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Growth factor activation of MAP kinase requires cell adhesion

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The MAP kinase pathway is a major regulator of both normal and oncogenic growth. We report that activation of the MAP kinase ERK2 by serum or purified growth factors is strongly dependent on cell adhesion to extracellular matrix proteins. This effect is specific to soluble growth factors, since suspended cells still activate ERK2 in response to plating on fibronectin, and is reversible. Analysis of endogenous Ras and Raf show that these proteins are still activated by serum in suspended cells, whereas MEK activity is inhibited. Conversely, activation of ERK2 by activated mutants of Ras and Raf is still adhesion-dependent but activation by MEK is not. Consistent with these results, activated MEK enhances growth of *ras*-transformed cells in suspension but not when adherent. These results identify a novel synergism between cell adhesion- and growth factor-regulated pathways, and explain how oncogenic activation of MAP kinases induces both serum- and anchorage-independent growth.

Keywords: cell adhesion/growth factor regulation/ integrin/signal transduction

Introduction

The MAP kinase pathway is a major cellular signaling pathway that mediates effects of growth factors on cell cycle progression (reviewed in Crews and Erikson, 1993; Cobb *et al*., 1994; Marshall, 1995). Growth factors induce conversion of c-Ras to its GTP-bound form, which then binds the protein kinase c-Raf. Localization of Raf to the plasma membrane leads to its activation via mechanisms that are incompletely understood but that appear to involve its phosphorylation (Morrison *et al*., 1988; Jelinek *et al*., 1996). Once activated, Raf phosphorylates and activates the protein kinase MEK1, which phosphorylates and activates the MAP kinases ERK1 and ERK2. These kinases phosphorylate a number of substrates that participate in cell cycle regulation, including the transcription factor Elk-1 (Janknecht *et al*., 1993), phospholipase A_2 (Lin *et al*., 1993) and $p90^{RSK}$ (Sturgill *et al*., 1988; Chung *et al*., 1991).

This pathway also plays a key role in oncogenic transformation, as activated forms of Ras are among the most common mutations found in human tumors (Cobb *et al*., 1994; Marshall, 1995) and expression of activated Ras, Raf (Leevers *et al*., 1994), or MEK1 (Crowley *et al*., 1994; Mansour *et al*., 1994) is transforming. Furthermore, inhibition of Ras, Raf, MEK1 or ERKs inhibits normal and transformed cell growth (Feramisco *et al*., 1985; Mulcahy *et al*., 1985; Feig and Cooper, 1988; Kolch *et al*., 1991; Crowley *et al*., 1994; Khosravi-Far *et al*., 1995). Ras, however, has a number of other effectors such as Rho family GTPases, PI 3-kinase and Ral GDS, which also make important contributions to cell cycle progression and transformation (Rodrigez-Viciana *et al*., 1994; Khosravi-Far *et al*., 1995; Qiu *et al*., 1995; White *et al*., 1996).

It is well established that normal cell growth requires cell adhesion to extracellular matrix proteins as well as stimulation by growth factors (reviewed in Clark and Brugge, 1995; Schwartz *et al*., 1995). The ability of integrins to transduce a variety of intracellular signals most likely mediates this effect. One such signaling pathway involves the MAP kinases ERK1 and ERK2. Plating of suspended cells on extracellular matrix proteins such as fibronectin (FN) triggers a rapid, transient activation of MAP kinases (Chen *et al*., 1994; Morino *et al*., 1995; Zhu and Assoian, 1995). However, it is not at all clear how this transient phenomenon contributes to growth of stably adherent cells.

Conversely, how constitutive activation of ERKs leads not only to serum-independent but also to anchorage-independent growth is poorly understood. Anchorage-independence is the *in vitro* characteristic that correlates best with tumorigenicity *in vivo* (Freedman and Shin, 1974). Two groups have analyzed the effect of cell adhesion on growth factor activation of MAP kinase and found that placing 3T3 cells in suspension had either a modest effect (Zhu and Assoian, 1995) or no effect (Hotchin and Hall, 1995) on activation of ERKs by growth factors. These results are paradoxical, since growth factors do not induce DNA synthesis in suspended cells, yet activated variants of Ras, Raf or MEK1 do.

To resolve this paradox, we investigated in greater detail the involvement of cell adhesion in growth factor activation of the MAP kinase pathway. We found that activation of ERK2 was in fact strongly dependent on adhesion to extracellular matrix. Furthermore, we identified activation of MEK by Raf as the adhesion-dependent step. Thus, constitutive activation of the MAP kinase pathway by oncogenes can bypass the requirement for both growth factors and cell adhesion.

Results

Inhibition of ERK2 activity in suspended cells

To test the possible dependence of the MAP kinase ERK2 on adhesion, cells were detached and either held in suspension in medium with 0.4% serum for 24 h or

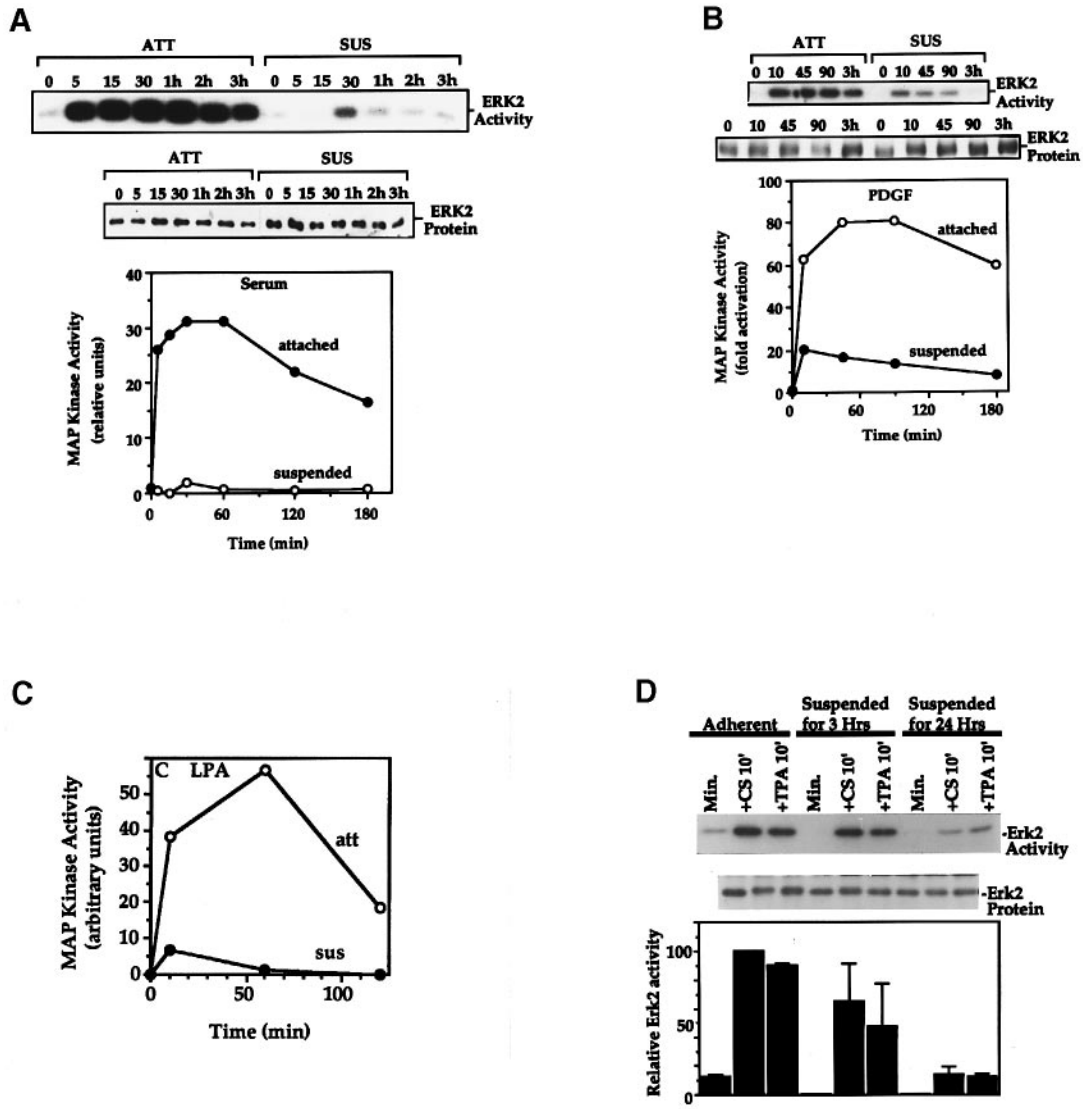


Fig. 1. ERK2 activity in adherent and suspended cells. (**A**) Stimulation by serum. Cells that had been incubated for 24 h in 0.4% serum while attached or suspended were stimulated by addition of 10% serum for the indicated times. Endogenous ERK was immunoprecipitated and analyzed for kinase activity using the in-gel assay and for ERK2 protein by Western blotting. Quantitation of the kinase assay is shown graphically. Data are representative of eight experiments. (**B**) Stimulation by PDGF. Attached or suspended cells that were incubated in 0.4% serum were stimulated by addition of 20 ng/ml PDGF for the indicated times. Endogenous ERK2 activity and amounts of immunoprecipitated protein were assayed as in (A). Quantitation of the kinase assay by densitometry is shown graphically. Data are representative of three experiments. (**C**) Stimulation by LPA. Attached or suspended cells were incubated in 0.4% serum, then stimulated with 2 μg/ml LPA for the indicated times. Endogenous ERK2 was immunoprecipitated and its activity determined in the immunoprecipitates. Kinase activity is shown graphically. Data are representative of four experiments. (**D**) Stimulation by TPA. Adherent cells kept in low serum for 24 h, serum-starved cells that were trypsinized and kept in suspension for 3 h, or cells kept in suspension in low serum for 24 h were stimulated with 100 nM TPA for 10 min. ERK2 kinase activity and immunoprecipitated protein amounts were assayed as before. The lower panel shows the quantitation of ERK2 kinase activity normalized for protein levels; values are means ± standard deviations from three experiments.

replated on tissue culture plastic in low serum for the same period. Subsequent stimulation with 10% serum induced strong activation of ERK2 in adherent cells but only a slight increase in suspended cells (Figure 1A). In eight experiments, inhibition varied between 97% and 77%, averaging 90.2 ± 9.8%. Platelet-derived growth factor (PDGF) and lysophosphatidic acid (LPA) are two of the major mitogens in serum. We therefore examined the stimulation of ERK2 activity by purified PDGF and LPA. Both mitogens strongly activated ERK2 in adherent cells but showed a substantially reduced response in non-adherent cells (Figure 1B and C)

Previous studies have shown that cells placed in suspension for short periods of time still activate MAP kinase in response to growth factors (Hotchin and Hall, 1995; Zhu and Assoian, 1995). To resolve the conflict between our results and these reports, we analyzed the effect of leaving cells in suspension for various lengths of time. We found that cells which were detached and held in suspension for short periods of time retained the ability to activate ERK2 in response to serum (Figure 2). With longer times in suspension, ERK2 activation diminished, with a half-time of ~6 h. These results indicate that loss of structures such as focal adhesions or dephosphorylation of focal adhesion proteins such as focal adhesion kinase, paxillin, $p130^{cas}$ or tensin, which occur rapidly upon loss

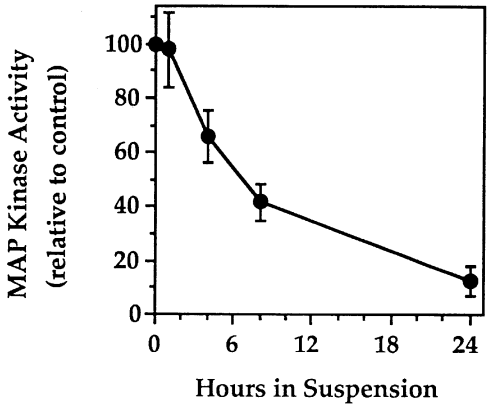
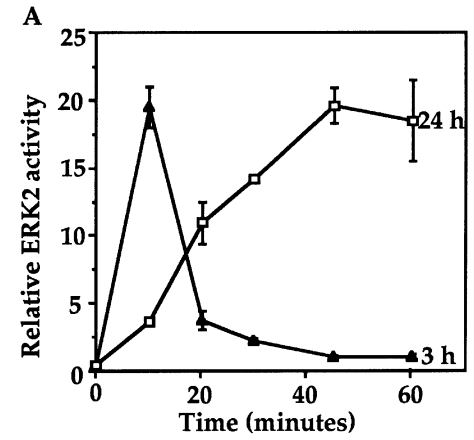


Fig. 2. Time in suspension. Cells were kept attached in 0.4% serum or were trypsinized and maintained in suspension for varying periods of time. Total time in 0.4% serum was kept constant by varying the length of starvation in low serum prior to detachment. Cells were then stimulated with 10% serum for 60 min and MAP kinase assayed. Values are means ± standard deviations from four experiments. Similar results were obtained with cells stimulated with serum for 15 min (not shown).



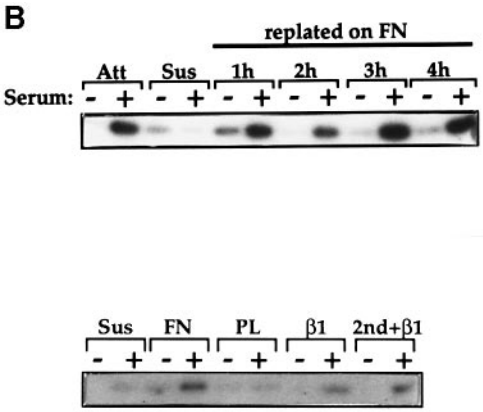


Fig. 3. Recovery of MAP kinase activation. (**A**) Replating in the absence of serum. Cells were incubated in suspension for 3 h or 24 h; total time in low serum was approximately constant. Cells were then replated on plastic coated with 25 μg/ml FN. ERK2 activity was assayed as before. Values are means ± standard deviations from three experiments. (**B**) Response to serum. Cells were maintained in suspension in low serum for 24 h, then replated on plastic coated with 20 μg/ml FN (upper panel). At the indicated times, cells were stimulated with serum for 15 min and MAP kinase assayed. Alternatively, as shown in the lower panel, cells were replated on plastic with different coating. These were: 20 μg/ml FN; 100 μg/ml polylysine (PL); 20 μg/ml anti-mouse β_1 integrin IgG (β1); 25 μg/ml goat anti-mouse IgG followed by 20 μg/ml anti-mouse β_1 IgG (2nd β1). In these experiments, the plastic was subsequently blocked with 10 mg/ml heat-denatured BSA to prevent adhesion via secreted FN. After 3 h, cells were stimulated with 10% serum for 15 min and MAP kinase assayed. Four experiments yielded similar results. Similar results were obtained with cells stimulated with serum for 60 min (not shown).

of cell adhesion, are not likely to account for the failure of suspended cells to activate the MAP kinase pathway. Consistent with this idea, treatment of cells with cytochalasin D for up to 90 min, which disrupts actin filaments and causes loss of focal adhesions, did not inhibit MAP kinase activation by serum (not shown). A prolonged period of 'adhesion deprivation' is required, analogous to the prolonged serum starvation that is required to de-activate growth factor-regulated pathways.

It is noteworthy that both LPA and PDGF, which bind a G protein-linked receptor and a protein tyrosine kinase receptor, respectively, are affected in the same manner by loss of cell adhesion. These observations argue against the likelihood that changes in receptor expression or function can account for the failure of non-adherent cells to activate ERK2. Consistent with this idea, previous work has shown that the PDGF receptor is still functional even after prolonged incubation of cells in suspension (McNamee *et al*., 1992). To test this idea further, we examined the activation of ERK2 by the phorbol ester TPA, which activates Raf via protein kinase C, bypassing Ras and the membrane receptors. The ability of TPA to activate ERK2 was also greatly attenuated in suspended cells in a time-dependent manner (Figure 1D).

Reversibility

To examine whether MAP kinase activation could be restored by replating cells on the extracellular matrix protein FN, two experiments were performed. First, cells that were kept in suspension for either 3 or 24 h were replated on FN. The cells that had been in suspension for 24 h adhered and spread at a significantly slower rate, though in both cases cells were well spread by 60 min (data not shown). Assay of ERK2 activity in these cells showed that the activation of ERK2 in cells kept in suspension for 24 h was delayed relative to cells kept in suspension for 3 h (Figure 3A). Presumably this delay is due to the delay in attachment and spreading. However, the extent of ERK2 activation was equivalent in the cells suspended for 24 h. This result shows that ERK2 can still be activated in 24 h suspended cells by an appropriate stimulus (adhesion). Thus, the failure to respond to serum is specific to soluble growth factors and cannot be due to general toxicity or apoptosis.

To test whether replating restores the ability to respond to serum, 24 h suspended cells were replated on FN in low serum for various times and then stimulated with serum. ERK2 activity was then assayed. We observed that upon replating, cells regained the ability to activate ERK2 in response to serum (Figure 3B). The activation by replating alone in the absence of serum was, as in Figure 3A, readily detectable at 1 h, followed by a return to baseline by 2 h. However, the increase in activity due to serum was readily detected at the 1 h time point. Interestingly, the serum response was diminished at the 2 h time point. This effect is most likely due to the fact that

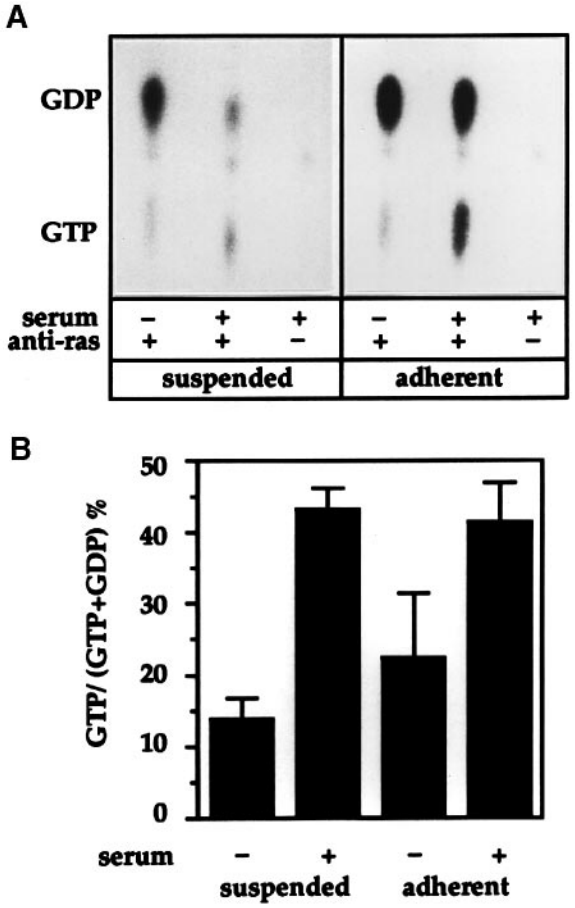


Fig. 4. Ras GTP loading. Attached or suspended cells in low serum were labeled with ^{32}P-orthophosphate and stimulated with serum for 8 min. Endogenous Ras was immunoprecipitated and the bound nucleotide was analyzed by chromatography (**A**). Values shown graphically (**B**) are means ± standard deviations from four experiments.

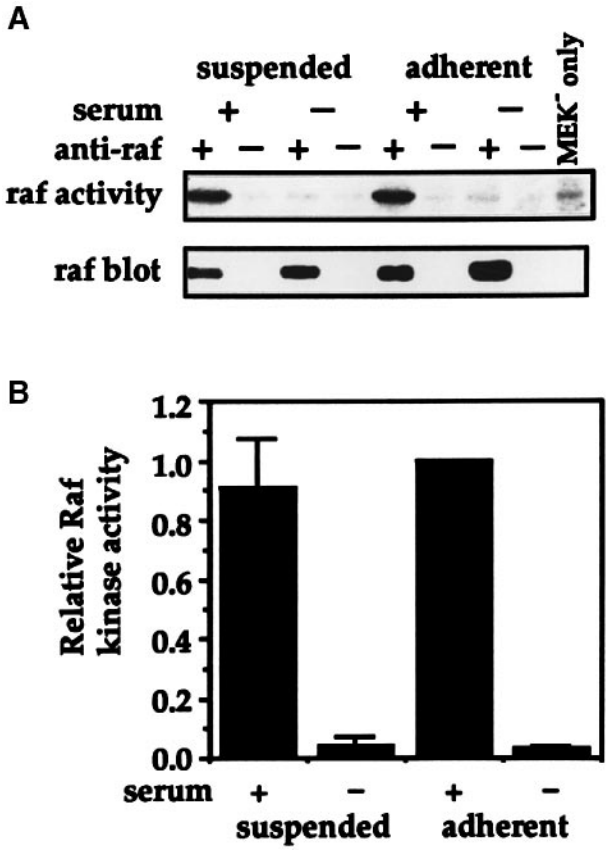


Fig. 5. Raf kinase activity. Attached or suspended cells incubated in low serum for 24 h were stimulated with 10% serum, then lysed and c-Raf immunoprecipitated. Raf kinase activity, using kinase-defective MEK-1 as a substrate, and Raf protein levels were assayed (**A**) as described in Materials and methods. Kinase activity was normalized for immunoprecipitated protein and data presented graphically (**B**). Values are means ± standard deviations from three experiments.

stimulation of MAP kinases by replating alone may render cells refractory to subsequent stimulation by serum, due to induction of the ERK phosphatase MKP-1 (Sun *et al*., 1993). Recovery of MAP kinase activation was also observed when cells were replated on an antibody to the integrin β1 subunit, but not on polylysine. Treatment of cells with cycloheximide did not block the recovery of MAP kinase activation (data not shown), but did reduce cell spreading and slightly reduced MAP kinase activation. These results show that the inhibition of ERK2 in suspended cells is reversible and is integrin-dependent.

Activation of intermediates

It is well established that growth factors activate ERK2 via a pathway that involves Ras, Raf and MEK1. To determine which step(s) in the pathway require cell adhesion, we assayed the activation of these components in adherent and suspended cells. Ras activation was assayed by immunoprecipitating endogenous Ras protein and analyzing the bound nucleotide. We observed that the baseline GTP content was somewhat lower in suspended cells; however, treatment with serum stimulated GTP loading to the same level in adherent and suspended cells (Figure 4). Thus, the adhesion-dependent step must lie downstream of Ras.

Next, we assayed Raf function by immunoprecipitating cellular Raf and carrying out an *in vitro* kinase assay using kinase-defective MEK1 as a substrate. These experiments showed that Raf kinase was still activated in suspended cells, with only a slight (~10%) decrease relative to adherent cells (Figure 5). Thus, activation of Raf is not significantly adhesion-dependent.

We then immunoprecipitated cellular MEK1, and examined its kinase activity using kinase-defective ERK2 as a substrate. These experiments revealed that activation of endogenous MEK1 in response to serum was greatly reduced in non-adherent cells (Figure 6), to an extent that was similar to the inhibition of ERK2. Thus, the ability of Raf to activate MEK1 appears to be the step that is sensitive to adhesion.

Activated components

To confirm the results of experiments analyzing the activities of cellular Ras, Raf and MEK1, we carried out a second series of experiments to study the activation of ERK2 by constitutively activated mutants. Cells were transiently transfected with *ras* G12V, which is constitutively activated by a mutation that reduces its GTPase; with *raf* BxB, in which the kinase is constitutively activated by truncation of the regulatory domain; or by ΔN3, S222 MEK1, which is activated by a deletion and a point

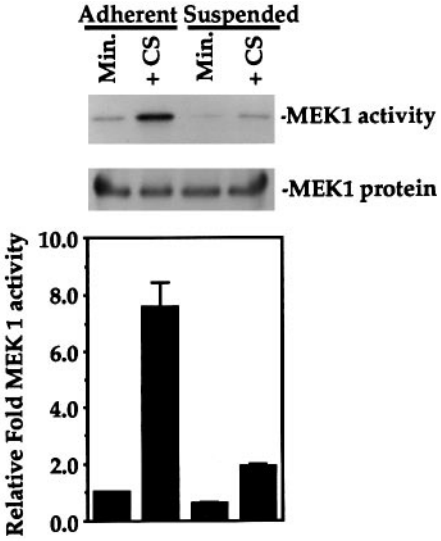


Fig. 6. MEK kinase activity. Attached or suspended cells incubated in low serum for 24 h were stimulated with 10% serum, then lysed and MEK-1 immunoprecipitated. MEK kinase activity was assayed using kinase-defective ERK2 as a substrate (top panel), and immunoprecipitated MEK-1 protein was analyzed by Western blotting (lower panel). For graphical data, kinase activity was normalized for immunoprecipitated MEK-1 protein. Values are means ± standard deviations from three experiments.

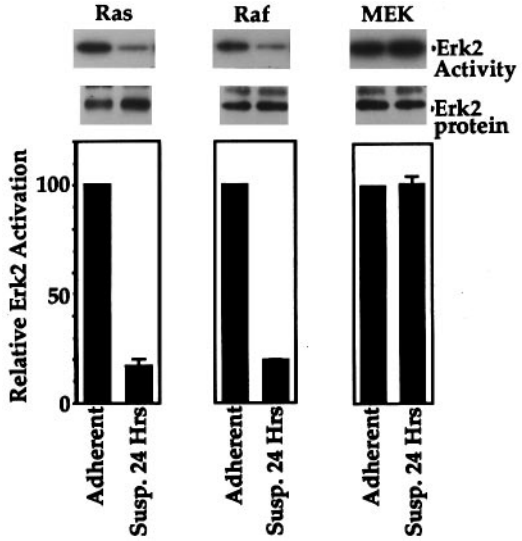


Fig. 7. Effect of activated mutants. NIH 3T3 cells were co-transfected with HA-tagged ERK2 together with activated mutants of Ras, Raf or MEK. At 24 h after transfection, cells were replated on tissue culture plastic in 0.4% serum or placed in suspension in 0.4% serum. After an additional 24 h, cells were harvested and the transfected ERK2 immunoprecipitated using anti-HA antibody. Both the kinase activity of the precipitated ERK2 and the ERK2 protein levels were assayed as described in Materials and methods. Negligible ERK2 activity was observed in the absence of co-transfected activated Ras, Raf or MEK. Values shown in the lower panels are kinase activity normalized for ERK2 protein, averaged from three experiments ± standard deviations.

mutation. In each case, the activated protein was co-transfected with an ERK2 that had been tagged with the hemagglutinin (HA) epitope. Control cells were co-transfected with HA–ERK2 plus empty vector. At 24 h after transfection, cells were placed in suspension or replated on tissue culture plastic in low serum, and incubated a further 24 h. The HA–ERK2 was then immunoprecipitated and its activity assayed. These results show that ERK2 activation by both Ras and Raf were decreased in suspended cells to ~15% of the levels in adherent cells (Figure 7). By contrast, activation of ERK2 by activated MEK1 remained high in non-adherent cells. These results confirm that the adhesion-dependent step lies between Raf and MEK. It should be noted, however, that the activation of ERK2 by these oncogenes in adherent cells is ~2.5-fold higher than that triggered by serum in adherent cells (not shown). Hence, the activation by oncogenes in suspended cells, while strongly attenuated, is ~40% of that found in adherent cells treated with serum.

Cell growth

The results obtained above make a surprising prediction. Present views of the MAP kinase pathway would argue against the idea that activated MEK1 should augment transformation by Ras. However, our data suggest that expression of MEK1 should enhance MAP kinase activity and growth in suspended cells. To test this hypothesis, cells were transfected with activated *ras* alone, activated MEK1 alone, or both. Growth of transfected cells in monolayer and in suspension was then examined.

As shown in Table I, *ras* induced a significant number of foci in monolayer culture and a similar number of colonies in soft agar. The number of foci and colonies was nearly equal to the total number of hygromycin-resistant colonies, suggesting that *ras* induced transformation of NIH 3T3 cells with high efficiency. Transfection with MEK alone induced foci rather weakly, as reported previously (Khosravi-Far *et al*., 1995; White *et al*., 1995). This result is consistent with the concept that *ras* induces transformation via combined effects on a number of effectors. Co-transfection of MEK with *ras* did not increase the number of foci or colonies, but this may be due to the already high efficiency of transformation by *ras* alone. However, MEK substantially increased the size of the colonies in soft agar (Table II). Measurement of their volume showed that MEK1 enhanced the rate of growth of cells in suspension by ~7-fold. By contrast, MEK1 did not increase the size of adherent *ras*-transformed colonies in low serum. These results show that preventing the decline in MAP kinase in non-adherent cells significantly augments their growth in suspension but does not enhance the ability of *ras* to promote serum-independent growth in adherent cells.

Discussion

Our results show that in NIH 3T3 cells, activation of the MAP kinase pathway by serum or growth factors is strongly dependent on integrin-mediated cell adhesion to extracellular matrix protein. Similar results were obtained with CHO cells (P.E.Hughes and M.H.Ginsberg, personal communication), but other cell types have yet to be examined. Several results demonstrate that this effect is due to a specific blockade rather than to general toxicity: suspended cells retain the ability to activate MAP kinase in response to adhesion, they retain the ability to activate Ras and Raf in response to growth factors, and the effect is reversed when cells are replated on FN.

Two independent lines of experimentation show that

Table I. Stable transformation assay

Vectors	HygR colonies	Foci in 10% CS	Foci in 0.5% CS	Soft agar colony
Control	1340 ± 60	0 ± 0	0 ± 0	0 ± 0
MEK	1120 ± 45	45 ± 11	11 ± 11	0 ± 0
Ras	1100 ± 45	1584 ± 88	1408 ± 220	1452 ± 44
Ras + Mek	1260 ± 98	1537 ± 227	1588 ± 151	1411 ± 101

NIH 3T3 cells were co-transfected with RSVHyg and combinations of the plasmids expressing the activated MEK or Ras mutant proteins or the empty expression vector (Control). Relative DNA amounts of the mutants were kept constant (0.8 μg) in the transfections, so in transfections containing only one of the mutants the remainder of the DNA was made up by the empty expression vector. Transfected cells were trypsinized and portions replated in media containing 10% CS, 10% CS with 200 μg/ml hygromycin, 0.5% CS, or soft agar to measure foci and colony formation. Values shown represent the total number of colonies or foci calculated for the entire transfection and represent values obtained from two separate experiments.

this effect occurs at the level of activation of MEK by Raf. First, as mentioned above, endogenous Ras and Raf are still maximally activated by serum in non-adherent cells, but MEK1 is not. Second, expression of activated mutants of these proteins showed that signals from oncogenic Ras and Raf were still attenuated in suspended cells, whereas the signal from activated MEK1 was undiminished. Thus, the ability of Raf to activate MEK1 maximally must require cell adhesion. Finally, the biological relevance of these effects was indicated by the result that activated MEK1 significantly enhanced the growth of *ras*-transformed cells in suspension, but had no effect on their growth when adherent.

Previous studies have been puzzling in that either no effect or a modest effect of cell adhesion on the activation of the MAP kinase pathway by growth factors was observed. Yet transforming activators of this pathway, such as mutated forms of Ras, Raf and MEK1, induce growth of cells in suspension. If MAP kinase were fully activated by growth factors in normal, suspended cells, then the activation of MAP kinase by oncoproteins would be redundant. Our results resolve these questions and suggest a model relating MAP kinase to anchorage-dependence and -independence of growth. Normal cells are anchorage-dependent in part because growth factor activation of MAP kinase depends on cell adhesion. Conversely, cells transformed by *ras* and other oncogenes that work through the MAP kinase pathway are anchorage-independent because activation of MAP kinase bypasses a requirement for both adhesion and growth factors.

This model, however, raises the question why activated Ras or Raf are able to induce growth in suspension at all. It must be noted that the reduction of the ERK2 signal in non-adherent cells is not complete (Figure 7). Indeed, the activation of the MAP kinase pathway by these oncogenes is so strong that the levels of ERK activity in suspended cells are still significant, ~40% of the level in adherent, serum-treated control cells. Thus, very strong activation of the pathway can at least partially overcome the requirement for adhesion.

The mechanism by which cell adhesion potentiates the activation of MEK by Raf is currently unknown. The existence of focal adhesions or the phosphorylation of focal adhesion proteins are not likely to be the immediate cause, since they are rapidly lost upon detachment whereas the decline in ERK2 activation occurs more slowly (Figure 1D). Possible explanations include the regulation of scaffolding proteins that control the proximity of Raf and MEK, analogous to the Ste5 protein in yeast (Choi *et al*., 1994), or as yet unidentified protein kinases or phosphatases that may regulate MEK1 activity. Further work will be required to resolve these issues.

Table II. Soft agar and minimal media colony size

Vector	Soft agar		Foci in 0.5% CS	
	Colony volume[a]	Relative	Foci size[b]	Relative
Ras	0.59 ± 0.17	1.00	87 ± 7	1.00
Ras + MEK	4.12 ± 1.07	6.99	81 ± 6	0.91

[a]Transfected cells from Table I were further analyzed by visually measuring soft agar colony size and calculating the volume ($4/3\pi r^3$). Values represent the mean ± standard error from 15 colonies each.
[b]Minimal media focus size was determined by trypsinizing the foci and replating in soft agar to determine the number of transformed cells per focus. Values represent the mean ± standard deviation from two separate experiments in which at least 35 foci from each transfection were trypsinized.

The majority of key cellular decisions about growth, differentiation and survival are made on the basis of input from both soluble mediators (growth factors, cytokines, hormones) and from extracellular matrix receptors. Thus, understanding how cellular pathways are controlled by both soluble factors and cell adhesion to extracellular matrix is a major goal. Our results identify a novel synergism between cell adhesion and growth factors in the activation of the MAP kinase pathway. These results are likely to be important in a variety of systems where gene expressions, cell growth and cell survival depend upon cell adhesion.

Materials and methods

Reagents

Anti-ERK2 polyclonal antibody (C-14) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LipofectAMINE, Dulbecco's modified Eagle's medium (DMEM), serum and other reagents for cell culture were from Gibco-BRL (Gaithersburg, MD). Anti-mouse β1 integrin IgG (HMβ1-1) was purchased from Pharmingen (San Diego, CA). Fibronectin was prepared from human plasma as described (Miekka *et al*., 1982). Myelin basic protein was prepared from bovine spinal cord as described (Deibler *et al*., 1972). Platelet-derived growth factor (PDGF), lysophosphatidic acid (LPA), agarose, methylcellulose and other reagents were purchased from Sigma Chemicals (St. Louis, MO) unless otherwise noted.

Cell culture

NIH 3T3 cells were cultured in DMEM supplemented with 10% bovine calf serum. For MAP kinase experiments, cells in DMEM with 0.4% serum were plated in 60 mm tissue culture plastic dishes at ~80% of confluence for adherent cells. Alternatively, equal numbers of cells in

DMEM with 0.4% serum and 0.5% methylcellulose were plated in 10 cm dishes coated with 1 ml of 1% agarose which was equilibrated with DMEM. Methyl cellulose reduces cell–cell aggregation but does not otherwise affect growth or MAP kinase activity (data not shown). Cells were incubated as described in the text, stimulated with 10% serum or other factors, harvested and analyzed as described below.

Plasmids and transfection

For transfections, cells were plated at a density of 4×10^5 cells per 6 cm dish 24 h before transfection. Cells were transfected with LipofectAMINE as described (Renshaw *et al*., 1996) using 0.2 μg of pCMV5 ERK2, 0.2 μg of pCMV5 βgal, and 1.6 mg of either pDCR *ras* G12V (White *et al*., 1995), pZIP Neo *raf* BXB (Bruder *et al*., 1992) or pMCL MEK ΔN3, S222D (Mansour *et al*., 1994) per plate. At 24 h after transfection, cells were transferred to medium containing 0.5% serum for an additional 24 h for adherent cells and for cells suspended for only 3 h. After 24 h in 0.5% CS, cells from indicated plates were then trypsinized and suspended for 3 h in serum-free DMEM containing 0.1% BSA (Calbiochem, nuclease-, protease-free), and 0.25 mg/ml soybean trypsin inhibitor (Sigma) over dishes which had been coated with 1% heat-denatured BSA (Sigma fraction V). Alternatively, for cells kept in suspension for 24 h, cells were trypsinized 24 h after transfection and placed in suspension in DMEM media containing 0.5% methyl cellulose and 0.4% calf serum in dishes coated with 1% agarose as above.

Cell growth and transformation assays

For stable transformation assays, cells were transfected with RSVHyg and 1.6 μg of either the empty control vector, pDCR Ras G12V, pMCL MEK ΔN3, S222D, or combinations thereof. After 24 h, the cells were changed to fresh media and allowed to grow for two more days. Cells were then trypsinized and a portion ($1/20^{th}$) replated to measure focus formation in normal media (with 10% serum), minimal media, or in soft agar (with 10% serum) as previously described (Renshaw *et al*., 1995). Cells were also replated in media containing 200 μg/ml Hygromycin (Boehringer-Mannheim) to determine the total number of stably transfected clones. Colonies were scored visually 2 weeks after transfections. Soft agar colony volume was determined by visually measuring colony diameter against a scale, from which colony volume was calculated. Size of foci in low serum ('minimal medium') was determined by trypsinizing the foci and replating them in soft agar. The number of soft agar colonies was then counted after 2 weeks to determine the number of transformed cells per original minimal medium focus.

Measurement of ERK2 activity

Cells were stimulated as described in the Results section, rinsed with cold phosphate-buffered saline (PBS) and extracted in ice-cold buffer containing 0.5% NP-40, 20 mM Tris, pH 7.6, 250 mM NaCl, 5 mM EDTA, 3 mM EGTA, 20 mM sodium phosphate, 20 mM sodium pyrophosphate, 3 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM NaF, and 10 μg/ml each of leupeptin and aprotinin. Lysates were centrifuged 15 min at maximum speed in a microfuge and the supernatants removed. For assays of transfected HA-ERK2 activity, 0.5 μg of anti-HA (12CA5) antibody purified over an HA affinity column was used for each immunoprecipitation. For endogenous ERK2, 0.5 μg of anti-ERK2 was used for each immunoprecipitation. Approximately 100 μg of cell protein was immunoprecipitated in each sample. For transfected cells, the amount of cell lysate used in the IP was normalized to βgal activity levels to account for transfection efficiencies. βgal activity levels were measured as previously described (Herbomel *et al*., 1984) using 20 μg of the cell lysate. For assays of endogenous ERK2 activity, samples were normalized to protein using the BCA kit from Pierce Chemicals (Rockford IL). For all immunoprecipitation, one-fifth of the samples were saved and run on a 10% SDS–polyacrylamide gels, transferred to Hybond C (Amersham), and immunoblotted using the anti-ERK2 antibody, to measure the amount of ERK2 protein immunoprecipitated. In some cases, activity of endogenous ERK2 was measured by carrying out kinase reactions in the immune precipitates using myelin basic protein (MBP) as a substrate (Minden *et al*., 1994). Phosphorylated MBP was separated by SDS–PAGE, bands cut from the gel and counted for radioactivity. Alternatively, in some experiments endogenous ERK2 activity was assayed using an in-gel kinase assay as previously described (Kamashita and Fujisawa, 1989). For transiently transfected ERK2, the in-gel kinase assay was used exclusively. Briefly, samples were run on 12.5% SDS–polyacrylamide gels containing 0.25 mg/ml myelin basic protein and kinase reactions were performed by soaking gels in buffer containing 25 mCi/ ml [γ-^{32}P]ATP with 10 mM non-radiolabeled ATP. Gels were washed exhaustively and analyzed by autoradiography and scanning densitometry using the deskscan software with a Scanjett IIP scanner (Hewlett Packard).

Measurement of MEK 1 activity

Endogenous MEK 1 was immunoprecipitated from 100 μg of cell lysate using 1 μg of anti MEK 1 (Santa Cruz Biotechnology). Immunoprecipitates were washed three times in lysis buffer, and then once in kinase buffer (Chen *et al*., 1996) 10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT. IPs were then split, using one-fifth to measure the amount of MEK 1 protein and four-fifths to measure kinase activity. MEK kinase activity was measured in kinase buffer containing 25 μM ATP, 5 μCi [γ-^{32}P]ATP and 2 μg of kinase-dead GST–ERK2 (Hipskind *et al*., 1994) for 30 min at room temperature. Samples were electrophoresed on 10% SDS–polyacrylamide gels, which were then dried and exposed to film. Autoradiographs were quantitated using a model I.S. 1000 digital imaging system from Alpha-Innotech Corp.

Ras-GTP loading

NIH 3T3 cells were maintained adherent in 0.4% serum or in suspension as before for 12 h. Cells were then transferred to phosphate-free DMEM containing 0.4% serum and 50 μCi/ml ^{32}P-labeled orthophosphate for an additional 12 h. Cells were then rinsed with cold Tris-buffered saline or phosphate-free DMEM and stimulated with 10% serum for 6–10 min at room temperature. (Preliminary experiments demonstrated that maximal GTP loading of Ras occurred at this time interval.) Cells were then rinsed three times with cold PBS, and lysed in 380 μl of 50 mM HEPES, pH 7.4, containing 1% NP40, 0.1% BSA, 100 mM NaCl, 15 mM $MgCl_2$, 0.1 mM GTP, 0.1 mM GDP, 1 mM ATP, 10 μg/ml aprotinin and leupeptin, 1 mM PMSF, and 2 μg of either a rat monoclonal anti-Ras antibody (Santa Cruz Biotechnology) or non-immune rat IgG as a control. Lysates were centrifuged at 13 000 r.p.m. for 12 min, the supernatants transferred to fresh tubes and adjusted to 0.5M NaCl, 0.5% deoxycholate, 0.05% SDS. They were incubated on ice for 30 min, then 10 μl protein G PLUS-Agarose (Santa Cruz Biotechnology) added and the samples rotated for 1 h at 4°C for 60 min. The beads were washed eight times, and the bound nucleotides extracted and chromatographed on polyethyleneimine paper as described (Downward *et al*., 1990). Chromatograms were analyzed by autoradiography on Kodak BioMax MS film and quantitated by densitometry.

Raf kinase assay

Adherent or suspended cells were stimulated with serum for 8 min, rinsed with cold PBS and lysed in buffer containing 50 mM HEPES, pH 7.4, 1% NP40, 100 mM NaCl, 2 mM EDTA, 10 μg/ml aprotinin and leupeptin, 1 mM PMSF, 5 mM sodium vanadate, 20 mM NaF, 10 mM sodium pyrophosphate and 3 mM β-glycerophosphate. Cell lysates were centrifuged at 13 000 r.p.m. in a microfuge for 15 min, and precleared with 10 μl of protein A–agarose beads (Pierce). Protein in each sample was assayed using the Pierce BCA Kit. Normalized samples were adjusted to 0.5 M NaCl, 0.1% SDS and 0.5% deoxycholate, and immunoprecipitated for 2 h at 4°C using 3 μg of mouse monoclonal anti-Raf1 IgG (Transduction Laboratories) and 10 μl of protein A–agarose beads prebound with 10 μg of rabbit anti-mouse IgG (Cappel). The beads were rinsed three times with lysis buffer containing 0.5 M NaCl and once with lysis buffer. The *in vitro* Raf kinase assay was carried out exactly as described (Morrison, 1995) using His-tagged kinase-defective MEK1 as a substrate (Gardner *et al*., 1994). A portion of each sample was analyzed by Western blotting with the monoclonal anti-Raf1 antibody to determine the amount of Raf in the immunoprecipitates.

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