Ecdysone-inducible expression of oncogenic Ha-Ras in NIH 3T3 cells leads to transient nuclear localization of activated extracellular signal-regulated kinase regulated by mitogen-activated protein kinase phosphatase-1

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The Ras family of GTP-binding proteins are key transducers of extracellular signals, particularly through the mitogen-activated protein kinase (MAPK) pathway. Constitutively active forms of Ras are found in a variety of tumours, suggesting an important role for this pathway in cancer. Here we report that initial cellular exposure to oncogenic Ras chronically activated the MAPK pathway in the cytoplasm, but transiently activated the same pathway in the nucleus. Nuclear-activated extracellular signal-regulated kinase (ERK) was rapidly dephosphorylated, with consequent short-term activation of the Elk-1 transcription factor and expression of the c-*fos* gene. Additional experiments suggested that the regulatory mechanism involved requires the calcium-dependent protein phosphotyrosine phosphatase MAPK phosphatase-1 (MKP-1). This is the first report on the ability of Ras, in the absence of growth factors, to transiently activate the MAPK pathway in the nucleus and show an involvement of MKP-1 in nuclear ERK2 regulation. In addition we show that transient activation of the MAPK pathway is sufficient to drive chronic cell-cycle progression. We conclude that, whereas the MAPK pathway is necessary to initiate cellular proliferation and transformation, the transient nature of the MAPK pathway activation suggests the involvement of additional signalling pathway(s) regulated by Ras.

Key words: cell cycle, ecdysone model, MAPK.

INTRODUCTION

The p21 Ras class of proteins cycle between an active GTP-bound state and an inactive GDP-bound state [1] and act as transducers of extracellular signals. Constitutively activated mutant forms of Ras are found in a variety of tumours [2], suggesting an important role in transformation. Activated Ras mediates its biological activity through interaction with various downstream effector targets [3,4]. Considerable evidence supports the critical role of the mitogen-activated protein kinase (MAPK) pathway and its upstream activator, Raf-1, as major targets in Ras function. Active Ras recruits Raf-1 to the plasma membrane, leading to its activation [5,6]. Raf-1 then activates the MAP/extracellular signal-regulated kinase (ERK) kinases (MEKs) 1 and 2, which in turn phosphorylate and activate ERKs 1 and 2, respectively [7–10]. Activated ERK1/2 translocate to the nucleus and phosphorylate a variety of targets, including the Ets transcription factor Elk-1 [11,12]. Phosphorylation of Elk-1 leads to the expression of a variety of genes such as c-*fos* [13,14]. The ability of dominant negative mutants of Raf-1, MEK-1 or ERK to block Ras transformation demonstrates that this cascade is necessary for transformation [15,16]. The ability of activated MEK-1 or Raf-1 to transform NIH 3T3 fibroblasts implies that activation of this pathway alone is sufficient [15,17–19]. However, it is unclear whether constitutive activation of the MAPK pathway is necessary for transformation. It has been reported that exposure to activated MEK-1 leads to the expression of activator protein 1 (AP-1) transcription-factor components [18] known to be essential in cellular transformation [20,21].

One aspect of Ras signalling important for transformation involves regulation of components of the cell-cycle machinery that are critical for progression through the G_1 phase [22]. Recent work to understand the involvement of the MAPK signalling pathway in cell-cycle initiation has focused on the use of *in vitro* models where components of the MAPK cascade are activated temporally. Utilizing inducible ΔRaf:ER constructs (containing the catalytic domain of Raf, fused to the hormone-binding domain of the oestrogen receptor), it has been shown that Raf-induced cell-cycle progression through a low-level activation of MAPK is associated with the ability of Raf to induce the expression and activation of cyclin D1–cdk4 and cyclin E–cdk2 activity and to decrease the expression of the cyclin kinase inhibitor $p27^{kip1}$ [19,23]. In contrast, Raf-induced cell-cycle arrest through high-level activation of the MAPK pathway is associated with the ability of Raf to induce the expression of cyclin kinase inhibitor $p21^{Cip1}$ [19]. These data are supported by experiments with an inducible oncogenic MEK-1 system (ΔMEK1:ER; using constructs containing the catalytic domain of MEK, fused to the hormone-binding domain of the oestrogen receptor), which demonstrated that when expressed in NIH 3T3 cells the MAPK pathway was activated weakly but that a robust proliferative response was observed [17]. However,

Abbreviations used: BAPTA, 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; MKP-1, MAPK phosphatase-1; MEK, MAP/ERK kinase; SRE, serum response element; SRF, serum response factor; PAP, potato acid phosphatase; EMSA, electrophoretic mobility-shift assay; BrdU, 5-bromo-2′-deoxyuridine; TCF, ternary complex factor; AP-1, activator protein 1; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione S-transferase.

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experiments by Pruitt and co-workers [24] have indicated that Raf-1-independent mechanisms are also important for Ras-mediated cell-cycle initiation in epithelial cells.

In this paper we describe a novel conditional oncogenic Ras-expression system in NIH 3T3 fibroblasts. We have examined the early effects of oncogenic Ras on the MAPK signalling pathway and the cell cycle. The data, in conjunction with data from chronic Ras-activation studies, will resolve the full spectrum of Ras-driven events in oncogenesis.

EXPERIMENTAL

Cell culture

Cells were maintained at 37 °C under 5% CO_2 and 95% humidity. NIH 3T3 fibroblasts expressing Ki-Ras (where Ki means Kirsten; clone R1), Ha-Ras (where Ha means Harvey; clone R6) and vector control (clone R5) were a kind gift from F. Mechta (Institute Pasteur, Paris, France). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 7% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Inducible clones were maintained under 200 μg/ml Zeocin (Invitrogen, Breda, The Netherlands) antibiotic selection.

Preparation of inducible clones

Ha-Ras cDNA containing a single coding change at amino acid number 12 (glutamine to valine; named the V12 mutant) was cloned into the ecdysone expression vector pIND (Invitrogen) after *Bam*HI/*Hin*dIII restriction endonuclease digestion. Parental NIH 3T3 cells were co-transfected with the Ras-containing pIND expression vector and a second plasmid, pVgRXR, carrying components of the ecdysone system. Clones were selected in 400 μg/ml G418 (Gibco–BRL) and 400 μg/ml Zeocin. Induction of Ras expression was attained by addition of Ponasterone A (Invitrogen) over a range of concentrations (0.1, 0.5 or 1 μM).

Soft-agar assays

Approx. 1×10^3 cells were suspended in 3 ml of agar [0.3% agar (Difco) in DMEM plus 7% FBS] and layered over a solidified cushion of 0.6% agar in DMEM plus 7% FBS. Every 2 days, 100 μl of DMEM containing 7% FBS and Ponasterone A (final concentration, 1 μM) was added. Individual macroscopic colonies were counted after 3 weeks of growth.

Cell growth in low serum

Culture dishes with six wells each were seeded in triplicate with approx. 1×10^3 cells, which were allowed to adhere overnight. Medium was replaced with DMEM plus 0.5% FBS and cells were incubated for 24 h. Induction was carried out by the daily addition of Ponasterone A at the indicated concentrations. At each time point the cell number was estimated by a Coulter counter.

Western-blot analysis and immunoprecipitation

Whole cell extracts and Western blotting were performed as described previously [21] after induction on serum-deprived cells. All solutions used for the preparation of cell extracts contained 5 μg/ml of each of the following inhibitors: aprotinin, pepstatin A, leupeptin and sodium orthovanadate (Sigma, St Louis, MO, U.S.A.), except when stated otherwise. Protein concentration was determined by the method of Bradford (Bio-Rad protein assay) and loading confirmed by Ponceau S staining after Western transfer. Antibodies specific for Ras (sc-29), phospho-ERK (sc-7383), Elk-1 (sc-355X), SAP-1a (sc-1426), MAPK phosphatase-1 (MKP-1; sc-1199), c-Fos (sc-7202), Raf-1 (sc-133) and serum response factor (SRF; sc-335) were purchased from Santa Cruz Biotechnology. Phospho-Elk (#9181), phospho-Raf-1 (#9421) and phospho-MEK-1 (#9121S) antibodies were purchased from New England Biolabs. Anti-Net antibody was described previously [25]. Anti-ERK2 and anti-MEK-1 antibodies were a kind gift from Professor Sir P. Cohen (Dundee, Scotland, U.K.). Blots were incubated with species-specific horseradish peroxidase-conjugated secondary antibodies followed by detection with enhanced chemiluminescence (Luminol detection system; Pierce). In additional experiments, nuclear and cytosolic fractions were prepared as described previously [21].

Immunoprecipitations were performed as described previously [26] using 80 μg of nuclear extracts incubated overnight at 4 °C with 1 μg of the anti-MKP-1 antibody. Immune complexes were pelleted with Protein A–Sepharose (Sigma), washed with lysis buffer, and resuspended in loading buffer. Samples were then subjected to Western blotting as described above.

Kinase assays

Solid-phase ERK assays were performed as a modification of the protocol described previously [27] on serum-deprived cells. Total protein (80 μg) was incubated for 2 h with 5 μg of glutathione S-transferase (GST)–Elk bound to glutathione–Sepharose beads (Sigma). Kinase–substrate complexes were washed five times in Tris-buffered saline plus 5 μg/ml of each of the following inhibitors: aprotinin, pepstatin A, leupeptin and sodium orthovanadate. They were then incubated in specific kinase reaction buffer containing 5 μM ATP for 20 min at 30 °C. Reactions were resolved by SDS/PAGE (15% gel), transferred by Western blotting to nitrocellulose and visualized by immunoblotting with phospho-Elk1-specific antibodies.

Electrophoretic mobility-shift assay (EMSA) supershift studies

Reaction mixtures (20 μl) containing 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 1 mM poly(dI-dC) and 25000 c.p.m. of ^{32}P-labelled DNA probe containing approx. 10 ng of c-*fos* serum response element (SRE) sequence [25] were incubated at 25 °C for 45 min and then the DNA–protein complexes separated on a 5% polyacrylamide/Tris/borate/EDTA (45 mM Tris/borate/1 mM EDTA) gel. After electrophoresis, gels were dried and autoradiographed. For SRE supershift analysis, protein samples were pre-incubated with specific antibodies (1 μg/ml) for 30 min at 25 °C prior to EMSA analysis. For potato acid phosphatase (PAP) treatment, protein samples were pre-treated with 1 μg of PAP (Sigma) on ice for 30 min, then the phosphatase activity was stopped by the addition of 5 μM of the following inhibitors prior to EMSA analysis: aprotinin, pepstatin A, leupeptin and sodium orthovanadate.

Immunofluorescence

Cells were fixed in ice-cold methanol for 10 min, washed in Tris-buffered saline for 30 min and blocked by incubation in 3% BSA in Tris-buffered saline for 30 min. Primary antibody incubations were performed overnight at 4 °C before incubation with appropriate fluorescent secondary antibodies (Sigma) for 1 h at room temperature. Stained preparations were mounted under glass coverslips and examined microscopically with a Zeiss epifluorescence microscope.

Flow cytometry analysis

Cell-cycle distribution experiments were performed on approx. 1×10^5 cells. Briefly, ethanol-fixed and propidium iodide- or 5-bromo-2′-deoxyuridine (BrdU)-stained cells were analysed by flow cytometry for their DNA content using a FACScaliber flow cytometer (Becton Dickinson). Distribution of cells in different phases of the cell cycle was quantified with the computer program CellQuest.

Reproducibility of results

At least two independently derived Ha-Ras clones were examined to confirm reproducibility. Results are from single experiments representative of several experiments giving similar data. Where possible, blots were stripped and reprobed with antibody recognizing both phosphorylated and non-phosphorylated forms of the target protein, to confirm Western-blot loading. In addition, Western-blot protein transfer efficiency was confirmed by visualization of the transferred proteins using Ponceau S stain.

RESULTS

Characterization of Ha-Ras expression

Three clones were selected for expansion and inclusion in this study (clones A6, A7 and A8). Analysis of Ras expression with or without induction with Ponasterone A for 24 h showed that levels of induced Ras protein were similar (Figure 1A). We examined the expression profile of Ras over time in clone A8 (Figure 1B). No Ras was detected at the earliest time points (0 and 1 h), presumably due to Ras protein levels present below the detection limit of the antibody. Ras was detectable after 2 h post-induction and accumulated over time. Similar expression profiles were observed in separately derived clones (results not shown). Finally, Ras expression level at 2 h was lower than the steady-state level of Ki-Ras-transformed cells (clone R1; Figure 1C). Serum stimulation failed to increase endogenous Ras levels above the detection threshold.

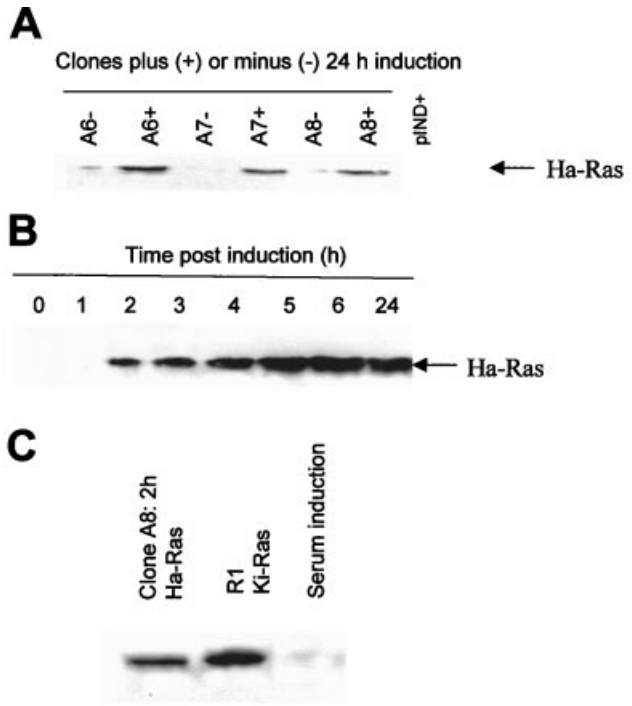

Figure 1 Inducible expression of Ha-Ras

(**A**) Western blotting of total Ha-Ras from three clones with (+) or without (−) 24 h of induction with 5 μM Ponasterone A. Vector pIND acted as a control. Loading was 150 μg of total cell extract and Ras was detected with a Ha-Ras-specific antibody. (**B**) Time-course analysis of Ras expression by Western blot from a representative clone, A8. Cells were induced with 5 μM Ponasterone A. (**C**) Comparison of Ras-expression levels by Western blot from clone A8 2 h post-induction with 5 μM Ponasterone A compared with Ki-Ras-transformed R1. Serum-stimulated non-induced clone A8 was included as a control. Similar results were obtained with additional clones in two to four separate experiments.

Induction of Ha-Ras leads to morphological transformation

Induction of Ras in the absence of exogenous growth factors led to a dramatic change in cell morphology after 48 h. Parental NIH 3T3 cells showed no morphological change upon induction (Figure 2A, panel 1), whereas R1 cells showed a fully transformed phenotype, such as condensed nuclei, increased refractability and spindle-like morphology (Figure 2A, panel 2). Non-induced clone A8 had the parental non-transformed phenotype (Figure 2A, panel 3), as did non-induced clones A6 and A7 (results not shown). Induced clones A6, A7 and A8 showed morphological changes, including the characteristics mentioned above (Figure 2A, panels 4–6). Induction of Ha-Ras enabled the formation of colonies in soft agar, albeit with varying low plating efficiencies and smaller colony sizes in comparison with clone R1 (Figure 2B). Some colony formation was observed in non-induced samples and control NIH 3T3 parental cells treated with the inducing agent, a feature reported for NIH 3T3 cells [21]. In comparison, constitutive Ras-expressing cell line R1 possessed the greatest ability to form colonies. Finally, clones were able to maintain limited expansion in the absence of growth factors for up to 6 days (Figure 2C). Interestingly, this growth was dependent on the level of Ha-Ras induction. When the same clones were untreated they were unable to proliferate in the absence of serum growth factors and declined steadily over time. Serum-independent growth could only be maintained for up to 6 days after high-level induction by Ponasterone A (1 μM; Figure 2C). Cells induced with a low concentration of Ponasterone A (0.1 μM), and hence showing a low level of Ha-Ras expression, were able to grow, although at a slower rate, but did not show a subsequent loss in cellular viability within the time limits of this experiment.

Molecular analysis of Ha-Ras-dependent MAPK activation

To investigate MAPK activation after initial exposure to oncogenic Ras, we isolated samples from serum-starved cells at and around the time point that Ras protein levels were first detected and looked for temporal changes in MAPK activation as determined by immunoblotting (Figure 3A). We detected an increase in Ras protein levels over time following induction. Analysis of the downstream MAPK components revealed that activation of Raf-1, MEK-1 and ERK1/2 proceeded rapidly and that signal intensity accumulated over time. However, activation of Elk-1 was delayed in comparison with its kinase ERK1/2, possibly reflecting a difference in the subcellular localization of the two proteins. Subsequent activation of the SRE transcription-factor complex was confirmed by expression of the product of the c-*fos* gene, which is known to be regulated by the SRE. Detection of the expressed c-Fos was transient, which is in agreement with the reported short half-life of this protein. Finally we confirmed that Ha-Ras-mediated MAPK activation could be blocked by the addition of the MEK-1 inhibitor PD98059 (results not shown).

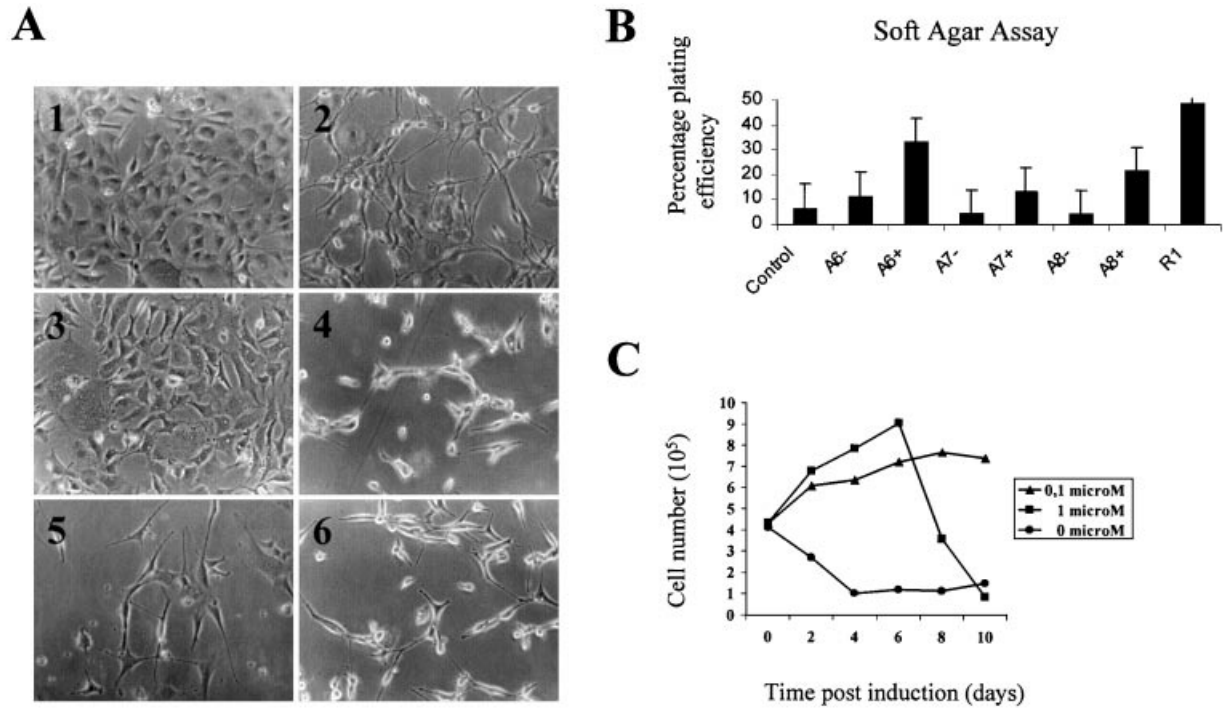

Figure 2 Induced oncogenic Ha-Ras is biologically active

(**A**) Morphological changes induced by Ras. Cells were serum-starved for 24 h and then induced with 1 μM Ponasterone A for 48 h. Morphology of parental NIH 3T3 cells is shown in panel 1, while Ki-Ras-expressing R1 cells are shown in panel 2 and non-induced clone A8 in panel 3. Morphology of induced clones A6, A7 and A8 is shown in panels 4–6, respectively. Original magnification, $\times$ 50. (**B**) Clonal growth in soft agar induced by Ha-Ras. Clones A6, A7 and A8, grown in the absence (−) or presence (+) of Ponasterone A, show limited colony-forming ability in a soft-agar growth assay, as shown by the percentage plating efficiency in the presence of 10% FBS after induction with 1 μM Ponasterone A. Parental NIH 3T3 cells and Ki-Ras-transformed R1 cells were included as controls ($n = 3$). (**C**) Growth in 0.5% sera after Ha-Ras induction showing a dose-dependent proliferation profile, from a single experiment representative of repeated analysis ($n = 4$). Cells were serum-depleted for 48 h prior to induction.

To investigate whether Ras-mediated activation of ERK1/2 was dose-dependent, we induced Ras expression to varying degrees by altering the amount of Ponasterone A inducing agent. We then analysed the MAPK output by both ERK1/2 immunoblot (Figure 3B; Western blot) and ERK1/2 kinase assay using GST–Elk-1 as a substrate (Figure 3B; ERK assay). We observed that the strength of MAPK activation was dependent on the level of Ha-Ras induction (Figure 3B; 0–1 μM) and was concurrent with detectable Ras protein expression at around 2 h post-induction (see Figure 1B). The induced activation of the MAPK pathway was increased markedly in comparison with clone R1, even though the levels of Ras were lower in the inducible cells (see Figure 1C). The increased ERK1/2 phosphorylation found in the serum-starved A8 clone (Figure 3B; 0 μM, Western blot) did not correspond to increased ERK activity when assayed by kinase assay (Figure 3B; 0 μM, ERK assay).

Active ERK1/2 translocates to the nucleus and is dephosphorylated rapidly

We suspected that the delay in activation of Elk-1 by ERK1/2 might be related to the difference in subcellular localization of these proteins. We therefore investigated nuclear translocation of active ERK1/2. Samples were taken and separated into nuclear and cytosolic fractions and analysed for ERK1/2 activation in several clones (Figures 4A and 4B). Western blotting of cytosolic extracts showed that ERK1/2 was detectable in the cytoplasm at around 2 h post-induction and increased steadily as a function of time. In contrast, nuclear phospho-ERK was detectable concurrent with the cytosolic presence of phospho-ERK, but peaked in intensity for a short time before signal loss. The transient localization of the active ERK1/2 was confirmed in one clone by re-probing the same blot with antibody specific for non-phosphorylated ERK1/2. Western-blot data were confirmed by kinase assay on independently derived samples from the same clones (Figures 4C and 4D). These data imply that nuclear translocation of active phosphorylated ERK1/2 occurred shortly after the ERK1/2 became phosphorylated and was de-activated rapidly by a negative control mechanism. Interestingly, we failed to detect nuclear phospho-ERK2 at any other time point outside of those points analysed, confirming that activation of ERK was rapid, transient and unlikely to be periodic.

One of the targets of the MAPK pathway is the ternary complex factor (TCF) protein Elk-1. Analysis of endogenous phosphorylated Elk-1 by immunofluorescence (Figure 4E, panel 1) showed activated Elk-1 at time points corresponding to nuclear translocation of phospho-ERK (Figure 4E, panel 2) and remaining unchanged through later time points (Figure 4E, panel 3).

Nuclear ERK1/2 is de-activated through a phosphatase-dependent mechanism; possible role for MKP-1

De-phosphorylation has been shown previously to be a major mechanism by which ERK1/2 is de-activated. We therefore looked at whether inhibitors of either tyrosine- or serine/

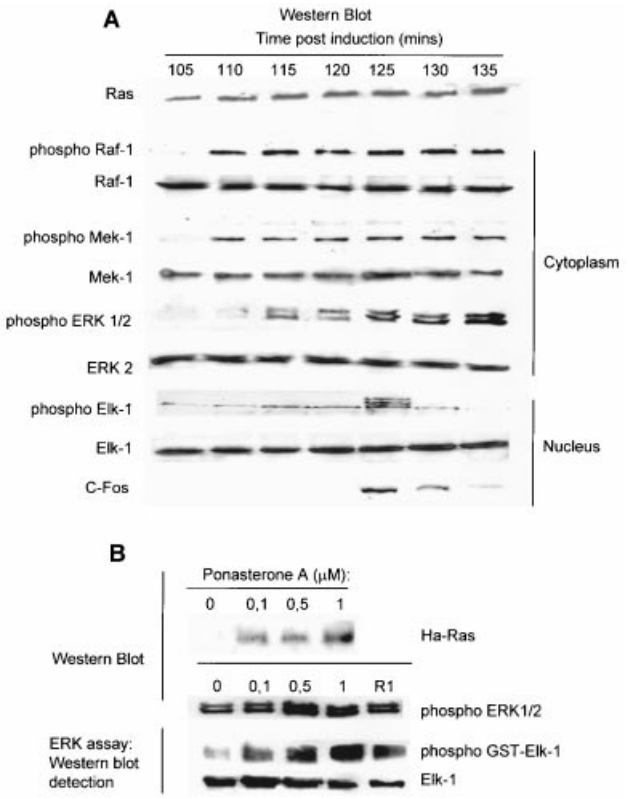

Figure 3 Activation of the MAPK pathway by Ha-Ras is dose-dependent

(**A**) Clone A8 was serum-starved for 24 h prior to induction with 1 μM Ponasterone A. Total cellular extract was collected at various times post-induction and then assayed for Raf-1, MEK-1, ERK1/2 and Elk-1 activation state by Western blotting with various phospho-specific antibodies. Antibodies specific for total protein were included as a control. (**B**) Cells were serum-starved for 24 h prior to induction and total cellular extract was taken after 120 min. Induced Ras protein is shown along with MAPK activation state assessed either by Western-blot analysis with a phospho-ERK1/2-specific antibody or by kinase assay using Western-blot detection of the phosphorylated Elk-1 substrate. Blotting for Elk-1 was used to confirm equal loading. Similar extracts from Ki-Ras-transformed clone R1 were included for comparison. Results are representative of two or three separate experiments.

threonine-directed phosphatases would stabilize the transient activity of nuclear ERK1/2. We selected a range of time points during which ERK nuclear translocation occurs. Analysis of nuclear fractions from untreated or okadaic acid-treated cells (a selective inhibitor of phosphatase types 1 and 2A) showed rapid and short-lived translocation of the phosphorylated ERK1/2 to the nucleus, similar to untreated cells. In contrast, phosphorylated ERK1/2 was retained in the nucleus when the same experiments were conducted in the presence of orthovanadate, a protein tyrosine phosphatase inhibitor (Figure 5A), suggesting that ERK1/2 inactivation in the nucleus was dependent on a tyrosine phosphatase. Similar results were observed in calcium-depleting 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA)-treated samples, suggesting that the involved phosphatase was influenced by calcium concentration (Figure 5A). No nuclear ERK1/2 signal was detected in non-induced control cells treated with orthovanadate or in sample cells pre-treated with the MEK-1 inhibitor PD98059 and then induced with 1 μM Ponasterone A (results not shown).

The data on the phosphatase inhibitor show that calcium concentration affects ERK dephosphorylation, implying the involvement of a calcium-dependent dual-specificity phosphatase in the de-activation of nuclear ERK1/2. One possible candidate is MKP-1. We therefore looked for evidence of association of MKP-1 with ERK1/2 at the appropriate time points. We detected an interaction between MKP-1 and nuclear ERK2 using immunoprecipitation of ERK2 by an anti-MKP-1 antibody from nuclear extracts at time points corresponding to ERK1/2 nuclear translocation (Figure 5B). A similar interaction was observed by immunoprecipitation of MKP-1 with the anti-ERK2 antibody. Thus we confirmed an association between a phosphatase and its known substrate in cellular extracts, suggesting strongly that MKP-1 was responsible for the de-activation of nuclear ERK1/2 in this system.

We used Ki-Ras (R1), Ha-Ras (R6) or vector control (R5) NIH 3T3 cells in parallel experiments to assess whether the putative MKP-1 control mechanism was general or specific to our model (Figure 5C). When NIH 3T3 cells overexpressed either Ras isoform, very little ERK2 could be immunoprecipitated by an MKP-1 antibody (Figure 5C; R1− and R6−). However, if the same experiment was repeated after MKP-1 inhibition by BAPTA treatment (Figure 5C; R1+ and R6+), there was a large increase in the amount of ERK2 immunoprecipitated. This effect was specific for Ras-expressing cells, as it could not be repeated in the vector control samples (Figure 5C; R5− and R5+). Co-immunoprecipitation of the upstream MAPK protein MEK-1 acted as an internal control. Thus we observed the interaction of MKP-1 with nuclear ERK2 in diverse Ras-expressing systems, suggesting that this is a general control mechanism.

Activation of the MAPK pathway leads to an increase in SRF-dependent SRE DNA-binding activity

To confirm that nuclear-activated ERK1/2 led to activation of the Elk-1 transcription factor, we analysed DNA binding on the c-*fos* SRE using EMSA at the same time points as investigated previously. We noted an increase in SRF–TCF–DNA ternary complex formation at the time of Elk-1 activation and immediately afterwards (Figure 6A). Follow-up experiments demonstrated that ternary complex activity remained for a short period before declining rapidly (results not shown). Composition analysis of the ternary complex by supershift assay with specific antibodies determined that the complex contained SRF and Elk-1 predominantly (Figure 6B). The observed increase in SRF–DNA-binding activity was not due to an increase in the levels of total SRF protein (Figure 6C), leaving the possibility that post-translational modifications might be responsible. We undertook dephosphorylation experiments prior to EMSA analysis (Figure 6D). Pre-treatment of SRE-reactive nuclear extracts with an anti-SRF antibody, but not an isotype-matched non-specific antibody (results not shown), supershifted both DNA–protein complexes, confirming that they contained SRF (Figure 6D; anti-SRF). Pre-treatment with PAP followed by EMSA (Figure 6D; PAP-treated) completely abolished the SRF–DNA complex and greatly reduced the ternary complex.

Inducible oncogenic Ras stimulates DNA synthesis and cell-cycle progression

Ras-inducible clones, like their parental NIH 3T3 cells, entered proliferation arrest when cultured in serum-free medium. To assess whether transient activation of the MAPK pathway was sufficient for cell-cycle re-entry, quiescent cells were induced with

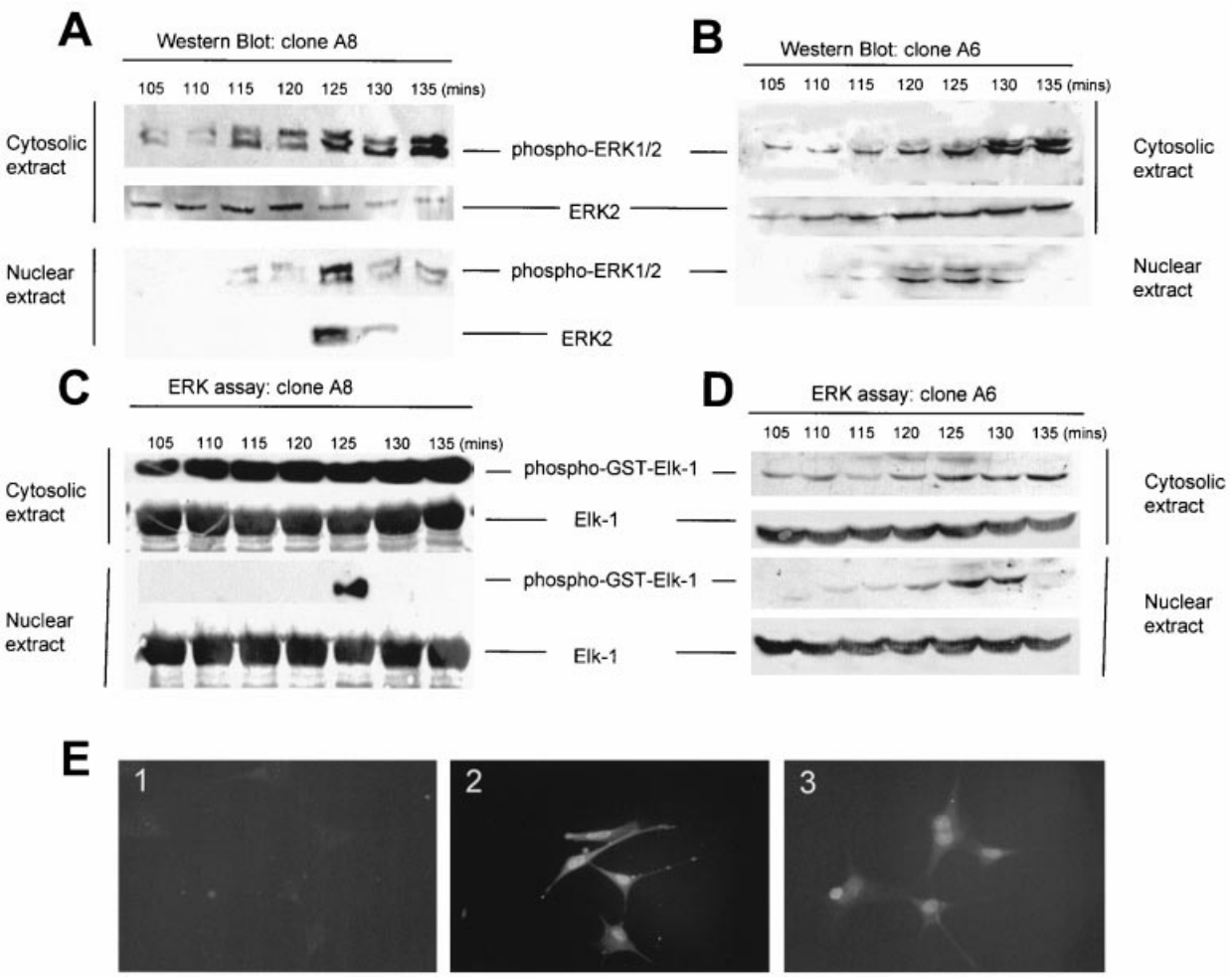

Figure 4 Analysis of MAPK translocation

Clones A8 (**A**) and A6 (**B**) were serum-starved for 24 h prior to induction with 1 μM Ponasterone A. Cellular extract was collected at various times post-induction, separated into cytosolic and nuclear fractions and assayed for phosphorylated ERK1/2 by Western blotting. Where possible, loading was confirmed using a specific antibody for ERK2. For the nuclear extracts, loading was confirmed by staining for total transferred protein with Ponceau S (results not shown). Western-blot data were confirmed by ERK kinase assay on a second set of independently derived lysates from identically treated clone A8 (**C**) and clone A6 (**D**), using GST–Elk-1 as the target substrate. Kinase activity was analysed by Western-blot detection of the phosphorylated substrate Elk-1. Loading was confirmed by analysing the level of GST–Elk-1 by Western blot for Elk-1 protein. (**E**) Analysis of phospho-Elk-1 localization by immunofluorescence. Clone A8 was serum-starved for 24 h prior to induction with 1 μM Ponasterone A. After induction, cells were methanol-fixed at time points representing before (panel 1; 105 min), during (panel 2; 125 min) or after (panel 3; 135 min) ERK nuclear translocation, as determined previously by Western blotting. Samples were examined for endogenous activated Elk-1 by immunofluorescence using a phospho-Elk-1-specific antibody; original magnification, $\times 250$.

1 μM Ponasterone A. To determine whether Ras could induce cellular proliferation, we pulse-stained the induced cells with the nucleotide analogue BrdU and examined the percentage of cells showing DNA replication 28 h post-induction. This time point was chosen because previous studies have shown that inducible activation of MAPK components showed DNA replication after 20 h post-induction but not before [18,28]. These data (Figure 7A) confirmed that oncogenic Ras was mitogenic. To analyse the entry into the cell cycle under more detail, we investigated the specific DNA content by propidium iodide staining and FACScan analysis at various time points after either Ha-Ras or serum stimulation (Figures 7B and 7C). These data were revealing in that, whereas there was no difference in cell DNA content between samples up to 12 h, a noticeable difference was detected after 24 h. At this time point there was almost a 2:1 ratio in the number of cells with increased DNA (the $S/G_2/M$ phase) when comparing Ha-Ras with serum induction (30.17% versus 18.59%). However, by 36 h the situation had been reversed. We detected only a slight increase in the number of $S/G_2/M$-phase cells after Ha-Ras induction (37.8%), whereas serum induction reached high levels of cells residing in the $S/G_2/M$ phase (62.1%). Thus the data suggested that exposure to oncogenic Ras led to a weak but sustained entry into the cell cycle, whereas serum stimulation of the same cells led to a strong response.

DISCUSSION

We have constructed a conditionally activated V12 mutant Ha-Ras expression system in NIH 3T3 fibroblasts and have used this model to investigate Ras-mediated events in MAPK activation and its influence on the cell cycle. It should be noted that, in

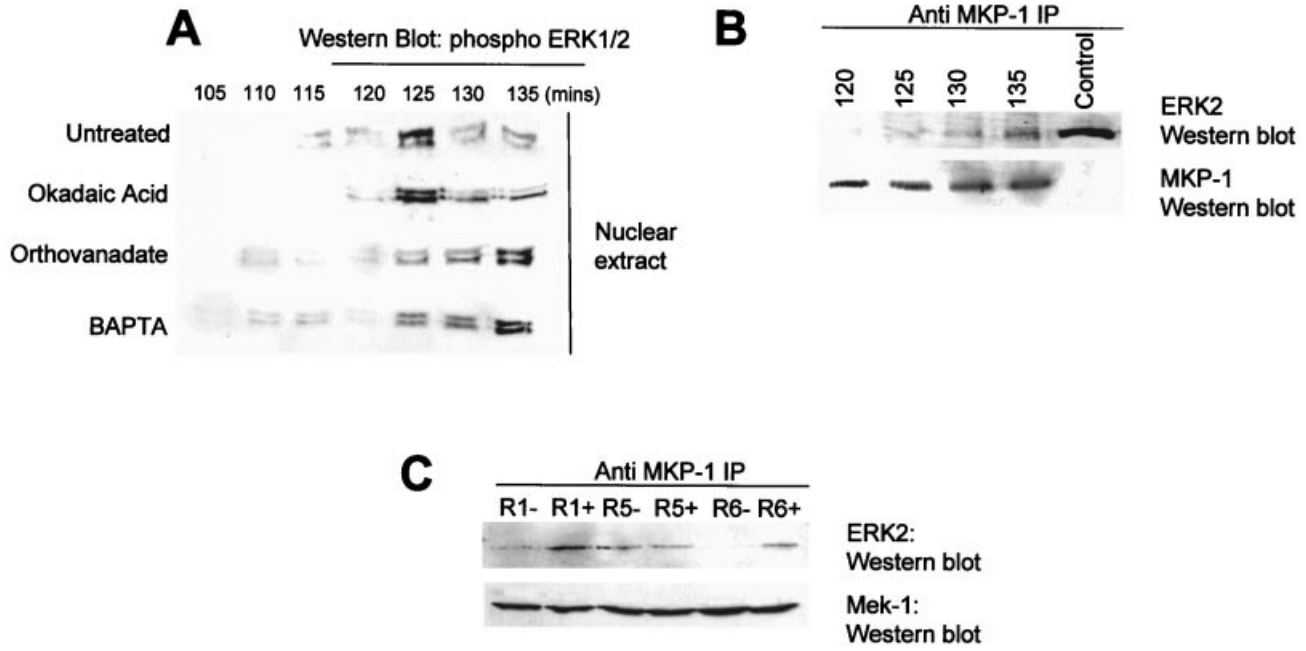

Figure 5 Potential regulatory mechanism of nuclear translocation of the active phospho-ERK1/2

(**A**) Nuclear export of ERK1/2 is blocked by orthovanadate or the calcium-depleting compound BAPTA, but not by okadaic acid. Clone A8 was serum-starved for 24 h, then pre-treated for 30 min with sodium orthovanadate (100 μM), okadaic acid (1 μM) or BAPTA (10 μM) prior to induction with 1 μM Ponasterone A. Nuclear extracts were then taken at 5 min intervals, starting 105 min after induction. As a control, nuclear lysates from untreated Ponasterone A-induced cells were used. Samples were analysed by Western blotting for phosphorylated ERK1/2. Loading was confirmed by staining of the total protein content using Ponceau S (results not shown). (**B**) Direct interaction of nuclear ERK1/2 with MKP-1. Clone A8 was serum-starved for 24 h prior to induction with 1 μM Ponasterone A and nuclear extracts obtained at the time points indicated (in min). Extracts were incubated with an anti-MKP-1 antibody prior to precipitation of the antibody–protein complex. Immunoprecipitated (IP) proteins were then analysed by Western blotting for the presence of ERK2. Cytosolic extract from Ki-Ras-transformed R1 cells was included as a positive control for ERK2. Loading was confirmed by Western blot with an antibody specific for MKP-1. (**C**) Direct interaction of nuclear ERK1/2 with MKP-1 is Ras-specific. Nuclear extracts were obtained from NIH 3T3 cells expressing Ki-Ras (R1), Ha-Ras (R6) or vector alone (R5) and either treated (+) or not (−) with BAPTA. Samples were then immunoprecipitated as above and the presence of ERK2 was detected by Western blot. Loading was confirmed by Western blot with an antibody specific for MEK-1. Similar results were obtained in two other experiments.

contrast with previous conditional systems that specifically activate the MAPK pathway, the conditional oncogenic Ha-Ras system activates diverse signalling pathways in addition to the MAPK pathway. It has been reported that activated Ras mutants with alterations in their effector domains (Val-12/Ser-35, Val-12/Gly-37 and Val-12/Cys-40) are deficient in specific effector function and activate specific signalling pathways [10,16]. In the future it will be interesting to use these and other mutant Ras proteins to analyse specific Ras-mediated signalling pathways and their biological functions.

Exposure to oncogenic Ras leads to transient nuclear ERK1/2 localization

As expected [29], inducibility of V12 Ras was dose-dependent, as was subsequent MAPK activation. However, MAPK activity after Ras induction was higher than MAPK activity in Ras-transformed R1 cells, despite lower levels of Ras. One possible explanation could be that the contribution of a specific c-Ras isoform may vary in its capacity to activate the MAPK pathway [30]. Alternatively, constitutive overexpression of Ras may lead to de-sensitization of the MAPK pathway [31], possibly through induced genomic instability [32].

Conditionally activated Ha-Ras expression led to transient activation of MAPK in the nucleus. Transient activation is in marked contrast with the response in other conditional systems, where it has been shown that induced activation of Raf-1 or MEK-1 (downstream components of the MAPK pathway) led to chronic activation of this pathway [17,18,33]. Evidence supporting our conclusion came from previous experiments where scrape loading of oncogenic Ras also led to transient activation of the MAPK pathway [34]. It is not clear why there should be a difference in the MAPK activation profile between conditional expression of upstream versus downstream components of this pathway. Induced expression of downstream MAPK components such as Raf-1 and MEK-1 has involved the use of deletion mutants, which might lack critical control elements or be insensitive to regulatory intervention. In light of this, it should be noted that there is some evidence that Ras-mediated signalling is tightly regulated by the phosphatase MKP-1, as reported here, or by activation of additional pathways such as p38 [28]. An alternative reason might be variations in experimental design. We assessed MAPK activation status by examining the expression or activation status of various MAPK-dependent proteins in fractionated samples. In contrast, the standard procedure is to assess ERK activity in unfractionated protein extract. It should be noted that we also detected an accumulative increase in ERK1/2 activity in cytosolic extract, but only transient ERK activity in nuclear extract (Figures 4A–4D). One possible way to address this question would be to examine the level of nuclear MAPK activity in other conditional systems.

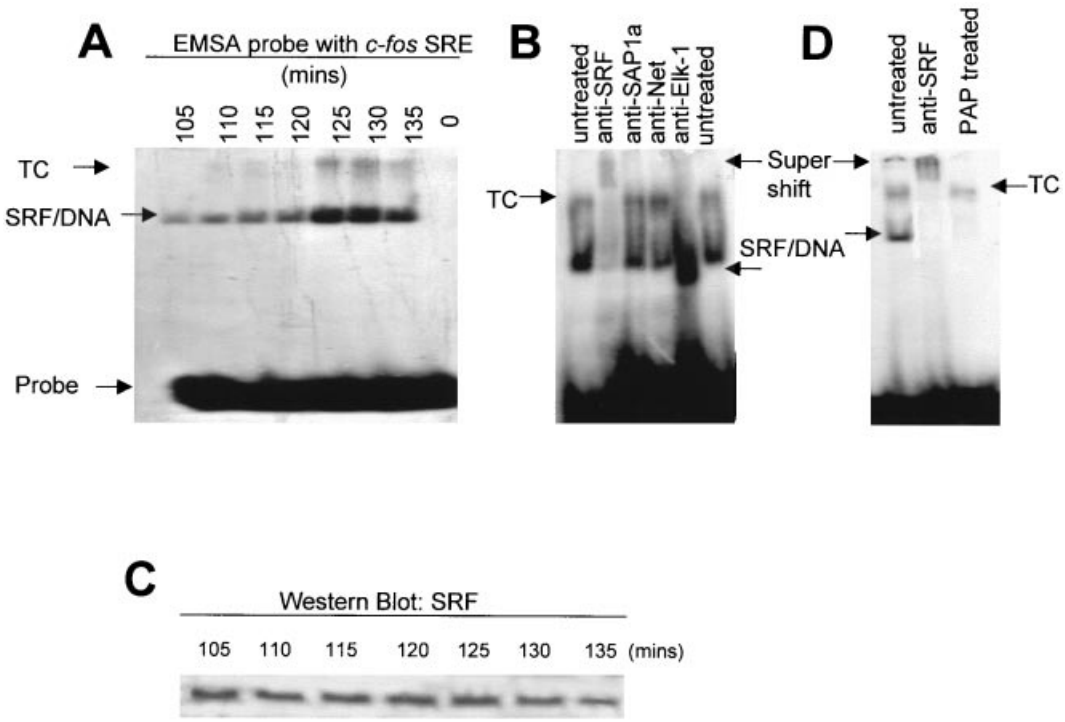

Figure 6 Transient MAPK activation leads to SRE activation

(**A**) Ras conditional cells were serum-starved for 24 h prior to induction with 1 μM Ponasterone A. Samples were harvested at 5 min intervals post-induction (105–135 min) and nuclear fractions were assayed for DNA-binding activity using an SRE-specific probe. Unstimulated cells (0 min) acted as a control. Ternary complex (TC) and SRF complexes on DNA are indicated. (**B**) Pre-incubation of SRE-reactive (130 min post-induction) nuclear proteins with specific antibodies prior to EMSA shows that the ternary complex is composed predominantly of SRF and Elk-1, as determined by disruption of the ternary complex. Untreated denotes nuclear protein without antibody, acting as a control. (**C**) Increase in SRF–DNA binding is not due to an increase in SRF protein expression. Western blot for SRF protein detection was performed on nuclear extracts used in the previous EMSA experiments using an SRF-specific antibody. Loading was confirmed by staining for total transferred protein with Ponceau S. (**D**) Increase in SRF–DNA binding is due to phosphorylation of SRF. SRE-reactive nuclear extract was pre-treated with either anti-SRF antibody or PAP prior to EMSA analysis. Treated samples were then compared with the untreated sample. Results are indicative of at least three separate experiments.

Potential regulatory mechanisms of the MAPK pathway and its components

The data suggested that Ras-mediated MAPK activation was tightly regulated under the conditions used in our experiments, thus emphasizing the importance of control mechanisms. Several classes of phosphatase have been suggested to play a role in MAPK regulation [35]. The ability of okadaic acid to block dephosphorylation of TCF has implied that MAPK control is through serine/threonine phosphatase activity [13]. This is supported by the finding that Elk-1 activation is negatively controlled by protein phosphatase 2B [36]. As reported here, however, dephosphorylation of Elk-1 and loss of its activity, with kinetics different from that observed for the dephosphorylation of nuclear ERK, suggests that negative control of ERK may use alternative mechanisms. Possible candidates are the tyrosine/threonine dual-specificity MKPs (MKP-1 and -2), which have been shown to be induced by the activation of ERK1/2 and then attenuate MAPK events in an inhibitory feedback loop [37,38]. The ability of orthovanadate and calcium-depleting BAPTA, rather than okadaic acid, to affect dephosphorylation and export of ERK in the nucleus in our model implies that control of ERK is mediated, at least in part, by calcium-dependent protein tyrosine phosphatase. The data presented here support the view that MKP-1 is involved in the negative regulation of nuclear ERK. It has been reported that orthovanadate has an inherent ability to activate the MAPK pathway [38]. However, this activation is rapid and transient, peaking after 30 min. In our experiments cells were treated with orthovanadate for 150 min prior to the collection of lysates. This, combined with the ability of BAPTA to give similar results to those of orthovanadate treatment, suggests that the observed retention of MAPK in the nucleus stems from the de-activation of the involved phosphatase and is not related to the experimental procedure.

A second potential mechanism of regulation of signalling-pathway activity could be through transcriptional repressors [26]; however, we did not address this point in this study.

Transient activation of Elk-1 and the possible role for post-translational modification of SRF

Once Elk-1 is activated in the nucleus, the SRE becomes active [39]. Using tightly defined time points, we were able to show that SRE–DNA-binding activity was concomitant with the kinetics of MAPK activation. Interestingly, we observed an increase in both ternary complex formation mediated through Elk-1 activation and an increase in SRF–DNA complex formation at the same time points. We found no increase in SRF protein. This was surprising since SRF expression is up-regulated by MAPK activation. However, at the time points analysed it is expected that only immediate early genes were expressed. It is possible that SRF undergoes some form of post-translational modification, such as phosphorylation, which enhances DNA binding. Indeed, the hyperphosphorylation of SRF has long been reported and would appear, at least in part, to be mediated by MAPK-

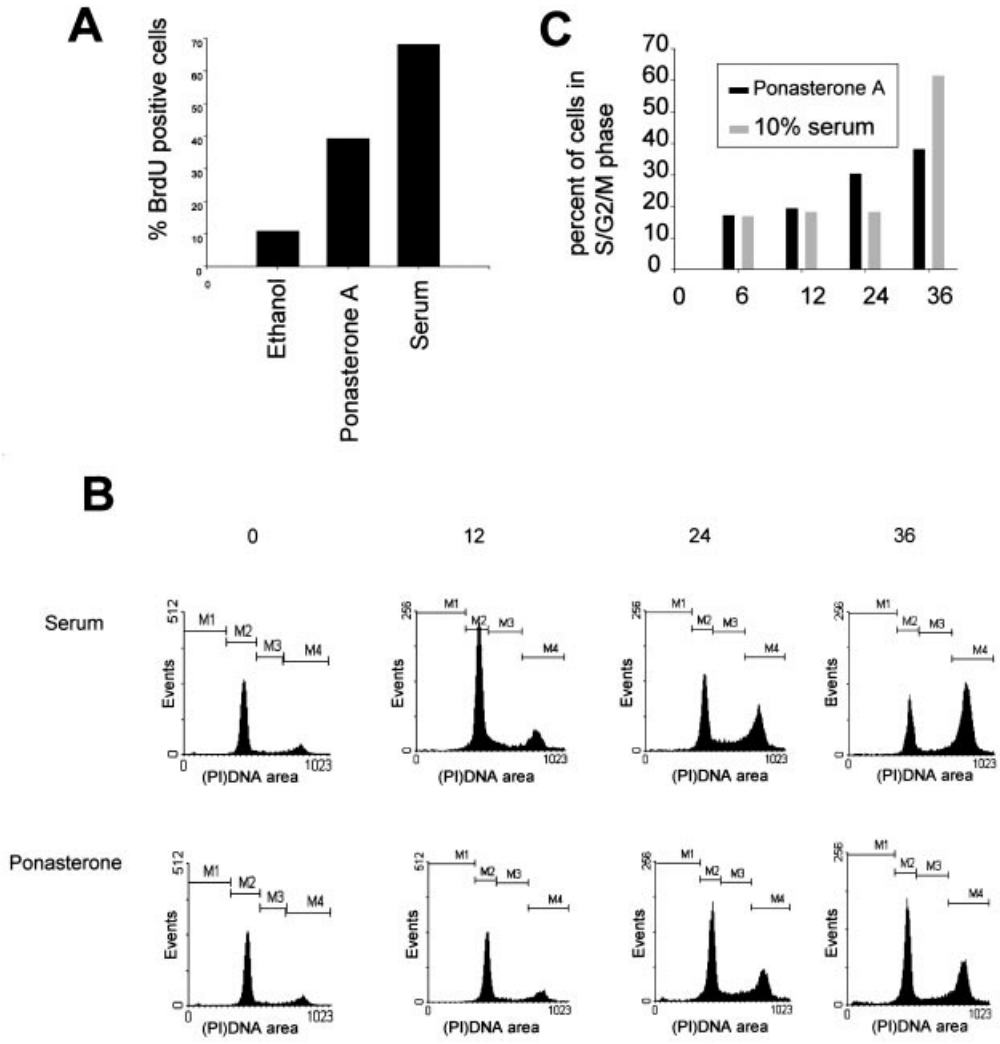

Figure 7 Transient MAPK activation leads to DNA synthesis

(**A**) Ha-Ras conditional cells were arrested by serum deprivation for 48 h and then stimulated with ethanol, Ponasterone A (1 μM) or 10% serum. After 16 and 18 h the cells were labelled with BrdU. Cells synthesizing DNA during this period integrated the BrdU into their chromosomal DNA and were detected by FACScan analysis. (**B**) Cell-cycle analysis of Ha-Ras conditional cells. Cells were arrested by serum deprivation for 48 h and then stimulated with either Ponasterone A (1 μM) or 10% serum. Samples were then harvested at the appropriate time points, fixed in ethanol and their DNA was stained with propidium iodide (PI). Cell populations were analysed for DNA content as described in the Experimental section. The raw FACS profile data is shown (*x*-axis = DNA content; *y*-axis = cell number). (**C**) The cell-cycle distribution of the profiles was quantified with the computer program Modfit (Verity Software) and is shown in a bar diagram representing the percentages of cells in the $S/G_2/M$ phase (> diploid DNA content) over time (h). Similar results were obtained in separate experiments ($n = 2$–3) with additional clones.

activated protein kinase 2 (MK2) [40]. Our data support the view that SRF phosphorylation is important in both TCF– and SRF–DNA complex formation and hence Ets factor signalling. Although we did not examine the activation state of the other known TCFs, SAP-1a (SRF accessory protein 1a) and Net/ERP/SAP-2, directly, supershift analysis of the TCF composition after MAPK activation showed a dominant role for SRF and Elk-1 in the formation of the ternary complex.

Oncogenic Ras and the cell cycle

It has been reported previously that activation of downstream components of the MAPK pathway allows cell-cycle re-entry after growth arrest by serum starvation [18,19]. Since induction of the V12 mutant Ras gave rise to transient activation of the MAPK pathway in our system, it was of interest to determine whether an early burst of MAPK activity was sufficient to induce DNA synthesis. Our data are in agreement with the observation that induction of upstream components of the MAPK pathway leads to cell-cycle re-entry of quiescent NIH 3T3 fibroblasts. Previous experiments with conditional MAPK components, such as inducible Raf-1 or inducible MEK-1, have also reported that MAPK initiates delayed cell-cycle re-entry in quiescent cells [18,19]. However, earlier experiments in NIH 3T3 cells, where oncogenic Ras was introduced by micro-injection [41], suggested that exposure to the oncogene led to accelerated cell-cycle re-entry and a shorter G_1 phase of the cell cycle. These experiments are supported partially by studies on alternative conditionally expressed Ras models, which report a shortened G_1 phase but

which disagree on whether cell-cycle progression occurred [42]. It is possible that the observed discrepancies between experimental systems may be due to the relative level of the Ras oncoprotein. Finally, whereas our data showed clearly that Ras-mediated activation of the MAPK pathway led to cell-cycle progression, experiments with conditionally expressed mutants of Ras reported that induction of DNA synthesis in quiescent NIH 3T3 cells was de-coupled from MAPK activation [43], implying the involvement of a non-MAPK pathway.

Oncogenic Ras and transformation

Upon expression of oncogenic Ha-Ras, cellular clones acquired the characteristics of oncogenic transformation, including colony formation in soft agar, morphological transformation and serum-free growth. Thus oncogenic Ras-induced transformation in this system would appear to be in agreement with effects observed upon overexpression of the Ha-Ras and Ki-Ras isoforms [15,21, 44]. It is therefore of interest why a short burst of MAPK activity can lead to transformation. Whereas activation of the Elk-1 transcription factor via the MAPK signalling pathway is involved in the initiation of transformation, probably through the initiation of G_1 entry in the cell cycle, it is possible that different Ras-dependent signalling pathways may be required to maintain transformation [45]. Although not reported here, we investigated the activation of signalling pathways such as c-Jun N-terminal kinase and p38 MAPK, known to activate AP-1 activity, and while we detected a delayed but sustained activation of c-Jun N-terminal kinase, no p38 MAPK activity was detectable at the time points analysed (D. Plows, unpublished work). However, MAPK may directly activate the AP-1 transcription factor [18]. Thus it is possible that transient activation of the MAPK pathway could lead to short-term activation of Elk-1, but to more sustained activation of the AP-1 transcription factor, which in turn leads to cellular transformation.

In conclusion, we report that initial exposure to oncogenic Ras caused transient MAPK activation, as determined by translocation of activated ERKs to the nucleus, which led to weak but sustained cell-cycle entry and morphological transformation. We present evidence that the rapid negative regulation of the active nuclear ERK1/2 may be through the action of MKP-1, a finding that highlights the benefits of using this novel system to obtain new insights into Ras-mediated effects.

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