



HAL
open science

Differential effect of Rac and Cdc42 on p38 kinase activity and cell cycle progression of nonadherent primary mouse fibroblasts

A. Philips, P. Roux, V. Coulon, J. M. Bellanger, A. Vié, M. L. Vignais, J. M. Blanchard

► **To cite this version:**

A. Philips, P. Roux, V. Coulon, J. M. Bellanger, A. Vié, et al.. Differential effect of Rac and Cdc42 on p38 kinase activity and cell cycle progression of nonadherent primary mouse fibroblasts. *Journal of Biological Chemistry*, 2000, 275 (8), pp.5911–5917. 10.1074/jbc.275.8.5911 . hal-02196830

HAL Id: hal-02196830

<https://hal.science/hal-02196830v1>

Submitted on 27 May 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

Differential Effect of Rac and Cdc42 on p38 Kinase Activity and Cell Cycle Progression of Nonadherent Primary Mouse Fibroblasts*

(Received for publication, October 18, 1999)

Alexandre Philips^{‡§}, Pierre Roux[¶], Vincent Coulon[‡], Jean-Michel Bellanger[¶], Annick Vié[‡], Marie-Luce Vignais[‡], and Jean Marie Blanchard[‡]

From the [‡]Institut de Génétique Moléculaire, CNRS, UMR 5535, IFR 24, and the [¶]Centre de Recherches de Biochimie Macromoléculaire, CNRS, UPR 1086, IFR 24, 1919 route de Mende, 34293 Montpellier cedex 5, France

The Rho GTPases play an important role in transducing signals linking plasma membrane receptors to the organization of the cytoskeleton and also regulate gene transcription. Here, we show that expression of constitutively active Ras or Cdc42, but not RhoA, RhoG, and Rac1, is sufficient to cause anchorage-independent cell cycle progression of mouse embryonic fibroblasts. However, in anchorage free conditions, whereas activation of either Cdc42 or Ras results in cyclin A transcription and cell cycle progression, Cdc42 is not required for Ras-mediated cyclin A induction, and the two proteins act in a synergistic manner in this process. Surprisingly, the ability of Cdc42 to induce p38 MAPK activity in suspended mouse embryonic fibroblast was impaired. Moreover, inhibition of p38 activity allowed Rac1 to induce anchorage-independent cyclin A transcription, indicating that p38 MAPK has an inhibitory function on cell cycle progression of primary fibroblasts. Finally, a Rac mutant, which is unable to induce lamellipodia and focal complex formation, promoted cyclin A transcription in the presence of SB203580, suggesting that the organization of the cytoskeleton is not required for anchorage-independent proliferation. This demonstrates a novel function for Cdc42, distinct from that of Rac1, in the control of cell proliferation.

The mechanisms by which anchorage-mediated cues are integrated at the level of the cell division cycle remain elusive. Constitutively active Ras mutants are frequently detected in human tumors and expression of mutated, oncogenic Ras in cultured rodent fibroblast cell lines induces alterations in cell morphology, loss of contact inhibition, changes in gene expression, decreased dependence on serum growth factors, and proliferation which is independent of adhesion to a substratum. Data accumulated during the past decade have shown that many of these Ras-promoted cellular transactions are mediated by Ras effectors, among which Rho family members play an important role, linking plasma membrane receptors to the organization of the actin cytoskeleton, cell adhesion, aggregation, motility, and proliferation (3).

The Rho family of small GTPases include various isoforms of Rac (1–3), Rho (A, B, C), RhoD, RhoE, RhoL, RhoH, RhoG, TC10, and Cdc42Hs. Like Ras, these GTPases oscillate between an active GTP-bound and inactive GDP-bound forms (4).

* This work was supported in part by grants from the BIOMED2 program, ARC, and the Ligue Nationale Contre le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a contract from the BIOMED2 program and INSERM. To whom correspondence should be addressed. Tel.: 33-4-67-61-36-49; Fax: 33-4-67-04-02-31; E-mail: philips@igm.cnrs-mop.fr.

Transient binding of guanine nucleotides is controlled by guanine nucleotide exchange factors, GTPase-activating proteins, and GDP dissociation inhibitory factors (5–7). In fibroblasts, Rho regulates the formation of focal adhesions and stress fibers, Rac mediates membrane ruffles and lamellipodia, and Cdc42Hs controls the formation of filopodia (8–11). Furthermore, Rac1 and Cdc42Hs have been shown to modulate the activity of the Jun N-terminal kinase (JNK)¹ and p38 stress-activated protein kinase signaling pathways (12, 13).

It has now become evident that Rho family proteins, in addition to regulating the organization of the actin cytoskeleton, play an important role in the control of cell proliferation (14, 15). RhoA, Rac1, and Cdc42 have been implicated in the regulation of transcription (16, 17) and DNA synthesis (14). Expression of activated forms of RhoA and Rac can transform fibroblasts and is essential for the establishment of a fully transformed phenotype following Ras activation (18–21). While these results suggest that RhoA and Rac may act downstream of Ras in the control of cell proliferation, the role of Cdc42Hs in this respect still remains unclear. Expression of an activated form of Cdc42 induces progression of adherent quiescent fibroblasts into S phase (14). However, Cdc42Hs has also been shown to inhibit cell cycle progression through a mechanism requiring p38 activation in NIH 3T3 cells (22). Cdc42Hs has also been implicated in the control of anchorage-independent growth (23). Indeed, the ability of Ras expressing fibroblastic cell lines to proliferate in soft agar depends on Cdc42 activity (23). However, the mechanisms of anchorage-independent growth control, modulated by Cdc42 and Ras are unknown. Extracellular signal response kinase (ERK) activation is not a limiting factor in this response (24), but a potential implication of the JNK/p38 stress-activated protein kinase signaling pathways remain to be established.

Most studies of the mechanisms underlying the control of anchorage-independent cell proliferation have been conducted on immortal or transformed cell lines in which many regulatory loops are altered as a consequence of the adaptation of the cells to *in vitro* culture. Alteration of these checkpoints might thus explain the existence of many apparent cell type-specific differences. Another important source of variability stems from the various experimental settings used for the study of anchorage-proliferation relationships. The ability of cells to form colonies within soft agar does not necessarily correlate with their ability to proliferate under other anchorage-free conditions, such as in liquid suspension on agar-coated dishes (24). Here, we have evaluated the role and putative interplay between Ras, RhoA, RhoG, Rac1, and Cdc42 on anchorage-independent

¹ The abbreviations used are: JNK, c-Jun NH₂-terminal kinase; ERK, extracellular signal-regulated kinase; MEF, mouse embryonic fibroblasts; BrdUrd, bromodeoxyuridine; MAPK, mitogen-activated protein kinase; WASP, Wiskott-Aldrich syndrome protein; HA, hemagglutinin.

proliferation of primary mouse embryonic fibroblasts (MEF) in liquid medium.

Expression of cyclin A is required for fibroblast proliferation. Through its association with cyclin-dependent protein kinases *cdc2/cdk1* and *cdk2*, cyclin A controls the normal progression through the S phase and the G₂/M transition. Even in the presence of growth factors, fibroblasts cultured without substratum contact fail to express cyclin A and, as a consequence, do not enter S phase. However, this situation can be overcome by ectopic expression of cyclin A. Since cyclin A expression is a marker of cell competence for proliferation, we assessed the role of the Rho GTPases in mediating proliferation in the absence of adhesion, through activation of cyclin A transcriptional activity. In the experiments reported here, we show that the anchorage requirement for cyclin A expression and subsequent cell cycle progression of MEF are alleviated by ectopic expression of the activated forms of either Ras or Cdc42, but not by Rac1, RhoA, or RhoG. Anchorage-independent proliferation is promoted by a synergy between Ras and Cdc42, thereby delineating two distinct signaling pathways. Finally, the level of p38 kinase activity seems to be a key regulator of anchorage-independent proliferation.

MATERIALS AND METHODS

Cell Culture and Transient Transfection Experiments—Early primary MEF were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum. Cells were transfected using the calcium phosphate procedure, cultured for 24 h in the presence of serum on agar-coated dishes, and processed for luciferase activity as described previously (1). Normalization was performed using the dual-luciferase reporter assay system from Promega. When required, cells were analyzed according to their DNA content with a FACScan (Becton Dickinson) after labeling with propidium iodide. The following plasmids were used: pCycA-luc is a pGL2-Basic vector containing murine cyclin A promoter sequences spanning nucleotides -177 to +100 relative to the 3' most transcription initiation site; pDCR-Ras (Val-12) is pDCR-based vector containing the Ras mutant under the control of the early cytomegalovirus promoter (gift from M. White); Myc epitope-tagged Rac-1, Cdc42Hs, RhoA, and their various mutants were obtained from Ph. Chavrier and expressed in pcDNA3. Expression vectors for wild type HA-tagged JNK1 (pSR α .3HA.JNK1), its dominant negative form (pcDNA3-HA-JNK1-APF), and wild type HA-ERK2 (pECE-HA-p44 MAPK) were gifts from A. Debant and B. Derijard. Wild type HA-p38 MAPK and its dominant negative form, HA-p38 MAPK-TA-YF, were expressed in a PECE-based expression vector (gift from A. Brunet). pCMV-WASP-GBD was constructed by insertion of an *EcoRI*-*XhoI* fragment of pGEX-KG-WASP (gift from A. Hall) into the same sites of pMYCS1 (gift from C. Sardet). As a result, the Cdc42-interacting domain of WASP extending from amino acid 201 to 321, is cloned in-frame with an upstream Myc-tag located between the *NcoI* and *EcoRI* sites of pcDNA3. 5 μ g of total DNA was used for 10⁵ cells per 3.5-cm diameter Petri dishes (0.5 μ g of pCycA-luc, 0.1 μ g of pRL-TK, expression vector as indicated in each figure legend and pBluescript SKII⁺ for a total amount of 5 μ g). To label cells with BrdUrd, MEF were cultured during 12 h in suspension and BrdUrd was then added for an additional 12 h. Cells were collected and fixed on glass slides before BrdUrd staining was monitored.

Protein Analysis and MAP Kinase Assays—After co-transfection of vectors expressing the various tagged MAP kinases and the small GTPases together with pCycA-luc, MEF were cultured for 24 h in the absence of adhesion. Cells were then lysed in Triton lysis buffer (TLB) containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml leupeptine as described (2). Soluble extracts were prepared by centrifugation at 15,000 rpm for 30 min at 4 °C and split into two fractions. The first one was used to test the expression of the tagged proteins, and the second to assay for ERK2, JNK1, or p38 activities. Extracts were incubated with 5 μ l of the 12CA5 anti-HA monoclonal antibody pre-bound to 20 μ l of protein-Sepharose G (Amesham Pharmacia Biotech). After a 2-h incubation at 4 °C, immunoprecipitates were washed three times with TLB and twice with kinase buffer containing 25 mM Hepes (pH 7.5), 25 mM MgCl₂, 25 mM β -glycerophosphate, 2 mM dithiothreitol, and 0.1 mM Na₃VO₄. Immunocomplex kinase assays were performed at 30 °C for 30

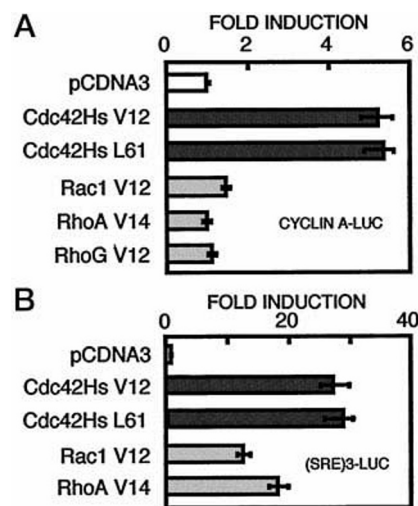


FIG. 1. Only activated Cdc42Hs (Val-12) or Cdc42Hs (Leu-61) and not Rac1 (Val-12), RhoA (Val-14) nor RhoG (Val-12) induce an anchorage-independent expression of a cyclin A reporter in MEF. Fibroblasts were transiently transfected while adherent with the indicated activated GTPase expressing vectors in combination with a Firefly luciferase reporter driven by either cyclin A promoter (A) or by serum responsive element (B), together with a cytomegalovirus-driven Renilla luciferase standard reporter. Cells were then replated on agar-coated dishes for 24 h, luciferase activities determined, and Firefly luciferase activity expressed relative to that of Renilla luciferase.

min in the presence of 50 μ M ATP and 2 μ Ci of [γ -³²P]ATP in 20 μ l of kinase buffer using 4 μ g of myelin basic protein (Sigma), GST-cJun (amino acids 1-79), and GST-ATF2 (amino acids 1-110) as substrates for ERK2, JNK1, and p38 kinase activities, respectively. Reactions were stopped with Laemmli sample buffer and the products resolved by gel electrophoresis through 10% (JNK1 and p38) or 15% (ERK2) polyacrylamide SDS-containing gels. Dried gels were exposed on imaging plates for 1 h, and signals were quantified with a PhosphorImager (Molecular Dynamics). Western blot analysis were carried out on total cellular extracts after transfer onto nitrocellulose filters, and ECL detection was performed with the following primary antibodies: cyclin A CY-A1, Myc 9E10 (Sigma), and HA12CA5 (Cold Spring Harbor Laboratory) mouse monoclonals, as well as an anti-glyceraldehyde-3-phosphate dehydrogenase polyclonal rabbit IgG (1).

RESULTS

Activation of the Cdc42Hs-mediated Pathway Promotes Anchorage-independent Cyclin A Expression and S-phase Entry of Nonadherent MEF—Fibroblasts lacking contact with the extracellular matrix are arrested in the G₁ phase of the cell cycle, as can be judged by most of the cells having a 2N DNA content and expressing cyclin D1 to a high level (data not shown) (25). It is well established that Rho family proteins play an essential role both in organizing the actin cytoskeleton (26) and in promoting cell cycle progression in adherent cells (14). We have investigated the effects of four Rho family members (RhoA, RhoG, Rac1, and Cdc42Hs) on cyclin A expression and S phase entry of nonadherent fibroblasts. The activated Myc-tagged mutants of the GTPases were co-expressed in transient transfection experiments with a transfected cyclin A promoter-driven luciferase reporter (pCycA-luc). In nonadherent MEFs, a constitutively activated form of Cdc42Hs (Cdc42Hs (Val-12) or Cdc42Hs (Leu-61)) activated pCycA-luc (Fig. 1A). In contrast, RhoA (Val-14), RhoG (Val-12), and Rac1 (Val-12) failed to stimulate the cyclin A-promoter (Fig. 1A). However, in a parallel experiment, Cdc42Hs, and to a lesser extent RhoA (Val-14) and Rac1 (Val-12), activated serum responsive element-driven transcription (Fig. 1B). We next investigated whether activated Rho proteins modulated the endogenous cyclin A expression in nonadherent MEF. We therefore analyzed endogenous cyclin A expression in nonadherent cells transfected with activated

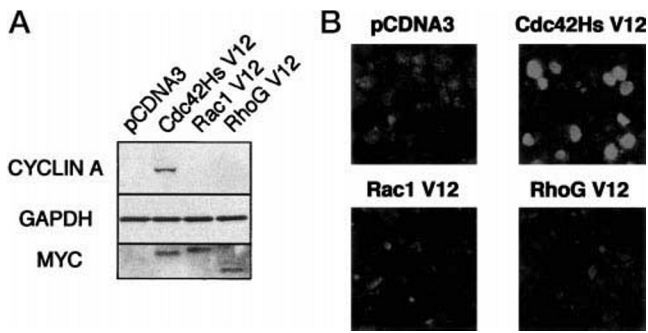


FIG. 2. Activated Cdc42 (Val-12) and not Rac1 (Val-12) nor RhoG (Val-12), induces cyclin A expression and S phase reentry of nonadherent MEF. Fibroblasts were transiently transfected as indicated in Fig. 1. *A*, expression of cyclin A together with the various Myc-tagged GTPases mutants was monitored by Western blotting on total cellular extracts performed successively with anticyclin A and anti-Myc monoclonals. Normalization was carried out with a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) rabbit polyclonal antibody. pCDNA3 indicates cells transfected with the empty vector. *B*, cells entering S phase were detected after BrdUrd incorporation by immunostaining with a fluorescein-conjugated anti BrdUrd monoclonal antibody (Amersham Pharmacia Biotech). A representative field is shown for each transfection.

forms of Cdc42Hs, Rac1, and RhoG. The different mutants were expressed at the same level as monitored by Western blot analysis performed with a monoclonal antibody directed against the Myc epitope (Fig. 2A). Nevertheless, activation of Cdc42Hs, but not RhoG nor Rac1, resulted in a significant expression of endogenous cyclin A in nonadherent MEF. Consistent with these data, BrdUrd labeling (Fig. 2B) indicative of S phase entry, were detected only in cells expressing activated Cdc42Hs. Moreover, using fluorescence-activated cell sorter analysis, we confirmed that these nonadherent MEF cells were competent for cell cycle progression since they accumulated in the S and G₂/M phases of the cell cycle (data not shown).

Cdc42Hs and Ras Promote Anchorage-independent Proliferation of MEF through Synergistic Signaling Pathways—Experiments aimed at determining whether Cdc42Hs is a key downstream mediator of Ras signaling have led to conflicting results. Thus we investigated in primary fibroblasts whether Ras was necessary for the resumption of cell proliferation as measured by cyclin A expression. MEF were transfected with cytomegalovirus-driven plasmid expressing the HA-tagged mutant of Ras which contains the activating Val-12 mutation. As shown in Fig. 3A, Ras (Val-12) gave rise to a 5-fold increase in cyclin A promoter activity. We next investigated the effect of Ras (Val-12) on endogenous cyclin A expression. Whereas cyclin A was not expressed in nonadherent cells transfected with the empty vector (pDCR) following exposure to serum, introduction of Ras (Val-12) resulted in the accumulation of endogenous cyclin A protein (Fig. 3B). As a consequence, cells entered S phase as monitored by BrdUrd staining (Fig. 3C) and progressed through cell cycle as observed using fluorescence-activated cell sorter analysis (data not shown). We next examined whether Cdc42Hs and Ras stimulate cyclin A expression through activation of the same signaling cascade. The dominant negative Asn-17 mutant form of Cdc42Hs did not block Ras (Val-12) induced cyclin A promoter activity in nonadherent MEF (Fig. 4A). Importantly, the dominant negative Asn-17 Rac mutant inhibited the Ras effect by 50% but did not alter the Cdc42Hs response (Fig. 4A). The latter result is consistent with previous data suggesting that pathways downstream of Rac1 are required for full Ras signaling (18–21). To address whether Cdc42Hs mediates Ras-induced cyclin A transcription in nonadherent MEF, we used the Cdc42Hs-interacting domain of the Wiskott-Aldrich syndrome protein (WASP), a specific effector

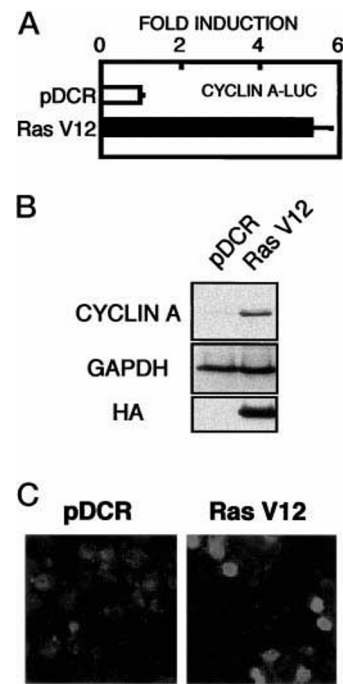


FIG. 3. An activated Ras (Val-12) can alleviate anchorage-requirement for cyclin A expression in MEF. Cells were transiently transfected while adherent with the indicated vectors and then replated on agar-coated dishes for 24 h. *A*, co-transfections were carried out with the mutant Ras-expressing vector together with a cyclin A promoter-driven luciferase reporter. Luciferase activity was normalized as described in the legend to Fig. 1. *B*, expression of cyclin A together with the HA-tagged Ras mutant was monitored by Western blotting on total cellular extracts performed successively with anti-cyclin A and anti-HA monoclonals. Normalization was carried out with a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) rabbit polyclonal antibody. pDCR indicates cells transfected with the empty vector. *C*, cells entering S phase were detected after BrdUrd incorporation as indicated in Fig. 2.

for Cdc42Hs involved in actin polymerization (27, 28). This fragment inhibits Cdc42Hs activity through competition with its effector binding site. Cdc42Hs, but not Ras (Val-12), induced cyclin A transcription was strongly inhibited upon expression of the WASP fragment (Fig. 4A). These results suggest that Ras and Cdc42Hs govern different pathways leading to adhesion-independent cyclin A induction. To determine whether Ras and Cdc42Hs activate co-operative pathways, we concomitantly expressed activated forms of Ras and Cdc42Hs, in nonadherent MEF cells. Separately, Ras (Val-12) and Cdc42Hs (Leu-61) activated cyclin A reporter activity to approximately the same extent (5-fold), whereas their co-expression affected cyclin A in a synergistic manner (15-fold) (Fig. 4B). In conclusion, these data suggest that Cdc42Hs and Ras cooperate to control anchorage-independent proliferation of MEF through parallel pathways.

JNK Is Not Required for Cdc42-mediated Anchorage-independent Proliferation of MEF—Activation of Cdc42Hs and Rac1 in adherent cells has previously been reported to activate the JNK and p38 stress kinases without any effect on the ERK pathway (12, 13, 29). We therefore assessed whether adhesion-independent cyclin A induction required the activation of any of these MAP kinases. MEF were transiently co-transfected with different combinations of HA-tagged versions of ERK2, p38, or JNK1 together with Ras (Val-12), Cdc42Hs (Leu-61), or Rac1 (Leu-61). As shown in Fig. 5A, Ras (Val-12) activated ERK and, to a much lesser extent, JNK in adherent cells. P38 activity was not induced by Ras (Val-12). In contrast, both Cdc42Hs (Leu-61) and Rac1 (Leu-61) had negligible effects on ERK activity whereas they activated p38 and JNK kinases. When the same

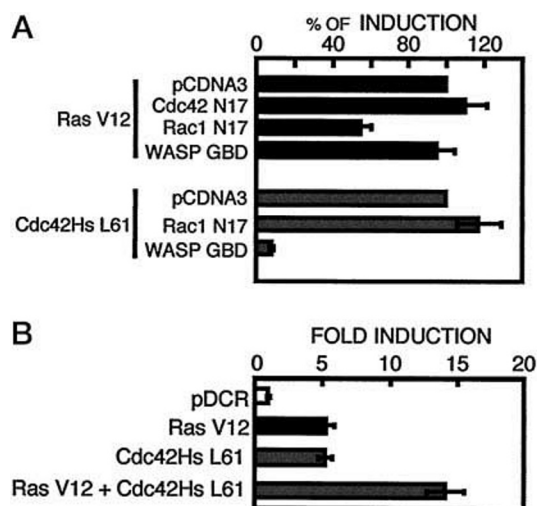


FIG. 4. Cdc42Hs (Leu-61) and Ras (Val-12) delineate two different pathways that cooperate to induce cyclin A transcription in nonadherent MEF. Fibroblasts were transiently transfected by the indicated combinations of expression plasmids with a cyclin A luciferase reporter, as described in the other figures. *A*, Ras (Val-12) or Cdc42Hs (Leu-61) were expressed in conjunction with either the empty pCDNA3 vector or the negative dominant forms of Cdc42, Rac1, or the WASP GTPase-binding domain (*WASP GBD*). In each case the measured luciferase activity is represented relative to that obtained with the empty vector. *B*, Cdc42Hs (Leu-61) was expressed alone or together with either Ras (Val-12). Activation of the cyclin A reporter is indicated relative to that obtained with the empty vectors (pDCR or pCDNA3).

experiment was carried out on nonadherent cells, Ras (Val-12) still activated ERK, albeit to a lower extent and had negligible effects on JNK. Interestingly, in a nonadherent context, the basal level of p38 activity was inhibited by Ras (Val-12) (Fig. 5*B*) and Cdc42Hs (Leu-61)- or Rac1 (Leu-61)-induced ERK and JNK activities were equivalent in adherent and nonadherent cells. Importantly, the Cdc42Hs-induced activation of p38 was significantly decreased in nonadherent cells as compared with adherent MEF (Fig. 5*A*).

In an attempt to identify the effector(s) involved, we used previously described Cdc42Hs and Rac1 variants, mutated in their effector domains, which are impaired in binding to specific effectors (30). Based on the work carried out on Ras, single amino acid substitutions were introduced within the effector loops of the constitutively active Cdc42Hs (Leu-61) and Rac1 (Leu-61) (30). The resulting Cdc42Hs (Q61L/Y40C) and Rac1 (Q61L/Y40C) mutants no longer interact with some Cdc42Hs or Rac1-interactive binding motif-containing proteins such as the Ser/Thr protein kinase p65^{PAK} or WASP (30). In contrast, the F37A substitution in Cdc42Hs (Q61L/F37A) and Rac1 (Q61L/F37A) does not affect the interactions with Cdc42Hs or Rac1-interactive binding domain-containing proteins, but Cdc42Hs (Q61L/F37A) lacks the ability to activate Rac1 (30). The experiments described above were repeated with these two Cdc42Hs (Leu-61) effector loop mutants. As expected, the Cdc42Hs (Q61L/Y40C) mutant did not induce JNK while the Cdc42Hs (Q61L/F37A) mutant weakly, but reproducibly, activated JNK (Fig. 6*A*). Neither form induced p38 activity in nonadherent cells (Fig. 6*B*). Importantly, the Cdc42Hs (Q61L/F37A) mutant was almost as potent as Ras (Val-12) or Cdc42Hs (Leu-61) in inducing cyclin A promoter activity after transient expression in nonadherent cells (Fig. 6*C*). Moreover, Cdc42Hs (Q61L/F37A) cooperated with Ras (Val-12) to the same level as Cdc42Hs (Leu-61).

One interpretation of these results is that Cdc42Hs induced JNK activity is required for cell cycle progression. To test this hypothesis, the dominant negative mutant of JNK (APF-JNK)

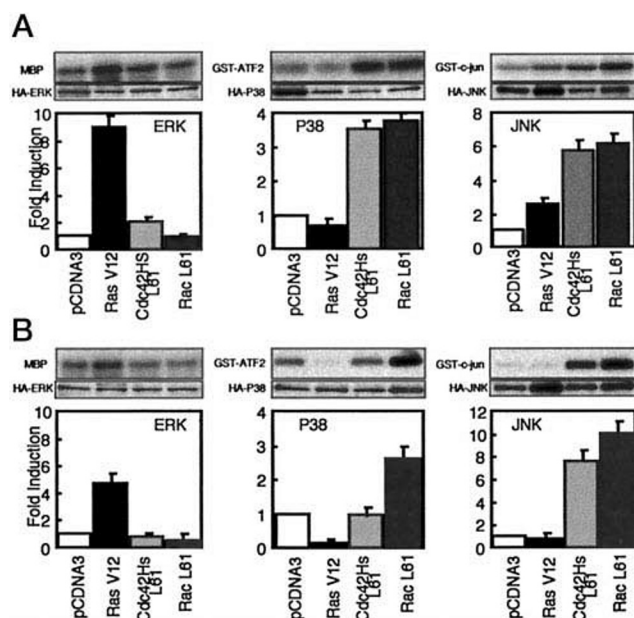


FIG. 5. Ras (Val-12), Cdc42Hs (Leu-61), and Rac1 (Leu-61) differentially activate the MAP kinases ERK2, p38, and JNK1. Cells were transiently co-transfected as indicated in the legend to Fig. 1 with the indicated activated forms of Ras, Cdc42, or Rac1, in the presence of vectors expressing the various HA-tagged MAP kinases. Immunoprecipitated ERK2, p38, and JNK1 activities from either adherent (*A*) or nonadherent (*B*) MEF were determined in the presence of [γ -³²P]ATP and, respectively, MBP, GST-ATF2, and GST-c-Jun recombinant proteins as substrates. Radioactive proteins were separated by electrophoresis through SDS-polyacrylamide gels and signal quantified with a PhosphorImager. Representative images are shown (*upper panels*) together with Western blots carried out on total extracts with anti-HA monoclonal antibodies and destined to correct for variations in the transfection efficiencies (*lower panels*). The fold induction is calculated as the ratio of the intensities of signals in the corresponding *upper* and *lower panels*.

was expressed in nonadherent cells. Although this dominant negative mutant abolished Cdc42Hs (Leu-61)-induced JNK activity (Fig. 7*A*), it did not inhibit Cdc42Hs-induced cyclin A expression (Fig. 7*B*). These data clearly demonstrate that JNK is not involved in Cdc42Hs-induced anchorage-independent proliferation.

Anchorage-independent Proliferation of MEF Is Associated with the Down-regulation of p38 Kinase Activity—Whereas Rac1 (Leu-61) was as potent as Cdc42Hs (Leu-61) in activating JNK, it did not induce cyclin A expression in nonadherent MEF. Interestingly, in contrast with Rac1 (Leu-61), Cdc42Hs did not activate p38 kinase in nonadherent cells (Fig. 5). Moreover, Ras (Val-12), which induced cyclin A expression in nonadherent MEF, strongly down-regulated p38 activity (Fig. 5). We therefore investigated whether the absence of p38 kinase activation was necessary for cyclin A expression in nonadherent MEF, and in particular, whether the ability of Rac1 to activate the p38 pathway could explain its inability to promote cyclin A expression. To test this hypothesis, Rac1 (Leu-61) effector loop mutants were transiently co-transfected into nonadherent MEF expressing HA-tagged versions of p38 or JNK. Rac1 (Q61L/F37A) activated both JNK and p38 kinases, while Rac1 (Q61L/Y40C) had no effect on JNK, and a reduced effect on p38 (Fig. 8, *A* and *B*). We next expressed these mutants with the cyclin A-luciferase reporter in nonadherent MEF. As previously observed for Rac1 (Leu-61), neither Rac1 (Q61L/F37A) nor Rac1 (Q61L/Y40C) were able to activate cyclin A promoter activity (Figs. 1*A* and 8*C*). However, addition of SB203580, a specific inhibitor of the p38 pathway (31, 32), resulted in strong cyclin A promoter activity in nonadherent Rac1 (Leu-61)-ex-

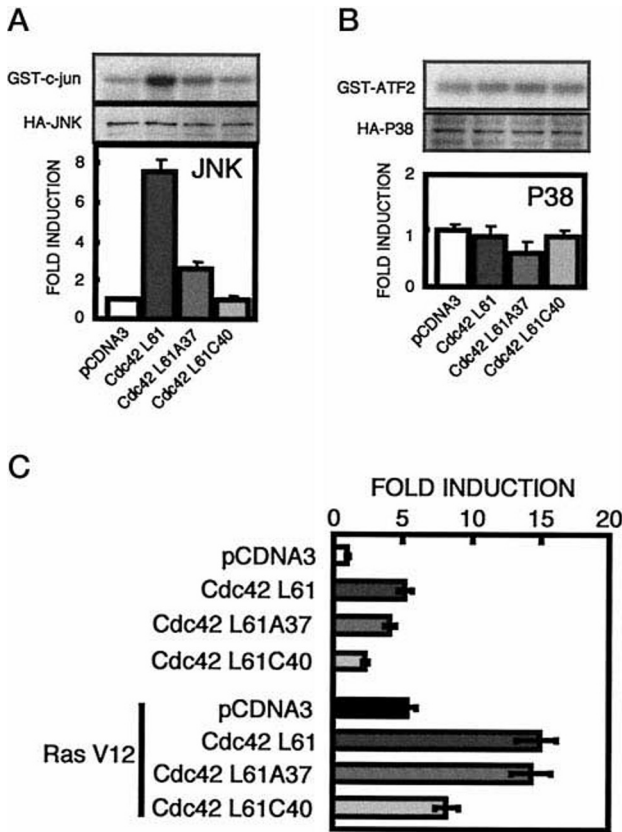


FIG. 6. An effector interacting with Cdc42Hs (Q61L/F37A) but not with Cdc42Hs (Q61L/Y40C) is required for Cdc42-induced cyclin A expression in nonadherent MEF. Cells were transiently co-transfected as indicated in Fig. 1 with the indicated effector loop mutants of Cdc42, in the presence of vectors expressing HA-tagged JNK1 and p38 kinases. Immunoprecipitated JNK1 (A) or p38 (B) kinases activities from nonadherent MEF were determined as described. Legends are as described in the legend to Fig. 5. C, the indicated effector loop mutants of Cdc42 were expressed alone or together with Ras (Val-12), in the presence of pCyc A-luc. Luciferase activity is represented relative to that obtained with the corresponding empty vector (pCDNA3).

pressing cells (Fig. 8C). Indeed, cyclin A expression was as high as that observed in the presence of Cdc42Hs (Leu-61). Similarly, the Rac1 double mutant (Rac1 (Q61L/F37A)) impaired in inducing actin polymerization and focal complex formation (30), activated cyclin A transcription upon inhibition of the p38 pathway (Fig. 8C). In contrast, the Rac1 (Q61L/Y40C) mutant did not activate cyclin A transcription under these conditions (Fig. 8C). This strongly suggests that inactivation of p38 kinase activity is required for the suppression of the block to proliferation as measured by activation of cyclin A transcription. This hypothesis is supported by experiments with a dominant negative form of p38 (YFp38), which led to an activation of cyclin A promoter activity when expressed in conjunction with Rac1 (Leu-61) (Fig. 8C).

In conclusion, inactivation of p38 pathway is required, but not sufficient, for the proliferation of nonadherent MEF. Therefore, anchorage-independent MEF proliferation might result from a dynamic balance involving inactivation of p38 kinase activity and activation of an effector distinct from JNK which interacts with Cdc42Hs.

DISCUSSION

Fibroblasts require both mitogens and integrin-mediated attachment for growth (26, 33, 34). When deprived of anchorage, many adherent cell lines fail to undergo several G₁-specific cell cycle-related transitions, even in the presence of growth factors

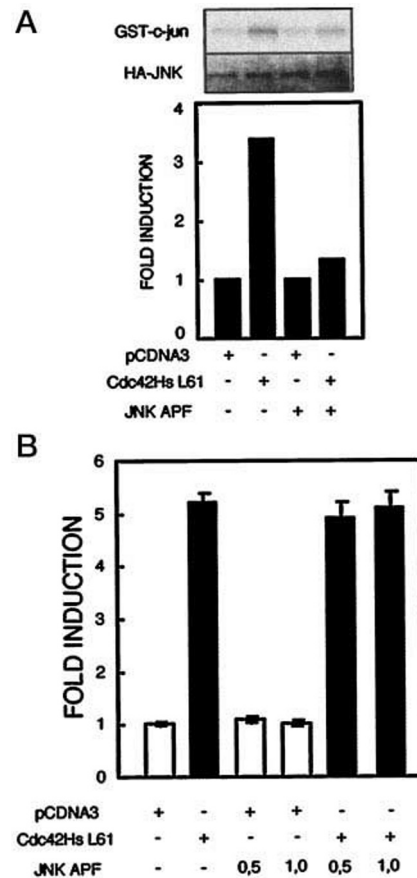


FIG. 7. JNK is not required for anchorage-independent expression of cyclin A in MEF. A, cells were transiently co-transfected as indicated in the previous figures with a vector expressing HA-tagged JNK1 in the presence of Cdc42Hs (Leu-61) and/or a negative dominant form of JNK (JNK-APF). Immunoprecipitated JNK1 kinase activity from nonadherent MEF was determined with GST-c-JUN as a substrate. Legends are as described in the legend to Fig. 5. B, Cdc42Hs (Leu-61) was expressed either alone or together with JNK-APF, in the presence of pCyc A-luc. Luciferase activity from nonadherent cells is represented relative to that obtained with the corresponding empty vector (pCDNA3).

(1, 35–39). However, no such studies have been carried out in primary cells. Stable expression of activated Ras or Cdc42Hs in rodent cell lines has been shown to overcome anchorage-dependent requirements to various extents. However, expression of a mutated Ras is known to induce a wide variety of responses including both gene expression and alterations in cellular morphology. Moreover, it has become clear that the great biological diversity observed within existing cell lines can at least in part be accounted for by the large array of integrin combinations which are expressed at the cell surface. As a result, many cell type-specific differences exist between normal and transformed cells, as well as between cell lines whose proliferation is strictly anchorage-dependent. The differences observed in established cell lines are probably the result of adaptive events that have occurred during cell culture. Thus, the use of primary cells as opposed to immortalized cell lines is particularly important for the investigation of pathways controlling anchorage-independent cell proliferation. Moreover, the use of different experimental conditions to study the links between anchorage and proliferation has also led to many apparent discrepancies. Specifically, growth on soft agar is different from growth in liquid suspension. Some mutants, selected for their ability to form colonies in soft agar are unable to proliferate in liquid suspension (24). In order to overcome these difficulties, we studied the requirements for anchorage-independent proliferation of pri-

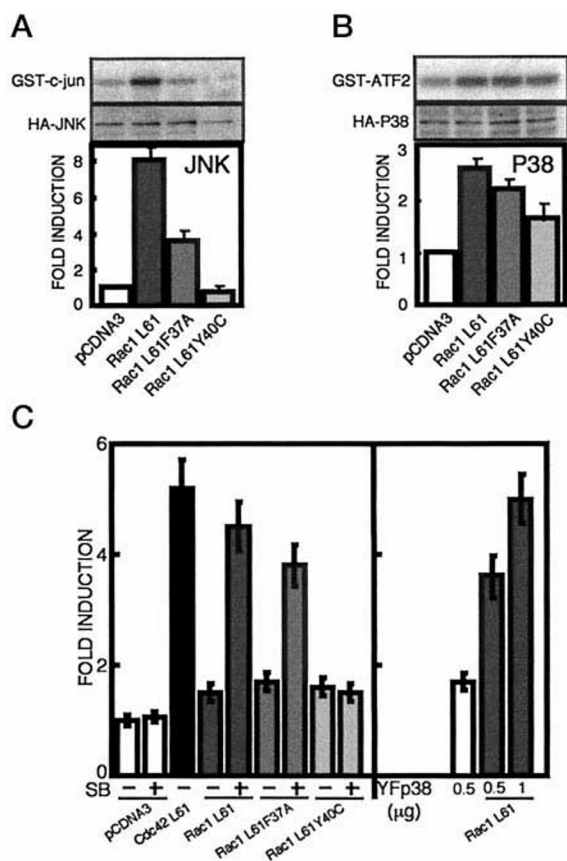


FIG. 8. Down-regulation of p38 kinase activity is required for Rac-induced cyclin A expression in nonadherent MEF. Cells were transiently co-transfected as indicated in the legend to Fig. 1 with the indicated effector loop mutants of Rac1, in the presence of vectors expressing HA-tagged JNK1 and p38 kinases. Immunoprecipitated JNK1 (A) or p38 (B) kinase activities from nonadherent MEF were determined as described. Legends are as described in the legend to Fig. 5. C, the indicated mutants of Rac1 were expressed either alone (C) or with the negative dominant YFp38 kinase (C), in the presence of pCyc A-luc. In the former case, cells were treated with SB203580 (+). Luciferase activity is represented relative to that obtained with the empty pCDNA3 vector.

mary mouse embryo fibroblasts grown in suspension following transient ectopic expression of activated Ras-related small GTPases. Mutants of Ras, Rac1, and Cdc42Hs were used in order to determine the individual contributions of these proteins in activating the cell cycle machinery. Finally, because cyclin A down-regulation is observed in all suspended cells (1, 38), we used cyclin A promoter activation as an index of the ability of a cell to resume cell cycle progression following anchorage deprivation.

The stable transfection of an activated Cdc42Hs has previously been shown to enable Rat 1 fibroblasts to grow in soft agar and proliferate in nude mice (23). Interestingly, coexpression of a dominant negative Cdc42 (Asn-17) inhibited Ras-mediated focus formation, growth in soft agar, and reversion of the transformed phenotype, indicating that Cdc42Hs is necessary for Ras transformation. Following transient transfection in the absence of cell adhesion, Cdc42Hs (Val-12), but not Rac1 (Val-12), RhoA (Val-14), or RhoG (Val-12), activated cyclin A promoter to the same extent as Ras (Val-12). Accordingly, transfected cells entered S phase as measured by BrdUrd labeling. We next addressed the possibility that Cdc42Hs could cooperate with Ras mutant in leading to cellular proliferation in a fully anchorage-independent context. A strong synergy was observed when either of two activated forms of Cdc42, Cdc42Hs (Val-12) or Cdc42Hs (Leu-61) were co-transfected with Ras

(Val-12). Importantly, a Cdc42 effector loop mutant (Cdc42Hs (Q61L/F37A)), which is able to interact with Cdc42Hs or Rac-interactive binding motif-containing proteins, synergizes with the Ras mutant. The importance of Cdc42 in this synergy was further demonstrated by an almost complete inhibition of cyclin A promoter activity following coexpression of activated Cdc42 with WASP-GBD. In contrast, neither the dominant negative form Cdc42 (Asn-17) nor WASP-GBD inhibited Ras-induced anchorage independent transcription of cyclin A, suggesting that Ras activates anchorage-independent cell proliferation through a Cdc42Hs-independent pathway.

Although expression of the constitutively active Rac1 was clearly not sufficient to induce anchorage-independent transcription of cyclin A, and thus to proliferation, the ability of the dominant negative Asn-17 form of Rac1 to partially inhibit Ras-induced cyclin A expression places it downstream of Ras in this pathway. Conversely, the inability of the Rac1 (Asn-17) to inhibit Cdc42-induced transcription of cyclin A indicates that the role of Cdc42 in transformation appears to be largely independent of Rac (18, 20). Furthermore, Rac and Cdc42Hs have been shown to delineate distinct pathways that cooperate to transform NIH 3T3 cells (2). Consistent with this, the Cdc42Hs double mutant Cdc42Hs (Q61L/F37A), which is unable to activate Rac (30), was still able to induce cyclin A transcription and cooperate with Ras. These observations suggest that the hierarchy established between Ras, Cdc42, and Rac in the control of cell morphology (40, 41) is not necessarily valid for other processes. Altogether, we show that pathways downstream of Rac1 are required for full Ras signaling, but are not required for Cdc42Hs signaling, in controlling cell cycle progression of MEF cultured under anchorage-free conditions.

Both Cdc42Hs and Rac (but not Rho) can activate the JNK/SAPK and the p38/HOG MAP kinases cascades in adherent cells (12, 13), thereby affecting gene transcription. JNK phosphorylates c-Jun at serines 63 and 73, and this transcription factor is implicated in G_1 progression by inducing cyclin D1 expression (42). Nevertheless, a recent study showed that phosphorylation of serines 63 and 73 of c-Jun was required for protection against UV-induced apoptosis but not for cell proliferation (42). Accordingly, JNK activity is not detected in cells growing on a solid support, and in our work cyclin D1 is normally induced and present in nonadherent MEF. Consistent with this, we show here that a dominant negative form of JNK was unable to inhibit Cdc42Hs-induced cyclin A transcription in MEF cultured under anchorage free conditions. In contrast, p38 MAPK activity has been proposed to participate in the control of the G_1/S transition of some cells (22, 43). Whereas it has been previously shown that Cdc42Hs inhibits proliferation of adherent NIH3T3 cells (22), we show here that it leads to the opposite effect in nonadherent MEFs. Interestingly, however, modulation of p38 kinase activity seems to be instrumental in both situations. In adherent NIH 3T3 cells, inhibition of proliferation is associated with an activation of p38 activity. In contrast, under anchorage-free conditions, we found that Cdc42Hs-mediated cyclin A induction and progression of MEF through G_1/S was associated with impaired p38 activity. Moreover, inhibition of p38 activity allowed Rac1-induced cyclin A transcription. These results indicate that Rac1 and Cdc42Hs use distinct pathways to activate p38 kinase. It is clear that inhibition of p38 kinase activity is not sufficient to trigger proliferation, and additional signals modulated by Cdc42Hs or Rac1 are necessary. It has been postulated that these events require actin polymerization and focal complex formation. However, in our study a Rac1 double mutant (Rac1 (Q61L/F37A)), unable to induce either membrane ruffling or the formation of focal complexes (30), was nevertheless able to induce

G₁ progression when p38 kinase activity was inhibited.

In summary, this study indicates a novel role for Cdc42 in the control of anchorage-independent cell cycle progression. Under these conditions, Cdc42 activity results in a down-regulation of p38 MAPK activity and the Cdc42 effector domain is redirected from JNK to another downstream effector. These findings suggest that unravelling the Cdc42 signaling cascade will shed some light on the mechanisms by which a cell integrates peripheral cues into a coordinated proliferation program.

Acknowledgments—M. White, Ph. Chavrier, A. Hall, C. Sardet, B. Dérijard, and A. Brunet are thanked for their gifts of plasmids. We thank Ph. Fort for helpful discussions and N. Taylor for carefully reading the manuscript.

REFERENCES

- Philips, A., Huet, X., Plet, A., Rech, J., Vie, A., and Blanchard, J. M. (1999) *Oncogene* **18**, 1819–1825
- Roux, P., Gauthier-Rouviere, C., Doucet-Brutin, S., and Fort, P. (1997) *Curr. Biol.* **7**, 629–637
- Hall, A. (1998) *Science* **279**, 509–514
- Boguski, M. S., and McCormick, F. (1993) *Nature* **366**, 643–654
- Lamarque, N., and Hall, A. (1994) *Trends Genet.* **10**, 436–440
- Van Aelst, L., and D'Souza-Schorey, C. (1997) *Genes Dev.* **11**, 2295–2322
- Whitehead, I. P., Campbell, S., Rossman, K. L., and Der, C. J. (1997) *Biochim. Biophys. Acta* **1332**, F1–23
- Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) *Cell* **70**, 401–410
- Ridley, A. J., and Hall, A. (1992) *Cell* **70**, 389–399
- Nobes, C. D., and Hall, A. (1995) *Biochem. Soc. Trans.* **23**, 456–459
- Nobes, C. D., Hawkins, P., Stephens, L., and Hall, A. (1995) *J. Cell Sci.* **108**, 225–233
- Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) *Cell* **81**, 1137–1146
- Minden, A., Lin, A., Claret, F. X., Abo, A., and Karin, M. (1995) *Cell* **81**, 1147–1157
- Olson, M. F., Ashworth, A., and Hall, A. (1995) *Science* **269**, 1270–1272
- Symons, M. (1995) *Curr. Opin. Biotechnol.* **6**, 668–674
- Hill, C. S., Wynne, J., and Treisman, R. (1995) *Cell* **81**, 1159–1170
- O'Hagan, R. C., Tozer, R. G., Symons, M., McCormick, F., and Hassell, J. A. (1996) *Oncogene* **13**, 1323–1333
- Khosravi-Far, R., Solski, P. A., Clark, G. J., Kinch, M. S., and Der, C. J. (1995) *Mol. Cell. Biol.* **15**, 6443–6453
- Prendergast, G. C., Khosravi-Far, R., Solski, P. A., Kurzawa, H., Lebowitz, P. F., and Der, C. J. (1995) *Oncogene* **10**, 2289–2296
- Qiu, R. G., Chen, J., Kirn, D., McCormick, F., and Symons, M. (1995) *Nature* **374**, 457–459
- Qiu, R. G., Chen, J., McCormick, F., and Symons, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 11781–11785
- Molnar, A., Theodoras, A. M., Zon, L. I., and Kyriakis, J. M. (1997) *J. Biol. Chem.* **272**, 13229–13235
- Qiu, R. G., Abo, A., McCormick, F., and Symons, M. (1997) *Mol. Cell. Biol.* **17**, 3449–3458
- Le Gall, M., Grall, D., Chambard, J. C., Pouyssegur, J., and Van Obberghen-Schilling, E. (1998) *Oncogene* **17**, 1271–1277
- Guadagno, T. M., and Assoian, R. K. (1991) *J. Cell Biol.* **115**, 1419–1425
- Tapon, N., and Hall, A. (1997) *Curr. Opin. Cell Biol.* **9**, 86–92
- Aspenstrom, P. (1999) *Exp. Cell Res.* **246**, 20–25
- Symons, M., Derry, J. M., Karlak, B., Jiang, S., Lemahieu, V., McCormick, F., Francke, U., and Abo, A. (1996) *Cell* **84**, 723–734
- Frost, J. A., Xu, S., Hutchison, M. R., Marcus, S., and Cobb, M. H. (1996) *Mol. Cell. Biol.* **16**, 3707–3713
- Lamarque, N., Tapon, N., Stowers, L., Burbelo, P. D., Aspenstrom, P., Bridges, T., Chant, J., and Hall, A. (1996) *Cell* **87**, 519–529
- Kumar, S., McDonnell, P. C., Gum, R. J., Hand, A. T., Lee, J. C., and Young, P. R. (1997) *Biochem. Biophys. Res. Commun.* **235**, 533–538
- Young, P. R., McLaughlin, M. M., Kumar, S., Kassiss, S., Doyle, M. L., McNulty, D., Gallagher, T. F., Fisher, S., McDonnell, P. C., Carr, S. A., Huddleston, M. J., Seibel, G., Porter, T. G., Livi, G. P., Adams, J. L., and Lee, J. C. (1997) *J. Biol. Chem.* **272**, 12116–12121
- Assoian, R. K., and Zhu, X. (1997) *Curr. Opin. Cell Biol.* **9**, 93–98
- Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J., and Parise, L. V. (1997) *Nature* **390**, 632–636
- Guadagno, T. M., Ohtsubo, M., Roberts, J. M., and Assoian, R. K. (1993) *Science* **262**, 1572–1575
- Bohmer, R. M., Scharf, E., and Assoian, R. K. (1996) *Mol. Biol. Cell* **7**, 101–111
- Zhu, X., Ohtsubo, M., Bohmer, R. M., Roberts, J. M., and Assoian, R. K. (1996) *J. Cell Biol.* **133**, 391–403
- Schulze, A., Zerfass-Thome, K., Berges, J., Middendorp, S., Jansen-Durr, P., and Henglein, B. (1996) *Mol. Cell. Biol.* **16**, 4632–4638
- Fang, F., Orend, G., Watanabe, N., Hunter, T., and Ruoslahti, E. (1996) *Science* **271**, 499–502
- Allen, W. E., Jones, G. E., Pollard, J. W., and Ridley, A. J. (1997) *J. Cell Sci.* **110**, 707–720
- Nobes, C. D., and Hall, A. (1995) *Cell* **81**, 53–62
- Wisdom, R., Johnson, R. S., and Moore, C. (1999) *EMBO J.* **18**, 188–197
- Wang, S., Nath, N., Minden, A., and Chellappan, S. (1999) *EMBO J.* **18**, 1559–1570