Cdc42Hs, but Not Rac1, Inhibits Serum-stimulated Cell Cycle Progression at G1/S through a Mechanism Requiring p38/RK*

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Antimitogenic stimuli such as environmental or genotoxic stress, transforming growth factor-β, and the inflammatory cytokines tumor necrosis factor and interleukin-1 activate two extracellular signal-regulated kinase (ERK)-based signaling pathways: the stress-activated protein kinase (SAPK/JNK) pathway and the p38 pathway. Activated p38 phosphorylates transcription factors important in the regulation of cell growth and apoptosis, including activating transcription factor 2 (ATF2), Max, cAMP response element-binding protein-homologous protein/growth arrest DNA damage 153 (CHDP/GADD153). In turn, p38 lies downstream of the Rho family GTTPases Cdc42Hs and Rac1, as well as at least three mitogen-activated protein kinase (MAPK)/ERK-kinases (MEKs): MAPK kinases-3, -6, and SAPK/ERK-kinase-1. Although many of the stimuli that activate p38 can also inhibit cell cycle progression, a clear-cut role for the p38 pathway in cell cycle regulation has not been established. Using a quantitative microinjection approach, we show here that Cdc42Hs, but not Rac1 or RhoA, can inhibit cell cycle progression at G1/S through a mechanism requiring activation of p38. These results suggest a novel role for Cdc42Hs in cell cycle inhibition. Furthermore, these results suggest that although both Cdc42Hs and Rac1 can activate p38 in situ, the effects of Cdc42Hs and Rac1 on cell cycle progression are, in fact, quite distinct.

Protein kinase signal transduction pathways that employ members of the extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK) family have been remarkably conserved in evolution (1–3). Typically these pathways consist of a three-tiered core of protein kinases wherein MAPK-kinase-kinases (MAPKKKs) activate MAPK/ERK kinases (MEKs), which, in turn, activate ERKs (1–3). In simple eukaryotes, these pathways are activated by a variety of stresses, including nutrient withdrawal and osmotic shock (3). In mammals and other multicellular eukaryotes, ERK/MAPK pathways are activated by both environmental stresses and more complex physiologic stimuli such as mitogens, developmental cues, neurotransmitters, vasoactive peptides, and inflammatory cytokines (1, 2, 4).

The stress-activated protein kinases (SAPKs, also referred to as JNKs) and p38 are the ERK components in two mammalian signaling pathways activated by a broad array of environmental stresses as well as by inflammatory cytokines (1, 2). Once activated, the SAPKs and p38 are responsible for the phosphorylation and activation of transcription factors necessary for the stress response including c-Jun, ATF2, Max, and CHOP (1, 2, 5).

The signaling components upstream of the SAPKs and p38 which have been identified thus far suggest a complex cell- and stimulus-dependent regulation consistent with the diversity of extracellular stimuli that activate these pathways. Several chromatographically distinct MEKs, including SEK1/MKK4, SAPK-kinase (SAPKK4), and SAPPK5 can activate the SAPKs in situ and in vitro. (6–8). SAPK4 and SAPKK5 are specific for the SAPKs, whereas SEK1 can also activate p38. By contrast, MKK3 and MKK6 are specific p38 activators (8–10).

Four families of MAPKKKs have also been implicated in SAPK and p38 regulation. MEK kinases (MEKs) 1 and 2, as well as the mixed lineage kinase SH3 domain-containing, proline-rich kinase (SPRK), can activate the SAPK pathway via direct activation of SEK1 (11–13). MEK2 can also activate the mitogenic MAPK/ERK1 and 2 pathway by directly phosphorylating the ERK1/2 upstream activator MEK1 (11). Tpl-2 the rat homolog of the human cot proto-oncoprotein product, like MEKK2, can activate both SEK1 and MEK1 (14). MEKK3 can activate both the SAPK and MAPK pathways, although SEK1 is not a MEKK3 substrate (11). In addition, transforming growth factor-β-activated kinase-1 (TAK1) can activate p38 in situ and has been implicated as a transforming growth factor-β-activated MAPKKK upstream of SEK1, MKK3, and MKK6 (15, 16). Finally, the p21-activated kinases (PAKs) and germinal center kinase (GCK), mammalian homologs of Saccharomyces cerevisiae STE20 and SPS1, respectively, can activate the SAPK and p38 pathways, although their mechanisms of action are unclear (17–20).

Genetic epistasis studies in yeast as well as biochemical and transfection experiments employing mammalian cells have identified members of the Ras superfamily as upstream elements regulating the core protein kinases in ERK signaling cascades (1, 2, 4, 19, 21–23). Thus, Ras is a critical upstream regulator of the Raf1 kinase, a MAPKKK in the MAPK pathway (4). Similarly, in mammalian cells, the Rho family...
GTPases Rac1 and Cdc42Hs can activate the p38 and SAPK pathways. This activation may involve the direct binding of Rac1 and Cdc42Hs to the PAKs. This binding results in PAK activation (18–20, 22–24).

A complete picture of the biological functions of these stress-activated pathways is only beginning to emerge. Recent data have implicated the SAPK and p38 pathways in the induction of apoptosis in response to nerve growth factor withdrawal in PC-12 cells, as well as heat shock, genotoxic chemotherapeutics (cis-platinum), and signaling pathways, which stimulate the generation of ceramide from membrane sphingomyelin (25–27). However, induction of apoptosis may not be the only biological consequence of activation of the SAPKs and p38. Moreover, the specific biological roles of the SAPK and p38 pathways in signaling mediated by Rac1 and Cdc42Hs have not been elucidated.

In this study, we used quantitative microinjection to begin to characterize the biological consequences of Cdc42Hs and Rac1 activation of p38. Here we report that Cdc42Hs is a potent inhibitor of cell cycle progression, arresting cells at the G1/S transition point. In addition, we demonstrate that this inhibition is mediated by elements of the p38 pathway. Rac1 and the SAPKs, although able to induce characteristic cellular and biochemical responses, do not inhibit cell cycle progression significantly.

EXPERIMENTAL PROCEDURES

Microinjection—NIH-3T3 cells were grown on glass coverslips and synchronized in G0 by serum withdrawal for 24 h. After this period, less than 1% of the cells incorporated bromodeoxyuridine (BrDUrd) during an additional 24 h of incubation. The arrested cells were released from serum starvation by readdition of 10% calf serum and were microinjected in early G1 with expression vectors harboring the HA-tagged or FLAG-tagged cDNAs, using a Zeiss automated microinjection system (28). As a control, cells were microinjected with the empty vector together with an inert rabbit antibody marker to identify the injected cells. S phase entry was monitored by BrDUrd (100 μM) incorporation into cellular DNA (28, 29). Cells were fixed at the indicated time after release from serum starvation as described. Cells stained for phospho-p38 or phospho-c-Jun were fixed with 4% paraformaldehyde according to the manufacturer’s suggestions. Injected DNA concentrations were: 100 ng/μl for the manufacturer’s suggestions. Injected DNA concentrations were: 100 ng/μl for the empty vector. Entry into S phase was monitored by incorporation of BrDUrd into DNA. After 24 h, expression of the relevant constructs was verified by immunostaining, and the expressing cells were scored blindly for S phase entry. Cells injected with the empty vector were identified by staining for coinjected rabbit IgG. Cells were also counterstained with bisbenzimide (Hoechst 33258) to visualize all nuclei and assess any abnormal nuclear morphology. Fig. 1 shows representative photomicrographs of the stained cells.

Expression of p38 resulted in a striking 77.5% inhibition of S phase entry compared with cells injected with empty plasmid (Fig. 2a). Similar results were obtained using mink lung Mv1Lu cells (data not shown). The inhibition of S phase entry by p38 is likely due to the presence of increased amounts of active p38 in the microinjected cells. Staining of the injected cells with an antibody specific for the active, phosphorylated form of p38 (1, 2) revealed that the p38-expressing cells contained greater amounts of phosphorylated p38 than did the uninjected cells (Fig. 2b). The ability of p38 to arrest cells in G1 was specific, with other signaling kinases having little or no effect on cell cycle progression. p44-MAPK is an integral component of the mitogenic pathway recruited by Ras, and constitutive activation of this pathway is sufficient to transform NIH-3T3 cells (4). Not surprisingly, expression of p44-MAPK modestly enhanced G1/S progression (91% of p44-MAPK-expressing cells in S phase versus 69% of empty plasmid-injected cells, Fig. 2a). p70 S6 kinase is also activated by mitogens through mechanisms involving phosphatidylinositol 3-kinase and FRAP/RAFT/TOR (32, 33). Inhibition of p70 S6 kinase blocks G1/S transition (34). Expression of p70 S6 kinase from microinjected plasmid had no significant effect on G1/S transition (Fig. 2a).

RESULTS

Effects of ERK Family Kinases on Cell Cycle Progression—Murine NIH-3T3 fibroblasts were rendered quiescent by serum withdrawal and synchronized in G0 by serum readduction. Cells were microinjected in early G1 with expression vectors encoding HA-tagged p38, SAPK-p46/1, p44-MAPK, p70 S6 kinase, or empty vector. Entry into S phase was monitored by incorporation of BrDUrd into DNA. After 24 h, expression of the relevant constructs was verified by immunostaining, and the expressing cells were scored blindly for S phase entry. Cells injected with the empty vector were identified by staining for coinjected rabbit IgG. Cells were also counterstained with bisbenzimide (Hoechst 33258) to visualize all nuclei and assess any abnormal nuclear morphology. Fig. 1 shows representative photomicrographs of the stained cells.

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SAPKs are preferentially activated by the same antimitogenic stimuli as is p38 (1, 2), to our surprise, however, expression of injected SAPK, which increased intracellular SAPK activity and, consequently, substantially elevated the level of phosphorylated c-Jun (a SAPK substrate) in the injected cells (Fig. 2b), resulted in only modest inhibition of S phase entry (55% in S phase; Fig. 2a). In
view of this result, we explored the effects on cell cycle progression of strong activation of the SAPKs.

GCK is a mammalian homolog of *S. cerevisiae* SPS1 which, when expressed transiently, is constitutively active (1, 2, 17, 35). Coexpression of GCK and SAPK results in potent SAPK activation in the absence of external stimuli. At low levels of expression, GCK activation of SAPK is specific, with no p38 or ERK1 activation seen (17). At high levels of expression, however, GCK can activate p38 modestly. Injection of GCK into NIH-3T3 cells resulted in inhibition of G1/S transition to a degree commensurate with that obtained upon injection of p38 alone (25% in S phase versus 67% in S phase for cells injected with empty vector, Fig. 3). If KR-MKK3, a kinase-dead dominant-inhibitory mutant of MKK3, which specifically blocks p38, but not SAPK activation (9 and see Fig. 4) is co-injected with GCK, this G1 arrest is abolished completely whether or not SAPK is co-injected with the GCK (Fig. 3). Thus, SAPK activation likely does not contribute to GCK-mediated cell cycle arrest.

**Effects of MEKs Upstream of p38 on Cell Cycle Progression**—To investigate further the role of activation of p38 in inhibition of G1/S transition, we asked if components known to lie upstream of p38 in stress signaling cascades could block S phase entry. MKK3, M KK6, and SEK1 are three mammalian MEKs that can activate p38 in *situ* and *in vitro* (9, 10). Although M KK3 and M KK6 are apparently specific for p38, SEK1 is also a strong SAPK activator (6, 9, 10). Expression of HA-tagged, wild-type SEK1, M KK3, or M KK6 from microinjected plasmids strongly inhibited S phase entry (14% in S phase for SEK1, 17% in S phase for M KK3, 15% in S phase for M KK6, 69% in S phase for empty plasmid). By contrast, expression of kinaseinactive mutant SEK1 or M KK3 plasmids, wherein the lysine residue critical for ATP binding has been mutated to arginine (KR mutants), was without significant effect (Fig. 4a).

Despite the lack of intrinsic activity, however, kinaseinactive SEK1 and M KK3 mutants can act as dominant inhibitors of coexpressed p38 activation and biological activity and can thus be used to assay the requirement for p38 activation for a particular biological function (6, 9, 10, 25–27). Accordingly, we tested whether activation of p38 was necessary for p38-mediated cell cycle arrest, we co-injected p38 with KR-MKK3 or KR-SEK1. Consistent with a requirement for active p38, both KR-SEK1 and KR-MKK3 completely inhibited the ability of p38 to block S phase entry (Fig. 4b) without preventing p38 expression (Fig. 4c).

**Effects of Rho Family GTPases on Cell Cycle Progression**—Cdc42Hs, a member of the Rho subfamily of small GTP-binding proteins, is a human homolog of *S. cerevisiae* CDC42 (36). Cdc42p activates Ste20p as part of the yeast mating pheromone signaling pathway. This pathway activates two ERKs, Fus3p and Kss1p, and culminates in G1 cell cycle arrest (3, 21). Likewise, GTP-charged Cdc42Hs or the related GTPase Rac1 can activate the mammalian STE20 homologs PAK1 or PAK3 in *situ* and *in vitro*; and recent evidence indicates that Cdc42Hs and Rac1, but not RhoA, can activate p38 (and the SAPKs) in *situ* via a mechanism requiring PAKs (18, 19, 22–24, 37). To investigate the potential effects of Rho family GTPases on cell cycle arrest, we microinjected expression plasmids encoding wild-type and mutant forms of Cdc42Hs, Rac1 or RhoA into synchronized NIH-3T3 cells. Expression of wt-Cdc42Hs resulted in a striking arrest of the cell cycle at G1/S, exceeding that seen for p38. Of the cells injected with empty plasmid, entry into S phase was apparent within 20 h after release from serum starvation; and 75% of the cells injected with empty plasmid had transitioned to S phase within 26 h. By contrast, less than 7% of the cells expressing wt-Cdc42Hs entered S phase within 30 h after serum readdition (Fig. 5a). This represents a dramatic 90.7% decrease in S phase entry (Fig. 5a and b). Expression of wt-Cdc42Hs from injected plasmid also elevated the level of activated, endogenous p38 phosphorylated at the
sites critical for activation, as determined by staining with an antibody specific for the activated form of p38 (Fig. 5c).

Coexpression of dominant interfering (KR) SEK1 and MKK3, although having no effect on Cdc42Hs expression (Fig. 5d), resulted in a partial reversal of the cell cycle inhibitory effects of Cdc42Hs with 37% of the cells progressing to S phase, 44% of control (Fig. 5b). These results suggest that p38 activation is a necessary component in the mechanism by which Cdc42Hs inhibits cell cycle progression.

To investigate further the role of Cdc42Hs in G1 arrest, we next sought to determine the effects of constitutively active and dominant interfering mutants of Cdc42Hs on cell cycle progression. V12-Cdc42Hs is a GTPase-defective mutant of Cdc42 which is constitutively active. Microinjection of V12-Cdc42Hs, although still strongly able to arrest cells in G1, is less effective than wild-type (Fig. 5b). We attribute this to the possible nonspecific activation of mitogenic pathways by the activated Cdc42Hs mutant. N17-Cdc42Hs is a dominant interfering mutant of Cdc42Hs which is thought to act by sequestering nucleotide exchangers for Cdc42Hs and other Rho family GTPases (38). Thus N17-Cdc42Hs can act either by blocking Cdc42Hs, Rac1 or RhoA activation (38). Consistent with this, microinjection of N17-Cdc42Hs does not activate endogenous p38, as detected by staining with antibodies to phospho-p38 (Fig. 5c).

However, to our surprise, N17-Cdc42Hs inhibited G1/S progression nearly one-third as effectively as wt-Cdc42Hs (Fig. 5b). This result supports the contention that the N17 mutant prevents activation of other Rho family GTPases, such as Rac1, which are required for cell growth.

It is conceivable that injection of wt-Cdc42Hs could nonspecifically recruit other Rho family GTPases that inhibit cell cycle progression. To investigate the specificity of cell cycle inhibition by Cdc42Hs, we assayed for cell cycle inhibition mediated by other members of the Rho family. To our surprise, injection of either a wild-type or constitutively active allele of Rac1 (V12-Rac1), either of which can activate p38 in situ (19), was far less effective at inhibition of G1/S transition than was wt-Cdc42Hs (50% in S phase for both Rac1 constructs versus 67% in S phase for cells injected with empty vector; Fig. 6a), even though injection of either wt- or V12-Rac1 was sufficient to activate p38 to an extent commensurate with that induced by Cdc42Hs, as judged by staining for activated endogenous p38 with anti-phospho-p38 antibodies (Fig. 6b). Moreover, injection of V12-Rac1 stimulated membrane ruffling, a characteristic response of the cell to activation of Rac1 (31) (Fig. 6c). This result is consistent with earlier observations indicating that Rac1 is in fact a necessary component for Ras transformation (39). Thus, although Rac and Cdc42Hs can both activate p38 in situ (18, 19, 23), each may also activate additional pathways that together result in distinct biological effects.

RhoA has not been shown to lie upstream of p38 or the SAPKs (22, 23). Injection of a constitutively active (V14) RhoA allele, although stimulating the characteristic formation of actin stress fibers (30) (Fig. 6c), yielded cell cycle results similar to those obtained upon injection of Rac1 (49% in S phase, Fig. 6a). Thus, among Rho family GTPases, cell cycle inhibition appears to be specific to Cdc42Hs.

**DISCUSSION**

p38 and the SAPKs are activated by an array of ligands that are known to be either antimitogenic or proapoptotic (1, 2). In contrast to the mitogenic pathways, which are regulated largely by polypeptide ligands coupled to tyrosine kinases, the stimuli that activate the stress pathways are remarkably diverse and include ionizing radiation, heat shock, chemical DNA damage, oxidative stress, reperfusion injury, and the inflammatory cytokines tumor necrosis factor-α and interleukin-1. Thus, the upstream molecular components that feed into the p38 and SAPK pathways are accordingly complex and diverse. The Rho family GTPases Cdc42Hs and Rac1 represent two
distinct mechanisms of p38 and SAPK activation with different biological functions.

We have demonstrated herein that activation of Cdc42Hs inhibits cell cycle progression at G1/S. Insofar as Cdc42Hs (and p38) inhibition of cell cycle progression can be at least partially reversed upon expression of kinase-dead MKK3 and SEK1, we conclude that recruitment of p38 is a critical component of Cdc42Hs-mediated cell cycle arrest. In addition, we have shown that p38 or elements immediately upstream of p38 (SEK1, MKK3, or MKK6) can also arrest cells in G1. By contrast, Rac1, which can also activate p38 (19, 23), fails to inhibit strongly cell cycle progression. Indeed, Rac1 has been implicated in Ras transformation (39) and may recruit additional signaling pathways that prevent inhibition of the cell cycle or promote cell cycle progression. Thus, although activation of p38 alone is sufficient to arrest cells in G1, we cannot rule out that p38, which can be activated weakly by mitogenic signals, could, in conjunction with other signaling pathways, lead to responses distinct from G1 arrest. These ideas are summarized in the model shown in Fig. 7.

Our results are in contrast with data implicating Rho family GTPases, notably Cdc42Hs, in cell growth and transformation (40). It is conceivable that the concomitant recruitment of Ras and several Rho family GTPase signaling pathways could result in cell growth, whereas activation of Cdc42Hs alone or in conjunction with other antimitogenic pathways results in cell cycle arrest. In this regard, care must be taken in the interpretation of results concerning Cdc42Hs mutants. Our results presented in Fig. 5 suggest that wt-Cdc42Hs is the most potent inhibitor of cell cycle progression, whereas a constitutively active mutant (V12-Cdc42Hs), although still growth inhibitory, is less so than wild-type. This result may reflect the ability of the overexpressed, constitutively active Cdc42Hs mutant to recruit nonspecifically mitogenic signaling mechanisms, possibly including those normally regulated by other Rho family GTPases, more effectively than the overexpressed wild-type Cdc42Hs. By the same token, overexpression of dominant negative Cdc42Hs, although unable to activate endogenous p38, was growth inhibitory, possibly because of nonspecific sequestering of the activation machinery for other Rho family GTPases. For example, Ost, a proto-oncogene, is a guanine nucleotide exchanger for Rho, Cdc42Hs, and Rac1 (38).

We do not believe that Cdc42Hs inhibition of G1/S transition is the result of nonspecific recruitment of p38 inasmuch as wild-type p38 itself, as well as MKK3, MKK6, and SEK1, all p38 activators, are able to arrest NIH-3T3 cells at G1/S. Moreover, recent evidence indicates that p38 overexpression inhibits mitogen induction of G1 cyclins (41). Thus it is not surprising that p38 activation, by a Cdc42Hs-dependent mechanism, could result in growth arrest. Moreover, precedent for Cdc42Hs as a G1 inhibitor has been observed in lower eukaryotes. Thus, S. cerevisiae Cdc42p is an essential component of the mating pheromone response pathway, which also results in G1 arrest (3, 21). Inasmuch as the experiments implicating Cdc42Hs in cell growth have employed Swiss 3T3 cells (40), our

staining (panels A and C, respectively). Staining for phospho-p38 is shown in panels B and D. Arrows indicate nuclear phospho-p38 staining (panel B) or mark the nuclei of the injected cells (panel D). Part d, expression of KR-SEK and KR-MKK3 has no effect on expression of coinjected Cdc42Hs. The expressed proteins (in red) were stained with anti-FLAG (panels A and B), and the rabbit IgG coinjected with the empty vectors were stained with anti-rabbit antibody (panel C). Nuclei of cells in S phase are stained with anti-BrdUrd and shown in green (panels A–C). All nuclei were stained with Hoechst 33258 (panels D–F). Injected cDNAs were: wt-Cdc42Hs (panels A and D), wt-Cdc42Hs plus KR-MMK3 and KR-SEK1 (panels B and F), empty vectors plus rabbit IgG (panels C and F).
The growth-promoting effects of Cdc42Hs in Swiss 3T3 cells are cell-specific.

The reasons for G1 arrest in response to stress signals have not been clearly defined; however, it is plausible to propose that cell cycle arrest would be followed by repair of stress-related damage. Alternatively, the cell could arrest in G1 and await restoration of a normal cellular milieu conducive to continued growth. The targets of Cdc42Hs and p38 which mediate cell cycle arrest are not known. However, inhibition of induction of G1 cyclins (41) is a logical candidate. Moreover, CHOP, a p38 substrate, promotes G1 arrest as part of the response to genotoxic stress (5, 42).

Finally, several recent studies indicate that recruitment of the SAPKs or p38 results in apoptosis. Withdrawal of nerve growth factor from PC-12 cells activates both the SAPKs and p38 and promotes apoptosis. Expression of KR-MKK3 or KR-SEK1 blocks this effect, and constitutively active mutants of MKK3 or SEK1 promote apoptosis in the presence of nerve growth factor (25). Likewise, thermotolerant fibroblasts display defective SAPK activation and cell death in response to heat shock. Expression of SEK1 restores heat sensitivity to these cells (26). Finally, treatment of macrophage cell lines with agonists that stimulate sphingomyelin hydrolysis (tumor necrosis factor, UV radiation, x-irradiation, oxidant stress) results in apoptosis, a response that can be reversed upon expression of dominant inhibitory SEK1 (27). Despite these findings, in none of the experiments shown in Figs. 1–6 did we see evidence of apoptosis. Thus, cycle arrest at G1/S, rather than apoptosis, appears to be the characteristic response of NIH-3T3 cells to activation of the Cdc42Hs/p38 pathway, indicating that different cells respond distinctly to p38 activation.

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Fig. 6. Rac1 and RhoA do not strongly block cell cycle progression. Part a, cells were microinjected with the indicated plasmids and quantitated for cell cycle progression. For each injection set the ratios of S phase expressing cells/total expressing cells were: empty plasmid: 53/85, 67/96, 80/117, 53/73; wt-Rac1: 24/53, 28/55, 58/113; V12-Rac1: 35/59, 30/64, 23/50; V14-RhoA: 19/35, 16/39, 39/101, 31/59. Part b, injection of wt- or V12-Rac1 activates p38 to the same degree as does wt-Cdc42Hs. Cells were injected with wt- (panels A and C) or V12-Rac1 (panels B and D) and stained for expression of the injected construct (panels A and B) or endogenous phospho-p38 (panels C and D). Part c, injection of V12-Rac induces membrane ruffling (panels A–D), whereas injection of V14-RhoA induces stress fibers (panels E–G). Panels A and F are stained for anti-M2-FLAG, panels C and D are stained for membrane ruffling, panel G is stained for actin stress fibers, and panels B and E are stained with Hoechst 33258. Arrows indicate the nuclei of the injected cells (panels B and E) and show membrane ruffling on panel D.

results with NIH-3T3 and Mv1Lu cells may indicate that the growth-promoting effects of Cdc42Hs in Swiss 3T3 cells are cell-specific.

Fig. 7. Model for cell cycle regulation by Rho family GTPases and the p38 pathway. Bold arrows indicate strong signaling inputs. Dashed arrows indicate weaker inputs.
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