Activation of either ERK1/2 or ERK5 MAP kinase pathways can lead to disruption of the actin cytoskeleton

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Accepted 3 February 2005
Journal of Cell Science 118, 1663-1671 Published by The Company of Biologists 2005
doi:10.1242/jcs.02308

Summary

Oncogenic transformation often leads to the disruption of the actin cytoskeleton. Activation of the classical Ras-Raf-MEK1/2-ERK1/2 signalling cascade has been implicated in the effects of oncogenes such as *Ras* and *Src* on the cytoskeleton. Many of the studies of the effects of oncogenes on the cytoskeleton have made use of chemical inhibitors of MEK1/2 but it is now clear that these inhibitors also inactivate MEK5 in the MEK5-ERK5 MAP kinase pathway raising the possibility that this pathway may also be involved in oncogenic transformation. We therefore investigated whether activation of ERK5 can lead to disruption of the actin cytoskeleton. We show that activation of ERK5 can lead to loss of actin stress fibres, but by a distinct mechanism to ERK1/2. We demonstrate that ERK5 is activated by oncogenic Src as demonstrated by translocation of endogenous ERK5 from the cytoplasm to nucleus and activation of an ERK5-dependent transcriptional reporter and that ERK5 activation is required for Src-mediated transformation. We also show that in *Src*-transformed cells inhibition of ERK1/2 signalling is not sufficient for reappearance of the actin cytoskeleton and that ERK5 activation contributes to cytoskeletal disruption by Src. Our results suggest that multiple MAP kinase pathways downstream of oncogenes participate in cytoskeletal alterations.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/118/8/1663/DC1

Key words: ERK5, ERK1/2, MAPK, Ras, Src, Cytoskeleton

Introduction

The organisation of the actin cytoskeleton is controlled by the Rho family of small GTPases. Rho controls the assembly of stress fibres, focal adhesions and actomyosin contractility, whereas Rac regulates actin assembly in lamellipodia and Cdc42 actin assembly in filopodia (Ridley and Hall, 1992a; Ridley and Hall, 1992b). Altered regulation of the actin cytoskeleton is a common feature of malignant transformation. Transformed cells frequently show evidence of downregulated Rho signalling by the loss of actin stress fibres and increased Rac signalling by enhanced membrane ruffling (Pollack et al., 1975; Frame and Brunton, 2002). These alterations to the cytoskeleton can lead to increased cell motility through changes in actin dynamics and decreased cell adhesion. Increased cell motility is an important component of the invasive phenotype of cancer cells. It is apparent from a number of studies that decreased Rho signalling through Rho kinase is required for some forms of cell motility (Nakahara et al., 1998; Arthur and Burridge, 2001; Sahai et al., 2001; Vial et al., 2003). Downregulation of Rho signalling to Rho kinase may increase cell motility via the enhancement of Rac-driven protrusions (Arthur and Burridge, 2001; Vial et al., 2003) and by decreased formation of focal adhesions (Nobes and Hall, 1994).

The mechanisms through which changes in the actin cytoskeleton occur in oncogenic transformation have been the focus of much investigation. One approach to this problem has been to delineate which signalling events downstream of oncogenes such as *Ras* or *Src* are involved in disruption of the cytoskeleton. Evidence has accumulated that both the classical extracellular activated kinase (ERK) 1/2 mitogen activated protein (MAP) kinase pathway and the phosphoinositide 3-kinase (PI 3-kinase) pathway can contribute to alterations in the actin cytoskeleton. The PI 3-kinase pathway has been shown to be responsible for RAC-dependent membrane ruffling downstream of Ras (Rodriguez-Viciana et al., 1997), although it is clear from other studies that ERK1/2 signalling can contribute to membrane ruffling downstream of oncogenic Ras through mechanisms such as transcriptional upregulation of urokinase plasminogen receptor (uPAR), an activator of Rac (Vial et al., 2003). ERK1/2 signalling has been shown to lead to disruption of actin stress fibres and loss of focal adhesions by several mechanisms. In *Ras*-transformed cells with high levels of Rho-GTP, ERK1/2 signalling leads to downregulation of Rho kinase levels (Sahai et al., 2001). Similar mechanisms may operate in *Src*-transformed cells (Pawlak and Helfman, 2002). In cells transformed by oncogenic *Ras* that have low levels of Rho-GTP, suppression of integrin signalling to Rho activation occurs through ERK1/2 transcriptional induction of Fra-1 (Vial et al., 2003). In *Src*-transformed cells decreased cell adhesion has been shown to result from activated ERK1/2 being recruited to focal adhesions (Fincham et al., 2000) and

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leading to their increased turnover and decreased adhesion through a calpain-dependent mechanism (Carragher et al., 2001). However other mechanisms such as activation of p190Rho-GAP through tyrosine phosphorylation (Fincham et al., 1999) can also contribute to disruption of the actin cytoskeleton in transformed cells.

As well as activating the classical ERK1/2 MAP kinase pathway it is now clear that a number of tyrosine kinases such as the epidermal growth factor (EGF) receptor (Kamakura et al., 1999), HER2/Neu (Esparis-Ogando et al., 2002) and Src (Abe et al., 1997) as well as oncogene products such as Ras (Kamakura et al., 1999) and COT (Chiariello et al., 2000) can activate another MAP kinase pathway: the MEK5-ERK5 pathway. This pathway has been much less extensively studied than the ERK1/2 pathway but it is apparent that it can contribute to the same cellular responses as ERK1/2 such as cell proliferation (Dong et al., 2001; Kato et al., 1998) and cell transformation (Pearson et al., 2001). Unlike other MAP kinases, ERK5 has a C-terminal transcription factor domain that is activated through autophosphorylation, thus ERK5 may signal both through itself (Kasler et al., 2000) and via the phosphorylation of substrates. Substrates of ERK5 include Sap1a (Kamakura et al., 1999) and myocyte enhancer factor 2 (MEF2) family members (Kato et al., 1998; Yang et al., 1998). Of considerable significance to understanding the roles of ERK1/2 and ERK5 in cellular responses is the demonstration that the commonly used MEK1/2 inhibitors also inhibit the activation of ERK5 (Kamakura et al., 1999; Mody et al., 2001), thus it is possible that some of the effects ascribed to ERK1/2 signalling may actually be a consequence of ERK5 signalling.

We therefore investigated the possibility that signalling to ERK5 contributes to oncogenic transformation by using one of the most widely studied oncogenes, the *Src* tyrosine kinase. We also wanted to determine whether this ERK5 signalling leads to disruption of the actin cytoskeleton during cell transformation by *Src*.

Materials and Methods

Plasmids

pGL2basicRSRF (luc2wt) (Woronicz et al., 1995) and pCI FL-MEF2D were gifts from A. Winoto (University of California, Berkeley, CA). HA-MEK5, HA-MEK5DD (Ser313 and Thr317 in MEK5 substituted with Asp) and HA-MEK5AA (Ser311 and Thr315 in MEK5 substituted with Ala) pCMV5 plasmids as well as Flag-ERK5AEF (Thr218 and Tyr220 in ERK5 substituted with Ala and Phe) pcDNA3 plasmid (Kato et al., 1997) were gifts from J. D. Lee. pDL SRα HA-ERK5 (Kamakura et al., 1999) was a gift from E. Nishida. pFlag-CMV2 ERK2 was a gift from S. Benjamin (King's College, London, UK). MEK1, MEK1A (Ser217 and Ser221 in MEK1 substituted with Ala) and MEK1EE (Ser217 and Ser221 in MEK1 substituted with Glu) pBabePuro plasmids were made by S. Cowley (Cowley et al., 1994). PEF RhoAV14 was a gift from R. Treisman (London Research Institute CR-UK, London, UK). pEF Y527F SRC and β-Gal expressing vector pON249 (Knowlton et al., 1991) were gifts from R. Marais (Institute of Cancer Research, London, UK). pEGFP-C1 was purchased from Clontech and was used as a transfection marker for immunofluorescence experiments. Myc-HRasV12 pEF6 vector was made by M. Rosario. Neu* (Val664 in the transmembrane domain has been substituted with Glu) and P1* (has a deletion of the C-terminal except for the most terminal tyrosine, Tyr1253) pLSV plasmids were made by R. Ben-Levy (Ben-Levy et al., 1994).

Antibodies

ERK2 polyclonal rabbit antibody (122) (Leevers and Marshall, 1992) was used to detect ERK2 expression. Mouse panERK and MEK1 antibodies were obtained from BD Transduction Laboratories. Phosphorylated ERK1 and ERK2 were detected with a phospho-specific antibody (clone MAP-YT) from Sigma. Mouse monoclonal antibody against MEK5 was purchased from BD Biosciences and the ERK5, Flag (M2), vinculin and β-tubulin antibodies were purchased from Sigma. Mouse Myc tag (9E10) (Evan et al., 1985) and HA tag (12CA5) antibodies were obtained from the Hybridoma Unit at the Institute of Cancer Research, Sutton, UK. The RhoA rabbit polyclonal (119) and mouse monoclonal (26C4) were obtained from Santa Cruz Biotechnology. All horseradish peroxidase (HRP)-conjugated antibodies were from Pierce Biotechnology. Fluorochrome-conjugated antibodies were acquired from Jackson ImmunoResearch Laboratories and Texas Red-conjugated phalloidin was from Molecular Probes. The MEK inhibitor PD184352 (Davies et al., 2000) was from Calbiochem.

Cell culture

All cells were grown at 37°C, 10% CO_2 and 99% relative humidity. Untransformed NIH3T3 as well as Ras-transformed 149169 and v-*Src*-transformed NIH3T3 were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% donor calf serum (DCS) for the untransformed and 5% for the transformed cells (Gibco BRL). DMEM media for serum starvation of transformed and untransformed cells was supplemented with insulin-transferrin-sodium selenite (ITS, Sigma-Aldrich). All media was supplemented with penicillin (60 μg/ml) and streptomycin (100 μg/ml). The 149169 cell line, expressing *N-Ras*, is a tertiary transfectant from transfection with DNA from human rhabdosarcoma cells. The *Src*-transformed cell line clones D4F2, D4F3, D4F6, D4F8, D4F9ac2M, D4F9ac2L are derived from a transfection with v-*Src*.

Cell lysates for analysis of ERK1/2 phosphorylation were prepared by scraping 30 mm plates with 40 μl extraction buffer [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5% (v/v) glycerol, 1% (v/v) Triton X-100, 1.5 M KCl, 5 mM NaF, 0.2 mM Na_3VO_4, 0.3% (v/v) β-mercaptoethanol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μM phenylmethylsulfonyl fluoride (PMSF)] followed by a dilution in 160 μl dilution buffer (as extraction buffer, but with 10% glycerol replacing the KCl). For ERK5 immunoblots, cells were harvested in 200 μl ERK5 lysis buffer consisting of 0.3% (v/v) β-mercaptoethanol, 1 mM Na_3VO_4, 1 mM PMSF, 1% aprotinin, 50 mM NaF and 5 mM $Na_4P_2O_7$. For western blot analysis, membranes were blocked with 3% BSA (Sigma) in TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and antibodies were diluted 1% BSA TBST.

Luciferase and β-galactosidase assay

Cell lysates used for determination of luciferase activity were prepared in 200 μl luciferase cell culture lysis reagent (Promega). The luciferase assay was carried out according to the manufacturer's instructions (Promega) and luciferase activity was determined in an AutoLumat LB953 luminometer. The results were subsequently adjusted with the β-galactosidase assay. The reaction mix for each β-galactosidase assay was made up of 400 μl LacZ buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 2.7 ml/l β-mercaptoethanol), 80 μl of 5 mg/ml O-nitro-phenyl-β-D-galactoside and 10 μl of 1 M Tris-HCl (pH 7.8). 40 μl of the cell lysate supernatant prepared for the luciferase assay was added to the reaction mix and incubated for up to 2 hours at 37°C. The reaction was stopped by addition of 250 μl of 1 M Na_2CO_3 and the absorbance measured at 420 nm.

Focus assays

Untransformed low-passage NIH3T3 parental cells were transfected

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in 30 mm six-well plates with the required DNA using Lipofectamine reagent and transferred to 10% DCS after 6 hours. 24 hours after the start of transfection, each well was trypsinised, divided equally over two 100 mm tissue culture dishes and cultured in DMEM media containing 5% DCS. Cells were grown for 11-16 days, with media changes every 3 days. Plates were fixed and stained with 1% (w/v) Crystal Violet solution in 70% ethanol, for around 20 seconds, followed by several washes with large volumes of H_2O. The plates were then blind scored for the appearance of foci of morphologically and growth-transformed cells.

Microinjections and immunofluorescence

All cell lines were seeded on plastic dishes and grown in the normal growth media for 16 hours prior to microinjection. Plasmids were diluted in phosphate-buffered saline (PBS) to a final concentration of 50 ng/ml. DNA for use in microinjections was prepared on a double caesium chloride gradient. To harvest, cells were washed with PBS, fixed with 4% formaldehyde, permeabilised in 0.2% Triton X-100 and blocked with 10% FCS. Samples were stained sequentially with the relevant primary antibody followed by fluorochrome-coupled secondary antibodies. All buffers and antibody dilutions were made in PBS. Samples were examined using a BioRad MRC 1024 confocal imaging system equipped with a Nikon eclipse 400 microscope. Detection of focal adhesions by vinculin staining required cells to be permeabilised before fixation, for this, cells were washed once in PBS and then once in buffer A (50 mM $MgCl_2$, 3 mM EGTA, pH 6.0). Cells were then permeabilised in buffer A containing 0.5% Triton X-100 for 2 minutes, followed by a wash with buffer A, and then fixed for 15 minutes in buffer A containing 4% formaldehyde. All the permeabilisation/fixation procedures for vinculin staining were carried out on ice using ice-cold solutions. Cells were then washed extensively in PBS and stained as described above.

Results

ERK1/2 and ERK5 disrupt the cytoskeleton by different mechanisms

In order to determine whether ERK5 pathway activation can cause the disruption of the actin cytoskeleton, we investigated the effects of constitutive activation of the ERK5 pathway on organisation of the actin cytoskeleton. Introduction into NIH3T3 cells of an activated version of MEK5, MEK5DD (English et al., 1999), in which the regulatory phosphorylation sites in the activation segment are replaced by negatively charged Asp residues, selectively activates ERK5 but not ERK1/2 as shown by the presence of a slower migrating phosphorylated band of ERK5 but not an activated phosphorylated band of ERK2 (Fig. 1A). To examine the effects of activation of ERK5 on the actin cytoskeleton, NIH3T3 cells, which have well-developed actin stress fibres, were microinjected with plasmids to express activated versions of the MEK5 protein (MEK5DD) and wild-type ERK5. Expression of MEK5DD with wild-type ERK5 protein resulted in a striking disruption of the actin stress fibres of serum-deprived NIH3T3 cells (Fig. 1B). Scoring microinjected cell showed that 34 out of 38 injected cells had a disrupted cytoskeleton. Microinjection of MEK5DD with wild-type ERK5 also disrupted the cytoskeleton in Swiss3T3 cells (data not shown). Although injection of MEK5DD alone disrupted the cytoskeleton in some cells (data not shown), the effects were less pronounced than when co-injected with ERK5. In a number of other studies investigating ERK5 signalling it has been noted that more pronounced effects are observed upon coexpression of activated MEK5 with ERK5 rather than just

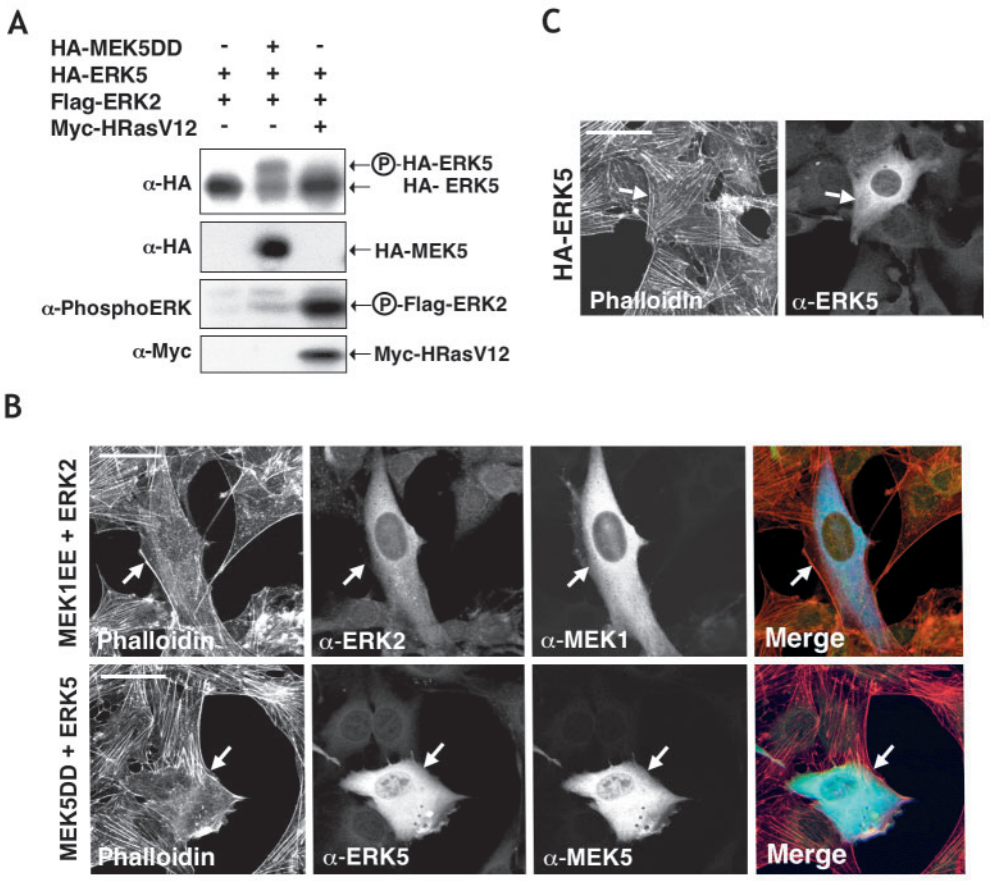

Fig. 1. Activation of ERK5 leads to disruption of the actin cytoskeleton. (A) MEK5DD activates ERK5 but not ERK1/2. Expression vectors for HA-tagged ERK5 and Flag-tagged ERK2 were co-transfected with expression plasmids for HA-MEK5DD, Myc-H-RasV12 or empty vector. After 24 hours cells were lysed and analysed by SDS-PAGE followed by western blotting for epitope tags, ERK5 or phospho-ERK. The two top arrows indicate the slower migrating phosphorylated form of ERK5 (phosphorylated) and non-phosphorylated ERK5. (B,C) NIH3T3 cells were microinjected, in the presence of serum, with expression vectors for activated MEK1 (MEK1EE) and ERK2, activated MEK5 (MEK5DD) and ERK5. 3 hours post-injection, cells were starved of serum and after 24 hours, plates were fixed and stained for polymerised actin with Texas Red-phalloidin and for the expressing proteins with antibodies against MEK1, ERK2, MEK5 and ERK5. Arrows indicate microinjected cells. Bar, 20 μm.

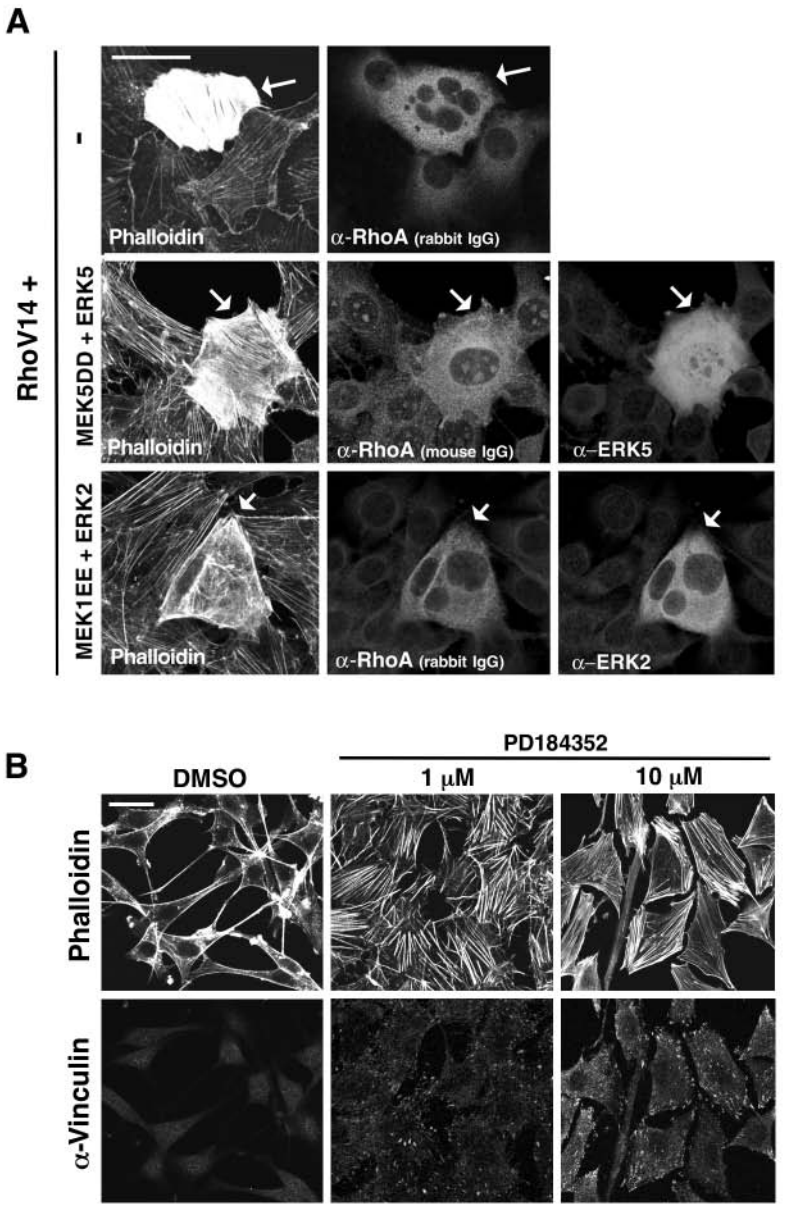

Fig. 2. ERK5 and ERK1/2 act differently to disrupt the actin cytoskeleton. Activation of ERK5 does not block the ability of constitutively activated RhoA to stimulate the formation of actin stress fibres. (A) NIH3T3 cells were microinjected, in the presence of serum, with expression vectors for constitutively activated RhoA (RhoAV14) alone or in combination with activated MEK1 (MEK1EE) + ERK2 or activated MEK5 (MEK5DD) + ERK5. 3 hours post-injection cells were serum starved and after 24 hours were fixed and stained with ERK2 and ERK5 antibodies. RhoAV14 expression was recognised with either a mouse or rabbit RhoA antibody and polymerised actin was detected using Texas Red-phalloidin. Arrows indicate injected cells; (B) treatment with 1 μM PD184352 restores the actin cytoskeleton in *Ras*-transformed NIH3T3 cells (clone 149169). 149169 cells were treated for 24 hours with 1 μM PD184352 or vehicle, permeabilised, fixed and stained for polymerised actin as in A and for vinculin, as a marker of focal adhesions, with a mouse monoclonal antibody followed by a anti-mouse FITC-coupled antibody. Bar, 20 μm.

activated MEK5 alone (Pearson et al., 2001; Yan et al., 2001; Mulloy et al., 2003). It is not clear why this is the case, but indicates that the levels of endogenous ERK5 are limiting for signalling by overexpressed MEK5DD. Expression of ERK5 alone did not cause any alteration of the actin cytoskeleton of the microinjected cells (Fig. 1C), indicating that ERK5 activity rather than just ERK5 overexpression is needed to disrupt the stress fibres in these conditions. As expected, expression of ERK2 and an activated version of MEK1 in which the regulatory phosphorylation sites are replaced by negatively charged Glu residues (MEK1EE) (Cowley et al., 1994) also led to the disruption of the cytoskeleton in NIH3T3 cells (12 out of 12 cells) (Fig. 1B).

Previously we have shown that the disruption of the actin cytoskeleton in Ras-transformed cells cannot be overcome by constitutively active RhoA but can be overcome by overexpression of the downstream kinase LIMK (Sahai et al., 2001). Therefore, as an approach to determine whether activation of the ERK5 pathway works in the same way as activation of ERK1/2 to disrupt the actin cytoskeleton we investigated whether activation of ERK5 could overcome signals from constitutively activated RhoA (RhoAV14) to actin organisation. In cells expressing MEK5DD and ERK5, coexpression of RhoAV14 leads to the formation of actin stress fibres whereas in cells expressing MEK1EE and ERK2, half (8/16) of the cells coexpressing RhoAV14 show disorganised stress fibres (Fig. 2A). The remaining cells have either a few stress fibres or an unusual stellate morphology. The difference in the effects of RhoAV14 on cells expressing MEK5DD compared to MEK1EE cannot be ascribed to changed activation of ERK5 or ERK1/2 as we find no effect of expression of RhoAV14 on the ability of MEK5DD or MEK1EE to activate signalling (see Fig. S1 in supplementary material). These results suggest that ERK5 activation acts differently to ERK1/2 activation to disrupt the actin cytoskeleton and that Ras signalling operates through the ERK1/2 pathway rather than the ERK5 pathway to disrupt the actin cytoskeleton. To test this hypothesis we examined the effects of treating Ras-transformed cells with 1 μM PD184352, a concentration that selectively blocks ERK1/2 activation but not ERK5 activation (Mody et al., 2001), which our own observations have confirmed. Treatment of Ras-transformed cells with this concentration of PD184352 leads to the restoration of actin stress fibres and focal adhesions as shown by vinculin staining (Fig. 2B). These data argue that it is the ERK1/2 pathway rather than the ERK5 pathway that is responsible for the disruption of the actin cytoskeleton by oncogenic Ras.

Role of ERK1/2 compared with ERK5 in cytoskeletal disruption by activated Src

Transformation by oncogenic Src has been described to result in a dramatic alteration of the shape and cytoskeletal architecture of the cells, leading to the disruption of actin stress

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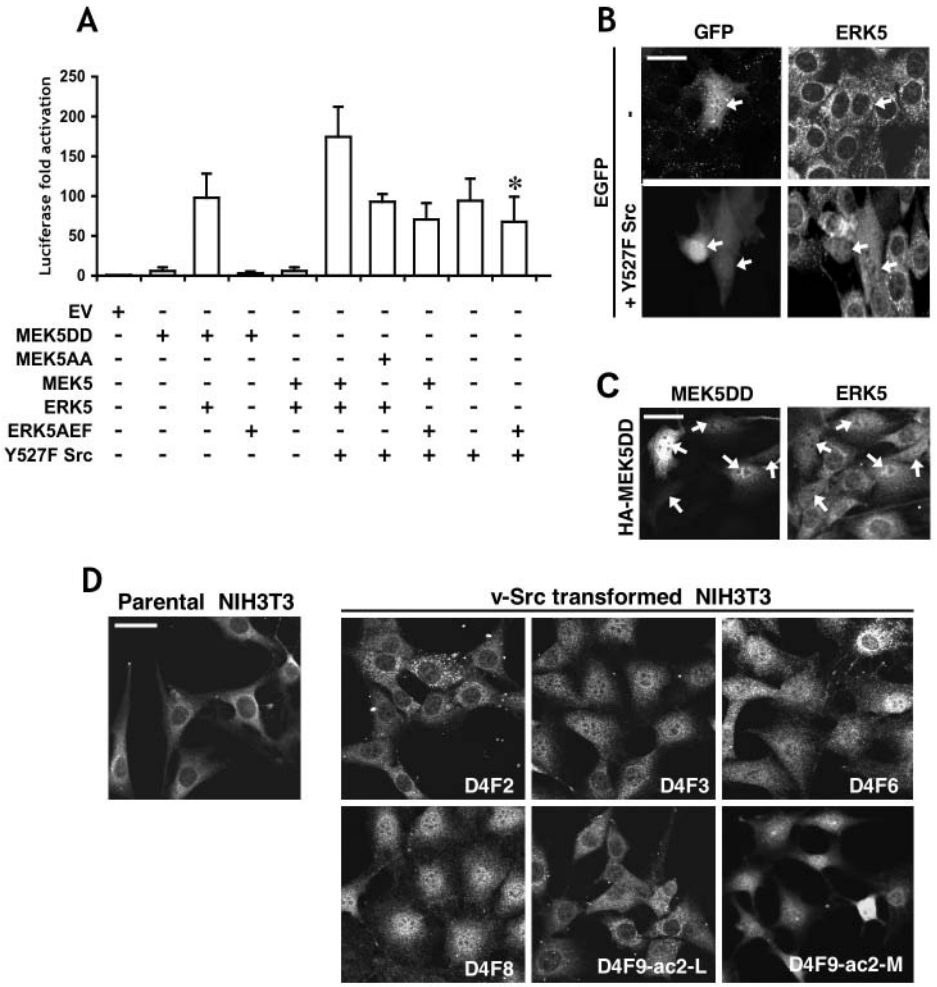

Fig. 3. Src activates the ERK5 pathway. (A) Activated Src (Y527F) was transfected together with different combinations of wild-type and mutant ERK5 and/or MEK5, the MEF2D luciferase reporter and a β-galactosidase plasmid for normalisation. Results are the mean±s.e. of eight independent experiments. The decrease in luciferase activation in the Y527F Src + ERK5AEF sample was statistically significant (*) by the Student's *t*-test (P=0.01). Arrows indicate nuclei of microinjected cells. (B) Activated Src causes translocation of ERK5 from the cytoplasm to the nucleus. NIH3T3 cells were microinjected at 5:1 ratio with empty vector and an EGFP expression plasmid (upper panels) or activated Y527F Src and EGFP (lower panels). Cells were then stained with ERK5 antibody to detect endogenous ERK5. Src protein expression was inferred by GFP fluorescence. Arrows indicate nuclei of microinjected cells. (C) NIH3T3 cells were microinjected with HA-MEK5DD and stained with anti-HA and anti-ERK5 to detect endogenous ERK5. (D) ERK5 is present in the nucleus of *Src*-transformed cell. Different *v-Src* transformed NIH3T3 clones were serum starved for 24 hours and then stained as in B. Bar, 20 μm.

fibres and focal contacts (Shriver and Rohrschneider, 1981; Parker et al., 1984). Several mechanisms have been suggested to account for these effects including Src-dependent tyrosine phosphorylation of Rho-GAP (Fincham et al., 1999), and downregulation of Rho kinases, ROCKI and ROCKII via ERK1/2 (Pawlak and Helfman, 2002). As several lines of evidence show that Src can signal through the activation of ERK5 (Abe et al., 1997; Suzaki et al., 2002; Scapoli et al., 2004) and we have shown above that activation of ERK5 can lead to disruption of the actin cytoskeleton we wished to determine whether activation of ERK5 plays a role in transformation by Src. First we determined whether oncogenic Src could activate the ERK5 pathway. After investigating a number of assays for ERK5 activity including antibodies to detect phosphorylated ERK5, we found that the most reliable and sensitive assay was to make use of the finding by Kasler and co-workers (Kasler et al., 2000) that activation of ERK5 leads to transcription from a myocyte enhancer factor 2 (MEF2) reporter construct. As expected, transfection of MEK5DD increases transcription from the MEF2 reporter (Fig. 3A). This could be considerably enhanced when wild-type ERK5 but not ERK5AEF was co-transfected with MEK5DD. Similarly Yan and colleagues have found that MEK5DD-induced transcription from a MEF2C reporter could be substantially enhanced if wild-type ERK5 was co-transfected (Yan et al., 2001). Transfection of Y527F Src increases activity of the MEF2 reporter. This activation was partially inhibited when Src was co-transfected with dominant-negative ERK5AEF and enhanced when transfected together with expression vectors for wild-type ERK5 and MEK5. This enhancement could be abolished if either MEK5 or ERK5 was substituted by the respective dominant-negatives. As expected, expression of wild-type MEK5 and ERK5 on their own did not result in any significant increase in transcription from the MEF2 reporter. These observations demonstrate that Src activates the ERK5 pathway in NIH3T3 cells. Additional evidence that activated Src leads to activation of ERK5 is provided by the observation that expression of activated Src results in translocation of ERK5 from the cytoplasm to the nucleus. Expression of Y527F Src protein in NIH3T3 cells results in partial translocation of endogenous ERK5 to the nucleus (Fig. 3B). The same incomplete translocation to the nucleus is caused by MEK5DD protein expression (Fig. 3C), indicating that even when ERK5 is potently activated, a proportion of ERK5 remains in the cytoplasm. Moreover we have found that four out of six Src-transformed NIH3T3 clones had nuclear staining for ERK5, unlike parental NIH3T3 cells (Fig. 3D) or *H-Ras*, *R-Ras* and *N-Ras*-transformed clones (data not shown). Recent work has suggested that endogenous ERK5 is always nuclear irrespective of its activation state (Raviv et

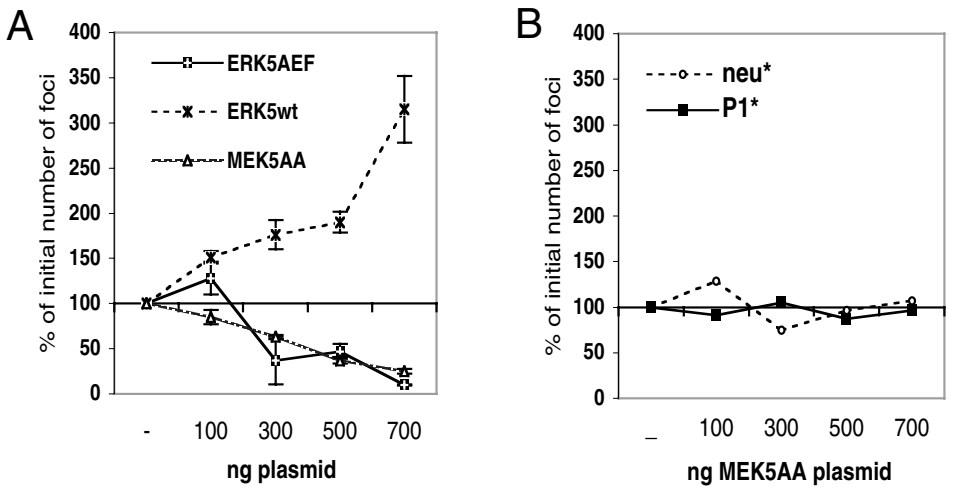

Fig. 4. Activation of ERK5 is required for transformation by oncogenic *Src*. (A) NIH3T3 cells were transfected with either Y527F Src alone, or with varying amounts of expression plasmids for MEK5AA, wild-type ERK5 or ERK5AEF. After 11-16 days transformed foci were blind scored. (B) NIH3T3 cells were transfected with either of two variants of oncogenic HER2/Neu (neu* or P1*) (see Materials and Methods) alone or with varying amounts of MEK5AA plasmid. Appearance of transformed foci was scored as in A.

al., 2004), however, using the same commercial antibody against ERK5 (Sigma) as used by Raviv and colleagues (Raviv et al., 2004), we consistently find that endogenous ERK5 is mainly cytoplasmic in control NIH3T3 cells but a proportion is translocated to the nucleus when activated Src is present. Indeed we see translocation of cytoplasmic ERK5 to the nucleus in EGF-stimulated NIH3T3 cells. (see Fig. S2 in supplementary material). It is possible that the differences that we have found compared with the study of Raviv and co-workers (Raviv et al., 2004) reflects cell type differences, however we have also observed that cellular stress such as from prolonged serum deprivation, as employed by these authors, can lead to increased levels of nuclear ERK5.

Next we used dominant-negative versions of MEK5 (MEK5AA) and ERK5 (ERK5AEF), in which the regulatory phosphorylation sites in the activation segment are replaced with non-phosphorylatable residues (Kato et al., 1997), to determine whether transformation by activated Src requires activation of ERK5. Varying amounts of ERK5AEF or MEK5AA expression plasmids were co-transfected with Y527F Src into NIH3T3 cells to determine whether blocking ERK5 activation would inhibit transformation by activated Src. Blocking ERK5 activation inhibits the ability of activated Src to produce transformed foci in NIH3T3 cells (Fig. 4A). Furthermore co-transfection of wild-type ERK5 with activated Src considerably enhanced the ability of Src to cause transformation. In contrast, expression of an interfering mutant of ERK5 activation (MEK5AA) had no effect on transformation by oncogenic variants of HER2/Neu. Consistent with the absence of an effect of expressing MEK5AA on transformation by oncogenic HER2/Neu, we have found no evidence that the oncogenic HER2/Neu plasmids used in these studies activate ERK5 (data not shown). We therefore demonstrate that the effect of blocking ERK5 activation on Src transformation is not due to a non-specific effect on NIH3T3 transformation.

Inhibition of ERK1/2 or ERK5 signalling alone is not sufficient to restore the actin cytoskeleton in Src-transformed cells

As we have shown that activation of either the ERK1/2 or ERK5 signalling pathway can lead to the disruption of the actin cytoskeleton and have demonstrated that transformation of NIH3T3 cells by activated Src requires signalling through the ERK5 pathway, we investigated whether these pathways are involved in disruption of the actin cytoskeleton in Src-transformed cells. For these experiments we again made use of the observation that concentrations of 1 μM PD184352 selectively inhibit activation of ERK1/2 without inhibiting activation of ERK5, whereas a concentration of 10 μM can inhibit ERK5 and ERK1/2 activation (Mody et al., 2001). Treatment with 1 μM PD184352 did not restore the actin cytoskeleton in Src-transformed cells (Fig. 5A), although ERK1/2 activation was totally blocked (Fig. 5B). This was true for all Src-transformed clones tested. Similarly, microinjection of an expression construct for MEK1A, a dominant-negative version of MEK1 in which Ser221 in the activation segment is replaced by an Ala residue (Cowley et al., 1994) failed to restore the actin cytoskeleton in Src-transformed cells (Fig. 5C). Treatment with higher concentrations of PD184352, when resulting in nuclear exclusion of ERK5, led to the restoration of Rho signalling to the actin cytoskeleton as shown by the formation of large stress fibres and large focal adhesions (Fig. 5A). In contrast, as previously shown (Fig. 2B), 1 μM PD184352 is sufficient to restore the actin cytoskeleton in Ras-transformed NIH3T3 cells. One interpretation of these results is that it is the ERK5 pathway rather than the ERK1/2 pathway that is responsible for the disruption of the actin cytoskeleton in cells transformed by activated Src. To examine this possibility, we microinjected the interfering mutants ERK5AEF and MEK5AA into Src-transformed cells, expression of neither of these mutants led to restoration of the actin cytoskeleton (Fig. 5C). Therefore we conclude that both ERK1/2 and ERK5 activation contribute to the disruption of the actin cytoskeleton in Src-transformed cells and that inhibition of both is required to restore the actin cytoskeleton.

Cross-regulation between ERK1/2 and ERK5

In conducting experiments with PD184352 and Src-transformed cells we noticed that treatment with 1 μM PD184352 enhanced nuclear accumulation of ERK5 suggesting that when ERK1/2 signalling is suppressed, ERK5 activation is enhanced. To extend this observation we used the MEF2 reporter assay to test whether ERK1/2 activation suppresses ERK5 signalling. Treatment with 1 μM PD184352 led to a twofold increase in MEF2 promoter driven luciferase activity in response to activated Src (Fig. 6). A similar effect

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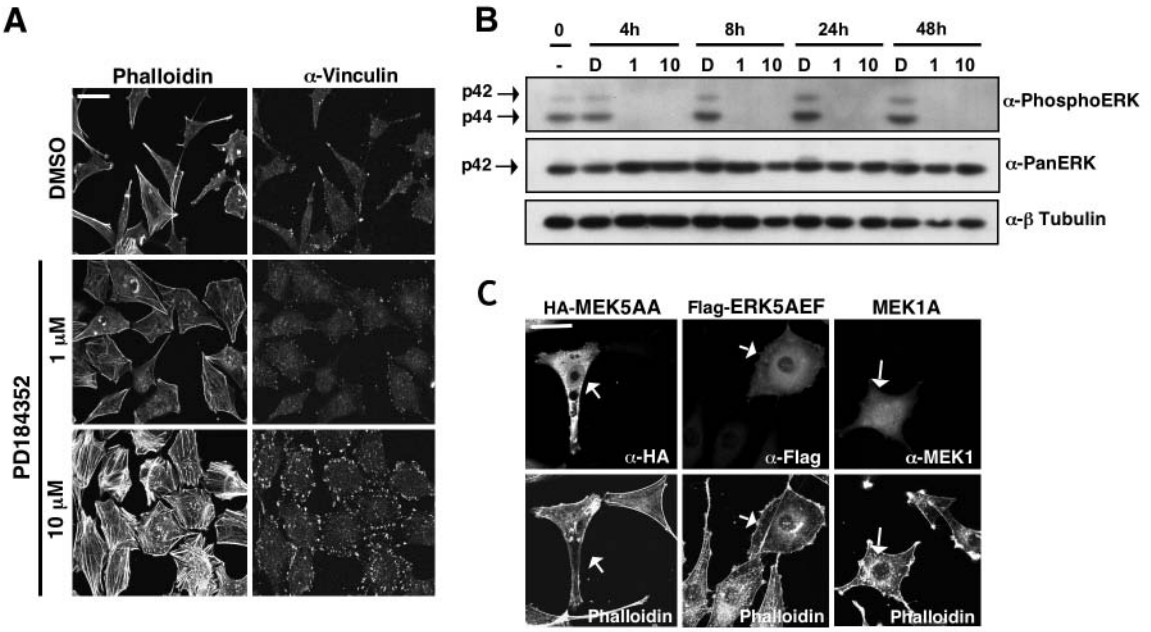

Fig. 5. Inhibition of both the ERK1/2 and ERK5 pathways is required to restore the cytoskeleton in Src-transformed cells. (A) Src-transformed NIH3T3 cells (D4F9-ac2M) were treated for 24 hours with PD184352 or solvent alone then fixed and stained with Texas Red-phalloidin to visualise filamentous actin and a vinculin antibody to visualise focal adhesions. (B) Lysates of Src-transformed NIH3T3 cells treated for up to 48 hours with PD184352 (1=1 μM and 10=10 μM PD184352) or solvent alone (D=DMSO) were western blotted with antibodies against activated dually phosphorylated ERK1/2 and β-tubulin and pan-ERK as loading controls. (C) *v-Src* transformed NIH3T3 cells (D4F9-ac2M) were microinjected with expression vectors for HA-MEK5AA, Flag-ERK5AEF or MEK1A. After 24 hours, cells were fixed and stained for HA-MEK5AA and Flag-ERK5AEF with HA (12CA5) and Flag (M2) directed antibodies. MEK1A expression was detected with a MEK1 antibody. Polymerised actin was stained with Texas Red-phalloidin. Bar, 20 μm.

was seen when activated Src was co-transfected with an expression vector for the MEK1-interfering mutant MEK1A, confirming that this effect is mediated through the ERK1/2 pathway. No potentiation of Src-stimulated MEF2 reporter activity was seen in cells treated with 10 μM PD184352 to inhibit ERK1/2 and ERK5 activation. Similarly the effect of inhibiting the ERK1/2 pathway, by either treatment with 1 μM PD184352 or expression of dominant-negative MEK1, in enhancing activation of the MEF2 reporter was ablated by expression of either of the interfering mutants MEK5AA or ERK5AEF. These results show that inhibiting activation of ERK1/2 enhances MEK5 signalling through ERK5. Evidence for cross-talk between the ERK1/2 pathway and ERK5 has previously been suggested by the observation of Mody and co-workers (Mody et al., 2001), that 1 μM PD184352 led to a more sustained activation of ERK5 following growth factor stimulation. Further studies will be needed to determine whether the increased activation of ERK5 by Src in cells where ERK1/2 activation has been blocked has the same underlying mechanism as the more sustained activation of ERK5 following growth factor stimulation observed by these authors (Mody et al., 2001).

Discussion

We have shown that activation of both the ERK1/2 and ERK5 pathways can lead to disruption of the actin cytoskeleton. Activation of these two distinct MAP kinase pathways appears to disrupt the actin cytoskeleton by different mechanisms because activation of the ERK1/2 pathway can overcome the effects of constitutively activated RhoA to organise the actin cytoskeleton whereas ERK5 signalling cannot. Previous work has shown that activation of ERK1/2 signalling in response to oncogenes can lead to disruption of the actin cytoskeleton either through suppressing Rho activation (Vial et al., 2003) or

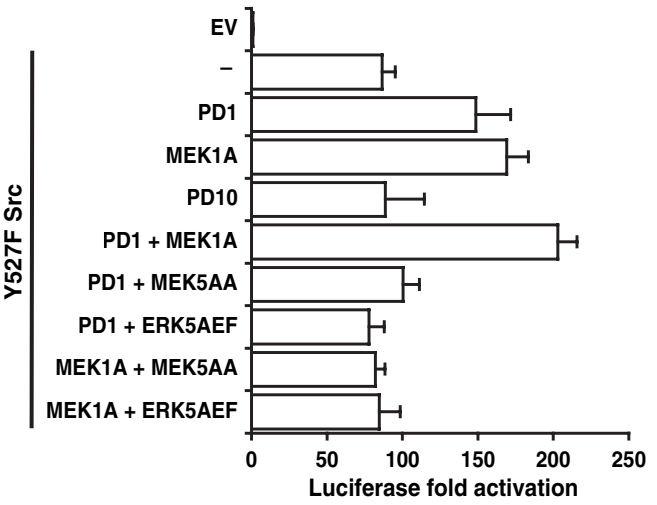

Fig. 6. Inhibition of ERK1/2 signalling enhances ERK5 activation by Src. NIH3T3 cells were transfected with expression vectors for Y527F Src, dominant-negative MEK1 (MEK1A), dominant-negative MEK5 (MEK5AA) or ERK5 (ERK5AEF), where indicated, cells were treated with 1 μM PD184352 (PD1) or 10 μM (PD10) for 24 hours.

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by downregulating the levels of the Rho effectors ROCK1 and ROCKII (Sahai et al., 2001; Pawlak and Helfman, 2002).

Our experiments show that activated oncogenic Src activates the ERK5 pathway and that activation of this pathway contributes to transformation by Src. We have also obtained evidence that activation of the ERK5 pathway is not required for cytoskeletal disruption by Ras (Fig. 2B). Together with data from a previous study demonstrating cooperation between the ERK5 signalling and activated Raf (English et al., 1999; Pearson et al., 2001) these studies show that ERK5 signalling may play a significant role in neoplastic transformation. This finding is potentially of some importance as in the past much use has been made of the MEK inhibitors PD098059 and U0126 to imply roles for ERK1/2 activation downstream of Ras and Raf in neoplastic transformation. It is now clear that the commonly used concentrations of these inhibitors block ERK5 activation as well (Kamakura et al., 1999; Mody et al., 2001), making it difficult to conclude that only ERK1/2 activation is involved. Important targets of ERK5 signalling in cell transformation may include Myc (English et al., 1998), Fos and NFκB (Pearson et al., 2001). As well as its role in signalling within the tumour cells themselves the ERK5 pathway may be important during tumour angiogenesis as demonstrated by the defects in embryonic angiogenesis when ERK5 signalling is blocked (Sohn et al., 2002).

Activation of both the ERK1/2 and ERK5 pathways appears to be involved in the disruption of the actin cytoskeleton resulting from cell transformation by activated Src. Inhibition of either ERK5 activation or ERK1/2 alone is insufficient to restore the actin cytoskeleton in Src-transformed cells suggesting that activation of each pathway is involved in disruption of the actin cytoskeleton. Our data suggest that ERK5 disrupts the actin cytoskeleton through a different mechanism to ERK1/2. Our conclusion that both ERK5 and ERK1/2 activation play a role in Src-mediated disruption of the cytoskeleton is in disagreement with the work of Pawlak and Helfman (Pawlak and Helfman, 2002), who concluded that the ERK1/2 signalling but not ERK5 signalling was responsible for disruption of the actin cytoskeleton by activated Src. The conclusion of these authors was based on the assumption that 50 μM PD098059 does not inhibit ERK5 activation, however data from two studies shows that this concentration of the inhibitor at least partially inhibits ERK5 activation (Kamakura et al., 1999; Mody et al., 2001). Furthermore, it should be noted that in examining a series of Src-transformed NIH3T3 clones we observed variability between clones in the degree to which the nuclear ERK5 was present (Fig. 3C) and the ability of 10 μM PD184352 treatment to redistribute ERK5 to the cytoplasm. This variability in ERK5 activation may account for the apparent discrepancies found by different investigators. However we found that in all Src-transformed NIH3T3 clones tested, treatment with 1 μM PD184352 did not restore the actin cytoskeleton providing very strong evidence that inhibition of ERK1/2 is insufficient to block disruption of the actin cytoskeleton in Src-transformed cells.

We have found that activation of ERK5 correlates with translocation from the cytoplasm to the nucleus. These results differ from those recently published by Raviv and co-workers (Raviv et al., 2004) who showed that in HeLa and Rat1 cells, endogenous ERK5 was always nuclear. The reason for the discrepancy between these studies is not clear, the same antibody were used and we have found that overexpressed epitope-tagged ERK5 shows the same staining pattern with the epitope tag antibody as with the ERK5 antibody (data not shown) further validating this antibody. It is possible that these differences represent differences in cell type or physiological conditions. In fact we have never seen a band-shift of the ERK5 protein of unstimulated NIH3T3 cells, whereas the authors see phosphorylated ERK5 even in unstimulated conditions (Raviv et al., 2004). This suggests that in HeLa and Rat-1 cells or under the experimental conditions used by these researchers, ERK5 is partially activated resulting in the higher nuclear staining observed. Interestingly another MAPK p38MAPK has been found to be nuclear in unstimulated cells and translocates to the cytoplasm on activation (Ben-Levy et al., 1998). These observations suggest that depending on the MAPK, cell type and physiological conditions, different patterns of MAPK localisation and translocation may be observed.

In conclusion, our findings point to a role of ERK5 in transformation by some oncogenes. ERK5 signalling can lead to the disruption of the actin cytoskeleton. However, further studies will be required to elucidate the mechanism by which ERK5 signalling disrupts the actin cytoskeleton. Our observations that both ERK1/2 and ERK5 signalling participate in oncogenic transformation and at the cellular level can each lead to disruption of normal cellular responses such as actin organisation highlights the desirability of blocking both as a therapeutic measure.

We thank the members of the laboratory for help and advice throughout this study, Hugh Paterson for microscopy advice and Clare Isacke for comments on the manuscript. We are grateful to numerous laboratories for their kind gifts of plasmids. J.C.B. was funded by Fundação para a Ciena e Techologia (FCT/FSE-III) Quatro Comunitário de Apoio and Fundação Calouste Gulbenkian and C.J.M. was funded by Cancer Research UK.

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