CNK1 Is a Scaffold Protein That Regulates Src-mediated Raf-1 Activation*

Algirdas Ziogas, Karin Moelling, and Gerald Radziwill‡

From the Institute of Medical Virology, University of Zurich, Gloriastrasse 30, CH-8006 Zurich, Switzerland

Received for publication, November 26, 2004, and in revised form, April 15, 2005
Published, JBC Papers in Press, April 21, 2005, DOI 10.1074/jbc.M413327200

Raf-1 is a regulator of cellular proliferation, differentiation, and apoptosis. Activation of the Raf-1 kinase activity is tightly regulated and involves targeting to the membrane by Ras and phosphorylation by various kinases, including the tyrosine kinase Src. Here we demonstrate that the connector enhancer of Ksr, CNK1, mediates Src-dependent tyrosine phosphorylation and activation of Raf-1. CNK1 binds preactivated Raf-1 and activated Src and forms a trimeric complex. CNK1 regulates the activation of Raf-1 by Src in a concentration-dependent manner typical for a scaffold protein. Down-regulation of endogenously expressed CNK1 by small inhibitory RNA interferes with Src-dependent activation of ERK. Thus, CNK1 allows cross-talk between Src and Raf-1 and is essential for the full activation of Raf-1.

The serine/threonine kinase Raf plays a key role in many different signaling cascades by transmitting proliferative, developmental, and anti-apoptotic signals from the plasma membrane to the nucleus (1–5). Vertebrate cells express three different Raf proteins, Raf-1, A-Raf, and B-Raf (Fig. 1A). In contrast to vertebrates, flies and worms express only one of the Raf isoforms corresponding to B-Raf. Genetic and biochemical studies have established that Raf kinases couple the small GTPase Ras to the mitogen-activated protein (MAP)3 kinase cascade (4). This cascade is conserved throughout evolution and consists of Raf, the dual specificity kinase MEK, and the MAP kinase ERK. Downstream of this cascade, activated ERK phosphorylates a number of cytoplasmic and nuclear targets (2, 5).

Activation of Raf is a complex process involving protein-protein interactions, lipid interactions, and phosphorylation (6, 7). Upon mitogenic stimulation, Raf is recruited to the plasma membrane by Ras (8, 9). In addition, phosphorylation of Raf is a critical step for Raf activation (10). In the case of Raf-1, negative regulation of the kinase activity involves phosphorylation of Ser-259 by Akt/protein kinase B (11) and of Ser-43 and Ser-233 by protein kinase A (12, 13). Full activation of Raf-1 requires the dephosphorylation of Ser-259 and the phosphorylation of several critical residues at the N-terminal side of the catalytic domain, including the phosphorylation of Ser-338 by p21-activated kinase (PAK) or by uncharacterized Ras-activated kinases and the phosphorylation of Tyr-341 by the non-receptor tyrosine kinase Src (2, 14–16). In addition, the phosphorylation of Raf-1 at Thr-491 and Ser-494 located in the kinase activation loop is necessary for Raf-1 activation (17). In the case of B-Raf there are two aspartic residues at the position equivalent to Tyr-340 and Tyr-341 in Raf-1, which were proposed to mimic Src-dependent phosphorylation (4, 15). In addition, the residue corresponding to the PAK-1 phosphorylation site of Raf-1 is constitutively phosphorylated in the case of B-Raf. These modifications lead to an elevated basal kinase activity of B-Raf compared with Raf-1. The simplified regulation of B-Raf activity may be the reason why a single point mutation can transfer B-Raf in a potent oncoprotein, as was shown recently in human melanomas (18, 19).

Recent studies have established that Raf is part of a large multiprotein complex. This complex includes highly abundant proteins such as 14-3-3 as well as heat shock proteins (20, 21). Apart from these proteins, genetic studies in yeast and invertebrates revealed the important role of scaffold proteins as organizing centers for signal transduction (22). Mammalian scaffold proteins include the positive regulators of the Raf/MEK/ERK pathway MP1 (MEK partner 1) and Ksr (kinase suppressor of Ras) (23, 24).

In flies, genetic screens for mutations that modify a ksr-dependent phenotype identified a novel gene, the connector enhancer of ksr (cnk). CNK is a multidomain protein and acts as regulator of Ras signaling (25) (Fig. 1A). The N-terminal portion of CNK strongly cooperates with RasV12G37, a Ras effector loop mutant known to activate the Raf pathway in mammalian cells. The C-terminal portion of CNK binds to D-Raf and blocks Ras- and Raf-dependent signaling, probably by titrating out specific signal molecules (26, 27). Thus, Drosophila CNK can act on several signaling pathways downstream of Ras and upstream or in parallel to Raf. The importance of CNK in the Ras cascade has been shown in insect cells by a down-regulation of CNK that correlates with the reduction of ERK-dependent insulin stimulation (28).

In contrast to Drosophila CNK, there exist three CNK proteins in mammals, namely CNK1, CNK2A, also designated as MAGUIN-1, and the spliced form CNK2B/MAGUIN-2 (29, 30). CNK2A, the homologue of Drosophila CNK, interacts with the Ras effector proteins Raf and Rlf and supports neuronal differentiation (31–33). Whereas the expression of CNK2A and CNK2B is restricted to brain tissue, CNK1 is expressed ubiquitously. CNK1 interacts with the GTPase Rho, several other proteins of the Rho-dependent c-Jun N-terminal kinase (JNK) pathway, and the Ras effector Raf-GDS (guanine nucleotide dissociation stimulator), thereby integrating different signal transduction pathways (34, 35).

* This work was supported by the Swiss National Fonds, the Swiss Cancer League, and the Krebsliga des Kantons Zürich. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 41-44-634-2787; Fax: 41-44-634-4906; E-mail: radziwill@immv.unizh.ch.

1 The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal regulated kinase; MEK, MAP kinase/ERK kinase; Ksr, kinase suppressor of Ras; CNK, connector enhancer of Ksr; GST, glutathione S-transferase; HA, hemagglutinin; PAK, p21-activated protein kinase; siRNA, small inhibitory RNA; VEGF, vascular endothelial growth factor; ct (suffix), C terminus; nt (suffix), N terminus; wt (suffix), wild-type.
Here we report on the direct interaction between human CNK1 and Raf-1. We demonstrate that CNK1 interacts with preactivated forms of Raf-1. In addition, CNK1 interacts with Src and is phosphorylated on tyrosine residue(s) via the Src kinase. CNK1 regulates the Src-mediated Raf-1 activation in the concentration-dependent manner typical for scaffold proteins. Knock-down of CNK1 by small inhibitory RNA (siRNA) resulted in reduction of vascular endothelial growth factor (VEGF)-dependent ERK stimulation, a pathway known to involve Src as an activator of Raf-1 (36). Thus, CNK1 allows a cross-talk between Src and Raf-1, which is essential for full activation of Raf-1.

MATERIALS AND METHODS

Plasmids—The cDNA of human CNK1 was subcloned from pBS-CNK1 (provided by M. Thérrien and G. M. Rubin) into pDNA3 (Invitrogen). The coding sequences for the HA and FLAG tags were inserted 5' to the start codon. CNKnt contains the sequence 5' to the Aat II restriction site (nucleotide position 959) and CNKct contains the sequence 3' to the Aat II site. Wild-type Src cDNA was purchased from Upstate Biotechnology. The Raf1, MEK, and Ras constructs were described previously (37, 38).

To express siRNA against CNK1 or firefly luciferase (GL2) as control, the following annealed oligonucleotides were cloned into pSUPER (39):

- Gtttttggaaa-3' and 5'-agcttttccaaaaaGTAGTACTGCTAGTCATGGttcaagagaCCATGACTAGTTCGAtctc-3' (the capital letters denote the sequences targeted in the 3' non-coding region of CNK1). The cassette coding for the H1 promoter and the siRNA sequence were subcloned into the self-inactivating retroviral vector pMSCVpuro 33 LTR that was obtained by deletion of the 3' LTR of pMSCVpuro (Clontech). All of the Raf-1 constructs used were described elsewhere (37, 38).

Antibodies—The following antibodies were used: anti-FLAG-M2 and anti-HA (12CA5), anti-FLAG-M2 peroxidase conjugate (Sigma); anti-HA (12CA5), and 5'-agcttttccaaaaaGTAGTACTGCTAGTCATGGttcaagagaCCATGACTAGTTCGAtctc-3' (the capital letters denote the sequences targeted in the 3' non-coding region of CNK1).

RESULTS

CNK1 Interacts with Preactivated Raf-1—Recent genetic studies in Drosophila identified the protein CNK as a novel molecule required for signaling via Ras effector proteins (25). Here we analyzed the role of human CNK1 in the Raf-1 signaling pathway.

First, we tested whether Raf-1 can directly interact with CNK1. HEK293T cells were cotransfected with plasmids encoding HA-tagged CNK and different FLAG-tagged Raf constructs (see Fig. 1A). Immunoprecipitates of Raf proteins were probed with the anti-HA antibody to demonstrate binding of CNK to Raf (Fig. 1B, top). The interaction between the wild-type proteins of Raf and CNK was detectable but weak. Coexpression of activated RasV12 enhanced the association between the two proteins, indicating that Raf-1 preactivated by RasV12 undergoes a conformational change that improves its binding to CNK1. The N terminus of Raf (RafNt) and the C terminus of Raf (Rafct) bound equally well to CNK1, indicating that there are two binding sites for CNK on Raf-1. Coexpression of RasV12 did not affect either one of these interactions. To mimic the activation of Raf-1, we analyzed the constitutively active Raf mutants Raf-S259A and Raf-Y340D (41) (Fig. 1B, bottom section). These mutants interacted with CNK without the help of RasV12.

Because B-Raf, the human homologue of Drosophila D-Raf, has an elevated basal kinase activity, we include this Raf protein in our study. In contrast to Raf-1, B-Raf bound to CNK1 without the coexpression of activated Ras (Fig. 1C). The interaction between CNK1 and B-Raf was preferentially exerted via the C terminus of CNK1 (Fig. 1C).

To support the biological relevance of the interaction between CNK1 and Raf-1, we analyzed endogenously expressed proteins. Expression of CNK1 was well detectable in different epithelial cell lines such as HEp2, MCf10A, and HeLa, and lower levels were present in HEK293 cells. Coexpression of endogenously expressed CNK1 and Raf-1 was well detectable in epithelial cells, but only after the coexpression of activated Ras (Fig. 1D). Replacing RasV12-dependent stimulation of cells by mitogens did not result in a detectable association between Raf1 and CNK1 (data not shown), perhaps due to the activation of only a small subpopulation of the cytosolic pool of Raf-1.
These data show that the interaction between CNK1 and Raf-1 depends on the activation state of the cells.

Dimerization of CNK1 Is Induced by Activation of the MAP Kinase Pathway—Genetic and biochemical data showed that scaffold proteins like Ste5 in yeast or the Jun-interacting protein JIP in mammals can form dimers or even oligomers, which are important for exerting their biological function (42, 43). To analyze whether the multidomain protein CNK1 can form dimers or oligomers, two differentially tagged CNK proteins, HA-CNKwt and FLAG-CNKwt, were coexpressed in HEK293T cells. This assay might lead to an underestimation of complex formation, because FLAG-CNK/HA-CNK heterodimers, but not homodimers, can be identified under these conditions. In the first set of the experiment the dimerization of CNK proteins was only weakly detectable. However, coexpression of activators of the MAP kinase pathway such as RasV12 or the constitutively active MEK mutant MEK-Glu-217/Glu-221 (MEK-EE in Fig. 2) strongly induced dimerization of the CNK proteins (2). CNK1 is phosphorylated through the MEK pathway, which may trigger CNK1 dimerization.2 MEK-dependent phosphorylation has been shown also for CNK2 (33). Transient activation of the MAP kinase pathway by treatment of cells with epidermal growth factor or phorbol 12-myristate 13-acetate did not induce dimerization of CNK proteins (data not shown). This result indicates that strong or prolonged activa-

2 A. Ziogas, K. Moelling, and G. Radziwill, unpublished observation.
tion of the MAP kinase pathway is necessary to trigger formation of CNK dimers or oligomers.

**CNK1 Interacts with Src**—The primary structure of CNK1 predicts a multidomain protein without a catalytic function, suggesting a function as a scaffold protein, e.g. for the MAP kinase pathway. To test this possibility, we searched for interacting partners of CNK1. Whereas the downstream effectors of Raf-1, MEK, and ERK did not bind, the upstream activator of Raf-1, Src, associated with HA-CNkwt (Fig. 3, top section). This interaction depended on the N-terminal part of CNK, because CNKwt and CNKnt, but not CNKct, retained interaction with Src. Src also induced phosphorylation of CNK1 on tyrosine residues, but, in contrast to binding, phosphorylation of CNK1 took place at its C terminus (Fig. 3, second section from top). A kinase-defective Src mutant, used as control, did not lead to tyrosine phosphorylation of CNK1 (data not shown). These results identified Src as a novel binding partner for CNK1, which could act thereby as an additional scaffold in Raf signaling.

**CNK1 Simultaneously Binds to Raf-1 and Src**—The maximal activation of Raf-1 requires cooperation of the Ras-dependent recruitment to the plasma membrane and the Src-dependent phosphorylation on tyrosine residues. Our interaction studies indicate that CNK might function as a scaffold protein for Raf-1 and Src. Therefore, we tested to determine whether CNK1 could bind Raf-1 and Src simultaneously in a trimeric complex. FLAG-Rafwt was coexpressed with Src-wt in the presence or absence of HA-tagged CNKwt. Raf was immunoprecipitated with the anti-FLAG antibody, and the immunoprecipitates were eluted from the antibody with the FLAG peptide (Fig. 4, top section). Eluates, which contained equal amounts of Raf, were immunoprecipitated with the anti-HA antibody and immunoblotted with the anti-Src antibody. These data suggested a function as a scaffold in Raf signaling.

**CNK1 Regulates Src-mediated Raf-1 Activation**—One function of scaffold proteins is to organize and thereby regulate signaling pathways. Therefore, we analyzed the effect of CNK1 on Raf-1 activation and Raf-1-dependent stimulation of MEK and ERK. Ectopic expression of CNK1 had no effect on the basal kinase activity of Raf-1, MEK, or ERK (data not shown). However, CNK1 interfered with the Src-dependent activation of Raf-1.

Constant amounts of Src and FLAG-Rafwt were coexpressed with an increasing amount of HA-CNkwt in HEK293T cells (Fig. 5A). Without coexpression of HA-CNkwt, Src induced a weak tyrosine phosphorylation of Raf. Coexpression of a low amount of HA-CNkwt increased tyrosine phosphorylation of Raf-1. Higher levels of HA-CNkwt led to the opposite effect, the loss of Src-dependent Raf-1 phosphorylation. ERK phosphorylation behaved similarly to Raf-1 tyrosine phosphorylation. Low levels of CNKwt increased ERK phosphorylation, whereas higher levels of CNKwt decreased it.
In addition, large amounts of CNKnt, but not CNKct, significantly decreased Src-dependent Raf activation (compare 0.5 μg and 2.5 μg of DNA), probably because CNKnt, but not CNKct, titrate out Src, which binds just to the N-terminal part of CNK1 (Fig. 3).

These results indicate that CNK1 regulates the Src-dependent activation of Raf-1 only under optimized conditions. Too low or too high amounts of CNK1 prevented the formation of the trimeric complex and promoted dimerization between CNK1 and Src or Raf-1, thereby preventing activation of Raf-1.

Down-regulation of CNK1 Impairs VEGF-dependent ERK Phosphorylation—We showed that CNK1 transmits the signal from active Src to the Raf pathway. The growth factor VEGF specifically induces tyrosine phosphorylation and activation of Raf-1 via Src (36). This pathway results in ERK stimulation and protects cells from apoptosis. Because CNK1 is well expressed in epithelial cells and because the expression of VEGF receptors is not restricted to endothelial cells (44, 45), we have chosen epithelial cells to analyze a putative function of CNK1 in VEGF-dependent signaling.

To knock-down CNK1 in Hep2 cells, we stably expressed siRNA specifically targeting CNK1 (Fig. 6, top section). For the control, siRNA against firefly luciferase (GL2) was used. VEGF stimulated ERK phosphorylation in control cells, but this stimulation was impaired in CNK1-depleted cells (Fig. 6, second section from the top, compare third and fourth lanes from the left). As expected, Src mediated VEGF-dependent stimulation of ERK, because pretreatment of cells with the Src inhibitor PP2 reduced ERK phosphorylation (Fig. 6, second section from the top, compare third and fifth lanes from the left). As shown in Fig. 5A, tyrosine phosphorylation and activation of Raf-1 by Src is modulated by the amount of the scaffold protein CNK1. In CNK1-depleted cells there is no VEGF-induced tyrosine phosphorylation of endogenous Raf-1 detectable, whereas this is the case in the control cells (Fig. 6, bottom two sections).

DISCUSSION

Raf-1 activation is a multistep process. It involves recruitment of Raf-1 to the plasma membrane by active Ras-GTP and the phosphorylation of Raf-1 at Ser-338 by Ras-activated kinases (15, 16). Full activation of Raf-1 also requires tyrosine phosphorylation of endogenous Raf-1 detectable, whereas this is the case in the control cells (Fig. 6, bottom two sections). These data show that CNK is involved in VEGF-dependent stimulation of ERK, a pathway mediated by Src and Raf-1.
phosphorylation of Raf-1 at Tyr-341. This is mediated by Src or Src-like kinases (15, 46). Activated Raf-1 can transmit the signal to MEK, ERK, and downstream effectors. This process is coordinated by two known scaffold proteins, Ksr and MP-1 (MEK partner 1), which connect MEK and ERK by directly binding to both of them (23, 24, 47). Here, we describe the multidomain protein CNK1 as a scaffold that connects Src and Raf-1 and thereby allows Src-dependent activation of Raf-1.

We demonstrate that CNK1 binds to preactivated Raf-1. Wild-type Raf-1 bound only weakly to CNK1, whereas Raf-1 activated by Ras bound strongly to CNK1. A preactivated state as a requirement for binding may explain why, in former studies, an interaction between ectopically expressed Raf-1 and CNK1 has not been detected (25). The biological relevance of the interaction between Raf-1 and CNK1 is supported by coimmunoprecipitation of both proteins endogenously expressed in epithelial cells. Also, under these conditions the complex formation between Raf-1 and CNK1 is strengthened by activation of the cells (Fig. 1D).

Raf-1 mutants that have an elevated basal kinase activity also showed an increased binding to CNK1. The mutant Raf-S259A does not bind 14-3-3 proteins at its N terminus and therefore lacks negative regulation of its kinase activity (41). This mutation leads to activation similar to that induced by Ras, which also displaces 14-3-3 from binding to Raf Ser-259. In both cases, a conformational change and pre-activation of Raf-1 is induced, which is a prerequisite for the binding of Raf-1 to CNK1.

The mutant Raf-Y340D, in which one tyrosine residue is substituted by the negatively charged aspartic acid, mimics Src-dependent activation (46). This mutant resembles B-Raf and Drosophila D-Raf, both of which contain two aspartic amino acid residues at the positions equivalent to Tyr-340 and Tyr-341 of Raf-1. These Raf proteins no longer require activation by Src but exhibit a constitutive elevated basal kinase activity. D-Raf has been shown to bind to the Drosophila-specific CNK in insect cells (25). This finding is in agreement with the result described here demonstrating that B-Raf, the homologue of D-Raf, can bind to mammalian CNK1. This interaction is not further influenced by coexpression of activated Ras, showing once more that pre-activation of Raf allows an efficient interaction with CNK1.

Preactivation of Raf-1 may depend on a conformational change induced by binding to activated Ras or by phosphorylation via Ras-activated kinases. Association of CNK1 with the kinase-defective mutant of Raf, RafK375E, and the mutant RafR89L, impaired for binding to active Ras, can be induced by coexpression of RasV12. This observation indicates that Ras-activated kinases, known to phosphorylate Ser-338 and possibly also Thr-491 and/or Ser-494 in the kinase activation loop of Raf-1, could be responsible for the postulated conformational change (16).

The binding region of Raf to mammalian CNK proteins is localized at the C-terminal part of CNK1 but is not further characterized. In Drosophila, D-CNK interacts via its C-terminal Raf-interacting and Raf inhibitory region (RIR) with D-Raf (26). This sequence is unique to D-CNK. In a very recent paper it has been shown that Drosophila Src42, independently of its catalytic function, binds near RIR and thereby counteracts the inhibitory effect of the RIR on D-Raf (48).

Our studies reveal another mechanism for Src activation of Raf-1 via CNK1 that involves a catalytic active Src. The simultaneous binding of active Src and preactivated Raf-1 to CNK1 suggests that CNK1 allows Src to phosphorylate and fully activate Raf-1. Thus, CNK1 acts as a scaffold protein that assembles and coordinates two kinases of a single signaling pathway. Typically, scaffold proteins function in a concentration-dependent manner (49, 50). Consequently increasing amounts of CNK1 first increase and then decrease Src-dependent activation of Raf-1 (Fig. 5A). A similar behavior has been described for other scaffold proteins such as the MEK partner protein MP-1 and Ksr. MP-1 connects MEK1 and ERK1 downstream of Raf-1 (23). Ksr acts as scaffold that connects MEK and ERK with activated Raf-1 and allows Raf-1 to phosphorylate and activate MEK, which, in turn, activates ERK (47, 51). The scaffold protein CNK1 acts even further upstream to activate Raf-1.

The contribution of Ras- and Src-dependent activation of Raf-1 depends on the stimulus and the cell type. It has been well documented for endothelial cells that VEGF signals via Src to Raf/MEK/ERK, whereas the basic fibroblast growth factor (bFGF) mainly involves Ras- and PAK-dependent activation of Raf-1 (36). Both pathways protected cells from apoptosis. The VEGF- and Src-dependent activation of Raf-1 results in phosphorylation of ERK and should involve CNK1 according to the results presented here. Indeed, depletion of CNK1 significantly reduced ERK activation by VEGF, indicating that CNK-dependent steps participate in VEGF-induced signaling. Activation of ERK by the basic fibroblast growth factor was not significantly affected in CNK1-depleted cells (data not shown), suggesting that the Ras- and PAK-dependent activation of the Raf/MEK/ERK pathway is not sensitive to CNK1 under these conditions. This is consistent with a recent observation that CNK1 does not contribute to epithelial growth factor-dependent activation of ERK in epithelial cells (31).

There is growing evidence obtained by knock-out studies that MEK is not the only substrate for Raf-1 activity (52, 53). Raf-1 exerts an anti-apoptotic response by phosphorylating and thereby inactivating the pro-apoptotic Bel-2 family member Bad (54). Recently it has been reported that overexpression of
CNK1 Mediates Raf-1 Activation by Src

CNK1 can support pro-apoptotic signaling under certain conditions, but it is unclear how endogenous levels of CNK1 will behave (55). Therefore, there could be a link between CNK and Raf in the regulation of apoptosis.

Src-dependent phosphorylation and activation is specific for Raf-1, but the binding of CNK1 to both Raf-1 or B-Raf suggests additional functions for the CNK-Raf complex. Recently it has been demonstrated that Raf-1 and B-Raf can heterodimerize (56). Furthermore, it was proposed that B-Raf mutants with impaired kinase activity can still activate ERK by stimulating Raf-1 (19). An intriguing possibility would be that CNK1 participates in Raf-1/B-Raf heterodimerization and Raf-1 activation by B-Raf because it interacts with both Raf family members (see Fig. 1).

As is the case for Raf proteins, CNK1 is cytosolic in unstimulated cells and recruited to the plasma membrane upon stimulation. Therefore, an additional function of a CNK1-Raf complex could be to shuttle activated membrane-bound Raf from the site of stimulation to the site of its substrate. Dimerization of CNK1 as shown here may be involved in this process, although this has still to be demonstrated.

Another function of CNK1 could be to allow a concerted action of active Src and fully activated Raf-1 or B-Raf for dual phosphorylation of downstream targets. A substrate that is phosphorylated at tyrosine residues by Src and on serine residues by the Raf/MEK/ERK pathway is cortactin. Phosphorylation of cortactin is linked to actin organization and cell motility (57). Interestingly, the focal adhesion complex protein paxillin forms a complex with Raf-1, MEK, and ERK to facilitate localized ERK activation (58). Binding and activation of ERK depends on prior tyrosine phosphorylation of paxillin by Src. CNK1 binds to Src and is phosphorylated via Src as shown here (Fig. 3). This tyrosine phosphorylation of CNK1 may create binding sites for substrates of CNK1-associated kinases as described here or for other signaling proteins.

Members of the CNK family are multimodular proteins. Only a few proteins interacting with CNK have been identified. Most of them may be signaling molecules such as GTPases and their effectors and protein kinases as shown here. In addition, CNK2A binds via its C-terminal PDZ domain binding motif to the synaptosomal PDZ proteins PSD-95, SCAMAC (synaptic scaffolding molecule), and densin-180 (29, 30). CNK1 also contains a putative PDZ domain binding motif at its very C-terminal end. Therefore, it would be of interest to know whether CNK1 itself, via its PDZ domain-binding motif, may form a complex with other scaffold proteins, which would open up novel aspects for Src and Raf kinases in such a large signal transduction complex.

Acknowledgments—We thank M. Therrien and G. Rubin for the cDNA of human CNK1, R. Fritz for cloning siRNA expression plasmids, J. Dennler and S. Schiper for excellent technical assistance, and A. Weiss, J. Heinrich, and V. Bruce-Winkler for critical reading of the manuscript.

REFERENCES
1. Moelling, K. (1998) Cell 95, 983–993
2. Schramm, K., Niehof, M., Radziwill, G., Rommel, C., and Moelling, K. (1994) Science 268, 632–638
3. Hallberg, B., Rayter, S. I., and Downward, J. (1994) J. Biol. Chem. 269, 3913–3916
4. Dhillon, A. S., and Kolch, W. (2002) Arch. Biochem. Biophys. 404, 3–9
5. Zimmermann, S., and Moelling, K. (1999) Science 286, 1741–1744
6. Schramm, K., Niehof, M., Radziwill, G., Rommel, C., and Moelling, K. (1994) Cancer Res. 54, 2524–2529
7. O'Neill, E., and Kolch, W. (2004) J. Biol. Chem. 279, 10485–10489
8. Hallberg, B., Rayter, S. I., and Downward, J. (1994) J. Biol. Chem. 269, 3913–3916
9. Baccarini, M. (2002) Cell Death Differ. 9, 783–785
10. Khan, R. R., Daar, I. O., and Morrison, D. K. (1993) Mol. Cell. Biol. 13, 1710–1719
11. Cotran, R. S., Robbins, S. L., and Wolters, J. W. (1999) Am. J. Pathol. 154, 1501–1511
12. Baccarini, M. (2002) Cell Death Differ. 9, 783–785
13. Aitken, A. (1995) Trends Biochem. Sci. 20, 85–97
14. King, A. J., Sun, H., Diaz, B., Barnard, D., Miao, W., Bagrodia, S., and Marais, R. (2004) Mol. Cell. Biol. 24, 454–458