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

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Rho 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 connector enhancer of ksr, 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.

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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, pRKS/myc or pRK5/FLAG 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 (pRhs-hCNK1) and pRetro-SUPER-control constructs were generated according to the manufacturer’s recommendations (Oligogene), using the following annealed oligonucleotides: pRhs-hCNK1 (5'-gageccGAGCTGCTGGA ACAGAAATcagagaCTTCCTCCGACAGCTGCTGAAggaag3' and 5'-gcttttc caaaaaAGAGCTGCTGGAACAGAAtctgtcaggACTCTTCCTCCGACAGCT G33gga3') and pRhs-control (5'-gageccCTTCCGAAATCCAGAAtcatgag aCTTCCTCGAGTTTGGAAGTTTggg3') (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-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, 1 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 MgCl2, and 0.1 mM diithiothreitol) and incubated for 5 min at 4°C with [32P]GTP-bound GST protein 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-32P]GTP, and the exchange reaction was stalled by addition of MgCl2 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-32P]GDP), while the other tube was left on ice to predominate [α-32P]GTP. The GST protein aliquots were used in a dot blot assay as described above, using RhoTektin 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 [15S]-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 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) CO2. Transfections were performed using GeneJuice (Novagen) according to manufacturer specifications. For hCNK1 depletion experiments, HeLa cells were transfected with the appropriate construct 24 h prior to transfection and passaged into puromycin-containing medium (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 (ScienCell).

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.

Microinjection and immunofluorescence. HeLa cells were plated onto glass coverslips and serum starved overnight prior to microinjection. Constructs were injected at 0.1 μg/ml 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 hCNK1 interacted with constitutively active RhoGDI as determined by GST pulldown assays (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
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 (CNKΔPH) and a point mutant of hCNK1 which has a W→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 phosphoinositides 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
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.
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).
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. 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 35S-labeled rhophilin. GST fusions, coupled to glutathione-Sepharose beads, of the indicated proteins were incubated with in vitro-synthesized, 35S-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.

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-
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 forRal. 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 P13K (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.

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