Cell cycle-dependent activation of Ras

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Background: Ras proteins play an essential role in the transduction of signals from a wide range of cell-surface receptors to the nucleus. These signals may promote cellular proliferation or differentiation, depending on the cell background. It is well established that Ras plays an important role in the transduction of mitogenic signals from activated growth-factor receptors, leading to cell-cycle entry. However, important questions remain as to whether Ras controls signalling events during cell-cycle progression and, if so, at which point in the cell-cycle it is activated.

Results: To address these questions we have developed a novel, functional assay for the detection of cellular activated Ras. Using this assay, we found that Ras was activated in HeLa cells, following release from mitosis, and in NIH 3T3 fibroblasts, following serum-stimulated cell-cycle entry. In each case, peak Ras activation occurred in mid-G1 phase. Ras activation in HeLa cells at mid-G1 phase was dependent on RNA and protein synthesis and was not associated with tyrosine phosphorylation of Shc proteins and their binding to Grb2. Significantly, activation of Ras and the extracellular-signal regulated (ERK) subgroup of mitogen-activated protein kinases were not temporally correlated during G1-phase progression.

Conclusions: Activation of Ras during mid-G1 phase appears to differ in many respects from its rapid activation by growth factors, suggesting a novel mechanism of regulation that may be intrinsic to cell-cycle progression. Furthermore, the temporal dissociation between Ras and ERK activation suggests that Ras targets alternate effector pathways during G1-phase progression.

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Background

Ras proteins act as molecular switches that relay proliferative signals from cell-surface receptors to the nucleus and cytoskeleton. Specifically, the activation of Ras by the binding of GTP is required for the ability of many growth factors and cytokines to induce non-proliferating cells to enter G1 phase of the cell cycle. Activation is the result of the stimulated exchange of GTP for GDP catalyzed by guanine nucleotide-exchange factors, such as Sos. The activation of membrane-bound Ras by growth-factor and cytokine receptors is generally achieved by the recruitment of Grb2–Sos complexes to the receptors themselves, or to adaptor proteins such as Shc. A primary target of activated Ras during growth-factor stimulation is Raf, which is the first component of a protein kinase cascade that leads to activation of the mitogen-activated protein (MAP) kinases ERK1 and ERK2 (ERK standing for extracellular signal-regulated kinase; [1]). The phosphorylation of transcription factors by these MAP kinases results in the expression of immediate–early response genes, such as *c-fos*, which are required for progression through early-G1 phase of the cell cycle.

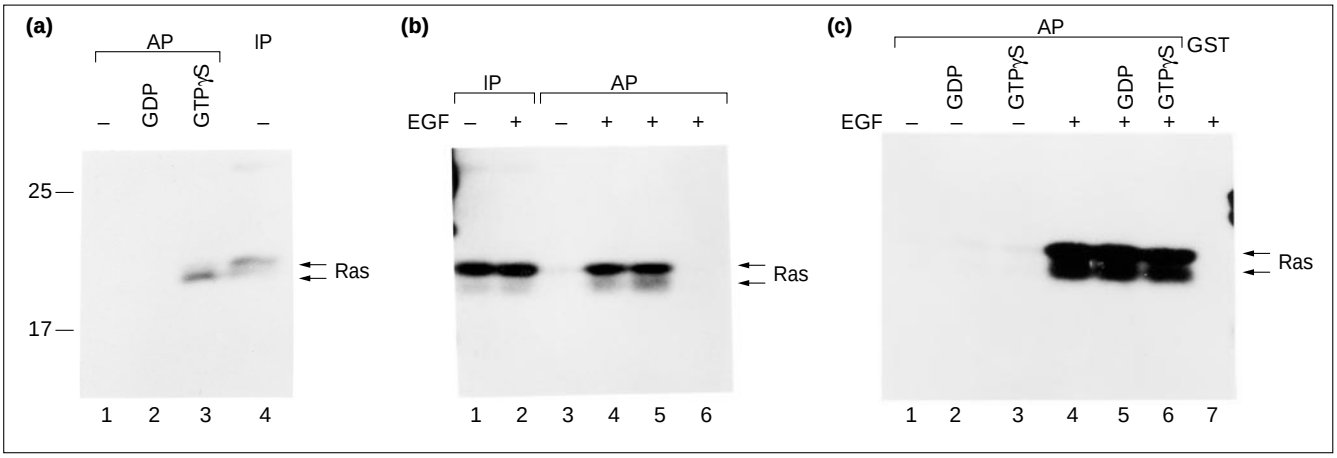
Although these signalling events occur within minutes of growth-factor stimulation, microinjection of neutralizing anti-Ras antibodies in late-G1 phase blocks progression of fibroblasts into S phase [2]. Furthermore, studies involving combinations of cell-cycle inhibitors and anti-Ras microinjection clearly demonstrate that there are multiple points in early and late-G1 phase at which Ras is required [3]. These findings, together with the observations that expression of oncogenic Ras increases cyclin D1 levels and shortens G1 phase [4,5], and that Ras and cyclin D1 cooperate in cellular transformation assays [6,7], point to an important role for Ras in regulating the progression from G1 into S phase.

Results and discussion

A novel assay for activated Ras

Most previous analyses of Ras activation have measured the GTP: GDP ratio of immunoprecipitated Ras following ^{32}P radiolabelling of cells [8–11]. The ability of even very low levels of radioisotopes to cause rapid cell-cycle arrest (within a few hours) or apoptosis [12–14], however, precludes the use of such assays to measure Ras activity in cycling cells. We therefore developed an assay that exploits the known specificity of the interaction between Ras–GTP and the Ras-binding domain (RBD) of Raf-1 [15–18] in order to detect activated Ras. Dialyzed detergent lysates of HeLa cells were treated with or without

Figure 1



An assay for activated Ras. (**a**) Dialyzed HeLa cell lysates were incubated in the absence (lanes 1,4) or presence of GDP (lane 2) or GTPγS (lane 3). Lysates were subjected to affinity precipitation (AP) with GST–RBD (lanes 1–3) or immunoprecipitation (IP) with anti-Ras antibodies (lane 4). Ras proteins were detected by immunoblotting with monoclonal anti-Ras antibodies. (**b**) Serum-starved HeLa cells were treated with (lanes 2,4–6) or without (lanes 1,3) 200 ng ml^{-1} EGF for 10 min before cell lysis. Lysates were subjected to immunoprecipitation with anti-Ras antibodies (lanes 1,2), or affinity precipitation with GST–RBD (lanes 3–6). Lanes 5,6: extracts were preincubated with 4 μg of anti-Ras antibody 238 (lane 5) or anti-Ras antibody 259 (lane 6) before precipitation. Ras proteins were detected as in (a). (**c**) Serum-starved HeLa cells were treated without (lanes 1–3) or with (lanes 4–7) 200 ng ml^{-1} EGF for 10 min, lysed in lysis buffer (lanes 1,4,7), or in lysis buffer containing 1 mM GDP (lanes 2,5) or 0.1 mM GTPγS (lanes 3,6), and precipitated with GST–RBD (lanes 1–6) or GST alone (lane 7). Ras proteins were detected as in (a).

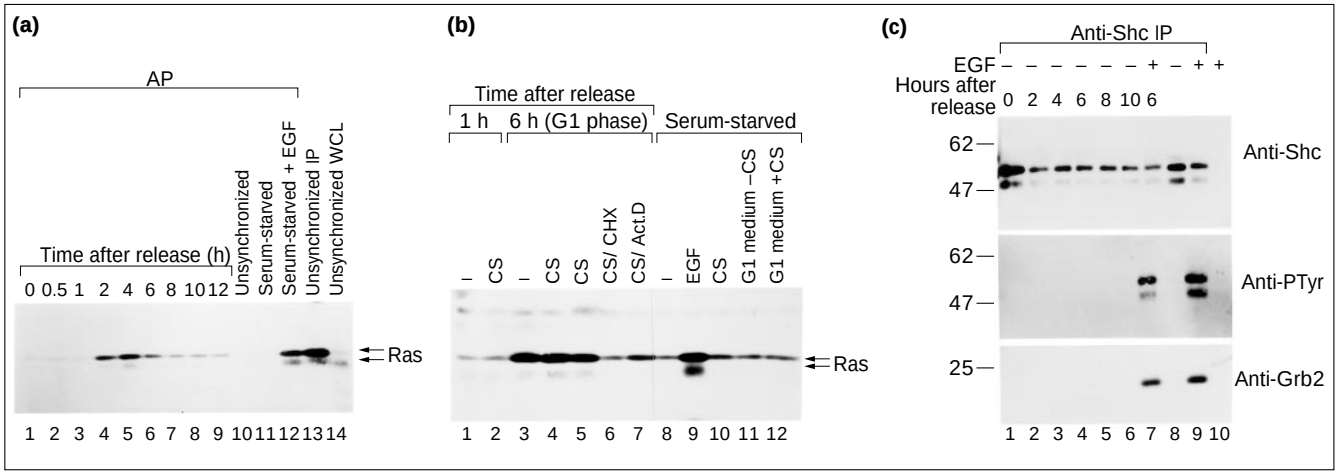
guanine nucleotides and then incubated with GST–RBD (a glutathione-S-transferase–RBD fusion protein) immobilized on glutathione–Sepharose. Bound proteins were eluted, and subjected to SDS–PAGE and western blotting using anti-pan Ras antibodies. GST–RBD affinity precipitated the GTPγS-bound form of Ras, but not GDP-bound or nucleotide-free forms (Fig. 1a, lanes 1–3). Therefore, as the Raf RBD has a significantly lower affinity for Ras–GDP than for Ras–GTP, only Ras–GTP is detected under the conditions of this assay.

Treatment of HeLa cells with epidermal growth factor (EGF) greatly increased the affinity precipitation of Ras by GST–RBD, whereas the recovery of Ras in anti-Ras immunoprecipitates was unaltered by EGF treatment (Fig. 1b, lanes 1–4). In this experiment, ~40–50 % of total cellular Ras was activated by EGF treatment, in close agreement with the level of Ras–GTP measured previously by ^{32}P incorporation in EGF-stimulated Rat-1 fibroblasts [19]. The anti-Ras antibody 259, which blocks the Ras–Raf interaction [20], blocked the affinity precipitation of Ras by GST–RBD in the EGF-stimulated cells, whereas antibody 238, which does not block Ras–Raf binding, did not (Fig. 1b, lanes 5,6). We did not detect co-immunoprecipitation of Ras and Raf after EGF treatment using either anti-Ras or anti-Raf antibodies under the conditions of this assay (data not shown); therefore endogenous Raf does not interfere with the binding of Ras–GTP to GST–RBD. Inclusion of GDP or GTP in the lysis buffer did not affect the affinity precipitation of Ras from EGF-treated or untreated cells (Fig. 1c, lanes 1–6), showing that GTP binding to Ras did not occur after lysis. In summary, the assay rapidly and specifically detects cellular Ras–GTP and changes in its levels in response to physiological stimuli.

Ras is activated during progression through G1 phase in HeLa cells

To determine whether Ras activity is modulated during progression through G1 phase, HeLa cells were released from a mitotic arrest and analyzed for Ras activation at time points thereafter. Figure 2a shows that the level of Ras–GTP increased several-fold following exit from mitosis, reaching a peak ~4 hours into G1 phase, and then decreased as the cells approached the onset of S phase (~10 h after mitosis, as determined by cell-sorting analysis). The total levels of cellular Ras remained constant during this period (data not shown). The pattern and extent of Ras activation in mid-G1 phase was the same when cells were grown in the presence or absence of added serum (Fig. 2b, lanes 1–4; data not shown), or in suspension or attached to plates (Fig. 2b, lanes 4,5; data not shown). Furthermore, Ras activation during G1 phase was blocked by inhibitors of protein synthesis and gene transcription (Fig. 2b, lanes 5–7) and was not restored by adding mid-G1 conditioned medium to serum-starved cells (Fig. 2b, lanes 8,11,12). These results point towards a mechanism of Ras activation that is integral to the cell-cycle machinery and is not solely linked to receptor activation.

Figure 2



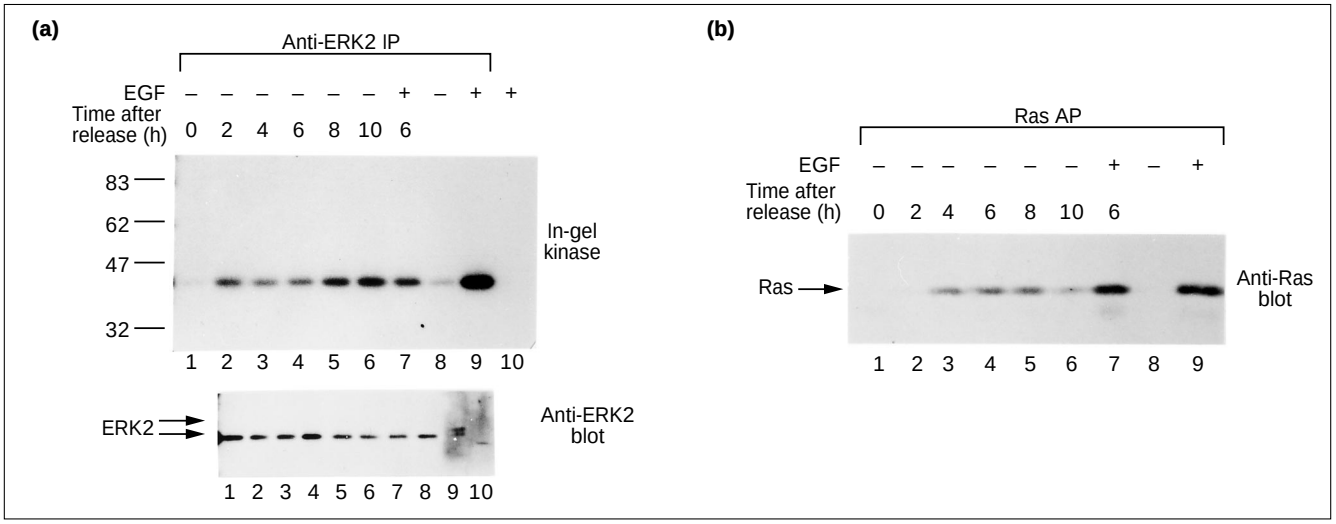
Activation of Ras during G1 phase in HeLa cells. Synchronized cells were produced by mitotic arrest using a thymidine–nocodazole double block and released into G1 phase (in suspension culture, unless otherwise indicated); unsynchronized cells were serum-starved and treated with agonists. Extracts were prepared and subjected to affinity precipitation or immunoprecipitation followed by immunoblot analysis using anti-Ras antibodies. **(a)** Lanes 1–9, GST–RBD affinity precipitates from cell lysates prepared at the indicated times after release from mitotic arrest; lanes 10–12, GST–RBD affinity precipitates from unsynchronized (lane 10) or serum-starved cells treated for 10 min without (lanes 10,11) or with (lane 12) 100 ng ml^{-1} EGF; lane 13, unsynchronized cell lysate (50 % of the volume used in affinity precipitations) subjected to immunoprecipitation using anti-Ras antibodies; lane 14, whole cell lysate (WCL; 5 % of the volume used in affinity precipitation). **(b)** Lanes 1–4, affinity precipitates from cells released for 1 h (lanes 1,2) or 6 h (lanes 3,4) from mitotic arrest in suspension culture in the presence (lanes 2,4) or absence (lanes 1,3) of 10 % calf serum (CS); see Materials and methods for details. Lanes 5–7, affinity precipitates from cells released from mitotic arrest for 6 h and allowed to attach to plates in the absence (lane 5) or presence of 25 $\mu g\ ml^{-1}$ cycloheximide (CHX; lane 6) or 5 $\mu g\ ml^{-1}$ actinomycin D (Act.D; lane 7), added 45 min after release. Lanes 8–12, affinity precipitates from serum-starved cells treated for 10 min in the absence (lane 8) or presence of 100 ng ml^{-1} EGF (lane 9), 10 % serum (CS; lane 10), or conditioned medium from the cells analyzed in lane 3 (G1 medium –CS; lane 11) or in lane 4 (G1 medium +CS; lane 12). **(c)** Anti-Shc immunoprecipitates from cells released from mitotic arrest for the indicated times (lanes 1–6), from cells relased from mitotic arrest for 6 h and treated with 100 ng ml^{-1} EGF (lane 7), or from serum-starved cells treated without (lane 8) or with (lanes 9,10) 100 ng ml^{-1} EGF. Lane 10, serum-starved cells treated with 100 ng ml^{-1} EGF and immunoprecipitated in the absence of anti-Shc antibodies. Immunoblots were probed with antibodies directed against Shc, phosphotyrosine (PTyr) or Grb2.

The activation of Ras by EGF and many other agonists involves the binding of Grb2–Sos complexes to tyrosine-phosphorylated Shc proteins. Treatment of serum-starved HeLa cells or cells synchronized in mid-G1 phase (6 h after mitotic release) with EGF resulted in rapid tyrosine phosphorylation of p46 and p52 Shc proteins (Fig. 2c, middle panel, lanes 7,9). This was accompanied by Shc–Grb2 complex formation, as shown by the co-immunoprecipitation of Grb2 in anti-Shc immunoprecipitates (Fig. 2c, lower panel, lanes 7,9). However, Ras activation during G1 progression was not associated with detectable tyrosine phosphorylation of Shc or with Shc–Grb2 complex formation (lanes 1–6). The mechanism of Ras activation in G1 phase therefore appears to differ from Ras activation induced by EGF and many other mitogens.

Activation of Ras in mid-G1 phase is uncoupled from ERK2 activation

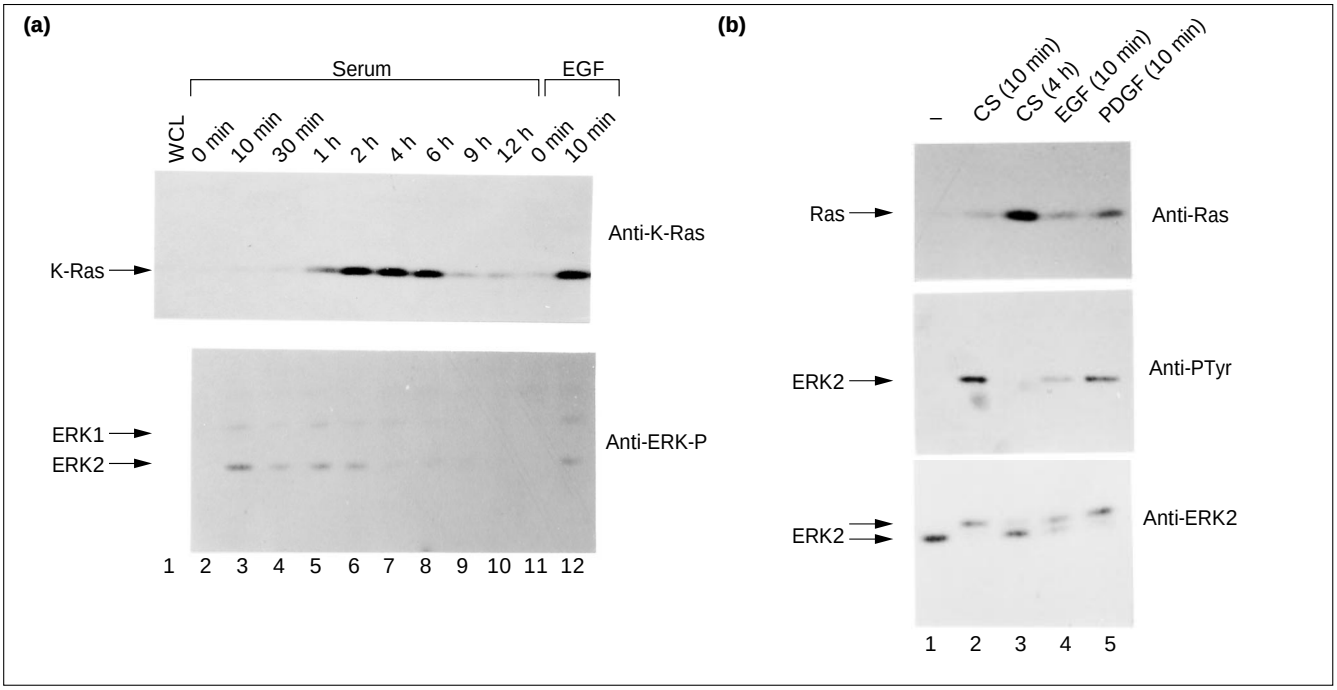
Activation of Ras by growth factors leads to the rapid activation of the MAP kinases ERK1 and ERK2. Activated RasL61, in which the glutamine at residue 61 is replaced by leucine, strongly activates co-expressed ERK2 in HeLa cells [21]. To determine whether ERK2 activity was stimulated in response to Ras activation during G1-phase progression, HeLa cell extracts were analyzed for Ras and ERK2 activity following mitotic release. ERK2 kinase activity — measured by in-gel kinase assay of anti-ERK2 immunoprecipitates using myelin basic protein as a substrate — increased within 2 hours of release from mitosis and maintained about the same level of activity throughout G1 phase, except for a small increase in activity near the G1/S transition (Fig. 3a, upper panel, lanes 1–9). In contrast, the level of Ras activation increased through early-G1 phase, again reaching maximal levels around mid-G1 (Fig. 3b). Interestingly, an in-gel kinase assay (Fig. 3a, upper panel) and a gel mobility-shift assay (Fig. 3a, lower panel) showed that the activation of ERK2 by EGF in mid-G1 cells was substantially lower than that induced by EGF in serum-starved cells (Fig. 3a, compare lanes 4 and 7 with 8 and 9), even though activation of Ras (Fig. 3b, lanes 4,7–9) and tyrosine phosphorylation of Shc (Fig. 2c) in response to EGF were similar in mid-G1 and serum-starved cells. Because only a small fraction of

Figure 3



Relative activation of Ras and ERK kinases in HeLa cells during G1 phase. (a) Lanes 1–10, anti-ERK2 immunoprecipitates from cells treated as described in lanes 1–10 in Fig. 2c. Immunoprecipitates were subjected to an in-gel kinase assay using myelin basic protein as substrate (upper panel), or immunoblotted with anti-ERK2 monoclonal antibodies (lower panel). (b) Lanes 1–9, GST–RBD affinity precipitates from the cells analyzed in lanes 1–9 of (a) immunoblotted with anti-Ras antibodies.

Figure 4



Relative activation of Ras and ERK kinases in NIH 3T3 cells during G1 phase. (a) NIH 3T3 cells were serum-starved 24 h and then treated without (lanes 2,11) or with 10 % calf serum (lanes 3–10) or 100 ng ml^{-1} EGF (lane 12) for the indicated times. Upper panel: GST–RBD affinity precipitates from cell lysates were immunoblotted with anti-K-Ras antibodies. Lane 1 contains whole cell lysate (WCL; 5 % of the volume used in the affinity precipitations). Lower panel: cell lysates were subjected to western immunoblotting using an antibody specific for tyrosine phosphorylated, activated ERK1 and ERK2 (anti-ERK-P). (b) NIH 3T3 cells were serum-starved and treated without (lane 1) or with 20 % calf serum for 10 min (lane 2) or 4 h (lane 3), or for 10 min with 50 ng ml^{-1} EGF (lane 4) or 10 ng ml^{-1} PDGF (lane 5). Upper panel: GST–RBD affinity precipitates probed with anti-K-Ras antibodies. Middle and lower panels: anti-ERK2 immunoprecipitates probed with anti-phosphotyrosine (anti-PTyr) or anti-ERK2 antibodies.

ERK2 was activated in G1 phase, as indicated by the low level of the mobility-shifted form on immunoblots (Fig. 3a, lower panel), these results indicate that Ras activation was significantly uncoupled from ERK activation in mid-G1 phase.

Ras is activated in mid-G1 phase in NIH 3T3 fibroblasts

To extend our observations, we examined Ras and ERK activities during G1 progression in NIH 3T3 fibroblasts released from quiescence by serum treatment. Under the conditions used, the cells entered S phase approximately 12 hours after serum addition, as determined by bromodeoxyuridine incorporation (data not shown). The levels of K-Ras (detected using a K-Ras-specific antibody which offers greater signal resolution in these cells) increased progressively during early-G1 phase, reaching maximal levels around 4 hours after serum addition, and then decreased towards basal levels as the cells approached S phase (Fig. 4a, upper panel). As in the mitotic-release experiments, maximal activation of ERK1 and ERK2 — as determined by immunoblotting of cell lysates with an antibody specific for tyrosine phosphorylated ERKs — preceded peak K-Ras activation, occurring within 10 minutes of serum treatment (Fig. 4a, lower panel, lanes 1,2). Thereafter, these activities decreased during early-G1 progression (Fig. 4a, lanes 2–10), in agreement with other studies [22,23], such that activated ERKs were barely detectable at the time of maximal K-Ras activation. Analysis of anti-ERK2 immunoprecipitates, using a gel mobility-shift assay and anti-phosphotyrosine antibodies (Fig. 4b) or an in-gel kinase assay (data not shown), revealed that treatment with serum, EGF or PDGF resulted in the substantial activation of ERK2 (>50 %, as determined by mobility shift) within 10 minutes, which correlated with minor activation of K-Ras (Fig. 4, lanes 1,2,4,5). In sharp contrast, only a small fraction of ERK2 was activated 4 hours after serum treatment, when K-Ras was maximally activated (Fig. 4, lane 3).

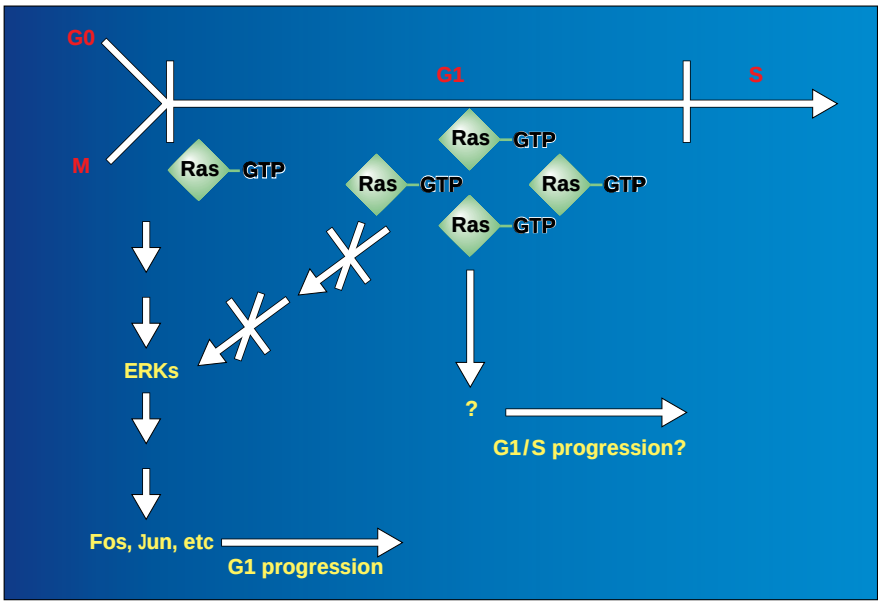
Conclusions

The assay described here rapidly and efficiently detects functionally active cellular Ras and should therefore find wide applicability in studies of normal and oncogenic Ras function. We have exploited this assay to measure Ras activation during progression through G1 phase and to temporally correlate this with established upstream and downstream events in Ras signalling. Following release from mitosis, Ras became maximally activated in mid-G1 phase. This activation was not associated with the recruitment of Grb2 by Shc, was dependent on gene transcription and protein synthesis, and was apparently independent of extracellular soluble ligands, suggesting that Ras may be regulated by the cell-cycle machinery as well as by receptor-mediated mechanisms. K-Ras activation also reached a maximum in mid-G1 phase in serum-stimulated fibroblasts. Importantly, this occurred after the largest increases in ERK activity, which occurred in very early-G1 phase, regardless of whether cells were released from mitosis or quiescence. This temporal disjunction between activation of Ras and a well established effector pathway raises the possibility that Ras targets other pathways in mid-G1 phase that may be required for progression into S phase (Fig. 5).

It has become clear that Ras uses multiple effectors to relay mitogenic signals to different cellular pathways [24]. For

Figure 5

A model for Ras function in G1 progression (see text for details).



instance, effector domain mutants of activated Ras have been identified that are defective for either ERK activation or cytoskeleton reorganization [25]. Neither type of mutant alone can stimulate DNA synthesis, but together they promote progression into S phase. How these distinct Ras effector pathways interact to result in mitogenesis is not yet clear. We suggest that these pathways may be temporally coordinated by Ras in a cell-cycle dependent manner.

Materials and methods

Cell culture and synchrony

HeLa S3 cells and NIH 3T3 fibroblasts were grown in DMEM containing 10 % calf serum. HeLa cells were arrested in mitosis using a thymidine–nocodazole double block: cells growing in DMEM containing 10 % calf serum were incubated in 2 mM thymidine for 20–24 h, washed with DMEM, trypsinized and replated; 0.1 μg ml^{-1} nocodazole was added after 2 h and mitotic cells were collected by shake-off 12–14 h later. For mitotic release, cells were washed twice with DMEM, resuspended in DMEM containing 10 % serum and incubated in suspension in spinner culture (HeLa cells grow synchronously in suspension; [26]) or allowed to re-attach to plates (in Fig. 2b, lanes 5–12). HeLa and NIH 3T3 cells were incubated in DMEM without serum for 24 h before treatment with growth factors or serum.

Cell lysis, immunoprecipitations and kinase assays

Suspension and adherent cells were washed twice with ice-cold HBS. In Fig. 1a, cells were lyzed in a solution containing 20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 % NP-40, 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} aprotinin, and the lysates were dialyzed extensively against the same buffer without protease inhibitors. After clearing by centrifugation, dialyzed lysates (0.3 ml) were incubated with or without 1 mM GDP or 0.5 mM GTPγS for 10 min, and then with 10 mM $MgCl_2$ for 60 min at room temperature. For affinity and immunoprecipitations in the other figures, cells were lyzed in Mg^{2+}-containing lysis buffer (25 mM Hepes (pH 7.5), 150 mM NaCl, 1 % NP-40, 0.25 % Na deoxycholate, 10 % glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM Na vanadate, 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} aprotinin) at 0.4–0.8 ml per plate. Lysates were either used immediately or frozen in liquid nitrogen and stored at –70 °C until use. In Fig. 1, equal volumes of lysate were used for precipitations (0.3 ml for affinity precipitations; 0.15 ml for immunoprecipitations). In other figures, the protein concentrations of lysates were determined and equal amounts of proteins were used. For immunoprecipitations, lysates were incubated with 1 μg anti-Ras monoclonal antibody 238 (Santa Cruz) for 60 min and then with Protein G–Sepharose (20 μl packed beads) for 40 min. Shc proteins were immunoprecipitated with 1 μg anti-Shc polyclonal antibody (Transduction Labs) for 4 h followed by collection on protein A–Sepharose beads. ERK2 was immunoprecipitated for 4 h with 0.5 μg anti-ERK R2 polyclonal antibody (UBI) plus 0.5 μg anti-ERK2 antibody (sc-154; Santa Cruz) followed by collection on Protein A–Sepharose beads. Although these antibodies recognize ERK1 and ERK2, we only detected ERK2 in immunoprecipitates. All precipitates were washed three times with lysis buffer containing 10 mM $MgCl_2$ and bound proteins were eluted with SDS–PAGE sample buffer. For anti-Shc immunoprecipitations, proteins were eluted in SDS-PAGE sample buffer containing 20 mM N-ethyl maleimide (to prevent IgG reduction and interference of heavy and light chains on blots). Proteins were resolved on 10 % or 11 % acrylamide gels and subjected to western blotting. Blots were probed with anti-pan Ras (Transduction Labs), anti-K-Ras (sc-30) or anti-H-Ras (sc-29) monoclonal antibodies (Santa Cruz), anti-Shc or anti-Grb2 monoclonal antibodies (Transduction Labs), anti-PTyr monoclonal antibody 4G10 (UBI), anti-ERK2 monoclonal antibody (Transduction Labs), or with anti-phospho-MAP kinase (anti-ERK-P) polyclonal antibodies (New England Biolabs); proteins were detected using peroxidase-conjugated anti-mouse or anti-rabbit antibodies and visualized by ECL.

In-gel kinase assays were performed on 10 % polyacrylamide mini-gels containing 0.4 mg ml^{-1} myelin basic protein essentially as described [27,28]. The kinase reaction was carried out for 40 min in 10 ml of 50 mM Tris–Cl (pH 7.5), 0.1 % NP-40, 1 mM DTT and 20 μM γ-[^{32}P]ATP (~2.5 mCi μmol^{-1}).

Assay for detection of activated Ras

To create an expression vector for the production of GST–RBD a *Bam*HI–*Hind*III fragment of plasmid pKScRaf1 (a gift from D. Morrison) was blunt-ended and ligated into the *Sma*I site of pGEX-2T. pGEX-RBD encodes amino acids 1–149 of cRaf-1 fused to GST. GST–RBD expression in transformed *Escherichia coli* was induced with 1 mM IPTG for 1–2 h and the fusion protein was purified on glutathione–Sepharose beads. The beads were washed in a solution containing 20 mM Hepes (pH 7.5), 120 mM NaCl, 10 % glycerol, 0.5 % NP-40, 2 mM EDTA, 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} aprotinin, stored in the same buffer at 4 °C and used within 2–3 days of preparation. For affinity precipitation lysates were incubated with GST–RBD pre-bound to glutathione–Sepharose (~15 μl packed beads, ~15–30 μg protein) for 30 min at 4 °C with rocking. Bound proteins were eluted with SDS–PAGE sample buffer, resolved on 11 % acrylamide gels and subjected to western blotting. Blots were probed with anti-pan Ras (Transduction Labs), anti-K-Ras (sc-30) or anti-H-Ras (sc-29) monoclonal antibodies (Santa Cruz).

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