPK. B shows a positive control in which NIH 3T3 cells were transiently cotransfected with 0.4 μg of EE-MEK and 0.1 μg of pZIP Ras (12V) and with 0.1 μg of pCGNraf 23-284 or pCGN empty vector (total DNA was supplemented with SP70 DNA to 2 μg/35-mm dish). pCGNraf 23-284 is also known as pCGNraf N4 (34). In C, NIH 3T3 cells were transiently co-transfected with 0.2 μg of EE-MEK construct and 1.8 μg of a construct (pZIP Ras (17N)) encoding the Ras (17N) dominant-negative protein, or with empty pZIP vector. The cells were allowed to attach to Fn for 10 min. In D, NIH 3T3 cells were co-transfected with 0.2 μg of EE-MEK, 0.2 μg of Sos F construct expressing the activated form of Sos-1, or with 0.2 μg of vector (a negative control), and 0.4, 0.8, or 1.6 μg of pZIP Ras (17N) (total DNA was supplemented with pZIP vector to 2 μg). EE-MEK activities were determined as described above. The lower inset in each set of panels shows EE-MEK expression levels measured by Western blot with a polyclonal antibody against MEK1 and -2 in the same cell lysates as for the kinase assay.

led to a robust activation of MAP kinase, as detected by a band shift assay.

We wished to ascertain possible cause and effect relationships between the proteins activated by integrin engagement and to determine whether those relationships were the same as for the consensus Ras cascade activated by receptor tyrosine kinases. To do this, we used pharmacological or molecular tools to selectively inhibit upstream events and then examined the downstream consequences. Thus, a recently described selective inhibitor of MEK (PD98059) (40) allowed us to demonstrate that integrin-mediated MAP kinase activation is blocked when MEK activation is inhibited (Fig. 3). This anticipated result confirms that this segment of the signaling cascades for receptor tyrosine kinases and for integrins are similar.

We looked for evidence of Ras activation in the integrin pathway by determining if a transient increase in active Ras GTP occurs in stimulated cells, using a well-established assay for this purpose (41). As seen in Fig. 4, transfection of cells with oncogenic (61L) Ras, or stimulation of cells with EGF, resulted in clear and substantial increases in GTP loading of Ras. However, integrin-mediated cell adhesion did not produce a significant increase in GTP loading of Ras over control levels. Thus, EGF stimulation increased Ras GTP loading by a factor of 3.1 ± 1.3 over control, while adhesion to fibronectin increased Ras GTP loading by a factor of only 1.3 ± 0.5 over control; the difference between the EGF and adhesion effects was significant at the p < 0.01 level. This suggested that Ras was not strongly activated in response to integrin ligation. These experiments cannot rule out the possibility of small increases in Ras GTP loading as a result of cell adhesion; however, minor changes may not be important in signaling to downstream effectors.

To further investigate the role of Ras in integrin signaling, we used a transient co-transfection assay that allowed measurement of MEK activation in the presence or absence of dominant inhibitors of the Ras pathway. As a positive control, we examined the effect of these dominant inhibitors on activation of MEK by oncogenic forms of Ras or Sos-1, proteins which clearly are integral components of the Ras cascade. We chose to use co-transfection with oncogenic Ras or Sos, rather than EGF stimulation, since the EGF receptor can trigger both Ras-dependent and Ras-independent activation of MAP kinase in fibroblasts (42). Ras-independent activation of Raf and of MAP kinases by tyrosine kinases has also been observed in other mammalian cell types (43, 44) and in Drosophila (45).

NIH 3T3 cells were transiently transfected with an epitope-tagged MEK construct (EE-MEK) whose expressed protein could be immunoprecipitated from the transfected cells and tested for its ability to phosphorylate a mutated, inactive form of MAP kinase. These cells were also co-transfected with constructs expressing a dominant interfering mutant of Raf-1 or with a control vector lacking this insert. The Raf 23–284 (17N) (total DNA was supplemented with pCGNempty vector to 2 μg) was transfected. The cells were allowed to attach to Fn for 10 min. In D, NIH 3T3 cells were co-transfected with 0.2 μg of EE-MEK, 0.2 μg of Sos F construct expressing the activated form of Sos-1, or with 0.2 μg of vector (a negative control), and 0.4, 0.8, or 1.6 μg of pZIP Ras (17N) (total DNA was supplemented with pZIP vector to 2 μg). EE-MEK activities were determined as described above. The lower inset in each set of panels shows EE-MEK expression levels measured by Western blot with a polyclonal antibody against MEK1 and -2 in the same cell lysates as for the kinase assay.

Fig. 5. Dominant-negative constructs that block Ras signaling do not affect integrin-mediated MEK activation. In A, NIH 3T3 cells were transiently co-transfected using LipofectAMINE with 0.4 μg of pCMV EE-MEK and 0.4, 0.8, or 1.6 μg of pCGNraf 23-284 (total amount DNA was supplemented with pCGN empty vector to 2 μg for each 35-mm tissue culture dish). Transfected cells were placed in suspension and then allowed to attach to a fibronectin-coated substratum as described in the legend of Fig. 1. EE-MEK was immunoprecipitated with an anti-polyEE monoclonal antibody, and EE-MEK activity was monitored by its ability to phosphorylate K-MAPK. B shows a positive control in which NIH 3T3 cells were transiently cotransfected with 0.4 μg of EE-MEK and 0.1 μg of pZIP Ras (12V) and with 0.1 μg of pCGNraf 23-284 or pCGN empty vector (total DNA was supplemented with SP70 DNA to 2 μg/35-mm dish). pCGNraf 23-284 is also known as pCGNraf N4 (34). In C, NIH 3T3 cells were transiently co-transfected with 0.2 μg of EE-MEK construct and 1.8 μg of a construct (pZIP Ras (17N)) encoding the Ras (17N) dominant-negative protein, or with empty pZIP vector. The cells were allowed to attach to Fn for 10 min. In D, NIH 3T3 cells were co-transfected with 0.2 μg of EE-MEK, 0.2 μg of Sos F construct expressing the activated form of Sos-1, or with 0.2 μg of vector (a negative control), and 0.4, 0.8, or 1.6 μg of pZIP Ras (17N) (total DNA was supplemented with pZIP vector to 2 μg). EE-MEK activities were determined as described above. The lower inset in each set of panels shows EE-MEK expression levels measured by Western blot with a polyclonal antibody against MEK1 and -2 in the same cell lysates as for the kinase assay.
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not affect the robust activation of EE-MEK caused by integrin-mediated adhesion of 3T3 cells (Fig. 5A). However, co-expression of Raf 23–284 very effectively inhibited activation of EE-MEK caused by simultaneous transfection with a construct expressing a constitutively activated Ras (12V) mutant (Fig. 5B). These results strongly suggest that integrin-mediated activation of MEK, and subsequent activation of MAP kinase in 3T3 cells, are largely independent of Ras activation.

To further rule out the involvement of Ras in integrin signaling, 3T3 cells were co-transfected with the EE-MEK construct and with a construct expressing a mutated form of Ras (Ras 17N) that can act in a dominant-negative manner by blocking guanine nucleotide exchange factors involved in the activation of endogenous Ras (24, 35). As seen in Fig. 5C, transfection with the Ras (17N) construct had no effect on the activation of EE-MEK caused by cell adhesion to fibronectin. However, transfection with the Ras (17N) construct strongly inhibited the activation of EE-MEK caused by expression of a membrane-targeted form (5'-Sos F) of the Sos-1 guanine nucleotide exchange factor for Ras (Fig. 5D). Once again, this suggests that integrin-mediated MEK activation does not involve Ras.

**DISCUSSION**

Treatment of NIH 3T3 cells with peptide mitogens such as EGF, or ligation of integrins, both lead to activation of tyrosine kinases; however, the patterns of tyrosine phosphorylation observed are quite different. EGF activates its cognate tyrosine kinases; however, the patterns of tyrosine phosphorylation observed for Ras, or ligation of integrins, both lead to activation of tyrosine kinases. As seen in Fig. 5A, ras activation of endogenous Ras (24, 35). As seen in Fig. 5C, transfection with the EE-MEK construct had no effect on the activation of EE-MEK caused by cell adhesion to fibronectin. However, transfection with the Ras (17N) construct strongly inhibited the activation of EE-MEK caused by expression of a membrane-targeted form (5'-Sos F) of the Sos-1 guanine nucleotide exchange factor for Ras (Fig. 5D). Once again, this suggests that integrin-mediated MEK activation does not involve Ras.

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