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Active ERK/MAP kinase is targeted to newly forming cell–matrix adhesions by integrin engagement and v-Src

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Integrin engagement generates cellular signals leading to the recruitment of structural and signalling molecules which, in concert with rearrangements of the actin cytoskeleton, leads to the formation of focal adhesion complexes. Using antisera reactive either with total ERK or with phosphorylated/activated forms of ERK, in rat embryo fibroblasts and embryonic avian cells that express v-Src, we found that active ERK is targeted to newly forming focal adhesions after integrin engagement or activation of v-Src. UO126, an inhibitor of MAP kinase kinase 1 (MEK1), suppressed focal adhesion targeting of active ERK and cell spreading. Also, integrin engagement and v-Src induced myosin light chain kinase (MLCK)-dependent phosphorylation of myosin light chain downstream of the MEK/ERK pathway, and MLCK and myosin activities are required for the focal adhesion targeting of ERK. The translocation of active ERK to newly forming focal adhesions may direct specificity towards appropriate downstream targets that influence adhesion assembly. These findings support a role for ERK in the regulation of the adhesion/cytoskeletal network and provide an explanation for the role of ERK in cell motility.

Keywords: cell adhesion/integrins/MAP kinase/signalling/Src

Introduction

Integrins are heterodimeric transmembrane cell adhesion receptors comprising α- and β-subunits. The differential association of more than 16 α-integrin and eight β-integrin subunits gives rise to at least 20 different α-integrin–β-integrin heterodimers (Hynes, 1992). These heterodimers are expressed in specific tissues or cell types and have differing specificity for extracellular matrix ligands such as laminin or fibronectin. While integrins do not possess intrinsic kinase, or other biochemical activity, integrin engagement to extracellular matrix (ECM) ligands nonetheless leads to the activation of a variety of intracellular signalling events via recruitment of signalling molecules to sites of integrin engagement or clustering. *In vitro*, the concerted actions of ECM-mediated clustering of integrin receptors, and associated signalling events, lead to the formation of defined cell–substrate contact sites known as focal adhesions or focal contacts (Jockusch *et al*., 1995).

How cell–ECM adhesions are assembled in response to integrin engagement, and the relationship between early integrin-induced biochemical signals and focal adhesion formation, the so-called 'inside out' signalling, remain relatively obscure. Some of the primary signalling events that occur concomitantly with cell adhesion include the phosphorylation of focal adhesion kinase (FAK) (Hanks *et al*., 1992; Schaller *et al*., 1992), Src-mediated tyrosine phosphorylation of adhesion proteins (Vuori *et al*., 1996; Schlaepfer *et al*., 1998) and stimulation of the mitogen-activated protein kinase (MAPK) [or extracellular signal-regulated kinase (ERK)] cascade (Chen *et al*., 1994; Schlaepfer *et al*., 1994; Morino *et al*., 1995; Zhu and Assoian, 1995; Clark and Hynes, 1996; Miyamoto *et al*., 1996; Renshaw *et al*., 1996). Early studies on FAK implied a role in regulating focal adhesion assembly, although the finding that fibroblasts derived from FAK-deficient mouse embryos were still able to assemble focal adhesions ruled out a critical role for FAK in adhesion formation (Ilic *et al*., 1995). Research principally from Hall's group (Ridley and Hall, 1992; Hotchin and Hall, 1995) has also implicated cytoskeletal organization, mediated by the Rho family of small GTPases, in regulating focal contact formation (see Tapon and Hall, 1997, for review). More recent studies have indicated that the effects of Rho are mediated by the downstream kinases, Dia1 and the Rho-dependent kinase ROCK, probably acting to increase myosin contractility leading to stress fibre bundling and consequent focal adhesion formation (Chrzanowska-Wodnicka and Burridge, 1996; Watanabe *et al*., 1999).

Despite this progress, a comprehensive understanding of the critical regulators of 'inside-out' signalling during focal adhesion formation is still lacking. Furthermore, the identity and origins of biochemical signals that arise at these newly formed structures remain unclear. Tyrosine phosphorylation is a long-held candidate mediator of focal adhesion formation, yet focal adhesions can still form in the absence of detectable tyrosine phosphorylation of focal adhesion components (Fincham *et al*., 1995; Gilmore and Romer, 1996). One explanation for this apparent paradox is that tyrosine phosphorylation at focal adhesions is required for the assembly of signalling complexes, mediated in part by Src homology 2 (SH2) domain–phosphotyrosine interactions, but that assembly of focal adhesion components into adhesive structures does not require tyrosine phosphorylation (see Schoenwaelder and Burridge, 1999, for

review). There is abundant evidence, however, that tyrosine phosphorylation of structural and signalling proteins, including FAK, is necessary for focal adhesion disassembly. In particular, v-Src-induced tyrosine phosphorylation events at focal adhesions stimulate focal adhesion disassembly and transformation (Nigg *et al*., 1986; Guan and Shalloway, 1992; Hanks *et al*., 1992; Turner, 1994; Calalb *et al*., 1995; Fincham *et al*., 1995).

Although it is not known whether ERK contributes to the regulation of focal adhesion dynamics, the evidence for ERK involvement in adhesion-mediated signalling has continued to accumulate. Adhesion-mediated activation of ERK is dependent on integrin engagement (Chen *et al*., 1994; Schlaepfer *et al*., 1994; Morino *et al*., 1995; Zhu and Assoian, 1995; Clark and Hynes, 1996; Miyamoto *et al*., 1996; Renshaw *et al*., 1996), and also occurs after v-Src activation (Wyke *et al*., 1995; Fincham *et al*., 1996). Cell adhesion is necessary for mitogen activation of the ERK cascade, although adhesion-dependent activation of ERK can occur independently of mitogens (Zhu and Assoian, 1995; Renshaw *et al*., 1997). In addition, ERK activity has been implicated in ECM-dependent cell migration (Klemke *et al*., 1997; Nguyen *et al*., 1999), consistent with a role for active ERK in the regulation of integrin-dependent adhesion/cytoskeletal organization. However, despite the link between integrin engagement, rapid ERK activation and integrin-dependent cell migration, and the implied connection between active ERK and focal adhesion dynamics, nothing is currently known about the spatial organization of ERK after stimuli that modulate focal adhesion assembly. In particular, how integrin engagement at the cell periphery is transmitted spatially to activate ERK that is presumed to be in the cytoplasm is not known.

To address this, we have used two independent, well characterized systems for studying focal adhesion assembly: (i) from outside the cell by integrin engagement in response to plating embryonic fibroblasts on ECM components; and (ii) from inside the cell by activating a conditional variant of v-Src which initially induces new focal adhesion assembly in adherent cells. We sought to define the spatial organization of ERK during focal adhesion assembly using a range of antisera specific for total ERK or for activated forms of ERK using high resolution immunofluorescence microscopy.

Results

We have used two well characterized cell culture model systems to study the spatial and temporal regulation of ERK upon induction of focal adhesion assembly. Rat embryo fibroblast REF52 cells, which spread rapidly after adhering, gave rise to flat cells with prominent focal adhesions and have been well studied with respect to cytoskeletal organization, cell spreading and focal adhesion formation (Pavalko and Burridge, 1991; Verkhovsky *et al*., 1995; Heidemann *et al*., 1999). v-Src-expressing chick embryo fibroblasts (CEF) are also well established as a model for studying Src-dependent focal adhesion assembly and turnover (Fincham *et al*., 1996; Fincham and Frame, 1998).

Phosphorylated ERK is localized to the cell periphery in newly spreading fibroblasts

Plating of cells on fibronectin with subsequent integrin engagement leads to rapid activation of ERK (see Aplin *et al*., 1998, for review). Treatment of REF52 cells with an inhibitor of the ERK-activating kinase mitogen-activated protein kinase kinase (MEK), UO126 (Favata *et al*., 1998), while not affecting cell adhesion *per se*, had a pronounced effect on the subsequent ability of these cells to spread on fibronectin (Figure 1A). Although the number of cells adhering to the fibronectin-coated dishes was not affected by UO126 treatment, their ability subsequently to make new surface contacts and form new lamellipodia was inhibited or substantially delayed. A similar effect has been reported previously for FG carcinoma cells treated with the MEK inhibitor PD098059 (Klemke *et al*., 1997). To confirm the specificity of the effect of the MEK inhibitor UO126, we also used a dominant-negative MEK construct (Cowley *et al*., 1994). REF52 cells were co-transfected with a dominant-negative MEK (or empty vector as control) and green fluorescent protein (GFP) to localize transfected cells and examined for their ability to adhere and spread on fibronectin. Cell spreading on fibronectin, but not cell adhesion, was inhibited by transient transfection of REF52 cells with a dominant-negative mutant of the ERK kinase MEK (Figure 1B). These simple observations confirm a role for ERK activation in the formation of new cell–substratum contacts during cell spreading.

In order to visualize low levels of active ERK present in focal adhesions and spreading lamellipodia, we established fixation and secondary detection conditions that led to clear visualization of ERK in both spreading lamellipodia and newly forming focal contacts, and in mature focal adhesion structures seen in adherent cells. We employed mild fixation of cells in cold methanol and, after incubation with primary antibody, we used biotin-conjugated secondary antibodies which were amplified with streptavidin coupled to the relevant fluor for detection. Using antisera specific for the dual phosphorylated and active forms of ERK1/2 (raised against the sequence pT^{183}-E-pY^{185} in the activation domain of ERK) we found that phosphorylated ERK was localized to newly spreading lamellipodia at the cell periphery in REF52 cells spreading on laminin or fibronectin (Figure 1C). In addition, we observed peripheral staining with a haemagglutinin (HA)-specific antibody in REF52 cells that transiently expressed exogenous HA-tagged ERK 1 and were plated on laminin; this peripheral staining was not evident when the cells were treated with the UO126 MEK inhibitor (Figure 1D). We also observed some peripheral phospho-ERK staining in CEF spreading on fibronectin (Figure 1E, upper left panel), which was not evident when they were spread on poly-L-lysine (Figure 1E, upper right panel). This is consistent with the targeting of active ERK to the cell periphery being dependent on integrins engaged by interaction with fibronectin or laminin, rather than as a consequence of cell adhesion *per se*. To address whether the impaired targeting of active ERK to the cell periphery when cells were plated on poly-L-lysine was indicative of a general defect in the peripheral targeting of focal adhesion components, we stained similarly treated cells with anti-paxillin. On poly-L-lysine, although paxillin was not

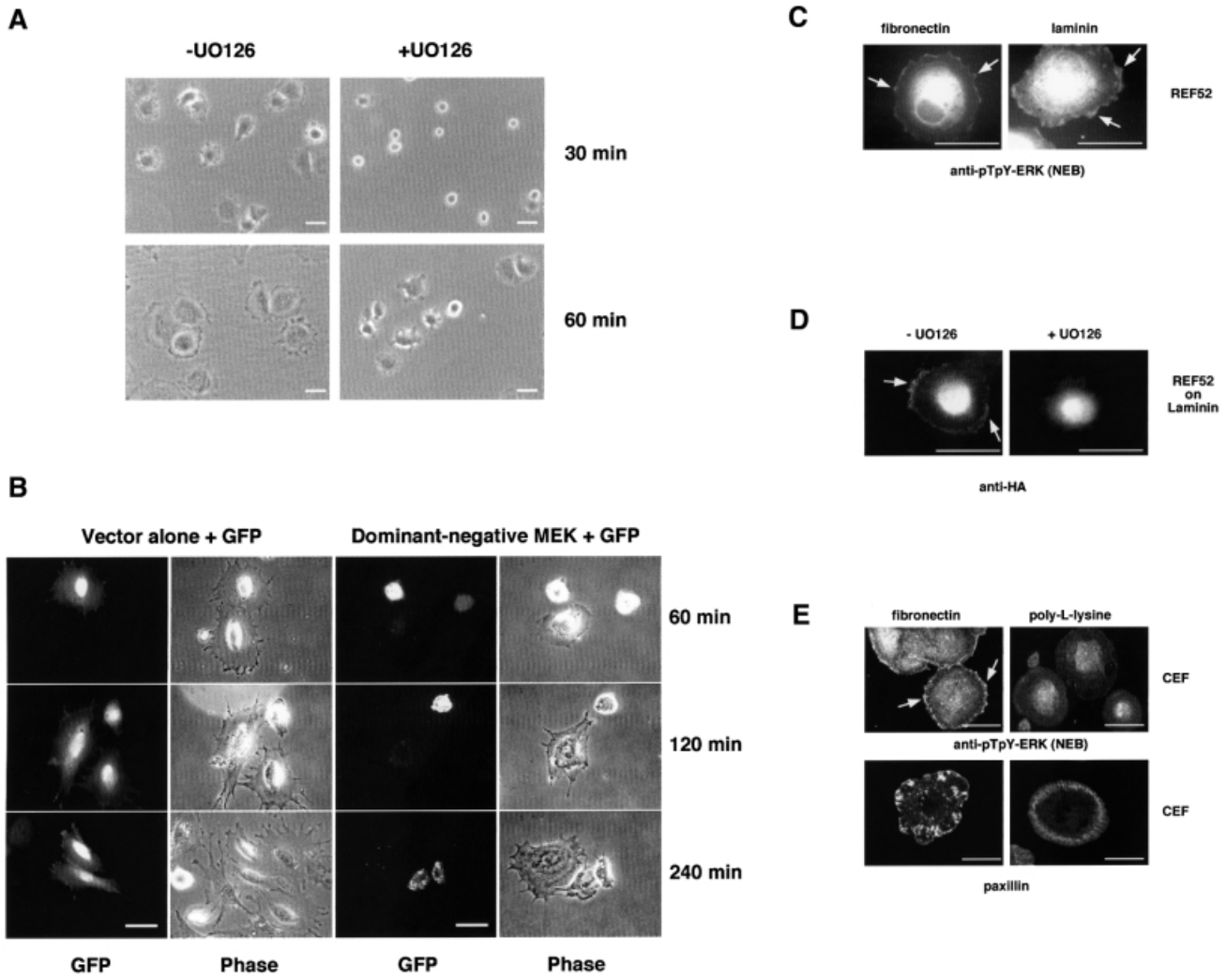

Fig. 1. Inhibition of ERK prevents cell spreading and active ERK localizes to the periphery of cells spreading on integrin ligands. (**A**) REF52 cells spreading on fibronectin in the absence (–) or presence (+) of the MEK inhibitor UO126 (50 μM), for 30 or 60 min. (**B**) REF52 cells spreading on fibronectin following transient transfection with dominant-negative MEK and GFP (right panels) or empty vector and GFP (left panels) for the indicated times. (**C**) Localization of active ERK stained with anti-pTpY-ERK (New England Biolabs) at 1:100, to the periphery of REF52 cells spreading on fibronectin (left) or spreading on laminin (right) for 30 min. Arrows indicate peripheral staining with anti-phospho-ERK serum. (**D**) Localization of HA–ERK (anti-HA; 1:100) in transiently transfected REF52 cells spreading on laminin. Arrows indicate peripheral staining with anti-HA (left), which is abolished by treatment with UO126 (right). Visualization and image capture were with an epifluorescence microscope. (**E**) Upper panels: localization of active ERK [anti-pTpY-ERK (New England Biolabs) at 1:100] to the periphery of CEF spreading on fibronectin for 30 min (left) and in CEF spreading on the non-specific substrate poly-L-lysine (right). Lower panels: localization of paxillin to the periphery of CEF spreading on fibronectin for 30 min (left) and in CEF spreading on the non-specific substrate poly-L-lysine (right). Visualization of a 2 μm section and image capture were with a confocal microscope. Bar represents 25 μm in all cases.

present in the clearly defined focal adhesion structures at the periphery of cells plated on fibronectin, it was concentrated in a region just inside the cell membrane (Figure 1E, lower panels). Thus, unlike active ERK, paxillin was targeted to the peripheral regions of cells after plating on poly-L-lysine, but the generation of well-defined adhesion structures that contain paxillin was dependent upon integrin ligand.

ERK is activated and targeted to focal adhesions upon v-Src activation

Like integrin engagement, v-Src induces the assembly of new focal adhesions at early times after activation (Fincham *et al*., 1996; Fincham and Frame, 1998), as well as the activation of ERK (Wang and Erickson, 1992; Cowley *et al*., 1994; Fincham *et al*., 1996). In previous work, we have used a conditional v-Src protein that is temperature-sensitive (ts) for translocation to peripheral focal adhesions (Fincham *et al*., 1996; Fincham and Frame, 1998), ERK activation (Wyke *et al*., 1995; Fincham *et al*., 1996) and cellular transformation (ts LA29 v-Src; Welham and Wyke, 1988). Focal adhesion formation induced by switching ts v-Src-expressing CEF to the permissive temperature requires an intact actin cytoskeleton that is organized by the Rho family of small G proteins (Fincham *et al*., 1996). Focal adhesion targeting is independent of Src catalytic activity, although the latter is required for focal adhesion turnover during cell motility and transformation (Fincham and Frame, 1998). Here we have tested whether focal adhesion assembly and ERK activation that is induced by ts v-Src at early times after activation is also accompanied by recruitment of activated

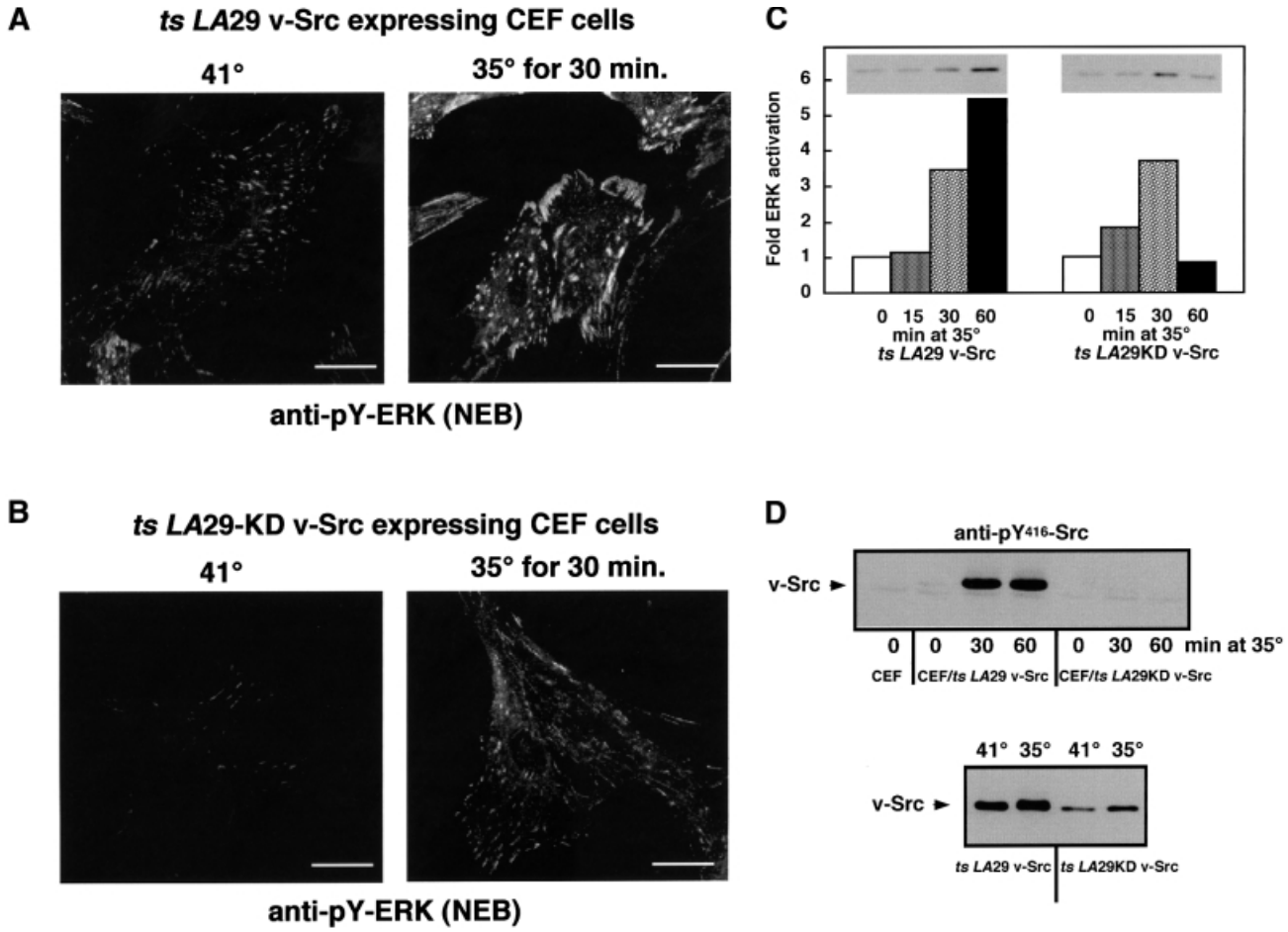

Fig. 2. Phospho-specific ERK antiserum staining of focal adhesions after v-Src activation. (**A**) Localization of phospho-ERK [anti-pY-ERK (New England Biolabs) at 1:100] to focal adhesions in adherent CEF after activation of ts LA29 v-Src by switching from the non-permissive (41°C) to the permissive temperature (35°C) for 30 min. (**B**) A kinase-defective mutant of ts v-Src (ts LA29-KD v-Src) also induced activation and targeting of ERK to focal adhesions on switch from the non-permissive (41°C) to the permissive temperature (35°C) for 30 min. Bar represents 25 μm. Visualization and image capture were with a confocal microscope. (**C**) Densitometric quantification of western blots for active ERK in response to activation of kinase-active (ts LA29) v-Src or kinase-defective (ts LA29KD) v-Src for the times indicated. The times after switching from the non-permissive (41°C) to the permissive temperature (35°C) are shown. Insets show actual data used in the quantification. (**D**) Immunoblots against autophosphorylated Y^{416} Src under the same conditions as in (C) Lower panel, loading controls for total v-Src at permissive and non-permissive temperatures.

ERK to the cell periphery. Thus, CEF expressing ts LA29 v-Src were stained with antiserum specific for the phosphorylated and activated form of ERK. We observed a temperature-dependent staining of phospho-ERK-containing focal adhesions after activation of ts v-Src. Although there was visible staining of cellular focal adhesions at the restrictive temperature (41°C), which was greater than in normal cells (not shown), the intensity of staining was increased after switching to the permissive temperature (35°C) for 30 min (Figure 2A). In cells expressing a kinase-defective variant of the ts LA29 v-Src protein (ts LA29-KD), which we have previously found to undergo temperature-dependent translocation to focal adhesions (Fincham and Frame, 1998), we also observed more intense staining of focal adhesions with anti-phospho-ERK after switching to the permissive temperature (Figure 2B). In this case, the residual staining of focal adhesions at the restrictive temperature was lower than that observed in cells expressing the kinase-active ts LA29 v-Src protein at the restrictive temperature (compare Figure 2A and B, left panels). Consistent with these findings, ERK activity, as measured by immunoblotting using phospho-specific sera, was stimulated by switching cells expressing either kinase-active v-Src or kinase-defective v-Src to the permissive temperature (quantified in Figure 2C). CEF growing in high serum have detectable ERK activity that is not attributable to Src activity; moreover, activation of v-Src can further stimulate this ERK activity. The ERK stimulation induced by kinase-defective v-Src, however, was transient, peaking ~30 min after temperature shift and returning to the unstimulated level by 60 min. In contrast, ERK activity induced by kinase-active v-Src continued to rise to 60 min, and is also sustained at later times after temperature shift of kinase active ts LA29 v-Src (Wyke *et al.*, 1995). Src activation was confirmed by western blotting with an antiserum specific for the autophosphorylated Y^{416} of v-Src. A robust signal was seen by 30 min at the permissive temperature in ts LA29 v-Src-expressing CEF whereas no signal was seen in ts LA29KD v-Src-expressing cells (Figure 2D). Loading levels for v-Src and kinase-defective v-Src are shown in the lower panel.

From these data, we conclude that the assembly of focal adhesions induced after activation of v-Src is accompanied

by the targeting of activated ERK to newly forming adhesions. Thus, as is the case with integrin engagement by plating on fibronectin (Figure 1C and E), v-Src induces early ERK activation and recruitment of activated ERK to cellular adhesions independently of Src catalytic activity.

Anti phospho-ERK sera are specific for active ERK

To validate the conclusion that the phosphorylation-specific anti-ERK sera were detecting phosphorylated and activated ERK at focal adhesions and to confirm that the observed reactivity was not due to spurious cross-reactivity of a particular anti-phospho-ERK serum, we used a range of commercial anti-ERK antisera to stain CEF in response to v-Src activation for 30 min: anti-pY^{185} ERK (New England Biolabs), anti-$pT^{183}pY^{185}$ ERK (New England Biolabs) and anti-$pT^{183}pY^{185}$ ERK (Promega). These anti-ERK sera stained focal adhesions in v-Src-expressing CEF at the permissive temperature (Figure 3A). In addition to examining the focal adhesion targeting of ERK using the anti-phospho-ERK antibodies, we have also used a mouse monoclonal antibody that specifically reacts with the phosphothreonine form of ERK, anti-pT^{183}-ERK (Sigma). Staining with this antibody, in addition to some nuclear staining, also revealed focal adhesion targeting of phosphorylated ERK after v-Src activation that was inhibited by UO126 (Figure 3B). This confirms that the anti-phospho-ERK serum staining of focal adhesions (Figures 2A, B and 3A) is not specific to antisera that recognize phosphotyrosine. We also used two general anti-ERK sera to stain ts v-Src-expressing CEF. As well as nuclear staining, these also diffusely stained the cytoplasm, with ~50% of cells also showing some peripheral adhesion staining at the restrictive temperature, 41°C (Figure 3C; left panels are representative of cells that show some adhesion staining and right panels are representative of cells that show only cytoplasmic and nuclear staining). On switching to the permissive temperature, 35°C, some ERK became more localized to the periphery of all cells, where it concentrated in focal adhesions, often at one edge of the cell (Figure 3C, arrows). In the areas of the cells where ERK was concentrated in focal adhesions, the diffuse cytoplasmic staining was considerably reduced (Figure 3C, 35°C). Thus, the general anti-ERK sera exhibited nuclear, cytoplasmic and peripheral staining, with an apparent net translocation to focal adhesions after activation of ts v-Src (Figure 3C). The detection of ERK in the nuclei of ts v-Src-expressing CEF at both temperatures (Figure 3B and C) is probably due to the presence of high concentrations of serum mitogens, which are known to

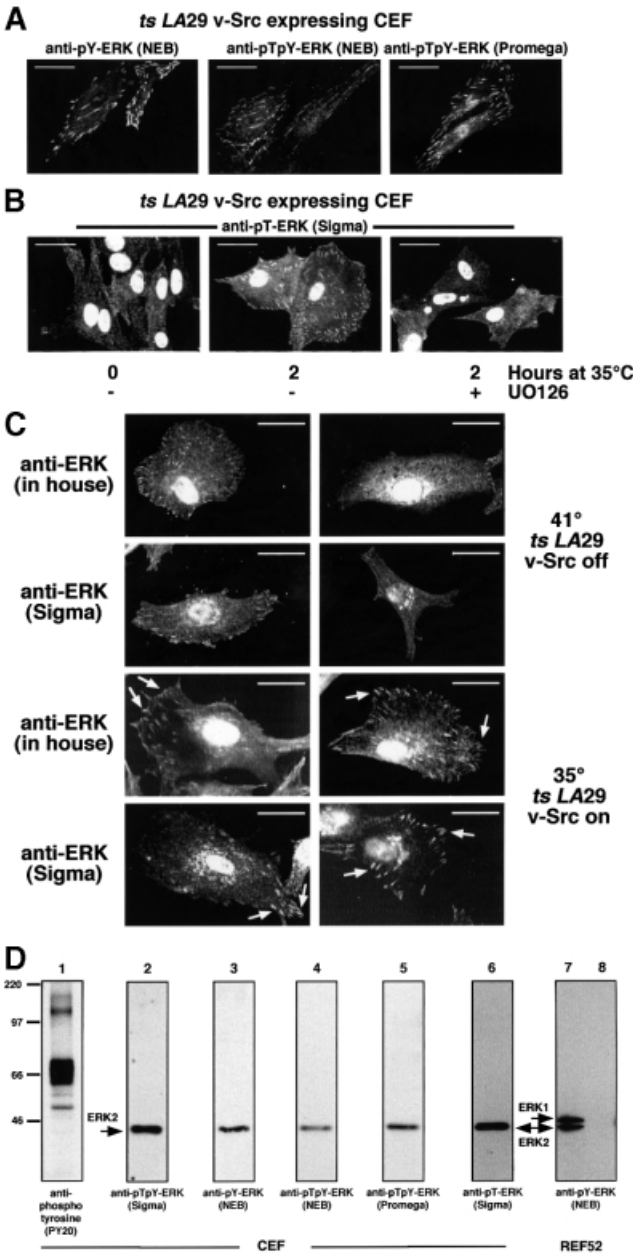

Fig. 3. Specificity of anti-ERK sera. (**A**) CEF were stained with the indicated ERK antiserum [anti-pY-ERK (New England Biolabs) at 1:100; anti-pTpY-ERK (New England Biolabs) at 1:100; anti-pTpY-ERK (Promega) at 1:500] after activation of ts v-Src by switching to the permissive temperature (35°C) for 30 min. (**B**) CEF were stained with anti-pT-ERK (1:100, Sigma) before (0 h) and after (2 h) activation of ts v-Src by switching to the permissive temperature (35°C) and in the absence (–) or presence (+) of 50 μM UO126. (**C**) CEF expressing ts v-Src were stained with pan-specific anti-ERK serum, an anti-ERK polyclonal antibody raised against a peptide from the C-terminus as described by Wyke *et al*. (1995) at 1:500 and anti-ERK polyclonal serum (Sigma) at 1:500, at the non-permissive temperature (top four panels) or 30 min after switch to the permissive temperature (bottom four panels). At 41°C, ~50% of cells showed some peripheral adhesion staining, but in all cases there was nuclear and diffuse cytoplasmic staining (top left panels). In the other ~50% of the cells, there was only nuclear and cytoplasmic staining (top right panels). On switching to 35°C, focal adhesion staining became more intense (arrows), with cytoplasmic clearing around the regions where ERK became concentrated at focal adhesions. Visualization and image capture were with a confocal microscope. Bar represents 25 μm in all cases. (**D**) Immunoblots of lysates of CEF or REF52. Lane 1 shows the profile of phosphotyrosine-containing proteins upon probing lysates of CEF after v-Src activation for 30 min with anti-phosphotyrosine (PY20 at 1:1000). Similar extracts were also immunoblotted with ERK antisera as shown in lanes 2–6 [anti-pTpY-ERK (Sigma) at 1:10 000, anti-pY-ERK (New England Biolabs) at 1:1000, anti-pTpY-ERK (New England Biolabs) at 1:1000, anti-pTpY-ERK (Promega) at 1:5000 or anti-pT-ERK (Sigma) at 1:1000]. In all cases only a single species of ERK2 immunoreactivity was detected (ERK1 is not expressed in CEF). Extracts from EGF-stimulated REF52 cells (100 ng/ml EGF for 30 min) (lane 7) or EGF stimulated REF52 cells also treated with 50μM UO126 (lane 8) were immunoblotted with anti-pY-ERK (New England Biolabs) at 1:1000. The antisera specifically recognized both ERK1 and ERK2 from REF52 cells and all immunoreactivity was abolished by the MEK inhibitor UO126. Numbers on the left of lane 1 represent the positions of molecular weight standards (kDa).

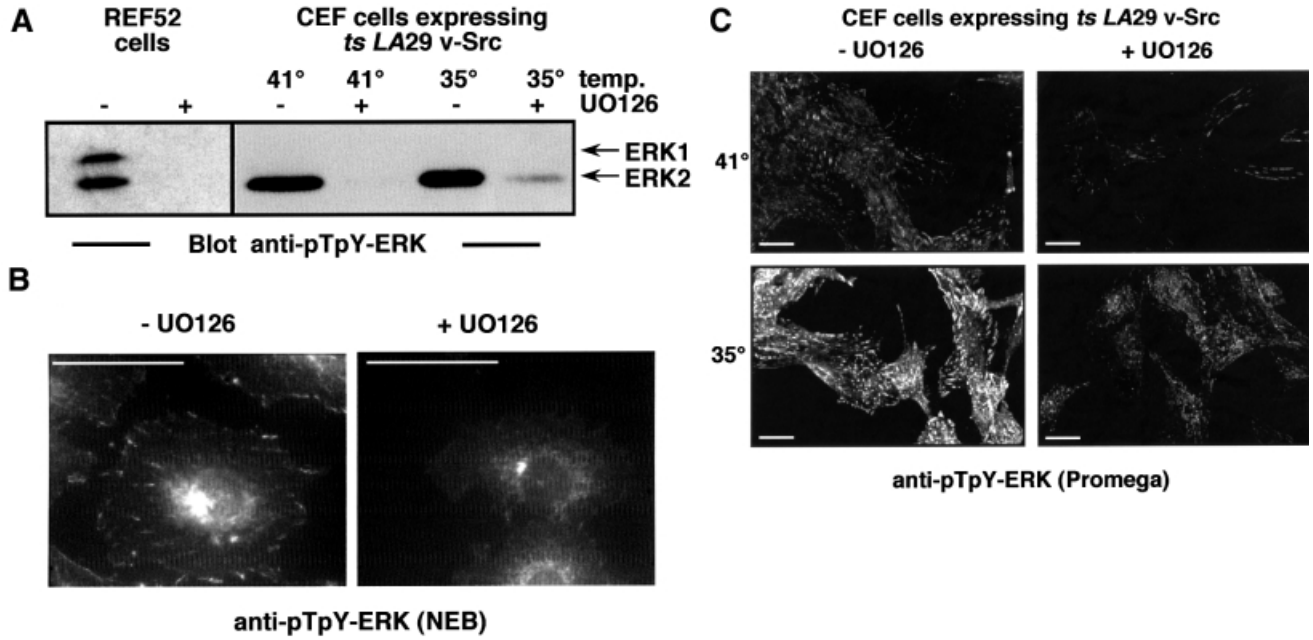

Fig. 4. Inhibition of active ERK staining at focal adhesions by the MEK inhibitor UO126. (**A**) Immunoblot showing effect of the MEK inhibitor (+ or –) UO126 (50 μM) on ERK activity in REF52 cells and ts LA29 v-Src-expressing CEF at restrictive (41°C) and permissive (35°C) temperatures. Blots were probed with anti-pTpY-ERK serum (Promega) at 1:5000; CEF blots are overexposed to show residual Src-dependent anti-phospho-ERK staining after UO126 treatment. (**B**) Localization of active ERK to focal adhesions in REF52 cells spreading on fibronectin for 2 h in the absence (–) or presence (+) of 50 μM UO126 [anti-pTpY-ERK serum (Promega); 1:500]. Visualization and image capture were with an epifluorescence microscope. (**C**) ts v-Src-expressing CEF were maintained at the restrictive temperature (41°C) or shifted to the permissive temperature (35°C) in the absence (–) or presence (+) of UO126 (50 μM). The intensity of active ERK staining [anti-pTpY-ERK serum (Promega) at 1:500] in focal adhesions in response to v-Src activation was reduced in the presence of UO126. Visualization and image capture were with a confocal microscope. Bar represents 25 μm in all cases.

support nuclear localization of ERK (Chen *et al*., 1994; Clark and Hynes, 1996; Lin *et al*., 1997; Renshaw *et al*., 1997; Brunet *et al*., 1999).

The data presented above support the conclusion that active phospho-ERK can be localized at focal adhesions and indicate that the detection of active ERK at focal adhesions by the phospho-specific antisera was not a consequence of non-specific reaction with a tyrosine phosphoprotein in focal adhesions. The latter was further supported by the demonstration that the anti-ERK sera used here specifically recognized ERK2 in immunoblots of proteins extracted from CEF in which ts v-Src had been activated for 30 min (Figure 3D, lanes 2–6). ERK1 is not expressed in CEF (Sanghera *et al*., 1992; Greulich *et al*., 1996). For comparison, an anti-phosphotyrosine immunoblot of the same proteins confirmed that the phospho-ERK antisera specifically reacted with ERK against a high background of other phosphotyrosine-containing proteins (Figure 3D, lane 1). Low stringency immunoblots carried out using the anti-pTpY-ERK (New England Biolabs or Promega) in the absence of any detergents did not reveal additional reactivities (data not shown). We also examined the specificity of anti-pY-ERK (New England Biolabs) and anti-pTpY-ERK (New England Biolabs and Promega) (not shown) against REF52 proteins extracted from cells after EGF treatment, in the absence or presence of the MEK inhibitor UO126. In EGF-stimulated REF52 cells, anti-pY-ERK only recognized active ERK1 and ERK2 (Figure 3D, lane 7), even after over-exposure of the enhanced chemiluminescence (ECL) blot (data not shown). This, together with the loss of reactivity in the presence of the MEK inhibitor UO126 (Figure 3D, lane 8), confirmed that the anti-phospho-ERK sera were specifically recognizing activated ERK.

The MEK inhibitor UO126 prevents ERK activation and targeting to focal adhesions

We next examined the effect of inhibiting ERK activation by the MEK inhibitor UO126 on the targeting of ERK to peripheral focal adhesions. UO126 inhibited ERK activity in REF52 cells, as judged by the loss of signal on protein immunoblots (Figure 4A, left panel, and Figure 3D), and immunoreactivity of anti-phospho-ERK sera at focal adhesions of cells spreading on fibronectin (Figure 4B). This was in contrast to other focal adhesion components, such as talin, which, although present at the cell periphery of REF52 cells spread on fibronectin in the presence of UO126, was not in robust focal adhesion structures (data not shown). Thus, UO126 suppressed integrin-dependent activation and peripheral targeting of ERK to focal adhesions in REF52 cells. In ts v-Src-expressing CEF, UO126 inhibited ERK activity at the restrictive temperature (41°C; Figure 4A, right), but was not able to inhibit ERK activity completely at the permissive temperature (35°C; Figure 4A, right); the blot was deliberately overexposed to highlight this observation. The reduced sensitivity to MEK inhibition when v-Src was active was also evident when the MEK inhibitor PD098059 (Dudley *et al*., 1995) was used, the latter being a less effective inhibitor of ERK activity in both CEF and REF52 (data not shown). The reason for the relative insensitivity of cells in which v-Src is active to inhibitors of MEK remains obscure, but raises the possibility that v-Src-induced ERK activation may be partially independent of the activity of

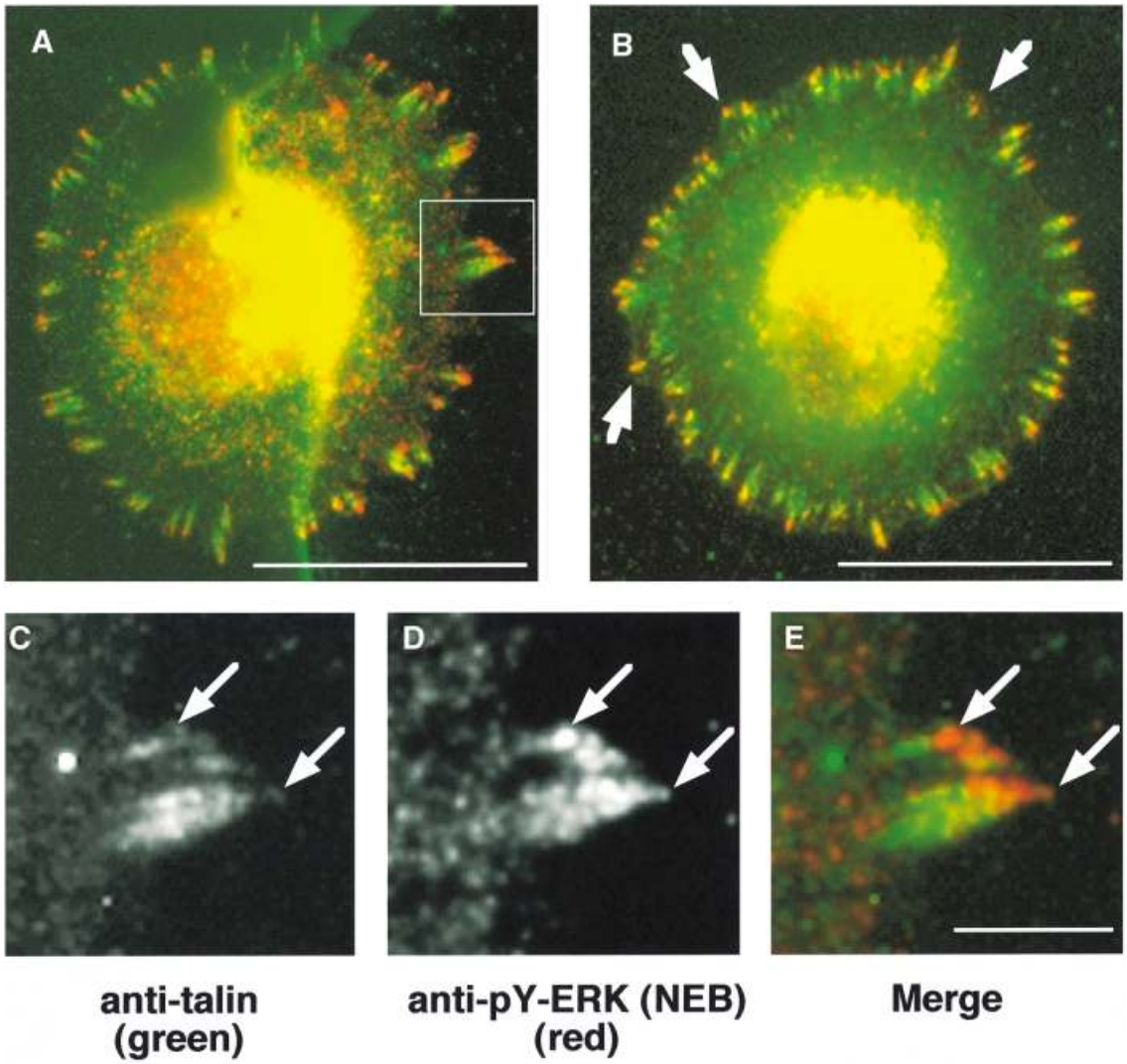

Fig. 5. Targeting of active ERK to new focal adhesions as cells spread. (**A** and **B**) REF52 cells spreading on laminin for 2 h were co-stained for active ERK [anti-pY-ERK antiserum (New England Biolabs) at 1:50], visualized as red, and talin, visualized as green. The active ERK antibody clearly stains the outside nuclear distal edge of the prominent focal adhesions delimited by talin staining. In other regions, active ERK staining is seen alone in the absence of talin [arrowheads in (B)]. (C–E) Individual and merged images of the boxed area in (A): (**C**), talin staining; (**D**) active ERK staining; (**E**) merged image. Arrows point to identical regions in all three images and highlight areas of intense active ERK staining at the cell periphery in the absence of talin staining. Bars represent 25 μm in (A) and (B) and 5 μm in (C)–(E). Visualization and image capture were with an epifluorescence microscope.

MEK. In this context, v-Src can induce phosphatidylinositol 3-kinase and protein kinase C activities, both of which have been proposed to activate ERK directly, independently of MEK (Grammer and Blenis, 1997). None the less, when CEF expressing ts v-Src were switched from the restrictive to the permissive temperature, addition of the MEK inhibitor UO126 suppressed the staining of cellular focal adhesions with an anti-phospho-ERK antibody (Figure 4C, lower panel). The residual staining of focal adhesions in the presence of the inhibitor (Figure 4C, lower right panel), was consistent with some v-Src-induced ERK activity remaining after treatment with the MEK inhibitor (Figure 4A, right).

Targeting and polarity of active ERK within newly forming focal adhesions

The inhibition of cell spreading on ECM components by inhibition of MEK, together with the localization of active ERK to the cell periphery in newly spreading fibroblasts and the activation and targeting of ERK to focal adhesions in response to v-Src, suggested a possible role for ERK in initiating focal adhesion formation. In order to investigate this further, we examined REF52 cells spreading on laminin in the presence of serum. This led to the formation of relatively large and well defined adhesions after ~2 h. Focal adhesions were detected by staining for the structural component talin, a protein that is essential for focal adhesion formation (Priddle *et al*., 1998). Talin delimits the whole focal adhesion and is thus considered a reliable marker of the full physical extent of the adhesion (DePasquale and Izzard, 1991). In addition, talin is rapidly recruited to sites of focal adhesion assembly (Miyamoto *et al*., 1995a) independently of the activation of intracellular signalling cascades (Miyamoto *et al*., 1995b). REF52 cells spreading on laminin, co-stained for talin (green) and phospho-ERK (red), revealed prominent talin-delimited focal adhesions which stained at their distal edges with anti-phospho-ERK (Figure 5A and B). Closer examination

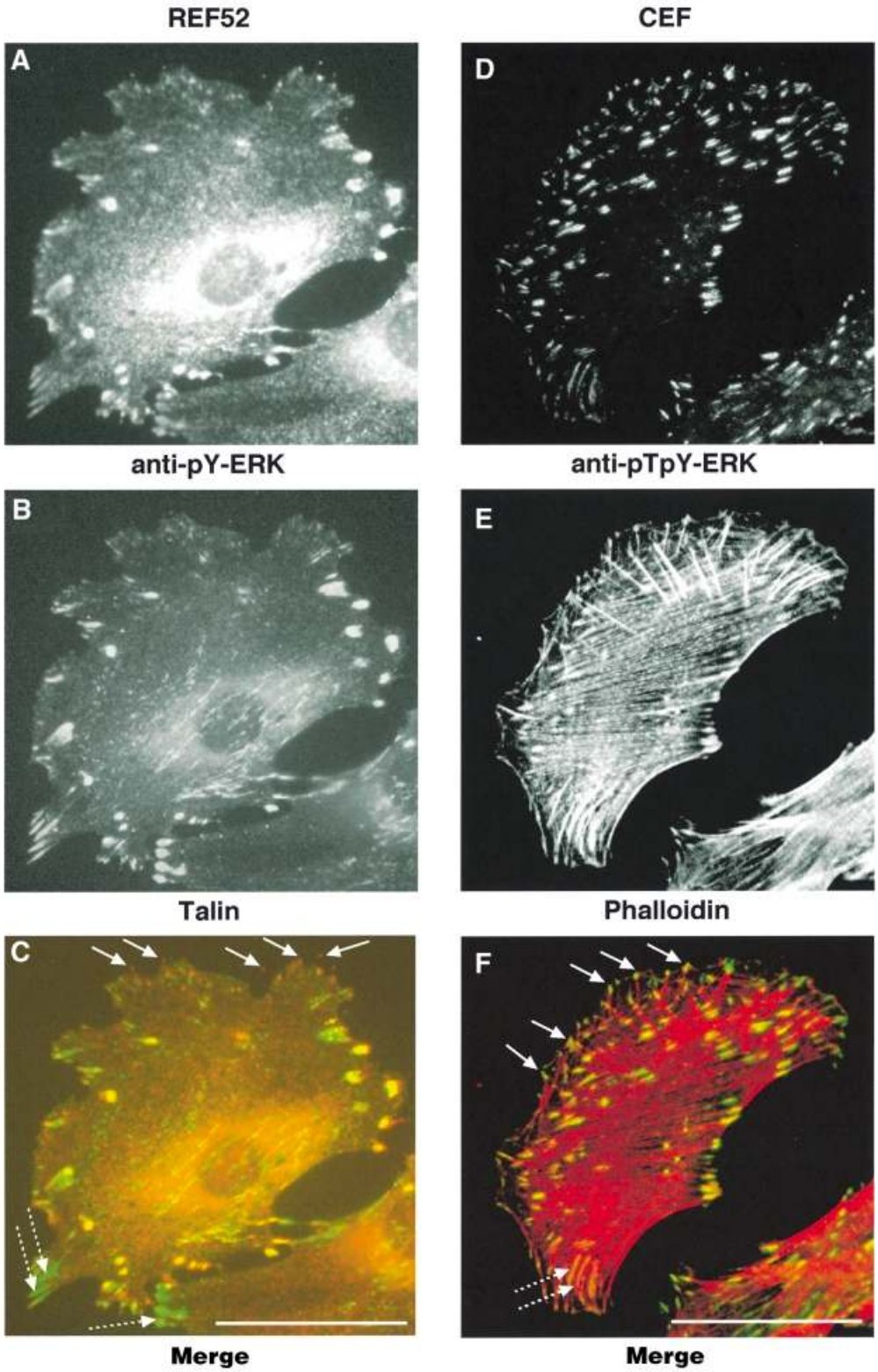

Fig. 6. Targeting of active ERK to lamellipodia. (A–C) REF52 cell spread on laminin and co-stained for active ERK [anti-pY-ERK serum (New England Biolabs) at 1:50], visualized in red channel, and talin, visualized in green channel. Active ERK is shown in (**A**), talin in (**B**) and the merged images in (**C**). Dashed arrows point to retracting or old cell adhesions, where focal adhesions are stained predominantly with talin alone; solid arrows point to new sites of focal adhesion formation at lamellipodia, staining predominantly with active ERK alone. Visualization was with an epifluorescence microscope. (D–F) Spreading CEF co-stained for active ERK [anti-pTpY-ERK serum (Promega) at 1:500], visualized in green channel, and actin filaments, visualized with TRITC–phalloidin (1 μg/ml) in red channel. Active ERK is shown in (**D**), actin in (**E**) and the merged images in (**F**). Dashed arrows point to the retracting region of the cell where focal adhesions are stained weakly by active ERK antisera; solid arrows point to new sites of focal adhesion formation at the tips of actin stress fibres at lamellipodia. Visualization was with a confocal microscope. Bars represent 25 μm.

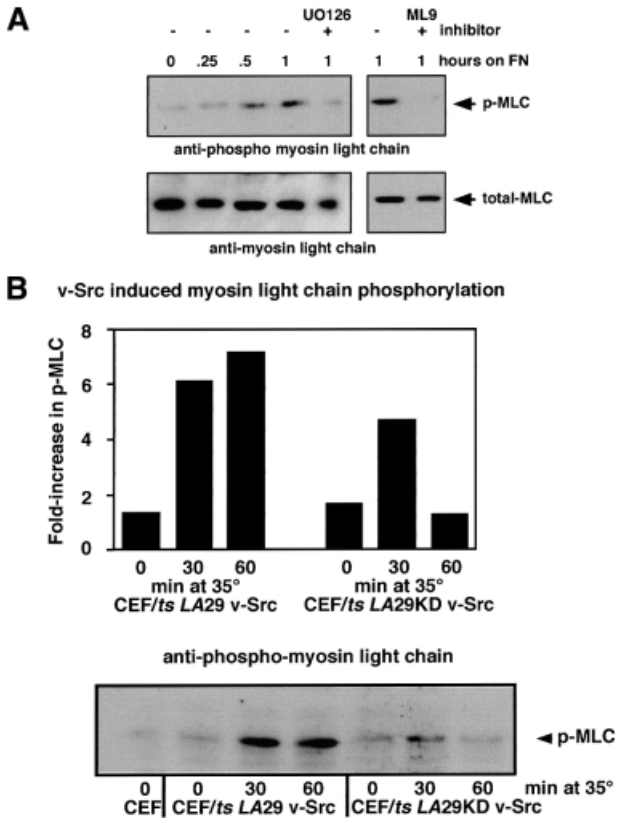

Fig. 7. Phosphorylation of MLC in response to cell adhesion. (**A**) CEF were allowed to adhere to fibronectin (FN)-coated plates for the indicated times in the absence (–) or presence (+) of UO126 (50 μM) or ML9 (7.6 μM). Immunoblots show the amount of diphosphorylated 20 kDa MLC (p-MLC, upper panel). Staining with a total MLC antiserum showed equivalency of protein loading (lower panel). (**B**) Densitometric quantification of immunoblots for phosphorylated MLC in response to activation of kinase-active (ts LA29) v-Src or kinase-defective (ts LA29KD) v-Src for the times indicated. Times after switching from the non-permissive (41°C) to the permissive temperature (35°C) are shown. Lower panel shows the corresponding immunoblots developed with anti-phospho-MLC antiserum.

of these focal adhesions (Figure 5C–E) indicated that as these cells were actively spreading with radial symmetry, as indicated by their round morphology, the active ERK staining appeared membrane-proximal to the talin staining at the very cell periphery. Comparison of the individual images for talin (Figure 5C), active ERK (Figure 5D) and the merged image (Figure 5E) shows the relative absence of talin in distal regions (arrows in Figure 5E) where ERK staining is abundant. Indeed, in some focal adhesions, active ERK staining appeared to be present at the cell periphery in the complete absence of talin (arrowed in Figure 5B), further suggesting that ERK might be recruited to presumptive sites of focal adhesion formation after activation of ERK, but prior to insertion of structural focal adhesion components such as talin.

Examination of active ERK localization in comparison with talin or actin filaments in more polarized cells supported the notion that active ERK was targeted to newly forming focal adhesions. Figure 6A–C shows individual and merged images for active ERK and talin staining in REF52 cells after spreading for 3 h on laminin. These cells have lost their radial symmetry and become more polarized. The overall cell morphology suggests that this cell has some lamellipodial extensions towards the top and retraction fibres are evident at its bottom left. Active ERK is localized to established focal adhesions around the cell periphery, but also as discrete regions of staining in the lamellipodial extensions in the absence of talin (solid arrows). However, ERK localization is not very intense in the focal contacts in retracting regions of the cell (dashed arrows). A similar type of distribution of active ERK was seen in CEF expressing ts v-Src. CEF were stained with Texas Red–phalloidin to visualize actin filament bundles (Figure 6E) and active ERK (Figure 6D). When individual images were merged (Figure 6F), it also became evident in these cells that, at the leading edge where new contacts were being formed, phospho-ERK was present in relatively small structures at the extreme tips of actin filaments (yellow in the merged image in Figure 6F; solid arrows). In contrast, anti-phospho-ERK staining was weakest in the region of cells where mature, more elongated adhesions were found (Figure 6F, dashed arrows). Taken together, these observations strongly suggest that active ERK is targeted to sites of presumptive focal adhesion formation at lamellipodia at the cell periphery, and is reduced or inactivated in old established or disassembling focal adhesions at the trailing edge of the cell. These findings are consistent with a role for ERK in regulating focal adhesion assembly in a polar fashion.

MLCK activity downstream of integrin- and v-Src-induced MEK/ERK activity is essential for peripheral targeting of ERK

Our observations presented above imply that the activation of ERK is spatially and temporally linked to the formation of new focal adhesions. This indicates that 'inside out' biochemical signalling involving ERK has an important role in the assembly of the adhesion structures, which also house components of 'outside in' signalling that is critical for cellular responses initiated at cell–ECM adhesions. We thus sought to define a downstream effector of ERK, and hence the biochemical pathway, that mediates ERK targeting and focal adhesion assembly. One candidate for this was the cytoplasmic ERK substrate, myosin light chain kinase (MLCK) (Klemke *et al*., 1997). As a reporter for MLCK activity, we used an antiserum specific for the diphosphorylated 20 kDa light chain of myosin (MLC) (Ikebe and Hartshorne, 1985; Sakurada *et al*., 1998). We found that MLC was phosphorylated after plating CEF on fibronectin (Figure 7A). Furthermore, using the MEK inhibitor UO126 or the MLCK inhibitor ML9 (Saitoh *et al*., 1987), we found that fibronectin-induced phosphorylation of the MLC was inhibited (Figure 7A). The reduction in MLC phosphorylation was consistent with a loss of MLCK activity and not a reduction in MLC stability. Thus, plating cells on fibronectin induced phosphorylation of MLC by MLCK that was at least partly dependent on MEK/ERK activity. Activation of v-Src also induced robust phosphorylation of MLC with similar kinetics to the phosphorylation of ERK induced by both kinase-active and kinase-defective v-Src (compare Figure 7B with Figure 2C). Thus, both stimuli we have used induce ERK phosphorylation and activation, MLCK-dependent

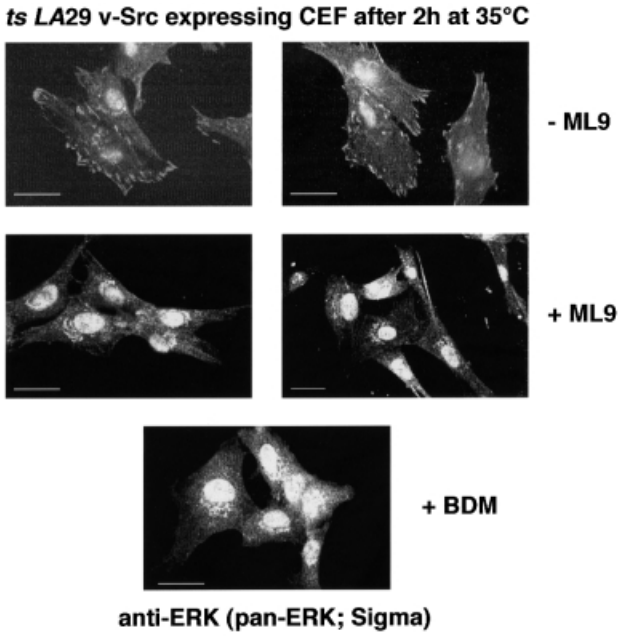

Fig. 8. ERK targeting to focal adhesions in response to Src activation is prevented by inhibitors of myosin contractility. ERK is targeted to focal adhesions in response to ts LA29 v-Src activation [pan-ERK, 1:500 (Sigma); upper panels]. The MLCK inhibitor ML9 (7.6 μM) and the myosin ATPase inhibitor, BDM (20 mM), both prevented the Src-induced targeting of ERK to focal adhesions (lower panels). Visualization was with a confocal microscope. Scale bar represents 25 μm.

phosphorylation of MLC and new focal adhesion assembly.

To determine whether MLCK and myosin activity downstream of MEK/ERK were involved in the peripheral targeting of ERK to newly forming focal adhesions, we used ML9, a pharmacological agent that specifically inhibits MLCK, or 2,3-butanedione 2-monoxime (BDM), which inhibits the ATPase activity of myosin and, hence, actomyosin contractility (Higuchi and Takemori, 1989; McKillop *et al*., 1994). Treatment of cells in which v-Src had been activated for 2 h with either agent was effective at suppressing the peripheral targeting of ERK as monitored using an antibody against total ERK (Figure 8). These data indicate that the activity of MLCK and myosin-induced contractility are required downstream of ERK activation for the peripheral targeting of active ERK.

Discussion

We have demonstrated for the first time that active ERK is present in cellular adhesions. While previous reports have indicated that the temporal mitogen- and integrin-induced activation of ERK is linked to its spatial organization, by stimulation of nuclear translocation (Chen *et al*., 1994; Davis, 1995; Zhu and Assoian, 1995), we have now shown that ERK can also be targeted to sites of cellular attachment where it is present in its active form. Although we did not vary the concentration of serum mitogens in our experiments, and so did not monitor nuclear translocation, there is the intriguing possibility of a direct shuttling of active ERK from peripheral focal adhesions to the nucleus in response to integrin engagement or other stimuli that induce focal adhesion assembly, ERK activation and targeting to newly forming adhesions, as well as a nuclear transcriptional response.

ERK translocation

Several factors, including the kinetics of ERK activation after adhesion and the nature of some potential ERK substrates, made it attractive to postulate that ERK might be located at integrin-dependent peripheral adhesions at appropriate times and perhaps have a role in adhesion assembly. It is clear that, in the absence of soluble growth factors or mitogens, simple cell adhesion and spreading on ligands that bind integrins are sufficient to activate the ERK cascade (Chen *et al*., 1994; Schlaepfer *et al*., 1994; Zhu and Assoian, 1995; Clark and Hynes, 1996; Renshaw *et al*., 1997). Also, substrates, such as poly-L-lysine, that support cell adhesion and spreading without engaging integrins, fail to activate ERK (Chen *et al*., 1994; Schlaepfer *et al*., 1994; Miyamoto *et al*., 1995b; Zhu and Assoian, 1995) and, as we show here, fail to target active ERK to the cell periphery (Figure 1E). Furthermore, in contrast to the robust but transient activation of ERK seen in response to mitogens such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF), typically lasting a few minutes (Zhu and Assoian, 1995; Aplin and Juliano, 1999), integrin engagement alone produces a modest but more sustained activation of ERK lasting up to 2 h (Chen *et al*., 1994; Clark and Hynes, 1996; Lin *et al*., 1997; Renshaw *et al*., 1997). This relatively slow activation of ERK in response to integrin engagement does not reflect the time required for cell attachment *per se*, but parallels time-dependent focal adhesion assembly associated with cell spreading subsequent to attachment (Zhu and Assoian, 1995; Lin *et al*., 1997). These observations are in agreement with the activation and localization of ERK to lamellipodia and focal adhesions in spreading cells (Figures 1 and 6) and the inhibition of cell spreading, but not attachment, by the MEK inhibitor UO126 and an inhibitory mutant of MEK (Figure 1A and B) (Klemke *et al*., 1997). Thus, ERK activation is temporally and spatially associated with integrin clustering during cell spreading and may play a role in regulating the focal adhesion assembly process and/or downstream events initiated at integrin-dependent focal adhesions, such as altered cytoskeletal organization or intracellular signalling. One other possibility raised by the finding that activated ERK localizes at focal adhesions is that ERK activation in response to integrin engagement comprises part of a negative feedback loop which may allow cells to sense, and modulate, their response to the extracellular matrix (proposed by Hughes *et al*., 1997); the target for ERK in the proposed negative regulatory loop is unknown.

MLCK is an ERK substrate that is required for focal adhesion targeting

Components of integrin-linked focal adhesions or the associated actin cytoskeleton are potential ERK substrates that might mediate peripherally located ERK-induced effects on cell adhesion. One of these is MLCK, which can be directly phosphorylated and activated by the sea star oocyte homologue of p44 ERK independently of calcium–calmodulin (Morrison *et al*., 1996). We showed here that MLCK phosphorylation and activity are stimulated down-

stream of MEK/ERK activity after integrin engagement or v-Src activation (Figure 7). Furthermore, MLCK and myosin activities are necessary for the focal adhesion targeting of ERK that is coincident with stimulation of focal adhesion assembly (Figure 8). Thus, our data define a substrate of ERK, and hence a biochemical pathway involving MEK/ERK/MLCK and myosin downstream of integrins and v-Src activity, that mediates the spatial targeting of active ERK to the cell periphery and integrin-induced focal adhesion assembly. Myosin activity, promoted by MLC phosphorylation, is known to be responsible for force generation associated with actomyosin contractility, and it has has been suggested that this drives integrin clustering and focal adhesion assembly from within the cell (Burridge *et al*., 1997). It therefore seems likely that the ERK/MLCK pathway activated rapidly after integrin engagement represents 'inside-out' signalling, which mediates the translocation of active ERK to newly forming sites of adhesion by a mechanism that is linked to actomyosin contractility and stress fibre assembly.

Previous work has shown that, in cells expressing a constitutively active variant of MEK, activation of ERK with subsequent phosphorylation of MLCK leads to enhanced cell migration (Klemke *et al*., 1997). Conversely, inhibition of MEK by PD098059 suppresses MLC phosphorylation and cell migration on collagen (Klemke *et al*., 1997). Further support for a role for ERK in cell migration has come from the observed dependence of integrin-stimulated urokinase-type plasminogen activator (uPA)-promoted cell migration on MLCK phosphorylation downstream of the Ras/ERK pathway (Nguyen *et al*., 1999). Our observations provide a plausible mechanistic explanation for the inferred role of ERK in cell motility, by indicating that ERK itself is targeted to the sites of new focal adhesion assembly, requiring MLCK and myosin activity, and is necessary at these sites for adhesion formation required in cell motility. How ERK activity at newly assembling adhesions influences the spatial organization of other focal adhesion components is unclear, although we have observed that ERK co-localizes and forms a complex with paxillin (data not shown), a multidomain adaptor protein that is implicated in cytoskeletal modelling and focal adhesion assembly (reviewed in Turner 1994, 1998).

Integrin signalling to ERK

One intriguing aspect of ERK signalling is how integrin-dependent adhesion leads to ERK activation. In this regard, integrin engagement induces FAK tyrosine phosphorylation, subsequent Src association and further phosphorylation of FAK on Src-specific tyrosine residues, leading to the binding of Grb2 and coupling to the Ras/Raf/ERK cascade via Son of sevenless (SOS) (Schlaepfer *et al*., 1994, 1998). However, the critical role of FAK or Ras in transmitting signals from integrins to ERK remains contentious, since FAK- or Ras-independent activation of ERK has also been demonstrated (Chen *et al*., 1996; Lin *et al*., 1997), implying that multiple adhesion-induced pathways can converge on the ERK cascade at different points. Thus, although the mechanism of ERK activation by the stimuli we have used here to induce new focal adhesion assembly, i.e. integrin engagement or activation of v-Src, is not well defined, what we have demonstrated is that ERK is recruited to these peripheral sites of signal initiation and is present there in an activated form. Although FAK is also present at newly forming focal adhesions induced by integrins and v-Src (Fincham and Frame, 1998), we have not yet determined whether other possible upstream components of the ERK activation pathways mentioned above are also recruited. In this regard, we note that activated mutants of Src can phosphorylate Raf-1 on tyrosine and contribute to its activation (Marais *et al*., 1995), although there are no reports of Raf-1 localization specifically at focal adhesions. However, in one recent report, MEK was found to localize to the cell periphery (Kranenburg *et al*., 1999), as was activated MAP kinase (Gonzalez *et al*., 1993), and MEKK1, an upstream activator of the c-Jun N-terminal kinase/stress-activated kinase (JNK/SAPK), has been shown to associate with actin stress fibres and localize at focal adhesions (Christerson *et al*., 1999). In addition, beads coated with fibronectin, RGD peptides or integrin antibodies are reported to bind cellular proteins enriched for focal adhesion signalling components, including Src, FAK, phosphatidylinositol 3-kinase and phospholipase Cγ (Plopper *et al*., 1995) and components of the MAPK cascade including ERK1 and 2 (Miyamoto *et al*., 1995b). Taken together with our observations, these findings indicate that focal adhesions physically house all the components of signalling cascades that mediate adhesion-induced cellular responses, including components of the ERK pathway.

In conclusion, we have demonstrated that active ERK is translocated to newly forming focal adhesions in response to integrin engagement or activation of v-Src, and that inhibition of its upstream activating kinase, MEK, suppresses peripheral targeting of ERK and integrin-dependent focal adhesion assembly. MLCK and myosin activities downstream of MEK/ERK are required for the focal adhesion targeting of ERK. Our data also provide an explanation for the recently reported requirement for MEK/ERK and MLCK activities during cell migration, by indicating that ERK itself undergoes MLCK-dependent targeting to newly forming adhesions and functions at these sites to regulate adhesion assembly, an important component of the cell motility process. The translocation of some active ERK to newly forming focal adhesions may serve to direct specificity toward appropriate downstream targets at cellular adhesions.

Materials and methods

Cell culture

Primary CEF were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% chick serum, 5% newborn calf serum and 10% tryptose phosphate and were maintained in 5% CO_2. Low density cultures were transfected with replication-competent avian retroviral ts LA29 v-Src construct or its kinase-defective variant (5 μg DNA per 25 cm^2 flask) or with retroviral vector alone, by the *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*,-trimethylammonium methylsulfate method as previously described (Fincham and Frame, 1998). Cultures were grown at the permissive temperature (35°C) until uniformly infected and expressing high levels of v-Src protein, and then maintained at the restrictive temperature (41°C) or shifted to the permissive temperature for the required times. The rat embryo fibroblast cell line REF52 was grown in DMEM supplemented with 10% fetal calf serum (FCS) in 5% CO_2. Where cells were plated on ECM, coverslips were incubated with

fibronectin (Gibco-BRL), laminin (Gibco-BRL) or poly-L-lysine (Sigma; all at 1 μg/ml) overnight at 4°C and washed extensively with phosphate-buffered saline (PBS) before use. When required, serum-free DMEM was supplemented with the MEK inhibitor UO126 (50 μM), myosin ATPase inhibitor BDM (20 mM) or the MLCK inhibitor ML-9 (7.6 μM) for the indicated periods of time. HA-tagged ERK (Meloche *et al*., 1992) was introduced into 50% confluent cultures of REF52 cells using Lipofectamine (Gibco-BRL; 6 μl/ml) and a final DNA concentration of 1 μg/ml in Optimem (Gibco-BRL) over 5 h. Cells were transferred to DMEM with serum and cultured for a further 15 h prior to assay. A dominant inhibitory mutant of MEK (Cowley *et al*., 1994) or the empty expression vector was co-transfected with pcDNA3.1–GFP (Fincham *et al*., 1999) at a ratio of 5:1 into REF52 cells as described above.

Protein immunoblotting

Subconfluent dishes of cells were washed twice with ice-cold PBS and lysed with modified radio-immunoprecipitation assay (RIPA) buffer [50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EGTA, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1% Nonidet P40 (NP-40), 10 mM sodium pyrophosphate, 0.5 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml each of aprotinin, leupeptin and benzamidine and 100 μM sodium vanadate] for 15 min on ice. The insoluble material was pelleted at 14 000 *g* for 30 min at 4°C. Protein concentrations were measured by the Micro BCA method (Pierce). For direct detection of proteins, 5 μg of total cell lysate (ERK blots), 20 μg (phosphotyrosine blot) or 50 μg (MLC or Src blots), were separated by SDS–PAGE, transferred to polyvinylidene difluoride and probed with 1:10 000 anti-ERK (Sigma), 1:1000 polyclonal anti-$pT^{183}pY^{185}$ ERK (New England Biolabs), 1:1000 polyclonal anti-pY^{185} ERK (New England Biolabs), 1:2000 anti-$pT^{183}pY^{185}$ ERK (Promega), 0.5 μg/ml anti-pT^{183} ERK (Sigma), 1:1000 anti-MLC (Sigma), 1:1000 anti-phospho-MLC (Sakurada *et al*., 1998), 0.25 μg/ml anti-pY^{416} Src (Biosource International); 1:1000 anti-Src (UBI) and 1:1000 anti-phosphotyrosine monoclonal antibody (PY20; Transduction Labs) as required. Detection was by reaction with horseradish peroxidase-conjugated secondary antibody and visualization was by ECL according to the manufacturer's instructions (Amersham).

Immunofluorescence

Cells were grown on glass coverslips and fixed for 10 min at –20°C in methanol (or occasionally with 3.7% paraformaldehyde in the case of v-Src-expressing cells). Fixed cells were blocked in 5% FCS, 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature and then incubated with primary antibody alone or in combination, using dilutions which were optimized for individual sera with the fixation conditions used and which are indicated in the figure legends: polyclonal anti-ERK serum (Sigma), anti-ERK polyclonal serum (Wyke *et al*., 1995); polyclonal anti-pT^{183} ERK serum (Sigma), polyclonal anti-pY^{185} ERK serum (New England Biolabs), polyclonal anti-$pT^{183}pY^{185}$ ERK serum (New England Biolabs), polyclonal anti-$pT^{183}pY^{185}$ ERK serum (Promega), anti-HA polyclonal serum (Santa Cruz Biotech) and anti-talin serum (Sigma). Talin was visualized with a fluorescein isothiocyanate (FITC)-coupled anti-mouse IgG (Vector Laboratories) at 1:100 and all ERK and anti-HA antisera were visualized by incubation with species-specific anti-IgG coupled to biotin (Vector Laboratories) at 1:750 followed by streptavidin coupled to FITC or TRITC (Vector Laboratories), as appropriate. Actin filaments were visualized with TRITC–phalloidin (1 μg/ml for 40 min). Specific details are summarized in the individual Figure legends where appropriate. Coverslips were mounted in Vectashield (Vector Laboratories) and images captured digitally by cooled CCD camera on an Olympus Provis epifluorescence microscope or using an MRC600 confocal microscope.

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