MOLECULAR AND CELLULAR BIOLOGY, Feb. 2004, p. 1736–1746 Vol. 24, No. 4
0270-7306/04/$08.00+0 DOI: 10.1128/MCB.24.4.1736–1746.2004

Human CNK1 Acts as a Scaffold Protein, Linking Rho and Ras Signal Transduction Pathways

Aron B. Jaffe,[1] Pontus Aspenström,[2] and Alan Hall[1]*

MRC Laboratory for Molecular Cell Biology and Cell Biology Unit, Cancer Research UK Oncogene and Signal Transduction Group, and Department of Biochemistry, University College London, London WC1E 6BT, United Kingdom,[1] and Ludwig Institute for Cancer Research, S-751 24 Uppsala, Sweden[2]

Received 9 April 2003/Returned for modification 22 May 2003/Accepted 17 November 2003

Rho family GTPases act as molecular switches to control a variety of cellular responses, including cytoskeletal rearrangements, changes in gene expression, and cell transformation. In the active, GTP-bound state, Rho interacts with an ever-growing number of effector molecules, which promote distinct biochemical pathways. Here, we describe the isolation of hCNK1, the human homologue of *Drosophila c*onnector enhancer of *k*sr, as an effector for Rho. hCNK1 contains several protein-protein interaction domains, and Rho interacts with one of these, the PH domain, in a GTP-dependent manner. A mutant hCNK1, which is unable to bind to Rho, or depletion of endogenous hCNK1 by using RNA interference inhibits Rho-induced gene expression via serum response factor but has no apparent effect on Rho-induced stress fiber formation, suggesting that it acts as a specific effector for transcriptional, but not cytoskeletal, activation pathways. Finally, hCNK1 associates with Rhophilin and RalGDS, Rho and Ras effector molecules, respectively, suggesting that it acts as a scaffold protein to mediate cross talk between the two pathways.

Rho GTPases constitute a family within the Ras superfamily of small GTPases, which regulate a wide range of cellular effects, such as rearrangement of the actin cytoskeleton, transcriptional regulation, cell cycle progression, and cellular transformation (8, 10). The best-characterized members of this family are Rho, Rac, and Cdc42. These GTPases respond to various extracellular stimuli, which promote switching of the GTPase from the inactive, GDP-bound form to the active, GTP-bound form via guanine nucleotide exchange factors (27, 39). GTPases transduce their signals by interacting with target proteins, termed effectors, which preferentially interact with the GTP-bound (active) form of the GTPase (2). More than 50 effectors for Rho GTPases have already been isolated, primarily based on their ability to interact with the active GTPase, but relatively few have been linked to a particular cellular response.

In a yeast two-hybrid screen using constitutively active (L63) RhoA as bait, we identified hCNK1, a protein previously implicated in Ras signaling. *CNK* was originally isolated in a genetic screen designed to identify factors that modify the function of *kinase suppressor of ras* (*ksr*), a positive regulator of Ras signaling (18, 31, 33). CNK has been found to be essential for Ras signaling in *Drosophila* (35). The *Drosophila cnk* gene encodes a 1,557-amino-acid (aa) protein which contains two protein-protein interaction motifs, a sterile alpha motif (SAM) domain (28) and a PSD-95/DLG-1/ZO-1 (PDZ) domain (24) at the N terminus and a pleckstrin homology (PH) domain (20) in the middle of the protein. CNK is evolutionarily conserved, and there are predicted orthologues in *Caenorhabditis elegans* and humans with a similar domain arrangement. A comparison of *Drosophila* CNK with orthologues from other species has revealed a fourth, highly conserved domain, conserved region in CNK (CRIC), which lies between the SAM and PDZ domains (35).

The role of CNK in Ras signaling is not entirely clear. The C-terminal region of CNK (aa 381 to 1554) can suppress the phenotype caused by expression of activated Ras or Raf in the *Drosophila* eye by its ability to physically associate with, and sequester, Raf (35). This interaction is evolutionarily conserved, since rat CNK, also called MAGUIN-1 (42), can interact with Raf in vivo (43). However, overexpression of full-length CNK, or the N-terminal 384 amino acids, which contain the SAM, CRIC, and PDZ domains, enhances the phenotype caused by expression of activated Ras, suggesting that CNK can cooperate with Ras in vivo (34, 35). Mutation of the SAM domain or the CRIC domain abolishes its ability to cooperate with activated Ras (34), suggesting that these two domains interact with components necessary to transduce the Ras signal. Studies using Ras mutants which selectively impair signaling through a subset of Ras effectors indicates that CNK is involved in the RalGDS branch of Ras signaling (34). Together, these data indicate that CNK may act as a scaffold protein involved in multiple Ras signaling pathways—the Raf/mitogen-activated protein kinase pathway via its ability to physically interact with Raf and the RalGDS pathway via the N-terminal SAM and CRIC domains.

We now report that the PH domain of hCNK1 interacts specifically with the GTP-bound form of Rho. Expression of hCNK1 containing a single amino acid substitution in the PH domain that abolishes Rho binding or depletion of cellular hCNK1 using RNA interference (RNAi) inhibits Rho-dependent transcriptional activation. Finally, hCNK1 physically associates with rhophilin, another Rho effector, and with RalGDS, a Ras effector, suggesting that it acts as a scaffold

* Corresponding author. Mailing address: MRC Laboratory for Molecular Cell Biology and Cell Biology Unit, Cancer Research UK Oncogene and Signal Transduction Group, and Department of Biochemistry, University College London, London WC1E 6BT, United Kingdom. Phone: 44 020 7679 7806. Fax: 44 020 7679 7805. E-mail: alan.hall@ucl.ac.uk.

protein and mediates cross talk between these two GTPase signaling pathways in vivo.

MATERIALS AND METHODS

Plasmids and antibodies. All hCNK1 deletion, truncation, and mutant constructs were made by PCR or restriction digestions using standard methods. The expressed sequence tag encoding full-length hCNK1 (IMAGE clone 4651001) was used as the starting material for all hCNK1 constructs. Various fragments of hCNK1 were cloned into the prey vector pACT2 (Clontech) for expression in yeast, pRK5myc or pRK5FLAG for expression in mammalian cells, and pGEX2T or pGEX4T-3 for expression of glutathione *S*-transferase (GST) fusion proteins in bacteria. Rho GTPase constructs for expression in mammalian cells, yeast, and bacteria have been described previously (13). The pRetro-SUPER-hCNK1 (pRS-hCNK1) and pRetro-SUPER-control constructs were generated according to the manufacturer's recommendations (Oligoengine), using the following annealed oligonucleotides: pRS-hCNK1 (5′gatccccGGAGCTGCTGGAACAGAAGttcaagagaCTTCTGTTCCAGCAGCTCCtttttggaaa3′ and 5′agcttttccaaaaaGGAGCTGCTGGAACAGAAGtctcttgaaCTTCTGTTCCAGCAGCTCCggg3′) and pRS-control (5′gatccccCCTGCAAAGCCTGACAGAGttcaagagaaCTCTGTCAGGCTTTGCAGGttttggaaa) (uppercase letters denote the double-strand region corresponding to the hCNK1 sequence targeted). Commercial antibodies used were mouse anti-FLAG M2 (Sigma), mouse anti-hCNK1 (Transduction Laboratories), and rat anti-α tubulin (Serotec).

Dot blot assay. The interactions between Rho GTPases and hCNK1 or various fragments of hCNK1 were assessed using a dot blot assay as previously described (6). In brief, 10 μg of GST fusion protein, full-length or the indicated fragment of hCNK1, p50rhoGAP, Rhotekin RBD, or RhoGDI, was spotted in a volume of 10 μl onto nitrocellulose membranes. The filter was air dried and incubated with blocking buffer (1 M glycine, 5% milk powder, 1% ovalbumin, 5% fetal calf serum) for 2 h at room temperature. The membrane was washed in buffer A (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM $MgCl_2$, and 0.1 mM dithiothreitol) and incubated for 5 min at 4°C with [γ-^{32}P]GTP-bound GTPase proteins in buffer A. The filters were quickly washed three times with cold buffer A containing 0.1% Tween, and interacting GTPases were visualized by autoradiography. To determine the relative binding of RhoGTP and RhoGDP to hCNK1, we used a modified dot blot protocol as previously described (32). Briefly, wild-type Rho was loaded with [α-^{32}P]GTP, and the exchange reaction was stopped by addition of $MgCl_2$ on ice. The sample was split in two, and 10 ng of RhoGAP was added to one of the tubes. This tube was incubated for 10 min at 30°C (to produce predominantly [α-^{32}P]GDP), while the other tube was left on ice (predominantly [α-^{32}P]GTP). The GTPase aliquots were used in a dot blot assay as described above, using Rhotekin RBD (which preferentially binds RhoGTP) and RhoGDI (which shows a slight preference for RhoGDP) as controls.

Immunoprecipitations. Cells expressing the appropriate constructs were washed with ice-cold phosphate-buffered saline and incubated at 4°C in Nonidet P-40 buffer (20 mM Tris-HCl [pH 8], 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). Cell debris was pelleted, and lysates were incubated for 1 h at 4°C with specific antibodies. Protein G-Sepharose was added, and the lysates were incubated for a further 1 h at 4°C. Immunoprecipitates were collected by centrifugation and extensively washed in Nonidet P-40 buffer containing 250 mM NaCl. Immunoprecipitated proteins were eluted with sodium dodecyl sulfate (SDS)-sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting.

GST pulldowns. Ten micrograms of bacterially produced GST fusion proteins, coupled to beads, were incubated with 10 μl of ^{35}S-labeled, in vitro-synthesized protein (TNT quick coupled transcription/translation system; Promega) in 200 μl of binding buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) for 2 h at 4°C. The beads were washed twice in 1 ml of binding buffer containing 0.5 M NaCl and then once in 1 ml of binding buffer. The beads were resuspended in Laemmli buffer and then resolved on by SDS-PAGE. The gel was stained with Coomassie to monitor the levels of the GST fusion proteins, dried, and visualized with a phosphorimaging screen.

Cell culture and transfections. HeLa and COS cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and penicillin-streptomycin (100 IU/ml and 100 μg/ml) and incubated at 37°C and either 10% (COS-7) or 5% (HeLa) CO_2. Transfections were performed using GeneJuice (Novagen) according to manufacturer specifications. For hCNK1 depletion experiments, HeLa cells were transfected with the appropriate construct (day zero) and passaged into puromycin-containing media (2 μg/ml) 24 h later (day 1). For transient luciferase assays, hCNK1-depleted cells were transfected with the appropriate reporter and expression constructs at day 3 and harvested and analyzed for luciferase activity at day 4. For analysis of stress fiber formation, expression constructs were introduced into hCNK1-depleted cells at day 3 or day 4 posttransfection. Stable cell lines were selected for with 2 μg of puromycin (InvivoGen)/ml, and individual clones were picked with sterile cloning disks (Scienceware).

Luciferase reporter assays. Cells were transfected with different expression plasmids together with 0.25 μg of pSRE-luc and 0.01 μg of pRL-CMV (a plasmid encoding *Renilla* luciferase under the control of the cytomegalovirus promoter) as an internal control. The total amount of DNA was adjusted with empty vector. Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's specifications. One-twentieth of the lysate was used to monitor expression levels of transfected constructs by Western blotting.

Microinjection and immunofluorescence. HeLa cells were plated onto glass coverslips and serum starved overnight prior to microinjection. Constructs were injected at 0.1 μg/μl into the nucleus of approximately 50 cells over a period of 15 min and allowed to express for 3 to 4 h. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature and stained for the epitope tag, injection marker, and actin, as previously described (21). Fluorescence images were recorded on a charge-coupled device camera and processed using Openlab software.

RESULTS

Identification of hCNK1 as a Rho effector. To identify proteins that mediate signaling downstream of RhoA, we performed a yeast two-hybrid screen with constitutively active (L63) RhoA as bait and a HeLa cDNA library as prey. One of the isolated clones encoded the last 394 aa of the human orthologue of *Drosophila* CNK, hCNK1 (35). An expressed sequence tag corresponding to full-length hCNK1 was subcloned into the yeast prey vector and tested for its ability to interact with constitutively active forms of RhoA, Rac1 (L61Rac), and Cdc42 (L61Cdc42). We found that hCNK1 associates with L63Rho but not L61Rac or L61Cdc42 (Fig. 1A), indicating that it is a Rho-specific binding protein. To verify this and to test whether this interaction is direct, we tested the ability of hCNK1 to associate with Rho GTPases in vitro. Full-length, recombinant hCNK1 produced in bacteria was unstable, so we used three fragments of hCNK1 (aa 1 to 314, 315 to 548, and 549 to 72) for our binding studies. We found that bacterially produced, recombinant hCNK1 (aa 315 to 548) directly associates with bacterially produced, recombinant L63Rho but not L61Rac or L61Cdc42 (Fig. 1B), confirming that hCNK1 specifically binds to Rho. To test whether hCNK1 and Rho can associate in vivo, we performed coimmunoprecipitation experiments using epitope-tagged forms of each protein, expressed in COS cells. We found that hCNK1 and L63Rho can associate in mammalian cells (Fig. 1C), indicating that this interaction occurs in vivo.

A hallmark of effectors of small GTPases is that they preferentially interact with the active, GTP-bound form rather than the inactive, GDP-bound form of the GTPase. In vitro dot blot analysis (Fig. 1D) revealed that hCNK1 interacts with GTP-Rho more strongly than with GDP-Rho, similar to another Rho effector, Rhotekin, but unlike RhoGDI, which has a slight binding preference towards GDP-Rho rather than GTP-Rho. These data indicate that hCNK1 is a Rho-specific effector protein.

The hCNK1 PH domain mediates Rho binding. Some Rho effector proteins share a common Rho binding motif (REM-1) (2), but this was not present in hCNK1. To identify the region of hCNK1 involved in binding to active Rho, we constructed a series of deletion mutants and tested their ability to interact

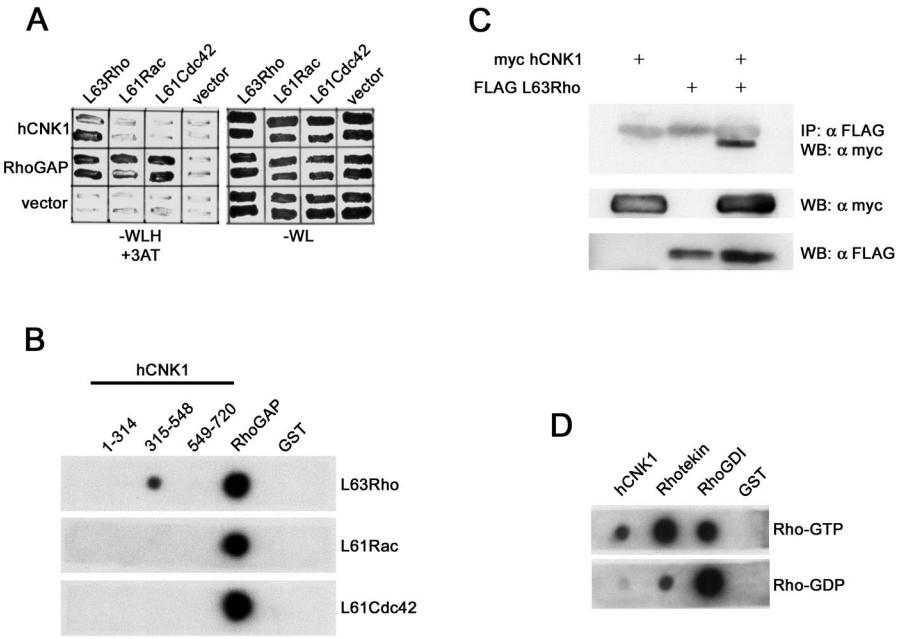

FIG. 1. Characterization of hCNK1-Rho interaction. (A) hCNK1 specifically interacts with Rho in yeast. Interaction of the indicated GAL4-DNA binding domain fusion (top) with the indicated GAL4-activation domain fusion (side) was examined by growth on triple selection plates (left). Presence of both plasmids was verified by growth on double selection plates (right). (B) hCNK1 interacts specifically with Rho in vitro. GST fusions of the indicated proteins were spotted onto nitrocellulose and probed with [γ-^{32}P]GTP-loaded L63Rho, L61Rac, or L61Cdc42. (C) Immunoprecipitation from extracts of cells expressing Myc-tagged hCNK1 and FLAG-tagged L63Rho, either individually or in combination. Expression of hCNK1 and Rho was monitored by Western blotting with anti-Myc or anti-FLAG antibodies, respectively. (D) hCNK1 interacts with Rho in a GTP-dependent manner. GST fusions of the indicated proteins were spotted onto nitrocellulose and probed with [α-^{32}P]GTP-loaded or [α-^{32}P]GDP-loaded wild-type Rho.

with L63Rho by using yeast two-hybrid and in vitro dot blot analyses (Fig. 2A). The minimal Rho binding domain of hCNK1 (CNK-RBD) mapped to amino acids 384 to 504, which includes the PH domain (aa 404 to 504). To directly test whether the PH domain of hCNK1 is required for binding to active Rho, we constructed a deletion mutant of hCNK1 which lacks the PH domain (CNK ΔPH) and a point mutant of hCNK1 which has a W$\rightarrow$A mutation in the C-terminal alpha helix of the PH domain (CNK W493A), a mutation previously shown to destabilize the PH domain structure and block binding to both lipids and proteins (23). Using yeast two-hybrid analysis, we found that either deletion or disruption of the PH domain blocks the ability of hCNK1 to bind to L63Rho (Fig. 2B). hCNK1 W493A was also unable to interact with L63Rho in an in vitro dot blot assay (Fig. 2C), indicating that the PH domain is essential for the hCNK1-Rho interaction. Since PH domains are more commonly associated with lipid binding, we tested whether the hCNK1 PH domain bound to specific phosphoinosides in vitro and found only weak, promiscuous binding by both the wild-type and W493A mutant PH domains (data not shown), suggesting that the function of the hCNK1 PH domain is to mediate the interaction between hCNK1 and Rho.

hCNK1 is not involved in Rho-mediated stress fiber formation. Since hCNK1 is likely a scaffold protein, we reasoned that expression of a mutant hCNK1, which is unable to interact with Rho (CNK W493A), would either promote or inhibit signal transduction pathways in which it normally participates. To identify a cell line, expressing endogenous hCNK1, which we could test for various cellular effects downstream of Rho, we performed Western analysis on extracts from several different human cell lines and found that hCNK1 is expressed in HeLa cells (Fig. 3A) as well as wild-type mammary epithelial cells (data not shown). Since HeLa cells have been previously used to study Rho GTPase function, we focused on this cell line for our analysis of hCNK1 function.

The best-characterized cellular effect of Rho activation is the rearrangement of the actin cytoskeleton to form stress fibers (8). To test whether hCNK1 is involved in this process, we microinjected either wild-type hCNK1 or hCNK1 W493A together with constitutively active L63Rho into serum-starved HeLa cells and stained for filamentous (F) actin to examine stress fiber formation. While wild-type hCNK1 blocked L63Rho-induced stress fibers in more than half of injected cells (Fig. 3D to F and J), hCNK1 W493A, which cannot bind to L63Rho, had no effect on stress fiber formation (Fig. 3G to J). These data indicate that hCNK1 blocks L63Rho-induced stress fibers through sequestration of active Rho and that hCNK1 W493A does not interact or interfere with proteins required for Rho-mediated stress fiber formation. In agreement with this, we have also observed a loss of stress fibers in growing fibroblasts overexpressing wild-type hCNK1 (unpublished observations). We conclude that hCNK1 is unlikely to participate in the signal transduction pathway downstream of Rho leading to stress fiber formation.

hCNK1 W493A blocks Rho-induced SRF activation. Another cellular response to Rho activation is modulation of gene expression. One of the most extensively investigated transcription factors activated downstream of Rho is serum response

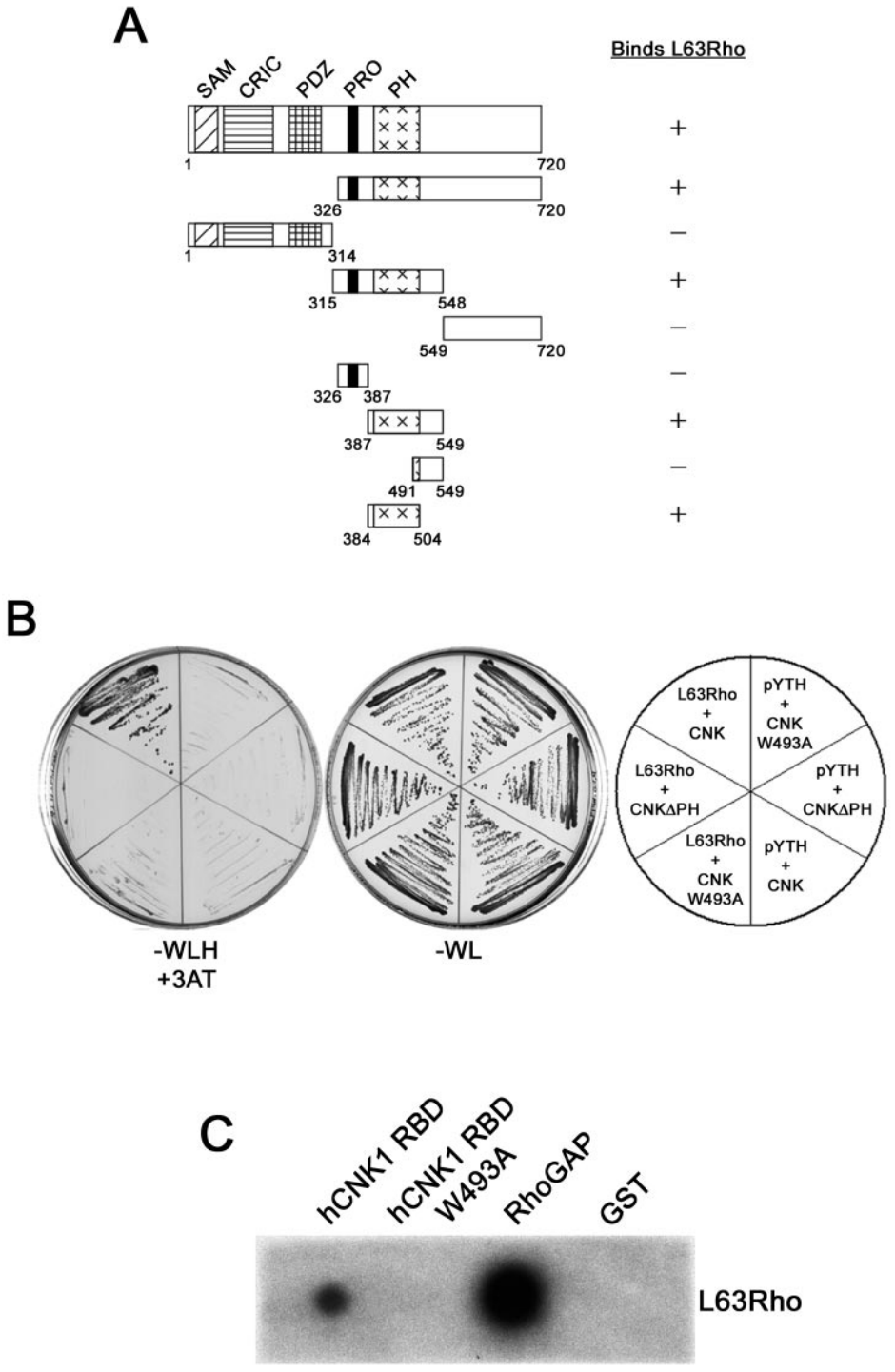

FIG. 2. Mapping the Rho-binding domain of hCNK1. (A) Schematic of hCNK1 deletion constructs. Interaction with L63Rho by yeast two-hybrid or dot blot analysis is indicated at right. (B) The PH domain of hCNK1 is required for Rho binding in yeast. Colonies expressing the indicated constructs (right) were streaked onto triple selection plates (left) and analyzed for growth to determine their ability to interact. The presence of both plasmids was verified by streaking onto double selection plates (middle). (C) The PH domain of hCNK1 is required for Rho binding in vitro. GST fusions of the indicated proteins were spotted out onto nitrocellulose filters and probed with [γ-^{32}P]GTP-loaded L63Rho, followed by autoradiography.

factor (SRF) (37). Rho is thought to regulate the activity of SRF by controlling levels of monomeric (G) actin via Rho kinase→LIM kinase and Diaphanous (mDia). However, mutational analysis of Rho suggests that there are other pathways leading to SRF activation which do not involve actin (see Discussion).

To address whether hCNK1 is involved in SRF activation by Rho, we examined the effect of hCNK1 W493A on Rho-in-

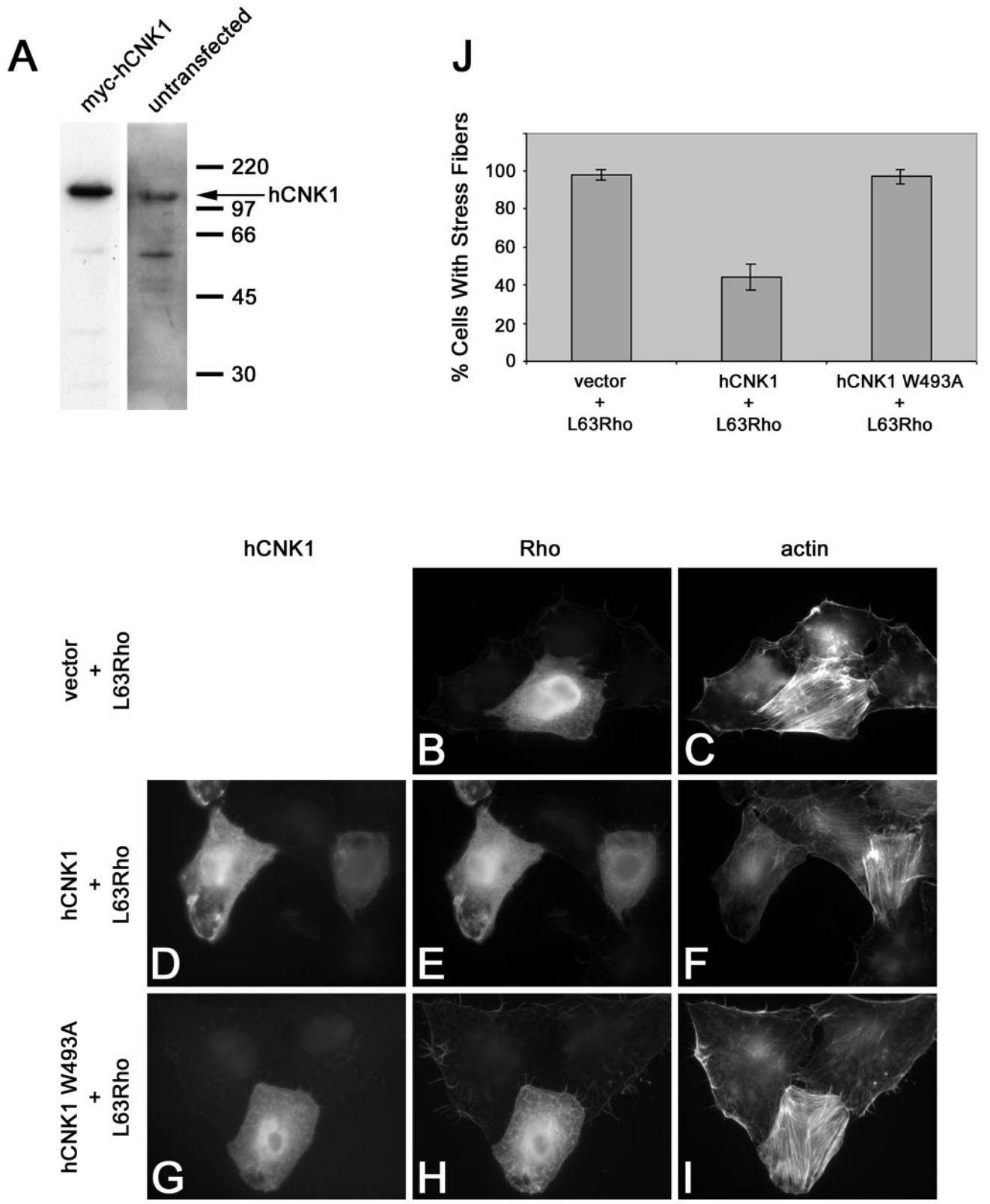

FIG. 3. hCNK1 does not affect Rho-mediated stress fiber formation. (A) hCNK1 expression in HeLa cells. Western blot analysis of cell lysate from HeLa cells transfected with Myc-tagged, full-length hCNK1 (lane 1) or untransfected (lane 2), probed with anti-Myc (lane 1) or an N-terminus-specific hCNK1 antibody (lane 2). (B to I) HeLa cells injected with L63Rho together with control vector (B and C), hCNK1 (D to F), or hCNK1 W493A (G to I) were fixed and analyzed for expression of CNK (D and G), expression of Rho (B, E, and H), and stress fiber formation (C, F, and I). (J) Quantitation of the effect of hCNK1 and hCNK1 W493A on L63Rho-mediated stress fiber formation. Data are expressed as means ± standard deviations for at least three experiments, in which a minimum of 40 expressing cells were counted per experiment.

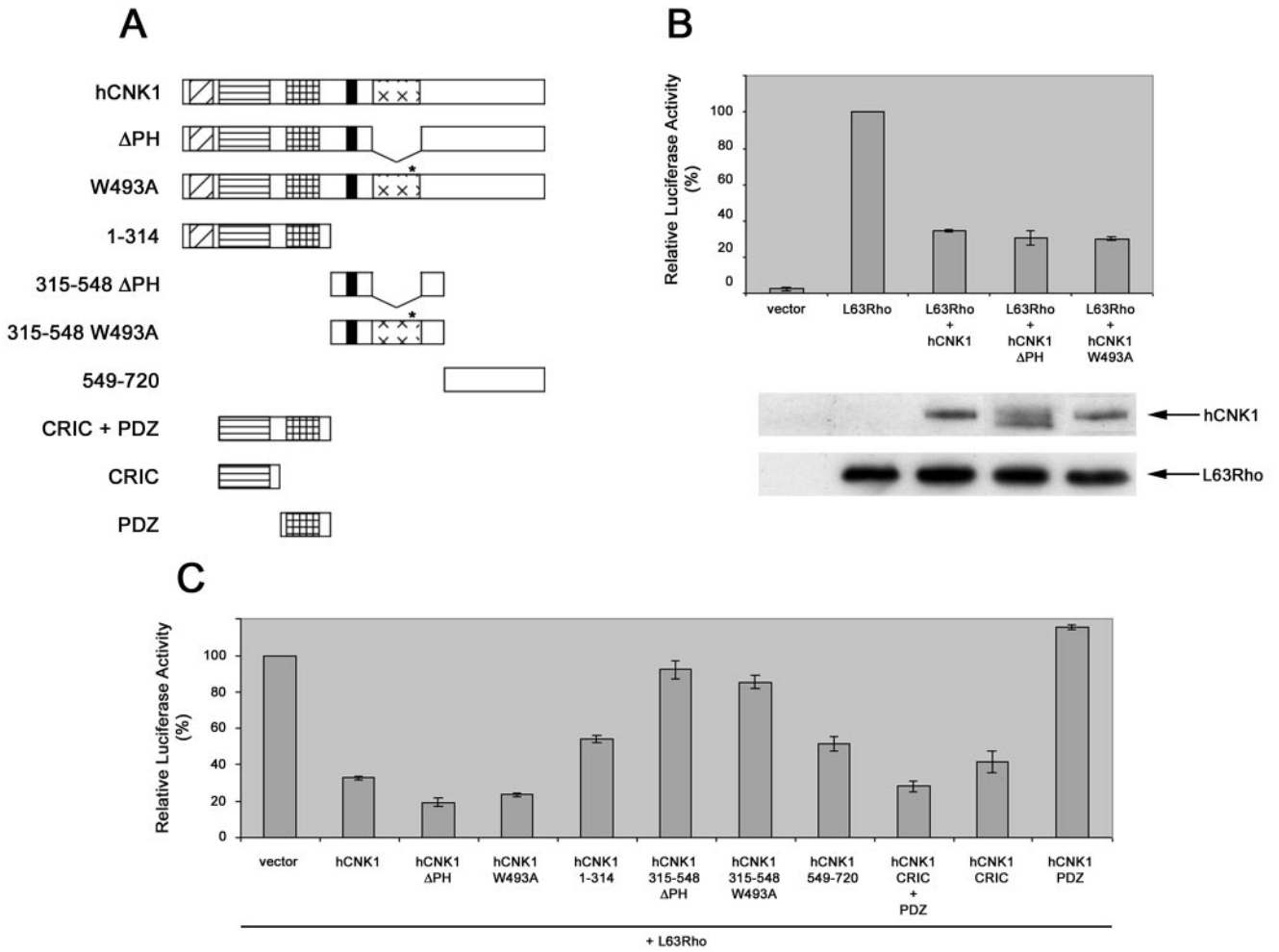

FIG. 4. Role of hCNK1 in Rho-mediated SRF activation (A) Schematic of hCNK1 constructs used for transcription analysis. (B) HeLa cells transfected with pSRE-luc, together with Myc-tagged expression plasmids encoding the indicated proteins, were harvested and subject to a luciferase assay. Luciferase activity is expressed relative to the activity of L63Rho, which is set at 100%. All constructs were analyzed in at least three independent experiments, each performed in duplicate. Shown is a representative example of each sample. Below is a Western blot of the cell lysates using an anti-Myc antibody to monitor expression levels of the transfected constructs. (C) HeLa cells transfected with expression plasmids encoding the indicated proteins and analyzed for luciferase activity as in panel A.

duced activation of a luciferase reporter under the control of an SRF response element (SRE-luc). Expression of L63Rho strongly activates SRE-luc in HeLa cells, and coexpression of wild-type hCNK1 represses this activation, similar to the effect seen on Rho-induced stress fiber formation (Fig. 4B). However, unlike in the stress fiber assay, hCNK1 W493A, which cannot bind to Rho, is still able to strongly repress Rho-mediated SRF activation (Fig. 4B). Expression of hCNK1 or hCNK1 W493A did not impair the expression of L63Rho (Fig. 4B). These results suggest that hCNK1 W493A interferes specifically with Rho-mediated transcriptional activation and that it is likely to interact with protein(s) involved in this signal transduction pathway.

To map the region of hCNK1 responsible for suppression of SRF activation, we made a series of deletion constructs (Fig. 4A) and examined their effect on Rho-mediated SRF activation (Fig. 4C). We found that both the C terminus (aa 549 to 720) and the N-terminal CRIC domain are each able to suppress SRF activation by Rho, suggesting that these regions bind to factors involved in the signaling pathway.

To verify that the inhibition of Rho-mediated SRF activation caused by overexpression of either hCNK1 W493A, hCNK1 CRIC, or the hCNK1 C terminus truly reflected a specific requirement for hCNK1 in Rho-mediated transcription, we used RNAi to knock down hCNK1 levels in HeLa cells. As shown in Fig. 5A and B, transient expression of an appropriate small interfering RNA (siRNA) expression vector for 3 days led to an approximately 70% reduction in hCNK1 protein levels and a corresponding 30% reduction in Rho-induced SRF activation. Since this is a mixed population of cells and therefore likely to show significant variation in expression of hCNK1 from cell to cell, we also generated two stable HeLa cell lines, one expressing an siRNA construct that causes an approximately 80% reduction in hCNK1 levels and another which has no effect on hCNK1 levels (Fig. 5C). Depletion of hCNK1 in this stable cell line leads to a 50% reduc-

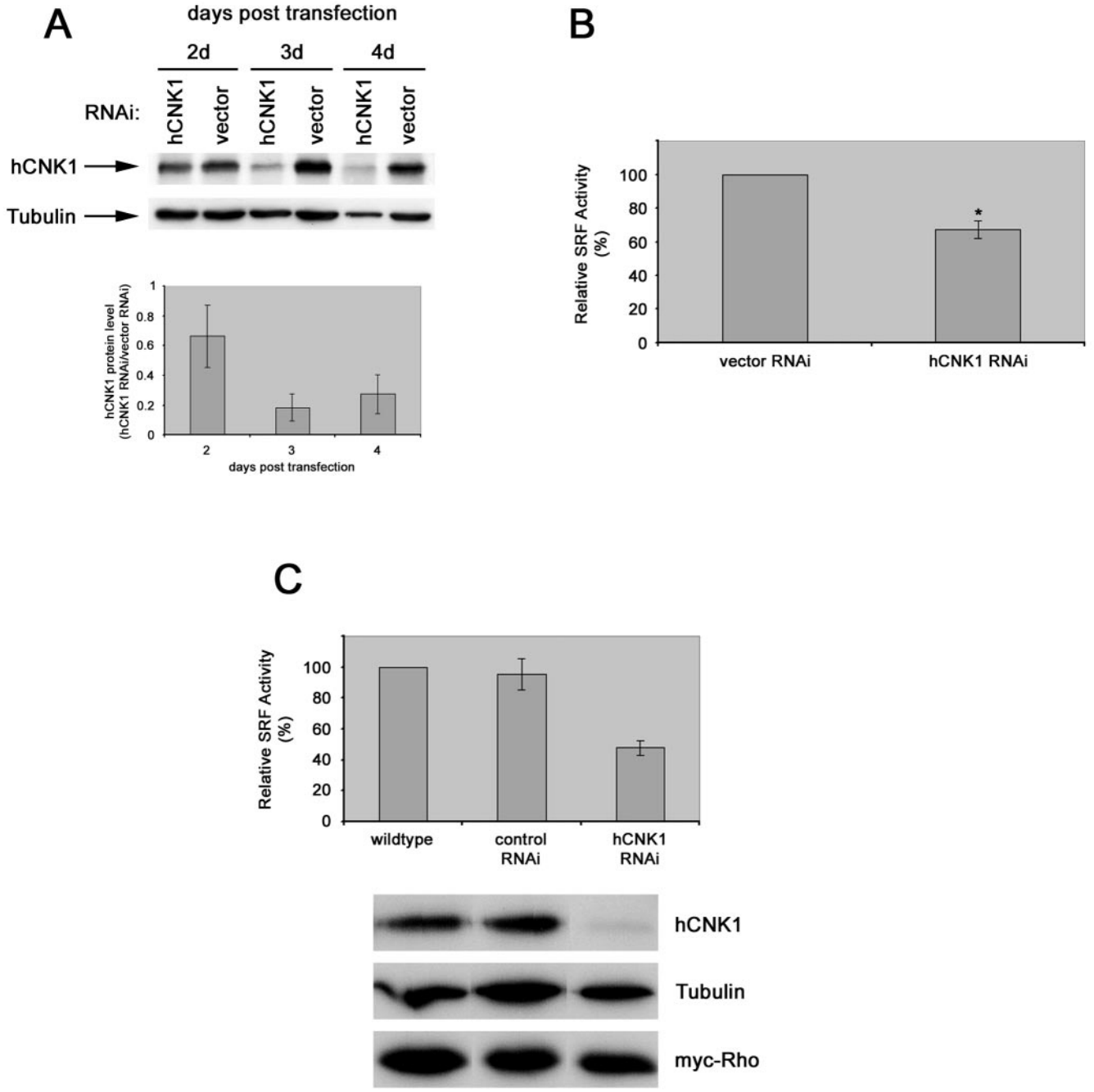

FIG. 5. hCNK1 depletion impairs Rho-mediated SRF activation. (A) Time course of hCNK1 depletion after transfection of pSUPER.retro constructs. Transfected cells were selected with 2 μg of puromycin/ml 24 h after transfection and analyzed at the indicated times for hCNK1 and tubulin protein levels. Quantitation of hCNK1 protein levels, normalized to tubulin levels, expressed as means ± standard deviations for three independent experiments, is shown below. hCNK1 proteins levels are indicated as percentages of expression in pSR-hCNK1-transfected cells relative to expression in pSR-transfected cells. (B) HeLa cells, either with or without hCNK1 depletion, were transfected with pSRE-luc, together with constitutively active Rho, and harvested and subject to a luciferase assay. Luciferase activity is expressed relative to the activity of L63Rho in hCNK1 wild-type cells, which is set at 100%. Data is presented as means ± standard errors of the means for three independent experiments, each performed in triplicate. *, Student *t* test ($P < 0.0001$). (C) HeLa cells, untransfected or stably transfected with pSUPER.retro constructs expressing either a control hairpin RNA or the hCNK1-specific hairpin RNA, were transfected with pSRE-luc, either with or without constitutively active Rho, and harvested and subject to luciferase assay. Luciferase activity is expressed relative to the activity of L63Rho in untransfected HeLa cells. Each experiment was performed in triplicate, and a representative experiment is shown. Data are presented as means ± standard errors of the means. Extracts used for luciferase analysis were also subjected to immunoblotting to monitor the expression levels of hCNK1, tubulin, and the transfected, Myc-tagged, constitutively active Rho (below).

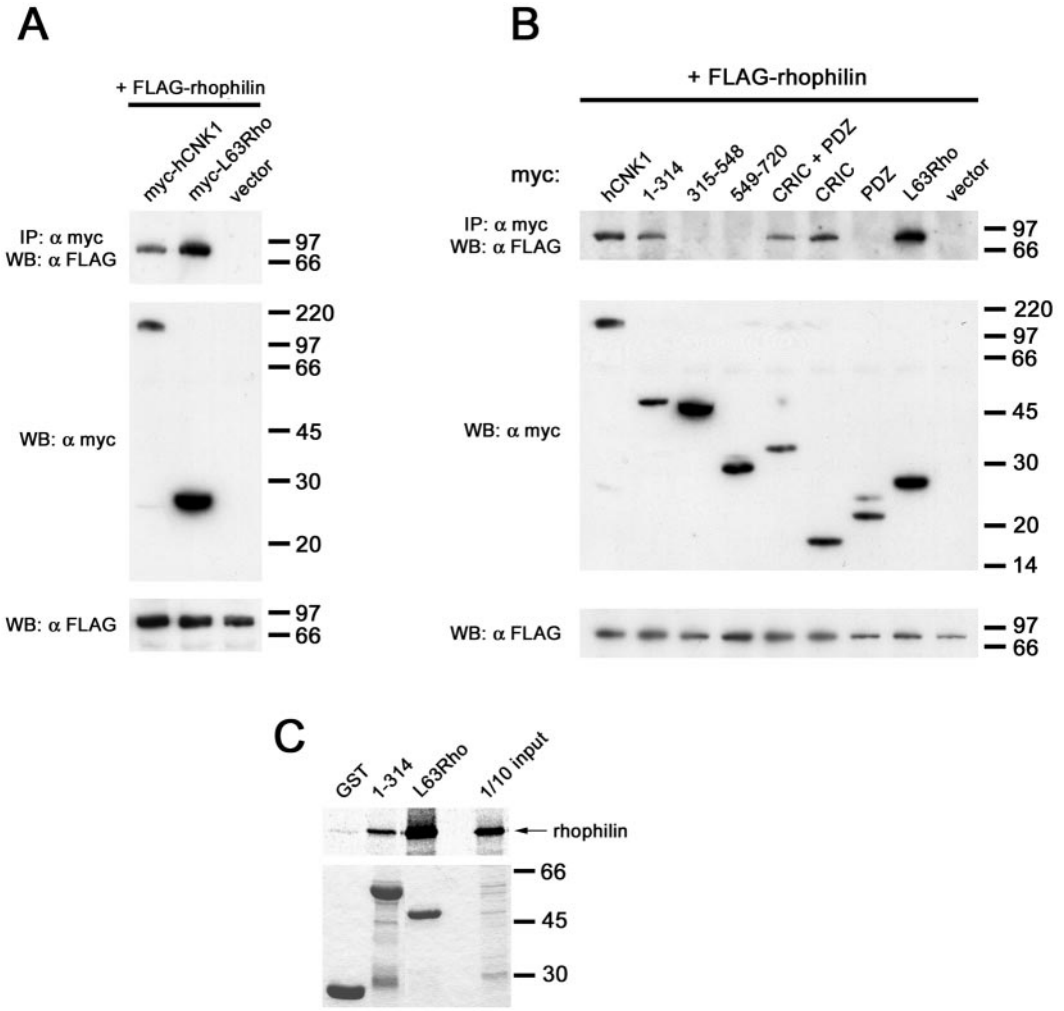

FIG. 6. hCNK1 physically associates with rhophilin via the CRIC domain. (A) Immunoprecipitation of extracts from cells expressing Myc-tagged hCNK1 or L63Rho, together with FLAG-tagged rhophilin. Expression of the transfected constructs was monitored by Western blotting with anti-Myc or anti-FLAG antibodies. (B) Immunoprecipitation of extracts from cells overexpressing the indicated Myc-tagged hCNK1 constructs (see Fig. 4A for schematic), L63Rho, or empty vector, together with FLAG-tagged rhophilin. Expression of the transfected constructs was monitored by Western blotting with anti-Myc or anti-FLAG antibodies. (C) GST pulldowns of ^{35}S-labeled rhophilin. GST fusions, coupled to glutathione-Sepharose beads, of the indicated proteins were incubated with in vitro-synthesized, ^{35}S-labeled rhophilin and resolved by SDS-PAGE. Top: autoradiograph of a representative pulldown experiment. Bottom: Coomassie-stained gel to visualize the indicated GST fusion proteins.

tion in Rho-induced SRF activation (Fig. 5C). Together, these data indicate that hCNK1 participates in SRF activation downstream of Rho and that overexpression of hCNK1 W493A recapitulates an hCNK1 loss-of-function phenotype.

The hCNK1 CRIC domain binds to rhophilin. To identify CNK-interacting proteins that might participate in Rho-dependent SRF activation, we first examined other known Rho effectors. As shown in Fig. 6A, hCNK1 interacts strongly with the Rho effector rhophilin when both are expressed in mammalian cells. Interestingly, rhophilin interacts specifically with the CRIC domain (Fig. 6B), one of the two regions of hCNK1 that block Rho-mediated SRF activation (Fig. 4C). To confirm that the interaction between hCNK1 and rhophilin is direct, we carried out GST pulldown experiments with bacterially purified hCNK1 and in vitro-synthesized, radiolabeled rhophilin. As shown in Fig. 6C, GST-hCNK1 1-314, which contains the CRIC domain, strongly associates with rhophilin in vitro. These results suggest that hCNK1 may modulate Rho-mediated transcriptional activation by forming a signaling complex containing Rho and rhophilin. There are two known rhophilin proteins in mammalian cells (22), and we are currently attempting to knock down both family members using RNAi, to further address their role in Rho-mediated transcription.

hCNK1 biochemically links Rho and Ras signaling pathways. Experiments with *Drosophila* CNK have suggested that it participates in Ras signaling partly through the RalGDS pathway. As shown in Fig. 7, we found that hCNK1 and RalGDS can be coimmunoprecipitated when expressed in mammalian cells. Attempts to identify the region of hCNK1 that interacts with RalGDS using deletion mutants (Fig. 4A) were unsuccessful. It may be that RalGDS binding occurs through a tertiary structural element rather than a linear protein sequence or that it requires additional proteins. To examine whether hCNK1 can form a complex with rhophilin and RalGDS, es-

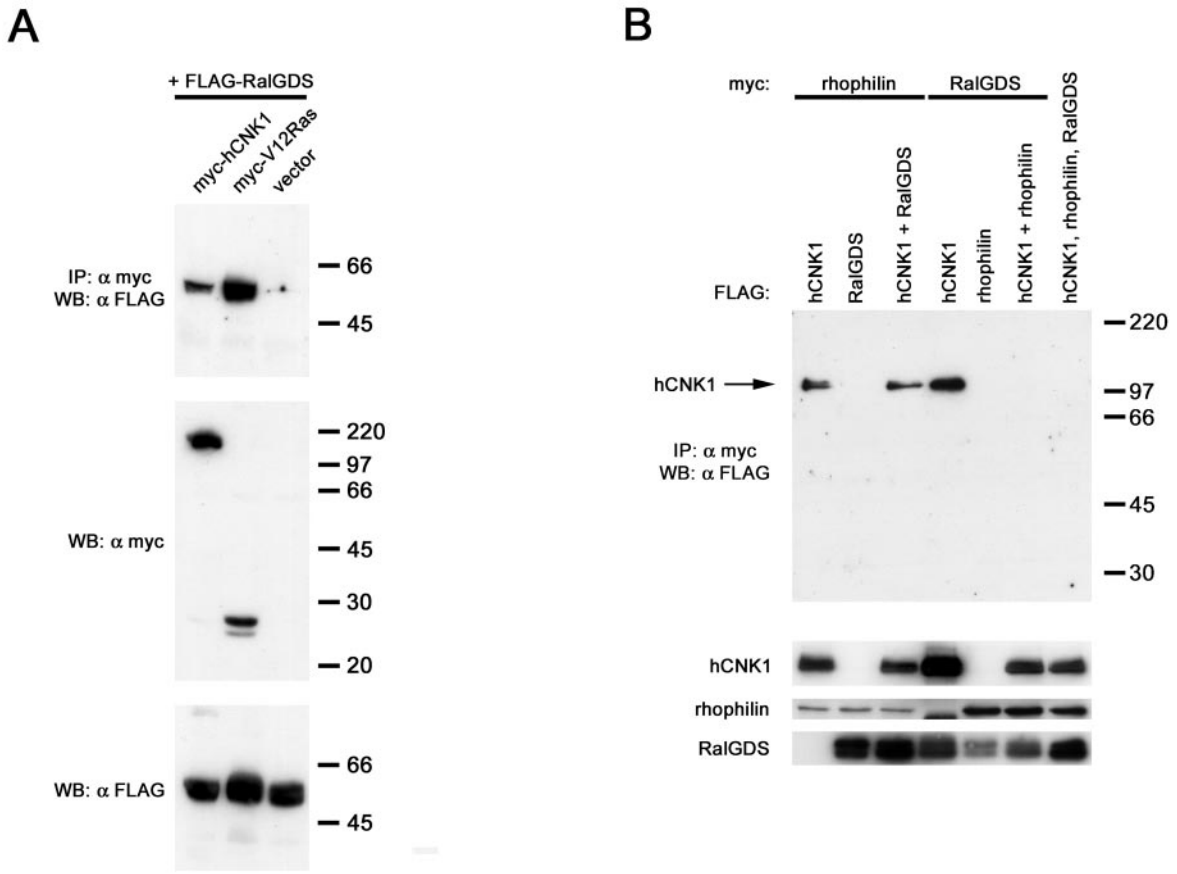

FIG. 7. CNK associates with RalGDS in mammalian cells. (A) Immunoprecipitation of extracts from cells expressing Myc-tagged hCNK1 or V12Ras, together with FLAG-tagged RalGDS. Expression of the transfected constructs was monitored by Western blotting with anti-Myc or anti-FLAG antibodies. (B) Immunoprecipitation of extracts from cells expressing Myc-tagged rhophilin, together with FLAG-tagged hCNK1, FLAG-tagged RalGDS, or both (lanes 1 to 3), Myc-tagged RalGDS, together with FLAG-tagged hCNK1, FLAG-tagged rhophilin, or both (lanes 4 to 6), or FLAG-tagged hCNK1, rhophilin, and RalGDS (lane 7). Expression of the transfected constructs was monitored by Western blotting with anti-Myc or anti-FLAG antibodies.

sentially acting as a molecular bridge linking Ras and Rho signaling pathways, we immunoprecipitated Myc-tagged rhophilin from cell extracts expressing Myc-rhophilin and FLAG-RalGDS, either with or without FLAG-hCNK1. We found that RalGDS could not be coimmunoprecipitated with rhophilin (Fig. 7B, lane 3). Moreover, when we performed immunoprecipitations from cell extracts expressing Myc-RalGDS and FLAG-rhophilin either with or without FLAG-hCNK1, we found that coexpression of rhophilin blocked the hCNK1-RalGDS interaction (Fig. 7B, compare lanes 4 and 6). These results suggest the following: (i) hCNK1-rhophilin and hCNK1-RalGDS complexes are mutually exclusive; and (ii) rhophilin and RalGDS compete for hCNK1 binding.

DISCUSSION

PH-GTPase interactions: a new small GTPase-binding domain? We show here that the PH domain of hCNK1 interacts specifically with the GTP-bound form of Rho. Pleckstrin homology domains are best known as mediators of protein-lipid interactions, but they also participate in protein-protein interactions. The physiological relevance of the majority of these interactions, however, remains unclear. Interestingly, some recent studies have reported that PH domains and structurally similar domains can interact with small GTPases (12, 40). RanBP2, for example, binds to the small GTPase Ran in a GTP-dependent manner (16). Although it does not contain a PH domain, crystal structure analysis of RanBP2 complexed with Ran-GTP reveals a remarkably high degree of structural similarity to PH domains (40). The tyrosine kinase Etk binds to Rho, but not Rac or Cdc42, through its PH domain, although in this case the interaction is not dependent on the nucleotide bound to Rho (12). Sequence analysis of effector proteins of Rho family GTPases has led to the identification of several consensus binding sites, most notably the Cdc42/Rac interactive binding motif (3). The PH domain of CNK, together with that of Etk and the PH-like domain of RanBP2, may represent another binding motif for interacting with small GTPases.

CNK acts as a specific effector for Rho-mediated transcription. Although many Rho effector molecules have been identified, primarily by their ability to physically associate with the active form of Rho, few have been implicated in specific Rho signaling events. We took advantage of our precise mapping of the Rho-binding domain to the PH domain to create a putative

dominant-negative, or interfering, CNK mutant, consisting of a single amino acid substitution in the context of the full-length protein. We found that hCNK1 W493A blocked Rho-mediated activation of SRF but not Rho-mediated stress fiber formation. Both transient and stable reduction of hCNK1 levels using siRNA expressed in HeLa cells verified its role in Rho-mediated SRF activation. These two complementary approaches suggest that hCNK1 is specifically involved in SRF activation. While we have not observed a role for hCNK1 in stress fiber formation using hCNK1 W493A overexpression or hCNK1 RNAi (Fig. 3 and data not shown), more quantitative analysis will be necessary to determine whether it plays a more subtle role in actin dynamics.

Actin rearrangements have been implicated in Rho-mediated SRF activation, and it appears that SRF activity is somehow responsive to the levels of monomeric actin (17, 29). Indeed, the Rho effectors so far implicated in SRF activation, mDia and Rho kinase (7, 36), also promote Rho-mediated actin rearrangements (36, 38, 41). However, mutants of Rho have been isolated which retain the ability to activate SRF but have lost their ability to induce actin polymerization (26, 44). Similarly, Rho mutants are known which can still bind to mDia, Rho kinase, or both but are either impaired or completely inhibited in their ability to activate SRF (26). The signaling pathway linking actin dynamics to SRF activation also appears to show some cell type specificity. In NIH 3T3 cells, for example, Rho-mediated SRF activation requires mDia but not Rho kinase or LIM kinase (29, 36). In rat aortic smooth muscle cells, Rho kinase is at least partially required for SRF activation (14), while in PC12 cells, both Rho kinase and mDia are required for SRF activation (7). Rho has also been reported to activate other transcription factors, such as GATA-4 and AP-1, but in these cases actin dynamics are not involved (4, 15). Our finding that expression of hCNK1 W493A or the reduction of cellular hCNK1 levels by RNAi impairs Rho-mediated SRF activation without having a pronounced effect on Rho-mediated stress-fiber formation raises the possibility that hCNK1 may play a more ubiquitous role in signaling from Rho to the nucleus. Furthermore, our observation that impairing hCNK1 function does not completely block Rho-mediated SRF activation is consistent with an additional, hCNK1-independent, actin-dependent mechanism for SRF activation in HeLa cells.

Since hCNK1 does not contain any known catalytic motifs and has multiple protein-protein interaction domains, it is likely acting as a scaffold molecule, transducing the signal from Rho by facilitating the formation of a multiprotein complex. In addition to active, GTP-bound Rho, we found that rhophilin, another Rho effector molecule, also interacts with hCNK1. Interestingly, rhophilin binds to the CRIC domain, which also participates in Rho-mediated SRF activation. This correlation raises the possibility that rhophilin may be required for Rho-mediated SRF activation, but how it would do so is unclear. Rhophilin is itself thought to be a scaffold protein, since it has no known catalytic motifs and contains only a PDZ domain in addition to its Rho-binding domain. Furthermore, overexpression of full-length rhophilin does not appear to affect the SRE-luc reporter (22), suggesting that at least it is not a limiting factor in SRF activation. The identification of other components of the hCNK1 complex should provide further insight into how it participates in Rho signaling to the nucleus.

CNK interacts with both Rho and Ras effector molecules: possible mediator of cross talk? In *Drosophila*, CNK participates in Ras signaling via a direct physical association with Raf, as well as a genetic interaction with the RalGDS effector pathway (35). CNK may be required for Raf activation by Ras, at least in some circumstances, since reducing CNK levels with RNAi in *Drosophila* tissue culture cells blocks Raf membrane association and activation following insulin treatment (1). The role of hCNK1 in signaling from Ras to Raf in vertebrate organisms, however, is unclear, since although a rat homologue of *Drosophila* CNK, MAGUIN-1, also associates with Raf-1 (43), hCNK1 does not (35). A second CNK (hCNK2A) protein is predicted in the human genome (accession number AF418269) which may interact with Raf and may also be involved in Rho signaling. Expression analysis, however, indicates that hCNK2A expression is restricted to the brain (11), suggesting that it does not play a role in Rho signaling in other tissues.

RalGDS is a guanine nucleotide exchange factor for Ral. The exact role of CNK in signaling via RalGDS downstream of Ras is unclear. Studies with *Drosophila* have revealed a genetic interaction between CNK and V12G37Ras, an effector loop mutant that stimulates RalGDS in mammalian cells, suggesting that it acts in Ral signaling downstream of Ras (34). However, other studies with *Drosophila* have indicated that the V12G37Ras mutant also activates PI3K (25). We show here that hCNK1 can associate with RalGDS in mammalian cells, supporting a role for it in signaling via this pathway. Ral signaling has been shown to be involved in vesicle trafficking, filopodia formation, transcriptional regulation, and cell transformation (5, 9, 19, 30), and we are currently examining whether hCNK1 is involved in one or more of these processes. Finally, the ability of hCNK1 to associate with signaling components from both Rho and Ras pathways raises the further possibility that it is involved in mediating cross talk between the two. It will be particularly interesting, given the importance of both pathways in cancer cells, to look at the role of hCNK1 in the process of transformation or metastasis.

ACKNOWLEDGMENTS

We thank G. Bokoch, M. Motri, and S. Narumiya for constructs, J. Moran for HeLa cells, M. Motri for advice on luciferase assays and the use of a luminometer, A. Self for advice on dot blots, A. Couve and J. Kittler for advice on GST pulldowns, C. Nobes and A. Schmidt for guidance on injections, N. Billon for discussing RNAi strategies, and the members of the Hall lab for helpful discussion.

This work was generously supported by a program grant from Cancer Research UK. Part of the work reported in this paper was undertaken during the tenure of a Research Training Fellowship awarded by the International Agency for Research on Cancer to A.B.J.

REFERENCES

1. **Anselmo, A. N., R. Bumeister, J. M. Thomas, and M. A. White.** 2002. Critical contribution of linker proteins to Raf kinase activation. J. Biol. Chem. **277:**5940–5943.
2. **Bishop, A. L., and A. Hall.** 2000. Rho GTPases and their effector proteins. Biochem. J. **348**(Part 2)**:**241–255.
3. **Burbelo, P. D., D. Drechsel, and A. Hall.** 1995. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. J. Biol. Chem. **270:**29071–29074.
4. **Charron, F., G. Tsimiklis, M. Arcand, L. Robitaille, Q. Liang, J. D. Molkentin, S. Meloche, and M. Nemer.** 2001. Tissue-specific GATA factors are transcriptional effectors of the small GTPase RhoA. Genes Dev. **15:**2702–2719.
5. **De Ruiter, N. D., B. M. Burgering, and J. L. Bos.** 2001. Regulation of the

Forkhead transcription factor AFX by Ral-dependent phosphorylation of threonines 447 and 451. Mol. Cell. Biol. **21:**8225–8235.
6. **Diekmann, D., and A. Hall.** 1995. In vitro binding assay for interactions of Rho and Rac with GTPase-activating proteins and effectors. Methods Enzymol. **256:**207–215.
7. **Geneste, O., J. W. Copeland, and R. Treisman.** 2002. LIM kinase and Diaphanous cooperate to regulate serum response factor and actin dynamics. J. Cell Biol. **157:**831–838.
8. **Hall, A.** 1998. Rho GTPases and the actin cytoskeleton. Science **279:**509–514.
9. **Hamad, N. M., J. H. Elconin, A. E. Karnoub, W. Bai, J. N. Rich, R. T. Abraham, C. J. Der, and C. M. Counter.** 2002. Distinct requirements for Ras oncogenesis in human versus mouse cells. Genes Dev. **16:**2045–2057.
10. **Jaffe, A. B., and A. Hall.** 2002. Rho GTPases in transformation and metastasis. Adv. Cancer Res. **84:**57–80.
11. **Kikuno, R., T. Nagase, M. Waki, and O. Ohara.** 2002. HUGE: a database for human large proteins identified in the Kazusa cDNA sequencing project. Nucleic Acids Res. **30:**166–168.
12. **Kim, O., J. Yang, and Y. Qiu.** 2002. Selective activation of small GTPase RhoA by tyrosine kinase Etk through its PH domain. J. Biol. Chem. **277:**30066–30071.
13. **Lamarche, N., N. Tapon, L. Stowers, P. D. Burbelo, P. Aspenstrom, T. Bridges, J. Chant, and A. Hall.** 1996. Rac and Cdc42 induce actin polymerization and G_1 cell cycle progression independently of $p65^{PAK}$ and the JNK/SAPK MAP kinase cascade. Cell **87:**519–529.
14. **Mack, C. P., A. V. Somlyo, M. Hautmann, A. P. Somlyo, and G. K. Owens.** 2001. Smooth muscle differentiation marker gene expression is regulated by RhoA-mediated actin polymerization. J. Biol. Chem. **276:**341–347.
15. **Marinissen, M. J., M. Chiariello, and J. S. Gutkind.** 2001. Regulation of gene expression by the small GTPase Rho through the ERK6 (p38 gamma) MAP kinase pathway. Genes Dev. **15:**535–553.
16. **Melchior, F., T. Guan, N. Yokoyama, T. Nishimoto, and L. Gerace.** 1995. GTP hydrolysis by Ran occurs at the nuclear pore complex in an early step of protein import. J. Cell Biol. **131:**571–581.
17. **Miralles, F., G. Posern, A. I. Zaromytidou, and R. Treisman.** 2003. Actin dynamics control SRF activity by regulation of its coactivator MAL. Cell **113:**329–342.
18. **Morrison, D. K.** 2001. KSR: a MAPK scaffold of the Ras pathway? J. Cell Sci. **114:**1609–1612.
19. **Moskalenko, S., D. O. Henry, C. Rosse, G. Mirey, J. H. Camonis, and M. A. White.** 2002. The exocyst is a Ral effector complex. Nat. Cell Biol. **4:**66–72.
20. **Musacchio, A., T. Gibson, P. Rice, J. Thompson, and M. Saraste.** 1993. The PH domain: a common piece in the structural patchwork of signalling proteins. Trends Biochem. Sci. **18:**343–348.
21. **Nobes, C. D., and A. Hall.** 1999. Rho GTPases control polarity, protrusion, and adhesion during cell movement. J. Cell Biol. **144:**1235–1244.
22. **Peck, J. W., M. Oberst, K. B. Bouker, E. Bowden, and P. D. Burbelo.** 2002. The RhoA-binding protein, Rhophilin-2, regulates actin cytoskeleton organization. J. Biol. Chem. **277:**43924–43932.
23. **Pitcher, J. A., K. Touhara, E. S. Payne, and R. J. Lefkowitz.** 1995. Pleckstrin homology domain-mediated membrane association and activation of the beta-adrenergic receptor kinase requires coordinate interaction with G beta gamma subunits and lipid. J. Biol. Chem. **270:**11707–11710.
24. **Ponting, C. P., C. Phillips, K. E. Davies, and D. J. Blake.** 1997. PDZ domains: targeting signalling molecules to sub-membranous sites. Bioessays **19:**469–479.
25. **Prober, D. A., and B. A. Edgar.** 2002. Interactions between Ras1, dMyc, and dPI3K signaling in the developing Drosophila wing. Genes Dev. **16:**2286–2299.
26. **Sahai, E., A. S. Alberts, and R. Treisman.** 1998. RhoA effector mutants reveal distinct effector pathways for cytoskeletal reorganization, SRF activation and transformation. EMBO J. **17:**1350–1361.
27. **Schmidt, A., and A. Hall.** 2002. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev. **16:**1587–1609.
28. **Schultz, J., C. P. Ponting, K. Hofmann, and P. Bork.** 1997. SAM as a protein interaction domain involved in developmental regulation. Protein Sci. **6:**249–253.
29. **Sotiropoulos, A., D. Gineitis, J. Copeland, and R. Treisman.** 1999. Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. Cell **98:**159–169.
30. **Sugihara, K., S. Asano, K. Tanaka, A. Iwamatsu, K. Okawa, and Y. Ohta.** 2002. The exocyst complex binds the small GTPase RalA to mediate filopodia formation. Nat. Cell Biol. **4:**73–78.
31. **Sundaram, M., and M. Han.** 1995. The C. elegans ksr-1 gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. Cell **83:**889–901.
32. **Tapon, N., K. Nagata, N. Lamarche, and A. Hall.** 1998. A new rac target POSH is an SH3-containing scaffold protein involved in the JNK and NF-kappaB signalling pathways. EMBO J. **17:**1395–1404.
33. **Therrien, M., H. C. Chang, N. M. Solomon, F. D. Karim, D. A. Wassarman, and G. M. Rubin.** 1995. KSR, a novel protein kinase required for RAS signal transduction. Cell **83:**879–888.
34. **Therrien, M., A. M. Wong, E. Kwan, and G. M. Rubin.** 1999. Functional analysis of CNK in RAS signaling. Proc. Natl. Acad. Sci. USA **96:**13259–13263.
35. **Therrien, M., A. M. Wong, and G. M. Rubin.** 1998. CNK, a RAF-binding multidomain protein required for RAS signaling. Cell **95:**343–353.
36. **Tominaga, T., E. Sahai, P. Chardin, F. McCormick, S. A. Courtneidge, and A. S. Alberts.** 2000. Diaphanous-related formins bridge Rho GTPase and Src tyrosine kinase signaling. Mol. Cell **5:**13–25.
37. **Treisman, R., A. S. Alberts, and E. Sahai.** 1998. Regulation of SRF activity by Rho family GTPases. Cold Spring Harb. Symp. Quant. Biol. **63:**643–651.
38. **Uehata, M., T. Ishizaki, H. Satoh, T. Ono, T. Kawahara, T. Morishita, H. Tamakawa, K. Yamagami, J. Inui, M. Maekawa, and S. Narumiya.** 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature **389:**990–994.
39. **Van Aelst, L., and C. D'Souza-Schorey.** 1997. Rho GTPases and signaling networks. Genes Dev. **11:**2295–2322.
40. **Vetter, I. R., C. Nowak, T. Nishimoto, J. Kuhlmann, and A. Wittinghofer.** 1999. Structure of a Ran-binding domain complexed with Ran bound to a GTP analogue: implications for nuclear transport. Nature **398:**39–46.
41. **Watanabe, N., T. Kato, A. Fujita, T. Ishizaki, and S. Narumiya.** 1999. Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. Nat. Cell Biol. **1:**136–143.
42. **Yao, I., Y. Hata, N. Ide, K. Hirao, M. Deguchi, H. Nishioka, A. Mizoguchi, and Y. Takai.** 1999. MAGUIN, a novel neuronal membrane-associated guanylate kinase-interacting protein. J. Biol. Chem. **274:**11889–11896.
43. **Yao, I., T. Ohtsuka, H. Kawabe, Y. Matsuura, Y. Takai, and Y. Hata.** 2000. Association of membrane-associated guanylate kinase-interacting protein-1 with Raf-1. Biochem. Biophys. Res. Commun. **270:**538–542.
44. **Zohar, M., H. Teramoto, B. Z. Katz, K. M. Yamada, and J. S. Gutkind.** 1998. Effector domain mutants of Rho dissociate cytoskeletal changes from nuclear signaling and cellular transformation. Oncogene.**17:**991–998.