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Integrin-dependent Activation of MAP Kinase: A Link to Shape-dependent Cell Proliferation

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Adhesion to extracellular matrix mediates cell cycle progression in mid-late G1; this effect involves an integrin-dependent organization of the cytoskeleton and a consequent change in cell shape. In an effort to identify potential signal-transducing agents that are associated with integrin-dependent shape changes, we looked for kinase activities that were stimulated by long-term adhesion of G0-synchronized NIH-3T3 cells to fibronectin-coated dishes. Several kinase activities were stimulated by this procedure, two of which migrated at 42 and 44 kDa and phosphorylated myelin basic protein in vitro. Blotting with anti-phosphotyrosine and anti-mitogen–activated protein (MAP) kinase antibodies identified these enzymes as ERK 1 and ERK 2. In contrast to the rapid and transient activation of these MAP kinases by platelet-derived growth factor, stimulation of MAP kinase activity by fibronectin was gradual, persistent, and associated with cell spreading rather than cell attachment itself. Cytochalasin D blocked the activation of MAP kinase activity that was induced by the binding of cells to fibronectin. Moreover, MAP kinase was also activated by adhesion of cells to vitronectin and type IV collagen; these effects were also associated with cell spreading. These results distinguish the regulation of G1 phase MAP kinase activity by soluble mitogens and extracellular matrix. They also implicate MAP kinase in shape-dependent cell cycle progression.

INTRODUCTION

Cell cycle progression in fibroblasts is regulated by growth factors and extracellular matrix proteins. Growth factors stimulate entry of quiescent (G0 synchronized) cells into the cycle, and they continue to mediate cell cycle progression through most of G1 (Pardee, 1989). Many subcellular events have been associated with growth factor-dependent entry into the cell cycle: autophosphorylation of receptor tyrosine kinases, stimulation of phospholipid turnover, activation of the Na/H antiporter, and activation of protein kinase C and mitogen-activated protein (MAP) kinases (Moolenaar, 1986; Ullrich and Schlessinger, 1990; Cantley *et al.*, 1991; Johnson and Vaillancourt, 1994). The induction of these events is typically rapid and transient.

Adhesion to the extracellular matrix is also required for cell cycle progression through G1 (Otsuka and Moskowitz, 1975; Shin *et al.*, 1975; Matsuhisa and Mori, 1981; Guadagno and Assoian, 1991; Guadagno *et al.*, 1993). The signals provided by extracellular matrix result from the interaction with integrins, a large family of specific matrix protein receptors that form by the combinatorial arrangement of distinct α and β subunits (Hynes, 1987, 1992; Albelda and Buck, 1990; Hemler, 1990; Ruoslahti, 1991). The major integrins expressed in fibroblasts include receptors for collagen ($\alpha1\beta1$ and $\alpha2\beta1$), fibronectin ($\alpha5\beta1$), and vitronectin ($\alpha V\beta3$). Although integrins transmit growth regulatory information, their structure is strikingly different from growth factor receptors; they contain very small cytosolic domains and they lack intrinsic tyrosine kinase activity.

Integrin-mediated signaling is required for cell cycle progression both at G0/G1 and in mid-late G1. However, the signals at G0/G1 seem to result from ligand-induced integrin clustering (Schwartz *et al.*, 1991; Schwartz and Lechene, 1992; McNamee *et al.*, 1993) whereas the signals at mid-late G1 require an integrin-

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dependent organization of the cytoskeleton and cell spreading (Folkman and Moscona, 1978; Hansen *et al.*, 1994). Several G0/G1 effects of cell adhesion have been described, and these include stimulation of the Na/H antiporter, phosphoinositol turnover, and protein kinase C (Schwartz *et al.*, 1991; Schwartz and Lechene, 1992; McNamee *et al.*, 1993; Vuori and Ruoslahti, 1993). Interestingly, these events are also growth factor dependent (see above); optimal induction likely requires the cooperative action of integrins and growth factor receptors (Schwartz and Lechene, 1992; McNamee *et al.*, 1993). Focal adhesion kinase (FAK) is also rapidly activated by integrins (Guan and Shalloway, 1992; Hanks *et al.*, 1992; Kornberg *et al.*, 1992; Schaller *et al.*, 1992).

In the studies described here, we looked for signal transduction events that might underlie the mid-late G1 effects of cell adhesion that result from integrin-dependent changes in cell shape. Consistent with the recent work of others (Chen *et al.*, 1994), we found that adhesion of cells to fibronectin results in the stimulation of MAP kinase. However, we also found that adhesion-dependent stimulation of MAP kinase activity in G0-synchronized NIH-3T3 cells is gradual, persistent, and results from an integrin-dependent organization of the cytoskeleton. These results distinguish the G1 phase stimulation of MAP kinase by growth factors and the extracellular matrix, and they implicate integrin-mediated stimulation of MAP kinase in shape-dependent cell cycle progression.

MATERIALS AND METHODS

Cell Culture

NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with gentamicin and 10% calf serum. Flasks containing half-confluent cultures were synchronized in G0 by incubation (18–24 h) in DMEM containing 0.1% calf serum. The synchronized cells were trypsinized, washed twice (in DMEM, 0.5 mg/ml soybean trypsin inhibitor), suspended in serum-free DMEM, and added (5×10^6 cells in 5 ml) to 100-mm dishes that had been coated with fibronectin (50 μg), vitronectin (50 μg), type IV collagen (100 μg), or poly-L-lysine (250 μg). The same procedures were used for NIH-3T3 cells transfected with the human α1 integrin subunit cDNA (α1–3T3; a generous gift of E.E. Marcantonio, Columbia University). In some experiments, the cultures also contained purified recombinant BB-platelet-derived growth factor (PDGF) (10 ng/ml; a generous gift of Gary Grotendorst, University of Miami).

The coated dishes were prepared similarly to the procedure described by Guan *et al.* (1991). Purified matrix protein or polylysine was dissolved in 5 ml phosphate-buffered saline (PBS), added to the dishes, and incubated overnight at 4°C. The medium was removed and 5 ml of heat-inactivated (70°C for 1 h) bovine serum albumin (BSA; 2 mg/ml in PBS) was added to block any remaining nonspecific adhesion sites. After 2 h at 37°C, the BSA solution was removed, and the dishes were washed with PBS before use in experiments. Dishes coated with BSA alone were used to maintain cells in suspension. Vitronectin and type IV collagen were purchased from Life Technologies (Gaithersburg, MD); the fibronectin was either purchased (Life Technologies) or prepared by affinity chromatography on gelatin Sepharose-4B as described by the manufacturer (Pharmacia, Piscataway, NJ).

To measure induction of DNA synthesis in response to PDGF and fibronectin, G0-synchronized NIH-3T3 cells (10^4 in 1 ml DMEM) were added to 6-well plates coated with BSA or fibronectin/BSA. The coating was performed as described above except that 10 μg of fibronectin and 2 mg of BSA were dissolved in 1 ml PBS and used for coating. Some samples also received purified PDGF (10 ng/ml final concentration) or fetal calf serum (5% final concentration). [^{3}H]thymidine (1 uCi) was added to each sample (either 0 or 12 h after seeding). After incubation for 18 h, the cells were fixed and washed in 5% trichloroacetic acid (TCA; Han *et al.*, 1993); the TCA-insoluble radioactivity was counted.

Immunoblotting

Cells attached to extracellular matrix proteins or polylysine were scraped and extracted in 50–100 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM sodium fluoride, and 0.1 mM sodium orthovanadate). Nonadherent cells were collected by centrifugation ($250 \times g$, 5 min, 4°C) and extracted as described above. After a 5-min incubation on ice, the cell lysates were cleared by centrifugation at $16,000 \times g$. The amount of protein in each lysate was determined by Coomassie binding (Bio-Rad protein assay; Richmond, CA). Either 100 or 200 μg of protein from each sample was fractionated on reducing sodium dodecyl sulfate-polyacrylamide gels (10% acrylamide) and electroeluted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH). For immunoblotting with the anti-phosphotyrosine monoclonal antibody (4G10; Upstate Biotechnology, Lake Placid, NY), the membranes were blocked with 3% BSA (fraction V, Life Technologies) in washing buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2.5 mM EDTA, and 0.1% Tween-20) for 1 h. For immunoblotting with the anti-MAP kinase monoclonal antibody (clone Z033; Zymed, South San Francisco, CA), the membranes were blocked with 5% non-fat milk in washing buffer. For all blots, the first antibody was diluted 1000-fold in 10 ml of fresh blocking solution and incubated with the filters for 1 h. The filters were washed for 30 min, incubated with horseradish peroxidase-conjugated anti-mouse IgG, and processed for enhanced chemiluminescent detection of immunoreactive protein using procedures similar to those described by the manufacturer (Amersham, Arlington Heights, IL). All incubations were performed at room temperature.

Immunofluorescence and Laser Scanning Confocal Microscopy

G0-synchronized NIH-3T3 cells (5×10^5 in 1 ml DMEM) were added to 6-well plates containing coverslips that had been coated with polylysine (50 μg) or fibronectin (20 μg). After 1 h at 37°C, the cells were fixed (with PBS, 3.7% formaldehyde, 5 min) and permeabilized (with 25 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Triton X-100, 2 min). The coverslips were incubated (1 h at room temperature) with a rabbit anti-MAP kinase antibody (diluted 100-fold into 0.5 ml PBS, 2% BSA; Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated (30 min at 4°C) with fluorescein isothiocyanate-conjugated goat antiserum to rabbit IgG (diluted 200-fold into 0.5 ml PBS, 2% BSA; Life Technologies). The coverslips were washed with PBS and mounted with Slowfade (Molecular Probes, Eugene, OR). The MAP kinase staining pattern was visualized by confocal microscopy as described (Bourguignon and Jin, 1995).

RESULTS

In an effort to identify kinases that are activated by cell adhesion, G0-synchronized NIH-3T3 cells were attached to fibronectin-coated dishes for 1 h in serum-free medium. The relatively long incubation period was chosen in an effort to screen out kinases that are

transiently activated in direct response to integrin binding and select for those associated with the longer term effects of adhesion on cytoskeletal organization and cell spreading. We fractionated lysates of the attached cells (FN) on sodium dodecyl sulfate gels containing myelin basic protein and performed in-gel kinase assays (Kameshita and Fujisawa, 1989). Lysates of suspended cells (cultured on BSA-coated dishes; negative control) were fractionated in parallel. As shown in Figure 1, we detected several kinases that were able to phosphorylate myelin basic protein (panel A). The enzymatic activity for two of these kinases (approximately 44 kDa and 42 kDa, arrowheads) was significantly induced in the cells attached to fibronectin. Enzymatic activity associated with the 44-kDa protein was always much less than that of the 42-kDa protein.

Aliquots of identically prepared lysates were subjected to an immunoblot analysis using anti-phosphotyrosine antibodies (Figure 1B). As expected, we observed an adhesion-dependent increase in tyrosine phosphorylation of a 120-kDa protein (arrowhead) that we identified as pp125FAK by immunoprecipitation with specific antibodies (our unpublished observations). However, we also observed an increase in the tyrosine phosphorylation of a 44-kDa and a 42-kDa protein in the cells attached to fibronectin. The phosphotyrosine signal at 44 kDa was always less than that at 42 kDa.

Based on the properties of the kinases characterized to date, the results described above suggested that adhesion of cells to fibronectin was resulting in the stimulation of MAP kinases (Cooper *et al.*, 1984; Anderson *et al.*, 1990; Erickson *et al.*, 1990). There are several members of the MAP kinase family of which ERK1 and ERK2 are the best characterized. These enzymes phosphorylate myelin basic protein on threonine in vitro, they have molecular masses of 44 and 42 kDa, respectively, and they are phosphorylated on tyrosine (and threonine) coincident with stimulation. Therefore, the blot used in Figure 1B was reprobed with an anti-MAP kinase antibody. As shown in Figure 1C, ERK2 was much more abundant than ERK1, and the presence or absence of substratum had no effect on the amounts of either protein. However, the ERK1 and ERK2 derived from adherent cells showed an upward gel-shift that is a hallmark of MAP kinase phosphorylation on threonine and tyrosine residues (Leevers and Marshall, 1992). In this experiment, we observed an almost complete activation of MAP kinase assessed by the stimulation of tyrosine phosphorylation and gel-shift, but the results of many independent experiments indicate that the extent of activation is typically 30–60% (see RESULTS below). Thus, we found that long-term adhesion of G0-synchronized NIH-3T3 cells to fibronectin stimulates the activities of MAP kinases ERK1 and ERK2. When taken together, the data in Figure 1 also show that the weaker enzymatic activity and

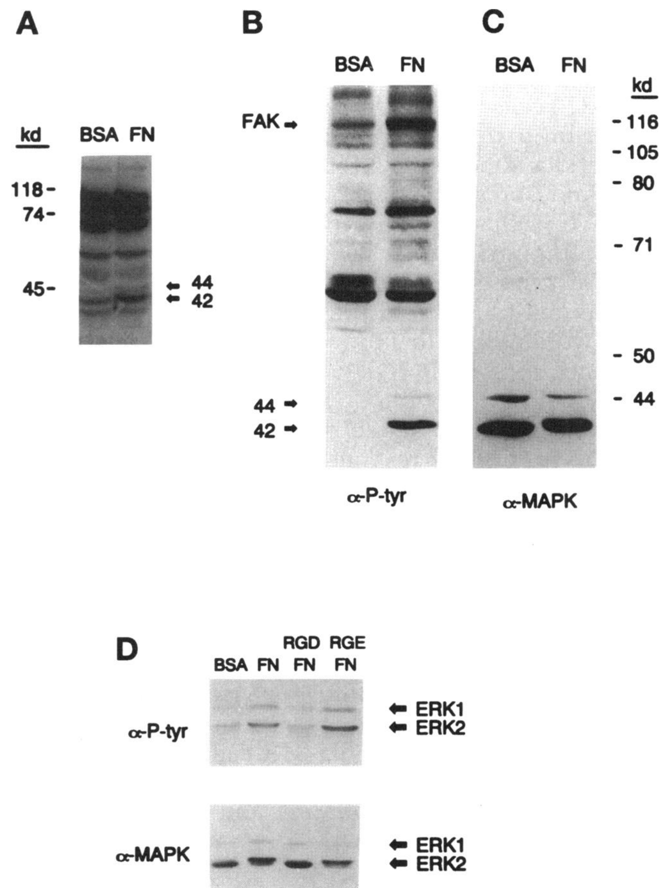


Figure 1. Activation of ERK-1 and ERK-2 by cell adhesion to fibronectin. G0-synchronized NIH-3T3 cells were added to dishes coated with fibronectin (FN; adherent cells) or BSA (nonadherent cells) in serum-free medium. After 1 h at 37°C, the cells were collected and extracted. (A) Cell lysates (50 μg) were analyzed for kinase activity toward myelin basic protein using an in-gel kinase assay performed as described (Kameshita and Fujisawa, 1989). (B) Cell lysates (200 μg) were analyzed for phosphotyrosyl-containing proteins by immunoblotting with anti-phosphotyrosine antibody (α-P-tyr). (C) The filter used in panel B was stripped and reprobed with an anti-MAP kinase antibody (α-MAPK). (D) G0-synchronized NIH-3T3 cells were added to fibronectin-coated dishes in the presence and absence of 1 mM RGDS or 1 mM RGES for 1 h at 37°C. An equal number of cells were also added to BSA-coated dishes as negative control. The cells were collected and extracted. (Adherent and nonadherent cells were pooled in the RGDS-treated culture.) Aliquots of the resulting lysates were subject to an immunoblot analysis using anti-phosphotyrosine and anti-MAP kinase antibodies. Under the experimental conditions we used, RGDS inhibited approximately two-thirds of cell adhesion and cell spreading. RGES affected neither adhesion nor spreading. Molecular weight standards are indicated on panels A-C; the positions of Pp 125FAK, 44-kDa ERK1, and 42-kDa ERK2 are indicated by the arrowheads.

phosphotyrosine signal of the 44-kDa protein (relative to the 42-kDa protein) merely reflects the relative steady-state levels of ERK1 and ERK2. (In some experiments [see below], the weak signal derived from ERK1 was not readily detected).

To demonstrate that the fibronectin-mediated stimulation of MAP kinase activity did not result from contamination by growth factors, G0-synchronized NIH-3T3 cells were added to fibronectin-coated dishes in serum-free medium supplemented with the tetrapeptides RGDS or RGES. RGDS inhibits cell adhesion and spreading on fibronectin whereas RGES is inactive in this regard (Pierschbacher and Ruoslahti, 1984). Immunoblotting with phosphotyrosine and anti-MAP kinase antibodies (Figure 1D) showed that activation of ERK1 and ERK2 was inhibited when the cells were added to fibronectin-coated dishes in the presence of soluble RGDS (Figure 1, compare lanes 1–3). RGES was without effect (Figure 1, compare lanes 1, 2, and 4). These results strongly argue that fibronectin itself is responsible for the stimulation of MAP kinase activity in this system. As expected, neither RGDS nor RGES inhibited the stimulation of MAP kinase activity when PDGF was added to nonadherent NIH-3T3 cells (our unpublished observations).

To compare the stimulation of MAP kinase activity in response to growth factors and fibronectin, we developed a serum-free culture system that exhibited both the mitogen and adhesion requirements for G1 cell cycle progression. NIH-3T3 cells were synchronized in G0 by serum starvation, trypsinized, suspended in serum-free medium, and added to dishes coated with fibronectin in the presence of PDGF (Figure 2). Incubation of these cells with [^{3}H]thymidine showed that the PDGF/fibronectin-treated cells entered S phase and synthesized DNA almost as efficiently as cells treated with serum. Cells exposed only to PDGF ("PDGF") or fibronectin ("FN") did not undergo efficient DNA synthesis. Using this system, G0-synchronized NIH-3T3 cells were cultured with PDGF (in suspension) or on fibronectin (in the absence of mitogen) for 0 to 3 h. Cells were collected, extracted, and the activation of MAP kinase was determined by immunoblotting with anti-phosphotyrosine and anti-MAP kinase antibodies.

As shown in Figure 3A, treatment of suspended cells with PDGF resulted in the activation of ERK2 as determined by tyrosine phosphorylation and gel-shift. Note that stimulation was rapid (maximal at approximately 10 min) and transient. These results are consistent with several previous studies (Ray and Sturgill, 1987; Rossomando *et al.*, 1989; Ahn *et al.*, 1990). In contrast, when cells were added to fibronectin-coated dishes in serum-free medium, the activation of MAP kinase (again determined by both phosphotyrosine content and gel-shift analysis of ERK2) was gradual and persistent. Stimulation was maximal at 1 h and remained elevated for at least 3 h (Figure 3B). No synergistic effect was detected when G0-synchronized NIH-3T3 cells were exposed simultaneously to both fibronectin and PDGF (our unpublished observations).

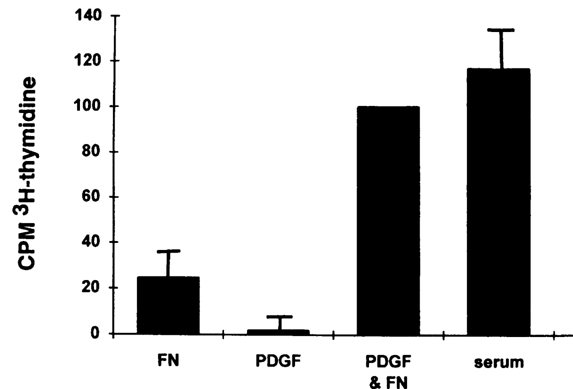
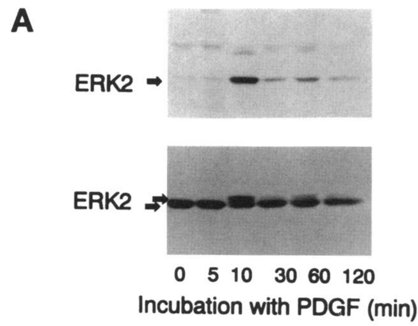


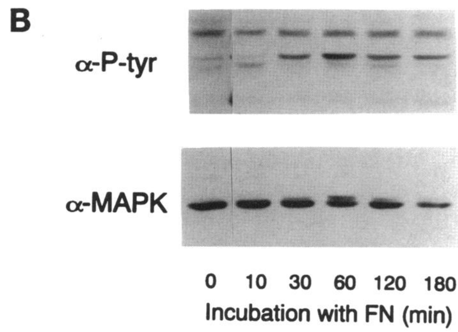
Figure 2. Induction of DNA synthesis in NIH-3T3 cells exposed to PDGF and fibronectin. G0-synchronized NIH-3T3 cells were added to 6-well plates coated with BSA or fibronectin in the presence and absence of PDGF (see MATERIALS AND METHODS). Cells treated with 5% FCS served as positive control. [^{3}H]thymidine was added to each culture to label newly synthesized DNA. After an 18-h incubation, the cells were fixed in 5% TCA and processed for TCA-insoluble radioactivity. The results presented show the mean ± S.E.M. for three separate experiments.

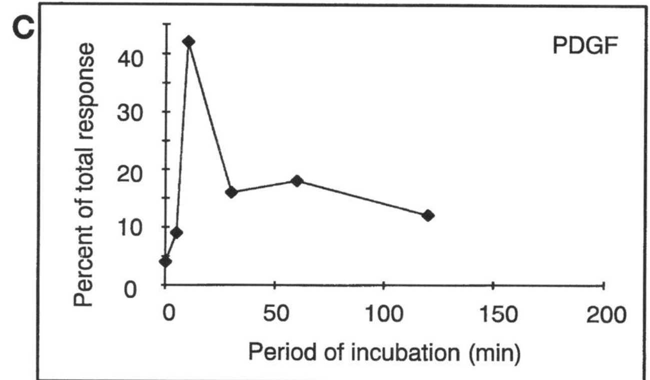
(In this experiment, the weak signal expected from ERK1 [refer to Figure 1] was detected only in the phosphotyrosine blot and only after a 10-min exposure to PDGF. The constitutively expressed signal in the phosphotyrosine blots of panels A and B migrates with a mobility that is slightly slower than ERK1 and represents nonspecific binding).

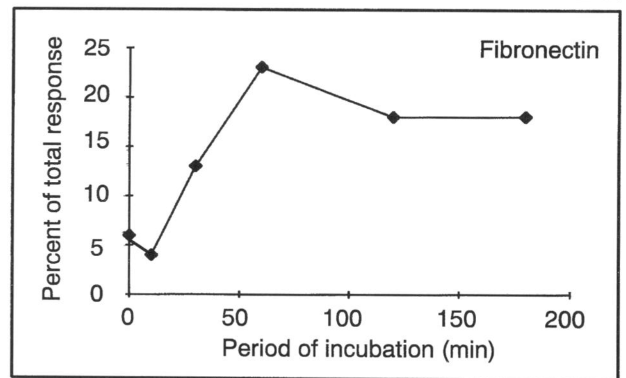
Densitometric scans of the ERK2 phosphotyrosine blots from panels A and B emphasize the very different kinetics by which PDGF and fibronectin activate MAP kinase (Figure 3C). Interestingly, the relatively slow activation of MAP kinase in response to fibronectin binding did not reflect the time required for cell attachment; in several separate experiments attachment was completed within 10–30 min. However, the time course of fibronectin-mediated MAP kinase activation did correspond to the time-dependent spreading (Figure 3D) that results from cell attachment to fibronectin.

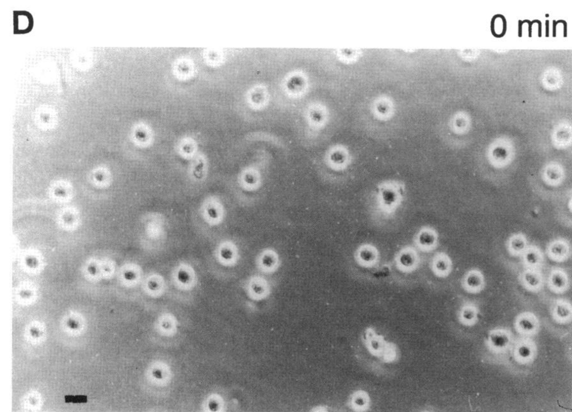
Figure 3. Sequential activation of MAP kinase by PDGF and fibronectin. For the experiment shown in panel A, G0-synchronized NIH-3T3 cells were added to BSA-coated dishes, and the resulting cell suspensions were exposed to PDGF for 0, 5, 10, 30, 60, and 120 min at 37°C. For the experiment shown in panel B, G0-synchronized NIH-3T3 cells were incubated in fibronectin-coated dishes for 0, 10, 30, 60, 120, and 180 min at 37°C. Cell lysates were analyzed by immunoblotting with anti-phosphotyrosine (α-P-tyr) and anti-MAP kinase (α-MAPK) antibodies. Arrowheads indicate the positions of 42-kDa ERK2 (α-P-tyr blot) and the gel-shift of activated ERK2 (α-MAPK blot). Panel C shows the results obtained when the anti-phosphotyrosine blots from panels A and B were scanned on a densitometer. The phase contrast micrographs in panel D show the time-dependent spreading of NIH-3T3 cells on dishes coated with fibronectin; the magnification bars correspond to 20 μm.

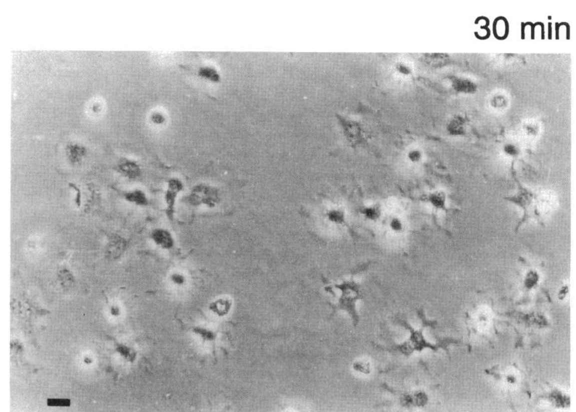

A
ERK2
ERK2
0 5 10 30 60 120
Incubation with PDGF (min)


B
α-P-tyr
α-MAPK
0 10 30 60 120 180
Incubation with FN (min)


C
PDGF
Percent of total response
Period of incubation (min)


Fibronectin
Percent of total response
Period of incubation (min)


D
0 min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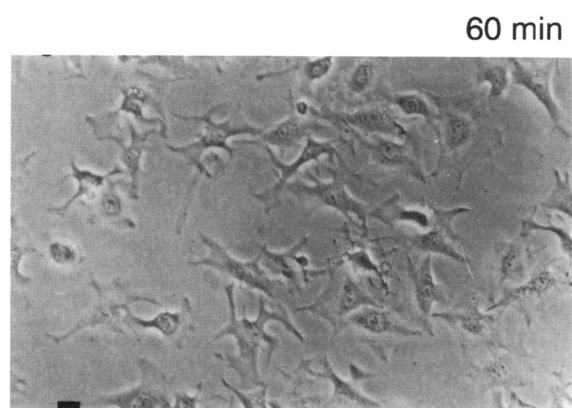
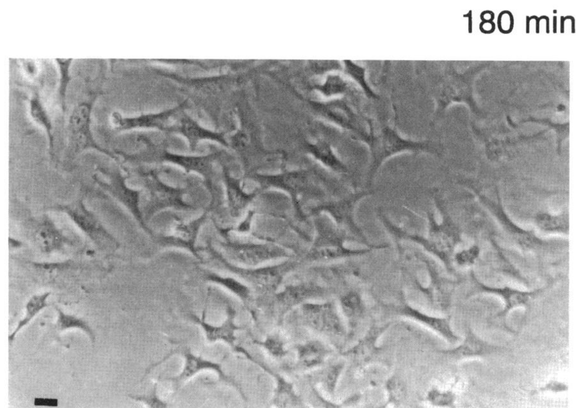

60 min


180 min

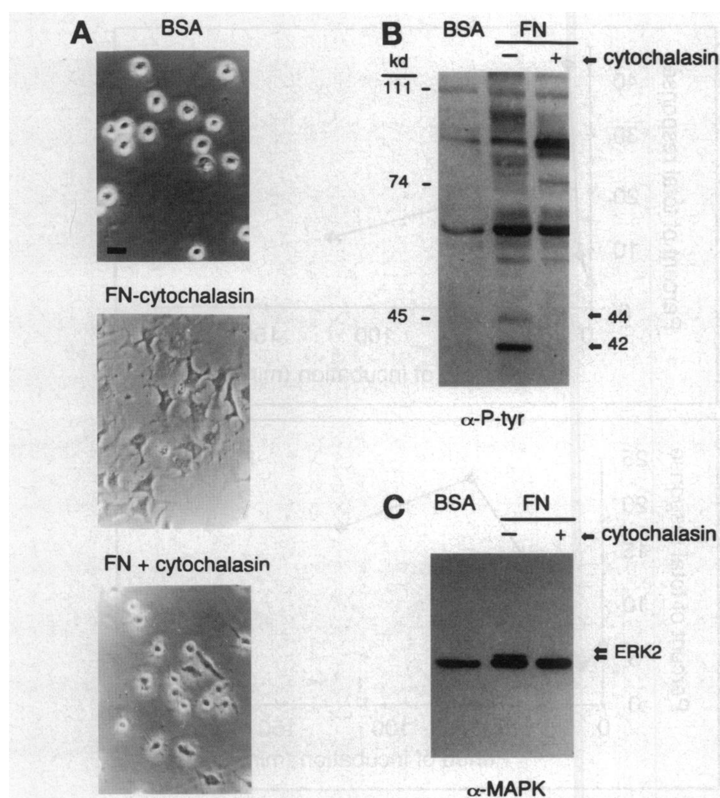


Figure 4. Cytochalasin D inhibits integrin-dependent activation of MAP kinase. G0-synchronized NIH-3T3 cells were added to fibronectin-coated dishes in the presence and absence of cytochalasin D (1 μM). Panel A shows phase contrast micrographs of the cells after 1 h of incubation at 37°C (the magnification bar corresponds to 15 μm). These cells were then collected and extracted. The resulting cell lysates were analyzed for activation of MAP kinase by immunoblotting with anti-phosphotyrosine (panel B) and anti-MAP kinase (panel C) antibodies. Arrowheads indicate the positions of 44-kDa ERK1 and 42-kDa ERK2 (α-P-tyr blot) and the gel-shift of activated ERK2 (α-MAPK blot). The migration of molecular weight standards is shown for panel B.

Considering the screening system we devised to identify adhesion-dependent kinase activities, the kinetic correlation between stimulation of MAP kinase and cell spreading was not unexpected. However, to determine if MAP kinase is activated in response to cell spreading, we blocked adhesion-dependent polymerization of actin by exposing cells to cytochalasin D during the 1-h incubation with fibronectin. The addition of cytochalasin had minimal effect on cell attachment, but it efficiently blocked the consequent change in cell shape (Figure 4A).

Control and cytochalasin-treated cells were extracted and analyzed for activation of MAP kinase by immunoblotting with anti-phosphotyrosine and anti-MAP kinase antibodies. As expected, fibronectin stimulated tyrosine phosphorylation of ERK1 and ERK2 (Figure 4B, arrowheads at 44 and 42 kDa) relative to cells maintained in suspension (BSA). Treatment with cytochalasin D blocked the stimulation of ERK1 and ERK2 tyrosine phosphorylation in response to fibronectin. This effect was specific because cytochalasin D did not inhibit the tyrosine phosphorylation of other proteins when NIH-3T3 cells were attached to fibronectin (e.g. refer to the proteins between 50 and 70 kDa in Figure 4B). As expected, cytochalasin D did block the activation of ERK2 as determined by gel-shift (Figure 4C; note that the weak protein signal for ERK1 was poorly detected in this experiment). We conclude that the polymerization of actin is required for adhesion-dependent activation of MAP kinase.

We also asked whether adhesion to fibronectin resulted in the translocation of MAP kinase from cytoplasm to nucleus (an additional characteristic of MAP kinase activation). G0-synchronized NIH-3T3 cells were incubated with coverslips coated with polylysine or fibronectin. Adhesion to fibronectin was also performed in the presence of cytochalasin D in an effort to examine the role of spreading in MAP kinase translocation. After a 1-h incubation, the coverslips were fixed, and the intracellular location of MAP kinase was determined histochemically using an anti-MAP kinase antibody. Conventional epifluorescent microscopy poorly resolved the cytoplasm from the nucleus in those samples (cells treated with cytochalasin or attached to polylysine) in which spreading was blocked. However, optical sectioning with confocal fluorescent microscopy enhanced the resolution of these subcellular compartments and permitted the examination of MAP kinase translocation. As shown by the confocal micrographs in Figure 5, MAP kinase immunofluorescence was associated primarily with the cytoplasm in cells attached to polylysine and primarily with the nucleus in cells attached to fibronectin. MAP kinase immunofluorescence was also primarily cytoplasmic when cells were attached to fibronectin in the presence of cytochalasin D. Analysis of multiple optical sections showed that MAP kinase immunofluorescence was directly proportional to the amount of nucleus only when cells were both attached and spread on fibronectin.

If the stimulation of MAP kinase reflects organization of the cytoskeleton rather than the signaling potential of specific integrins, then adhesion of cells to other extracellular matrix proteins should be able to mimic the effects we observe with fibronectin. To address this issue, G0-synchronized NIH-3T3 cells were added to dishes coated with fibronectin, collagen, or vitronectin in serum-free medium. The cells were also added to dishes coated with polylysine, which mediates nonspecific adhesion that is independent of integrins. Our NIH-3T3 cells do not attach and spread on dishes coated with either type I or IV collagens, so these experiments were performed with NIH-3T3 cells transfected with the complete cDNA for human α1 integrin subunit (α1–3T3 cells). As determined by surface radioiodination and

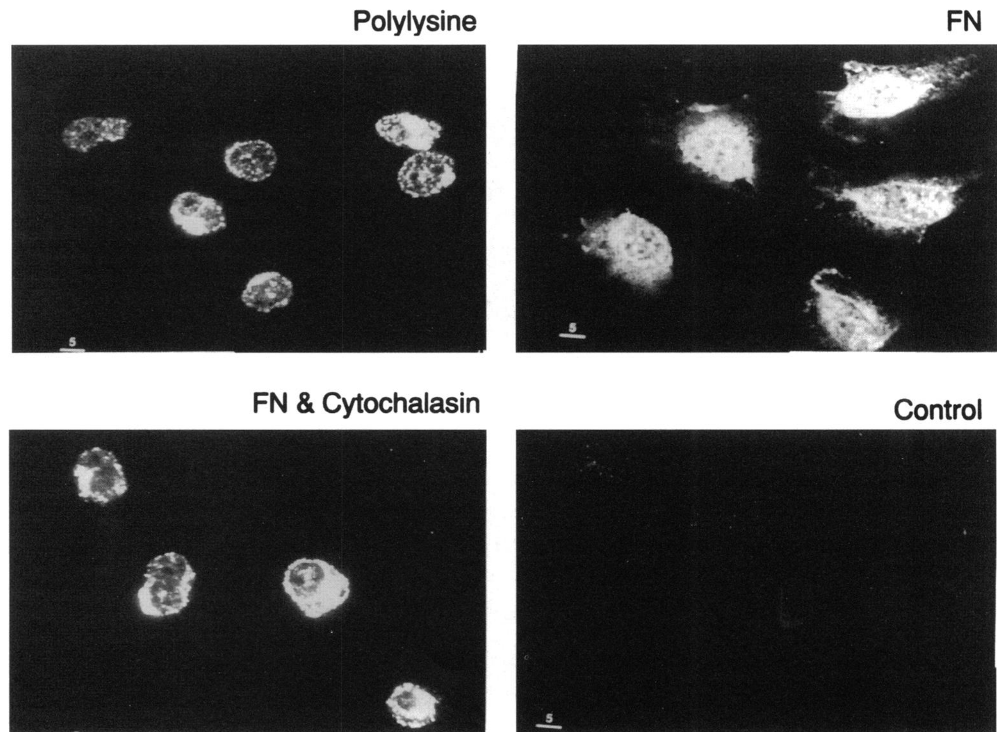


Figure 5. Cell spreading on fibronectin alters the subcellular localization of MAP kinase. G0-synchronized NIH-3T3 cells were incubated for 1 h with coverslips that had been coated with polylysine or fibronectin. Adhesion to fibronectin was performed in the absence and presence of 1 μM cytochalasin D. The subcellular location of MAP kinase was determined by immunofluoresence using laser scanning confocal microscopy. Cells attached to fibronectin (in the absence of cytochalasin) were also incubated with rabbit anti-mouse IgG and analyzed in parallel to control for nonspecific immunofluorescence. Cell thickness was 4–6 μm for NIH-3T3 cells attached to fibronectin and 10–14 μm for cells attached to polylysine or cells attached to fibronectin in the presence of cytochalasin D. For each panel, images were obtained with multiple optical sections of 0.6 μm. A representative optical section is shown for each panel. The magnification bars correspond to 5 μm.

immunoprecipitation, these transfectants express readily detectable amounts of $\alpha1\beta1$ (a type IV collagen receptor), about two- to threefold more $\alpha5\beta1$ integrin (the classic fibronectin receptor), and about threefold less $\alpha V\beta3$ integrin (the classic vitronectin receptor) on their cell surface (Hynes, 1987, 1992; Albelda and Buck, 1990; Hemler, 1990; Ruoslahti, 1991; Vandenberg *et al.*, 1991) We found no detectable $\alpha V\beta1$ integrin on the surface of α1–3T3 cells.

Consistent with the results in parental NIH-3T3 cells, a 1-h incubation of G0-synchronized α1–3T3 cells on fibronectin (FN) resulted in the activation of ERK1 and ERK2; assessed by immunoblotting with antibodies directed against phosphotyrosine (Figure 6A; refer to the arrowheads at 44 and 42 kDa) and MAP kinase (Figure 6B; refer to the gel-shift of ERK2). Vitronectin (VN) or type IV collagen (COLL) did not activate MAP kinase in this same time period (Figure 6, A and B) despite the fact that at least 70% of the cells were attached to these matrix proteins. However, we observed that α1–3T3 cells spread faster on fibronectin than on either vitronectin or type IV collagen, and that a 3-h incubation period on vitronectin and type IV collagen resulted in cell spreading that was comparable to that seen at 1 h with fibronectin (Figure 6B). In fact, when extracts were prepared from cells incubated for 3 h, the analysis of phosphotyrosine content and ERK2 gel-shifts (panels A and B, respectively) showed that all three of the extracellular matrix proteins we tested were able to activate MAP kinase. Importantly, MAP kinase was not activated by either a 1-h or a 3-h incubation on polylysine (Figure 6A), and treatment of the cells with cycloheximide (10 μg/ml; 1 h before and then throughout the incubation period) failed to block stimulation of MAP kinase in response to type IV collagen (our unpublished observations). These controls indicate that the delayed stimulation of MAP kinase did not result from the secretion and deposition of cellular fibronectin by α1–3T3 cells. We conclude that (i) multiple integrins can activate MAP kinase, (ii) this stimulation is not specific to the $\alpha\beta1$ family, and (iii) stimulation is associated with spreading rather than attachment itself.

DISCUSSION

Many recent studies have examined the activation and properties of the MAP kinases, which are now established mediators of growth factor action (Erickson *et al.*, 1990; Payne *et al.*, 1991; Pelech and Sanghera, 1992; Avruch *et al.*, 1994; Blumer and Johnson, 1994; Johnson and Vaillancourt, 1994). There are several members of the MAP kinase family; ERK1 and ERK2 are the most studied. MAP kinases phosphorylate myelin basic protein on threonine, the kinases themselves are activated by phosphorylation (on threonine 183 and tyrosine 185 for ERK2) and these phosphorylations are catalyzed by the dual specificity enzyme MEK. MEK can be activated by c-raf, and c-raf activity is regulated (positively and negatively) through a number of path-

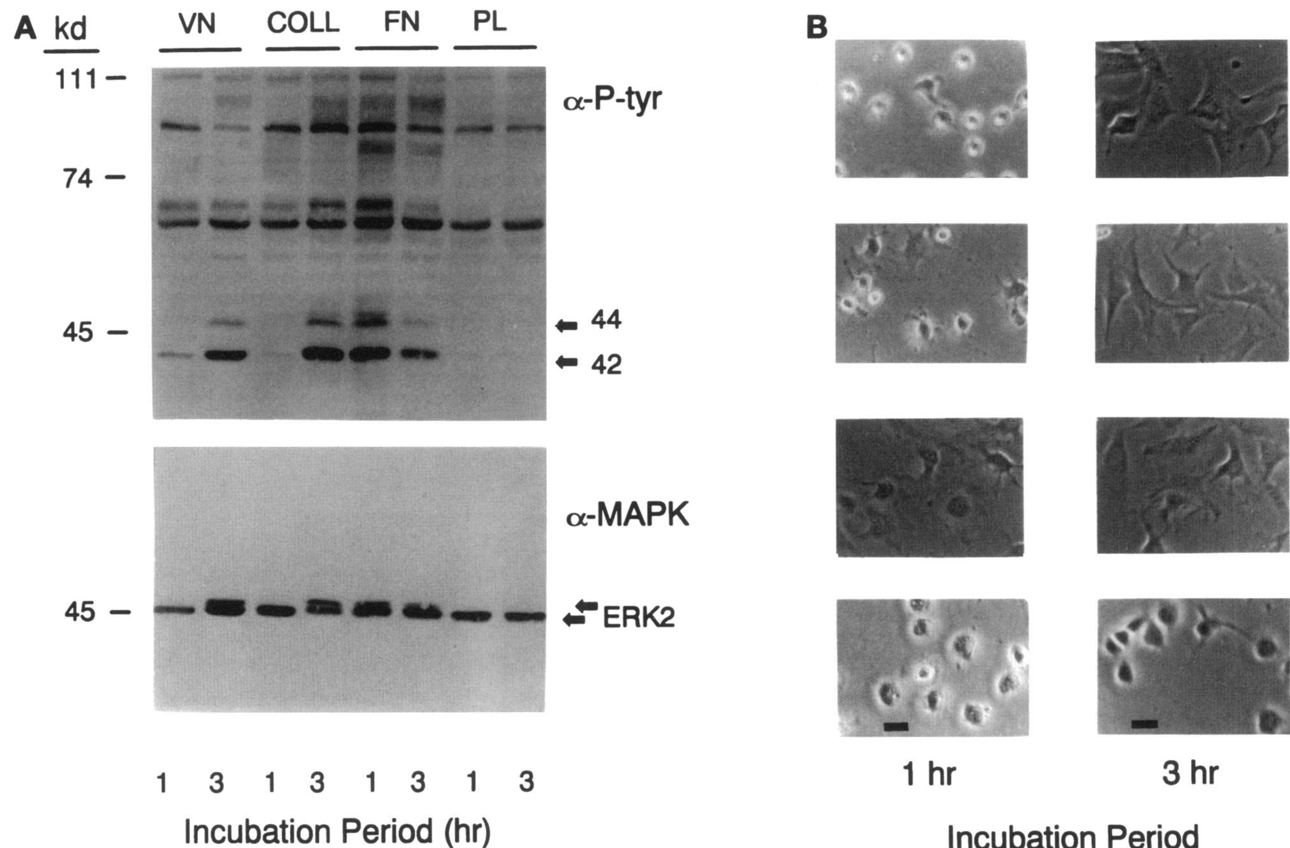


Figure 6. Activation of MAP kinase in response to cell spreading on vitronectin and collagen. G0-synchronized α1–3T3 cells were suspended in serum-free medium and added to dishes coated with vitronectin (VN), type IV collagen (COLL), fibronectin (FN), or polylysine (PL) for 1 and 3 h at 37°C. After collection and extraction, the resulting lysates were analyzed by immunoblotting with anti-phosphotyrosine and anti-MAP kinase antibodies (A). Arrowheads indicate the positions of 44-kDa ERK1 and 42-kDa ERK2 (α-P-tyr blot) and the gel-shift of activated ERK2 (α-MAPK blot). Panel B shows phase contrast micrographs taken at 1 and 3 h after addition of α1–3T3 cells to dishes coated with vitronectin, type IV collagen, fibronectin, and polylysine (magnification bars correspond to 20 μm). The positions taken by molecular weight standards are shown on the left side of panel A.

ways involving receptor tyrosine kinases, heterotrimeric G-proteins, ras, protein kinase A, and protein kinase C. Because MAP kinase is believed to regulate the activity of certain transcription factors such as c-myc and c-jun, activation of MAP kinase in response to extracellular matrix links integrin signaling to adhesion-dependent cell cycle control.

Despite a shared ability to activate MAP kinase, our results show that the effects of PDGF and extracellular matrix on MAP kinase differ. Growth factor-dependent activation of MAP kinase is rapid and transient whereas the activation in response to extracellular matrix protein is gradual and persistent. Because most studies on MAP kinases use adherent, G0-synchronized cells, results obtained under those conditions likely represent a composite of the effects induced individually by mitogens and adhesion. Our data indicate that MAP kinase is induced sequentially, first by mitogens and then by matrix, during the course of G1 cell cycle progression.

A second difference between growth factor and matrix-dependent stimulation of MAP kinase is that growth factor receptors interact directly with the signal-transducing agents that lead to MAP kinase stimulation (Schlessinger, 1993; Johnson and Vaillancourt, 1994) whereas stimulation in response to integrins is either coincident with or downstream of cytoskeletal organization. We base this conclusion on the kinetic relationship between the stimulation of MAP kinase and cell spreading (shown for fibronectin, type IV collagen, and vitronectin), and by the fact that cytochalasin D blocked the stimulation of MAP kinase in response to fibronectin (determined by phosphotyrosine content, gel-shift, and nuclear translocation).

Interestingly, organization of the cytoskeleton is also required for integrin-stimulated tyrosine phosphorylation of focal adhesion kinase (Guan *et al.*, 1991). Tyrosine phosphorylation of FAK (resulting from the adhesion of cells to fibronectin) generates a binding site for GRB2 and can result in the activation of MAP

kinase (Schlaepfer *et al.* 1994). A FAK/GRB2-dependent activation of MAP kinase is consistent with our data, especially because the tyrosine phosphorylation of FAK is also stimulated by the binding of cells to collagen and vitronectin (Burridge *et al.*, 1992; Kornberg *et al.*, 1992). However, the cytosolic domain of $\alpha V\beta 3$ integrin (the classic vitronectin receptor) can also interact with IRS-1 (Vuori and Ruoslahti, 1994); this association also has the potential to activate MAP kinase (Schlessinger, 1993). Thus, the particular mechanism used to activate MAP kinase in response to cell adhesion may vary in different cells or under different situations. Perhaps tyrosine phosphorylation of paxillin (Burridge *et al.*, 1992; Birge *et al.*, 1993) or the SH2 domain of tensin (Davis *et al.*, 1991) may also play roles in adhesion-dependent MAP kinase activation.

Chen *et al.* (1994) have used Swiss 3T3 and REF52 fibroblasts to show that adhesion of cells to fibronectin or laminin results in the stimulation of MAP kinase and that this effect is blocked by cytochalasin. In general, our data support those of Chen *et al.* However, we found that adhesion induces a gradual and persistent stimulation of MAP kinase that is associated with cell spreading whereas Chen *et al.* found that MAP kinase stimulation by fibronectin is rapid and transient. The basis for these differences is not clear, but they could result from the different cell lines used or the fact that the cells were in different states of synchrony (serum starved vs. confluent) before use.

Studies examining growth factor–dependent activation of the Na/H antiporter and stimulation of phosphoinositol turnover have shown that these events also require signals provided by integrins. However, optimal induction of these G0/G1 markers requires concomitant signaling from integrins and growth factor receptors (Schwartz and Lechene, 1992; McNamee *et al.*, 1993); the activation of MAP kinase by growth factors and adhesion seems sequential. Perhaps proper activation of early G1 cell cycle events requires simultaneous signals from growth factor receptors and integrins whereas the shape-dependent cell cycle events at mid-late G1 do not. Considering its close relationship to cell spreading and its established role in transducing proliferative signals, we are currently attempting to characterize shape-dependent cell cycle events that are regulated by the integrin-dependent activation of MAP kinase.

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REFERENCES

Ahn, N.G., Weiel, J.E., Chan, C.P., and Kreb, E.G. (1990). Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from Swiss 3T3 cells. J. Biol. Chem. *265*, 11487–11494.

Albelda, S.M., and Buck, C.A. (1990). Integrins and other cell adhesion molecules. FASEB J. *4*, 2868–2880.

Anderson, N.G., Maller, J.L., Tonks, N.K., and Sturgill, T.W. (1990). Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature *343*, 651–653.

Avruch, J., Zhang, X., and Kyriakis, J.M. (1994). Raf meets ras: completing the framework of a signal transduction pathway. Trends. Biochem. Sci. *19*, 279–283.

Birge, R.B., Fajardo, J.E., Reichman, C., Shoelson, S.E., Songyang, Z., Cantley, L.C., and Hanafusa, H. (1993). Identification and characterization of a high-affinity interaction between v-crk and tyrosine-phosphorylated paxillin in CT10-transformed fibroblasts. Mol. Cell. Biol. *13*, 4648–4656.

Blumer, K.J., and Johnson, G.L. (1994). Diversity in function and regulation of MAP kinase pathway. Trends. Biochem. Sci. *19*, 236–240.

Bourguignon, L.Y.W., and Jin, H. (1995). Identification of the ankyrin-binding domain of the mouse T-lymphoma cell IP_3 receptor and its role in the regulation of IP_3-mediated internal Ca^{2+} release. J. Biol. Chem. *270*, 7257–7260.

Burridge, K., Turner, C.E., and Romer, L.H. (1992). Tyrosine phosphorylation of paxillin and Pp 125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. J. Cell. Biol. *119*, 893–903.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991). Oncogenes and signal transduction. Cell *64*, 281–302.

Chen, Q., Kinch, M.S., Lin, T.S., Burridge, K., and Juliano, R.L. (1994). Integrin-mediated cell adhesion activates mitogen-activated protein kinases. J. Biol. Chem. *269*, 26602–26605.

Cooper, J.A., Sefton, B.M., and Hunter, T. (1984). Diverse mitogenic agents induce the phosphorylation of two related 42000-Dalton proteins on tyrosine in quiescent chick cells. Mol. Cell. Biol. *4*, 30–37.

Davis, S., Lu, M.L., Lo, S.H., Lin, S., Butler, J.A., Druker, B.J., Roberts, T.M., An, Q., and Chen, L.B. (1991). Presence of an SH2 domain in the actin-binding protein tensin. Science *252*, 712–715.

Erickson, A.K., Payne, D.M., Martino, P.A., Rossomando, A.J., Shabinowitz, J., Weber, M.J., Hunt, D.F., and Sturgill, T.W. (1990). Identification by mass spectrometry of threonine 97 in bovine myelin basic protein as a specific phosphorylation site for mitogen-activated protein kinase. J. Biol. Chem. *265*, 19728–19735.

Folkman, J., and Moscona, A. (1978). Role of cell shape in growth control. Nature *273*, 345–349.

Guadagno, T.M., and Assoian, R.K. (1991). G1/S control of anchorage-independent growth in the fibroblast cell cycle. J. Cell. Biol. *115*, 1419–1425.

Guadagno, T.M., Ohtsubo, M., Roberts, J.M., and Assoian, R.K. (1993). A link between cyclin A expression and adhesion-dependent cell cycle progression. Science *262*, 1572–1575.

Guan, J., and Shalloway, D. (1992). Regulation of focal adhesion-associated protein kinase by both cellular adhesion and oncogenic transformation. Nature *358*, 690–692.

Guan, J., Trevethick, J.E., and Hynes, R.O. (1991). Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kD protein. Cell. Regul. *2*, 951–964.

Han, E.K., Guadagno, T.M., Dalton, S.L., and Assoian, R.K. (1993). A cell cycle and mutational analysis of anchorage-independent growth: cell adhesion and TGF-β1 control G1/S transit specifically. J. Cell. Biol. *122*, 461–471.

Hanks, S.K., Calalb, M.B., Harper, M.C., and Patel, S.K. (1992). Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. Proc. Natl. Acad. Sci. USA *89*, 8487–8491.

Hansen, L.K., Mooney, D.J., Vacanti, J.P., and Ingber, E. (1994). Integrin binding and cell spreading on extracellular matrix act at different points in the cell cycle to promote hepatocyte growth. Mol. Biol. Cell *5*, 967–975.

Hemler, M. (1990). VLA proteins in the integrin family. Annu. Rev. Immunol. *8*, 365–400.

Hynes, R.O. (1987). Integrins: a family of cell surface receptors. Cell *48*, 549–554.

Hynes, R.O. (1992). Integrins: versatility, modulation and signaling in cell adhesion. Cell *69*, 11–25.

Johnson, G.L., and Vaillancourt, R.R. (1994). Sequential protein kinase reactions controlling cell growth and differentiation. Curr. Opin. Cell Biol. *6*, 230–238.

Kameshita, I., and Fujisawa, H. (1989). A sensitive method for detection of calmodulin-dependent protein kinase II activity in sodium dodecyl sulfate-polyacrylamide gel. Anal. Biochem. *183*, 139–143.

Kornberg, L., Earp, H.S., Parsons, J.T., Schaller, M., and Juliano, R.L. (1992). Cell adhesion or integrin clustering increase phosphorylation of a focal adhesion-associated tyrosine kinase. J. Biol. Chem. *267*, 23439–23442.

Leevers, S.J., and Marshall, C.J. (1992). Activation of extracellular signal regulated kinase, ERK2 by p21ras oncoprotein. EMBO J. *11*, 569–574.

McNamee, H.P., Ingber, D.E., and Schwartz, M.A. (1993). Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. J. Cell. Biol. *121*, 673–678.

Matsuhisa, T., and Mori, Y. (1981). An anchorage-dependent locus in the cell cycle for the growth of 3T3 cells. Exp. Cell Res. *135*, 393–398.

Moolenaar, W.H. (1986). Effects of growth factors on intracellular pH regulation. Annu. Rev. Physiol. *48*, 363–376.

Otsuka, H., and Moskowitz, M.J. (1975). Arrest of 3T3 cells in G1 phase in suspension culture. J. Cell Physiol. *87*, 213–220.

Pardee, A.B. (1989). G1 events and regulation of cell proliferation. Science *246*, 603–608.

Payne, D.W., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J.H., Shabanowitz, J., Humt, D.F., Weber, M.J., and Sturgill, T.W. (1991). Identification of the regulatory phosphorylation sites in Pp 42/mitogen-activated protein kinase (MAP kinase). EMBO J. *10*, 885–892.

Pelech, S.L., and Sanghera, J.S. (1992). Mitogen-activated protein kinases: versatile transducers for cell signaling. Trends Biochem. Sci. *17*, 233–238.

Pierschbacher, M.D., and Ruoslahti, E. (1984). Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature *309*, 30–33.

Ray, L.B., and Sturgill, T.W. (1987). Rapid stimulation by insulin of a serine/threonine kinase in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 in vitro. Proc. Natl. Acad. Sci. USA *84*, 1502–1506.

Rossomando, A.J., Payne, D.M., Weber, M.J., and Sturgill, T.W. (1989). Evidence that Pp 42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. Proc. Natl. Acad. Sci. USA *86*, 6940–6943.

Ruoslahti, E. (1991). Integrins. J. Clin. Invest. *87*, 1–5.

Schaller, M.D., Borgman, C.A., Cobb, B.S., Vines, R.R., Reynolds, A.B., and Parsons, J.T. (1992). Pp 125FAK, a structurally distinctive protein-tyrosine kinase associated with focal adhesion. Proc. Natl. Acad. Sci. USA *89*, 5192–5196.

Schlaepfer, D.D., Hanks, S.K., Hunter, T., and Van der Geer, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature *372*, 786–790.

Schlessinger, J. (1993). How receptor tyrosine kinases activate ras. Trends Biochem. Sci. *212*, 273–275.

Schwartz, M.A., and Lechene, C. (1992). Adhesion is required for protein kinase C-dependent activation of the Na-H antiporter by PDGF. Proc. Natl. Acad. Sci. USA *89*, 6138–6141.

Schwartz, M.A., Lechene, C., and Ingber, D.E. (1991). Insoluble fibronectin activates the Na^+/H^+ antiporter by clustering and immobilizing integrin $\alpha 5\beta 1$, independent of cell shape. Proc. Natl. Acad. Sci. USA *88*, 7849–7853.

Shin, S., Freedman, V.H., Risser, R., and Pollack, R. (1975). Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage-independent growth in vitro. Proc. Natl. Acad. Sci. USA *72*, 4435–4439.

Ullrich, A., and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. Cell *61*, 203–212.

Vandenberg, P., Kern, A., Ries, A., Luckenbill-Edds, L., Mann, K., and Kühn, K. (1991). Characterization of a type IV collagen major cell binding site with affinity to the $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins. J. Cell Biol. *113*, 1475–1483.

Vuori, K., and Ruoslahti, E. (1993). Activation of protein kinase C precedes $\alpha 5\beta 1$ integrin-mediated cell spreading on fibronectin. J. Biol. Chem. *268*, 21459–21462.

Vuori, K., and Ruoslahti, E. (1994). Association of insulin receptor substrate-1 with integrins. Science *266*, 1576–1578.