Human homologue of *Drosophila* CNK interacts with Ras effector proteins Raf and Rlf[1]

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ABSTRACT **Connector enhancer of KSR (CNK) is a multidomain protein that participates in Ras signaling in *Drosophila* eye development. In this report we identify the human homologue of CNK, termed CNK2A, and a truncated alternatively spliced variant, CNK2B. We characterize CNK2 phosphorylation, membrane localization, and interaction with Ras effector molecules. Our results show that MAPK signaling appears to play a role in the phosphorylation of CNK2 in vivo. CNK2 is found in both membrane and cytoplasmic fractions of the cell. In MDCK cells, full-length CNK2 is localized to the lateral plasma membrane. Consistent with previous reports, we show CNK2 interacts with Raf. CNK2 interaction was mapped to the regulatory and kinase domains of Raf, as well as to the carboxyl-terminal half of CNK2. CNK2 also interacts with the Ral signaling components, Ral GTPase, and the Ral-GDS family member Rlf. CNK2 interaction was mapped to the GEF domain of Rlf. The ability of CNK2 to interact with both Ras effector proteins Raf and Rlf suggests that CNK2 may integrate signals between MAPK and Ral pathways through a complex interplay of components.—Lanigan, T. M., Liu, A., Huang, Y. Z., Mei, L., Margolis, B., Guan, K.-L. Human homologue of *Drosophila* CNK interacts with Ras effector proteins Raf and Rlf. *FASEB J.* 17, 2048–2060 (2003)**

The small GTPase Ras oncogene is central in integrating and transmitting extracellular signals to a variety of cellular processes such as proliferation, differentiation and survival through downstream signaling pathways (1, 2). Ras controls these pathways by directly interacting with "effector" proteins, which then activate specific downstream pathways. Numerous putative Ras effectors have been identified, but Raf kinase, PI3 kinase, and the family of Ral GTP exchange proteins called RalGDS have been shown to be involved in Ras signaling (1, 2). Raf kinase activates the mitogen-activated protein kinase (MAPK) cascade that controls cell proliferation and differentiation. Genetic studies in *Caenorhabditis elegans* and *Drosophila* show that Raf plays a major role in Ras signaling (3, 4). PI3 kinase seems to mediate some of the Ras-dependent actin cytoskeleton remodeling, protection against apoptosis and oncogenic transformation. Last, RalGDS activates the Ral GTPase protein that regulates multiple pathways including receptor endocytosis, cytoskeletal changes, and DNA synthesis. Recently a number of proteins have been identified by genetic studies that appear to modulate Ras signaling in the cell. These modulators include kinase suppressor of Ras (KSR), suppressor of Ras (SUR-8), and connector enhancer of KSR (CNK). KSR was identified in both *Drosophila* and *C. elegans* as a positive regulator for MAPK signaling (5–7). Epistatic studies place KSR upstream or parallel to Raf. KSR is structurally similar to Raf kinase. Biochemical studies have shown that KSR interacts with components of the MAPK signaling pathway and may act as a scaffolding protein (8–11). Another modulator of Ras signaling is SUR-8, originally identified in *C. elegans*. Epistatic studies placed SUR-8 downstream or parallel to Ras (12). Biochemical studies have shown that mammalian SUR-8 enhances suboptimal stimulation of Ras and interacts with Ras and Raf simultaneously (13). It is possible that KSR and SUR-8 act in tandem to bring Ras and the MAPK signaling components together upon cell stimulation. Biochemical studies have also identified MP-1 as a positive modulator of the MAPK cascade (14). MP1 was shown to interact with MEK and ERK. These data indicate that the MAPK pathway may be modulated by multiple scaffold-like proteins. CNK was identified in *Drosophila* eye development by genetic screens designed to identify genes involved in KSR function (15). A dominant-negative KSR mutant caused a slight rough eye phenotype due to blocking Ras-dependent photoreceptor cell differentiation. A loss-of-function CNK mutation enhanced the rough eye phenotype, suggesting its role as a positive regulator in Ras signaling. The deduced CNK sequence contains several putative protein–protein binding domains but none containing known enzymatic functions. This has led to

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the hypothesis that CNK may function as an adaptor molecule, similar to KSR and SUR-8. Consistent with this notion, down-regulation of CNK1 in *Drosophila* S2 cells leads to decrease of ERK activation (16). Epistatic studies place CNK upstream or parallel to Raf in eye development (15). Overexpression of wild-type CNK enhances signaling of activated Ras, but attenuates activated Raf. The finding that CNK interacts with Raf supports the premise that CNK regulates Raf function in some capacity, but it is likely that CNK acts through additional Ras signaling pathways (15, 17). Furthermore, CNK positively acts in a cooperative fashion with Ras effector loop mutant RasV12G37, which selectively activates the Ral pathway (18), but not with effector loop mutants that activate MAPK or PI3K pathways (18, 19), suggesting that CNK may modulate the Ral pathway in *Drosophila* (17). The rat homologue of CNK, membrane-associated guanylate kinase-interacting protein (MAGUIN-1), was identified in two-hybrid screens using domains of the neuronal membrane-associated guanylate kinase protein synaptic scaffolding molecules (S-SCAM) as bait (20). An alternatively spliced truncated form was identified, named MAGUIN-2, which lacks the carboxyl-terminal region of MAGUIN-1. MAGUIN-1 was shown to have neural-specific expression and to coimmunoprecipitate with postsynaptic density (PSD) 95/synapse-associated protein (SAP) 90 and S-SCAM from rat synaptosomes. MAGUIN-1 was also shown to interact with Raf kinase suggesting a functional homologue of CNK (21). However, MAGUIN-1 did not affect Raf kinase activity or Raf translocation. We identified the human homologue of CNK (termed CNK2A) and an alternatively spliced truncated version, CNK2B. In this report, we characterize CNK2 phosphorylation, membrane localization, and Raf interaction. Overexpression of CNK2 inhibits MAPK signaling. CNK2 interacts with the Ras effector protein Rlf, a RalGDS homologue, and the Ral GTPase protein, both components of the Ral signaling pathway. Overexpression of CNK2 did not significantly activate Ral or cause Rlf to translocate to the membrane. Taken together, these results suggest that CNK2 is a functional homologue of *Drosophila* CNK that interacts with Ras effector proteins. We propose CNK2 may function as an adaptor protein or regulator of multiple Ras signaling pathways.

MATERIALS AND METHODS

Plasmid construction

CNK2 was isolated from a fetal human brain cDNA/lambda ZAPII library (Stratagene, San Diego, CA, USA) using standard library hybridization screening techniques as described in the manual (Stratagene). The human EST R13297, which shares sequence similarity with the *Drosophila* cDNA based on computer search analysis, was used as a probe in the screen. Several overlapping cDNAs were isolated, sequenced, and used to construct the full-length CNK2 cDNA. The full-length CNK2 cDNA was then subcloned into hemagglutinin antigen (HA) -tagged mammalian expression vector pcDNA3 (22) and FLAG-tagged pcDNA3 (23). CNK2-N-term consisting of residues 1 to 405 was subcloned into HA-pcDNA3 and FLAG-pcDNA3 vectors. CNK2A-C-term and CNK2B-C-term consisting of residues 389 to 1034 and 389 to 899, respectively, were subcloned into HA-pcDNA3 and pGEX-KG (24) vectors. HA-Ral (25) and HA-Rlf (26) were generously provided by Dr. J. Bos (Utrecht University). HA-Rlf N-term, HA-Rlf-GN, and HA-Rlf-GEF consisting of residues 1 to 527, 1 to 213, and 218 to 528, respectively, were subcloned into HA-pcDNA3 vector. FLAG-C-Raf was generously provided by Dr. Kevin Pumiglia (University of Michigan), while B-Raf and HA-C-Raf constructs were generously provided by Dr. A. Vojtek (University of Michigan). B-Raf was subsequently cloned into HA-tagged mammalian expression vector pcDNA3. FLAG-C-Raf 1-269 aa and FLAG-C-Raf 325-648 aa were constructed by deleting residues 270-649 and 1-324, respectively, from FLAG-C-Raf. FLAG-p85 and HA-p110 (27) were generously provided by Dr. E. Skolnik (New York University). FLAG-SOS (28), HA-smgGDS, HA-SUR8 (13), and FLAG-FKHR (29) were generously provided by Dr. J. Pessin (University of Iowa), H. Vikis (University of Michigan), Dr. Q. Li (University of Michigan), and E. Tang (University of Michigan), respectively. DNA constructs KRasV12, myc-ERK, Gal4-Elk, Gal4-LUC, myc-KSR, and pCMV-lacZ have been described (22).

Cell culture and transfection

HEK293 and MDCK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL). NIH3T3 cells were cultured the same as HEK293 cells, except 10% calf serum was used in place of FBS. HEK293 cells and NIH3T3 cells were transfected using the LipofectAMINE (Life Technologies, Inc., Grand Island, NY, USA) method and MDCK cells were transfected using Fugene 6 (Roche) method, both as recommended by the manufacturer.

In vitro kinase assays

HEK293 cells were transfected with 100 ng Myc-ERK, 10 ng K-RasV12, or 50–250 ng HA-CNK2B, as indicated. Cells were maintained in 10% FBS medium for 24 h and starved in 0.1% FBS for 15 h. Cells were lysed in RIPA lysis buffer (50 mM tris-HCl pH7.5, 150 mM NaCl, 1% Triton 100×, 0.5% deoxycholate, 1 mM DDT, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin) and Myc-ERK was immunoprecipitated with monoclonal antibody 9E10 (anti-myc, BABCO, Richmond, CA, USA) and protein G-Sepharose. The immunocomplexes were then washed and assayed for 20 min at 30°C in kinase assay buffer containing 10 μCi of [γ-^{32}P]-ATP and 1 μg GST-Elk. GST-Elk were expressed in *Escherichia coli* and purified as described previously (22).

Coimmunoprecipitation and pull-down analysis

For all but the Ral coimmunoprecipitation experiments, HEK293 cells grown in 6-well plates were transfected with 0.5 μg of each plasmid and equalized with empty vector. Forty-eight hours after transfection, cells were treated with 25 ng/mL EGF (where applicable), lysed in NP-40 buffer (10 mM tris-HCl pH7.5, 100 mM NaCl, 1% Nonidet P40, 50 mM NaF, 2 mM EDTA, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin), and immunoprecipitated with either anti-M2 or anti-HA (BABCO) antibodies and protein-G Sepharose beads. Glutathione-coupled beads were used in the pull-down

assay to purify GST fusion protein complexes. In the HA-Ral coimmunoprecipitation experiment, HEK293 cells were transfected with 10 ng K-RasV12, 100 ng HA-Rlf, 250 ng FLAG-CNK2, and 500 ng HA-Ral. In the endogenous Ral coimmunoprecipitation experiment, the brain from a 6-wk-old rat was homogenized in a Dounce homogenizer. In both Ral experiments, the cells were resuspended in lysis buffer (20 mM Tris, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 5% glycerol, 1% Triton 100×, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin) and immunoprecipitated as above using either anti-M2 (Sigma, St. Louis, MO, USA anti-tuberin (Santa Cruz, Santa Cruz, CA, USA), or anti-Maguin N-term (generously provided by Y. Takai) and protein-G Sepharose beads. The complexes were resolved by SDS-PAGE and transferred onto PVDF membranes. The precipitated proteins were detected with anti-HA, 9E10 antibody (anti-myc), anti-MEK (30), anti-GST (Zymed, San Francisco, CA, USA), anti-C-Raf (Transduction Laboratories, Lexington, KY, USA), anti-Ral (Transduction Laboratories), and anti-Maguin N-term antibodies.

Immunoblot analysis with anti-phospho antibodies and fractionation experiments

HEK293 cells were transfected with 0.5 μg of each plasmid unless otherwise noted. In experiments using anti-phospho antibodies, transfected cells were maintained overnight in 10% FBS medium and starved in 0.1% FBS for 3 h prior to cell lysis with RIPA buffer. In some cases, cells were treated with 25 ng/mL EGF, neuregulins, or 0.1 mM pervanadate (2 μL/mL of 50 mM metavanadate, 50 mM H2O2, made fresh) 5–10 min prior to cell lysis. HA-CNK2B and FLAG-ERK were immunoprecipitated as mentioned above, resolved by SDS-PAGE, and transferred to PVDF membranes. Membranes were blotted with anti-tyrosine or an anti-phospho-ERK specific antibody (Promega, Madison, WI, USA). In the fractionation experiment, cells were resuspended in PBS containing 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin and lysed by sonication. The insoluble material was pelleted from cell lysate by centrifugation for 10 min at 8000 × *g* and then membranes were pelleted from lysate by ultracentrifugation for 40 min at 100,000 × *g*. The cytosol was removed and the membranes resuspended in RIPA buffer at equal volume with cytosol. The membranes and cytosol were resolved and blotted as described above.

In vivo ^{32}P-labeling

HEK293 cells were transfected with 0.5 μg HA-CNK2A and 50 ng KRasV12 as indicated; 36 h after transfection, cells were washed twice with phosphate-free medium and incubated with 0.5 mCi/mL ^{32}P-labeled inorganic ^{32}P (ICN, Irvine, CA, USA) at 37°C for 4 h. Cells were treated with 50 μM Mek inhibitor 098059 (Calbiochem, San Diego, CA, USA) or 50 μM PI3K inhibitor ly294002 (Calbiochem) for 30 min prior to cell lysis. Cells were washed with PBS extensively and lysed in RIPA buffer. The HA-CNK2A was immunoprecipitated, resolved on SDS-PAGE and transferred to a membrane. Phosphorylated HA-CNK2B was exposed to a PhosphorImager.

Reporter Analysis

Luciferase reporter analysis was performed as described (22). HEK293 cells were cotransfected with 25 ng Gal4-Elk1, Gal4-luciferase (Gal4-LUC), and pCMV-lacZ, 5 ng KRasV12, 100 ng HA-CNK2B, HA-CNK2-N-term, and HA-smgGDS, or 500 ng HA-CNK2A; 24 h after transfection the cells were harvested and assayed. Luciferase activity was determined and normalized against the cotransfected β-galactosidase activity as described in figure legends and elsewhere (22).

Transformation assays

Cell transformation was assessed by focus formation assay. NIH3T3 mouse fibroblasts were cotransfected with 10–25 ng HRasV12, 50–100 ng pCMV-lacZ gene, and 250–500 ng CNK2 constructs; 24 h post-transfection, cells were trypsinized and 1/10th of the cells were taken for β-galactosidase activity assay to check the transfection efficiency among culture plates. The remaining cells were plated onto 10 cm dishes and maintained in 5% calf serum medium with medium change every 3 days; 14 days later, cells were stained with crystal violets and the number of transformed foci was counted.

Immunofluorescence

MDCK cells were cultured on 60 mm plates and transfected with 3 μg HA-tagged CNK2A, CNK2B, or CNK2 constructs; 48 h post-transfection, cells were trypsinized and plated onto 10 cm dishes and stable cells were selected with media containing 500 μg/mL G418 (Roche). Five days after selection, the cells were cultured on glass coverslips to near confluency. Cells were then washed in PBS, fixed with 4% paraformaldehyde, permeablized with 0.1% Triton 100× in PBS with 2% goat serum, and blocked with goat serum. Cells were incubated with either mouse anti-HA (BABCO) or rat anti-HA (Roche) antibody, followed by incubation with an anti-rat or anti-mouse IgG Texas red conjugated (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). myc-PASL 1 A deletion was generously provided by Dr. B. Margolis (University of Michigan). Myc-PALS A deletion was stained with anti-myc antibody (9E10) and goat anti-mouse IgG-FITC conjugated (Jackson ImmunoResearch Laboratories, Inc.). Fluorescent cells were viewed with an inverted fluorescent microscope (Leica) or imaged using a confocal fluorescent microscope (Noran OZ Confocal Laser Scanning Imaging System).

Ral-GTP pull-down assay

HEK293 cells were cultured in 6-well plates and transfected with 500 ng HA-Ral, 10–250 ng HA-Rlf, 100–500 ng HA-CNK2A, or 50 ng K-RasV12. The cells were harvested and Ral-GTP isolated essentially as described (31). RalBD containing amino acids 397 to 518 of human RLIP76 was obtained using RT-PCR techniques and cloned into pGEX-KG vector (24).

RESULTS

Isolation of human CNK2

Genetic data implicate a role of CNK in Ras signaling. Previous biochemical studies have shown that CNK is a tyrosine phosphorylated, membrane localized protein and can interact with *Drosophila* Raf (D-Raf) (15). Surprisingly, the human homologue of CNK, CNK1, identified by Therrien et al. (15) was not capable of interacting with Raf kinase. This observation leads us to speculate that the isolated human CNKs are not the functional homologue of the *Drosophila* CNK. Using human EST sequences with homology to CNK as probes, we screened a human fetal

brain cDNA library. From these screens we obtained two cDNA clones with homology to CNK, termed CNK2A and CNK2B (**Fig. 1**). The predicted protein product of CNK2A is 1034 amino acids; an alternatively spliced truncated form (CNK2B) consists of 899 amino acids. The putative protein–protein binding domains SAM, CRIC, PDZ, and PH of CNK are all conserved in CNK2 with a higher degree of homology than that of human CNK1 when compared with *Drosophila* CNK (Fig. 1). This observation indicates that CNK2 may be the functional homologue of *Drosophila* CNK. CNK2A has a carboxyl terminus PDZ binding motif that is not present in CNK2B or CNK. We also isolated an alternatively spliced form of CNK2 that is missing part of the PDZ domain (Fig. 1). This predicts that the alternative splicing form of CNK2 has a nonfunctional PDZ domain that cannot interact with target proteins containing PDZ binding sites, therefore suggesting a potentially different function. CNK2A and CNK2B have amino acid sequences nearly identical to rat MAGUIN-1 and MAGUIN-2, respectively. We termed our genes after CNK to keep in step with the previously identified human homologue CNK1.

MAPK pathway dependent phosphorylation of CNK2

We predicted a functional homologue of CNK would be a membrane-bound phosphoprotein capable of interacting with Raf kinase. Therefore, we asked whether CNK2 was phosphorylated in vivo. Since CNK was shown to be tyrosine phosphorylated in a receptor tyrosine kinase-dependent manner (15), we tested CNK2 for tyrosine phosphorylation. HA-CNK2B was expressed in HEK293 cells. Before cell lysis, cells were treated with EGF to stimulate receptor tyrosine kinases or pervanadate to block all tyrosine phosphatases. HA-CNK2B was immunoprecipitated from the cell lysate and immunoblotted with anti-phosphotyrosine (**Fig. 2*A***). We found that tyrosine phosphorylation of CNK2B could be detected only when the cells were pretreated with pervanadate. In the absence of pervanadate, tyrosine phosphorylation was not observed even when cells were treated with EGF, although EGF stimulation of ERK was observed (data not shown).

We also tested CNK2 phosphorylation by an in vivo labeling assay. HA-CNK2A was expressed in HEK293

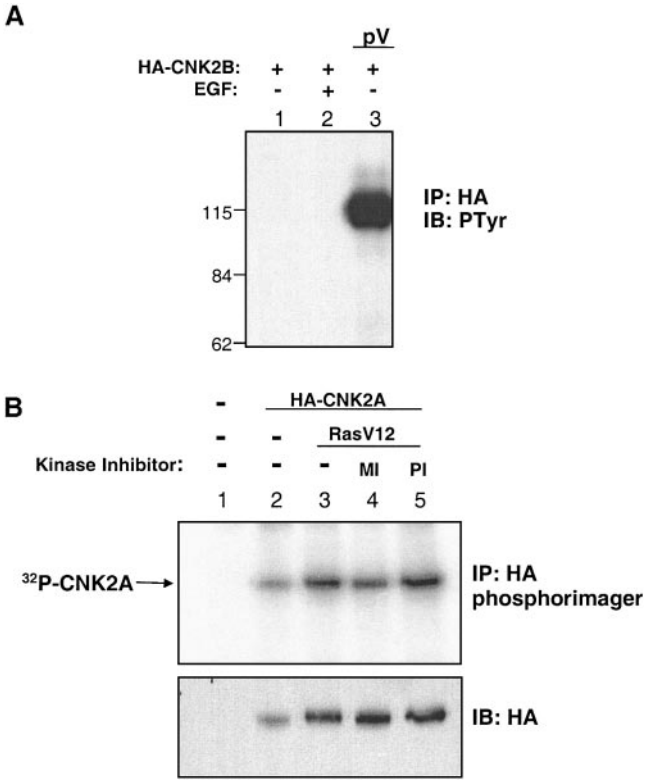

Figure 2. CNK2 phosphorylation in HEK293 cells. *A)* Tyrosine phosphorylation of CNK2B from cells pretreated with pervanadate. HA-CNK2B was expressed for 24 h, then starved in 0.1% FBS for overnight. Before lysis, cells were treated with EGF (25 ng/mL) for 5 min or with pervanadate (pV) for 30 min, as indicated. HA-CNK2B was then immunoprecipitated with anti-HA, resolved by SDS polyacrylamide gel, and immunoblotted with anti-phosphotyrosine. *B)* In vivo phosphorylation of CNK2A by Ras-stimulated signaling pathways. Expression of CNK2A in the presence or absence of RasV12 for 20 h. 4 h before lysis, media was changed to serum-free conditions minus phosphate and supplemented with 0.5 μCi/mL inorganic ^{32}P; 30 min prior to lysis, the cells were treated with MEK inhibitor (MI) or PI3K inhibitor (PI). After lysis, HA-CNK2A was immunoprecipitated and resolved on SDS polyacrylamide gel, blotted, and exposed on a PhosphorImager. The blot was subsequently immunoblotted with anti-HA as shown.

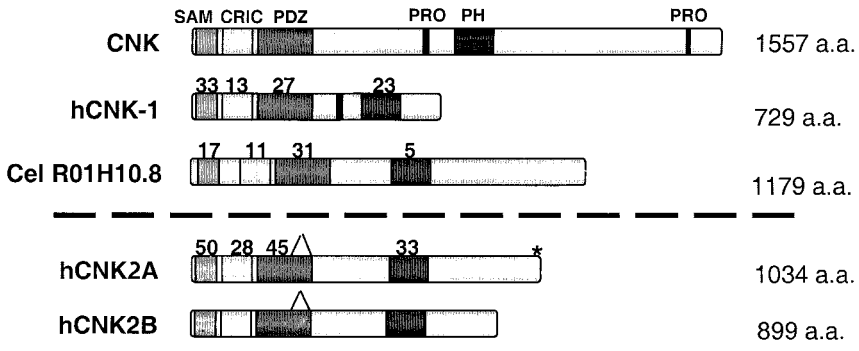

Figure 1. Schematic representation of human CNK2. Homologues of CNK2 have been identified across species from *Drosophila* (CNK), *C. elegans* (Cel R01H10.8), and mammals (hCNK1, CNK2, and MAGUIN; not shown). The numbers of the last amino acid residues are shown on the right. The conserved SAM, CRIC, PDZ, and PH domains are represented with shaded boxes and indicated above. An asterisk represents a PDZ binding site present in CNK2A. Amino acid identity percentage of the conserved domains compared with CNK is shown. Alternatively spliced variants of CNK2 that remove part of the PDZ domain is shown. Accession numbers for CNK2A and CNK2B are AF418269 and AF418270, respectively.

cells in the presence or absence of constitutively active RasV12. The Ras signaling pathways MAPK and PI3K were blocked with the addition of a MEK inhibitor (MI) and a PI3K inhibitor (PI), respectively, prior to cell lysis. HA-CNK2A was immunoprecipitated and incorporation of radiolabeled phosphate shown (Fig. 2*B*, upper panel). As a control, the amount of HA-CNK2A was determined with an immunoblot using anti-HA (Fig. 2*B*, lower panel). It is clear that CNK2A is phosphorylated in vivo. The MEK inhibitor reduced phosphorylation of CNK2A ~twofold when normalized to protein levels (compare lanes 3 and 4). PI3K inhibitor had no significant effect on CNK2A phosphorylation (compare lanes 3 and 5). These results suggest that CNK2 is phosphorylated by some factor downstream of MEK and that the MAP kinase signal transduction pathway plays a role in CNK2 phosphorylation.

CNK2 interacts with Raf but not MEK, KSR, or SUR8

To test whether CNK2 interacts with Raf kinase and other components of Ras signaling, coimmunoprecipitation experiments were performed with proteins known to be involved in the MAPK pathway. HEK293 cells were cotransfected with epitope-tagged CNK2B and either FLAG-C-Raf, MEK, myc-KSR, or HA-SUR8 (**Fig. 3**). Immunoprecipitation of HA-CNK2B was able to copurify FLAG-C-Raf (Fig. 3*A*). Stimulation of the MAPK pathway with EGF does not affect the interaction between HA-CNK2B and FLAG-C-Raf. Similarly, immunoprecipitation of FLAG-C-Raf was able to copurify HA-CNK2B (data not shown). There was no difference detected in the ability of CNK2A or CNK2B to interact with either C-Raf or B-Raf, suggesting a conserved region of interaction between these proteins (data not shown). However, immunoprecipitation of epitope-tagged CNK2B does not appear to copurify MEK, myc-KSR, or HA-SUR8 (Fig. 3*B*). Preliminary data suggest that CNK2B cannot coimmunoprecipitate Ras or ERK (data not shown). These results suggest that CNK2 interaction is specific for Raf kinase.

To map the regions of interaction in C-Raf and CNK2, domains of each protein were expressed and assayed for interaction by coimmunoprecipitation assay in HEK293 cells (**Fig. 4*A***). Immunoprecipitation of HA-CNK2B was able to copurify both FLAG-Raf-1-269 (regulatory domain) and FLAG-Raf-325-648 (kinase domain) (Fig. 4*B*). It was previously reported that MAGUIN-1 interacts with the kinase domain of C-Raf (21). We confirm this observation and extend these findings to show that CNK2 also interacts with the regulatory domain of C-Raf. To further map the regions of interactions, various deletion mutants of the C-Raf regulatory domain were coexpressed with a GST-CNK2B-C-term fusion construct in HEK293 cells and assayed for binding by pull-down experiment (Fig. 4*C*). The regulatory and catalytic domains of C-Raf were pulled down with the carboxyl-terminal part of CNK2B. However, it appears that an intact regulatory domain is required for full interaction with CNK2 (Fig. 4*C*).

Overexpression of CNK2 inhibits MAPK signaling

Using several approaches, we tested whether CNK2 mediates MAPK signaling. Using ERK activity as a readout for Ras signaling, HEK293 cells were transfected with HA-ERK, RasV12 and increasing amounts of

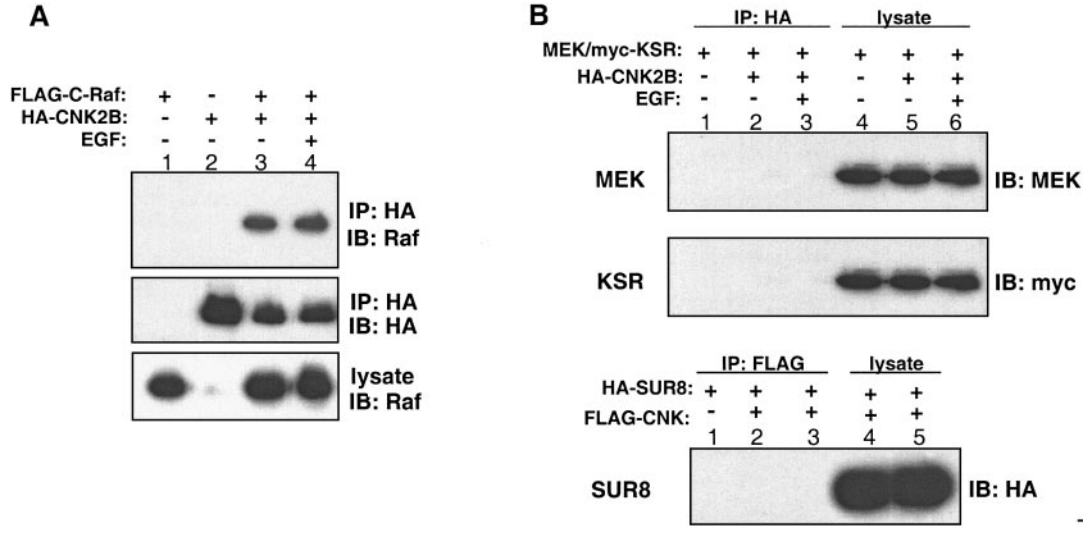

Figure 3. FLAG-C-Raf, but not MEK, myc-KSR, or HA-SUR8 coimmunoprecipitates with CNK2B. *A)* FLAG-C-Raf and HA-CNK2B are expressed either together or independently in HEK293 cells for 48 h and treated with EGF 5 min prior to lysis. HA-CNK2B was immunoprecipitated by anti-HA. The precipitates and cell lysate were resolved by SDS polyacrylamide gel and immunoblotted with anti-FLAG. The immunoblot was then stripped and reprobed with anti-HA. *B)* HA-CNK2B was expressed as in panel *A* with either MEK or myc-KSR. FLAG-CNK2B was expressed with HA-SUR8. CNK2B was immunoprecipitated with anti-HA or anti-FLAG. Precipitates and cell lysates were resolved as in panel *A* and immunoblots were probed with anti-MEK, anti-myc, or anti-HA.

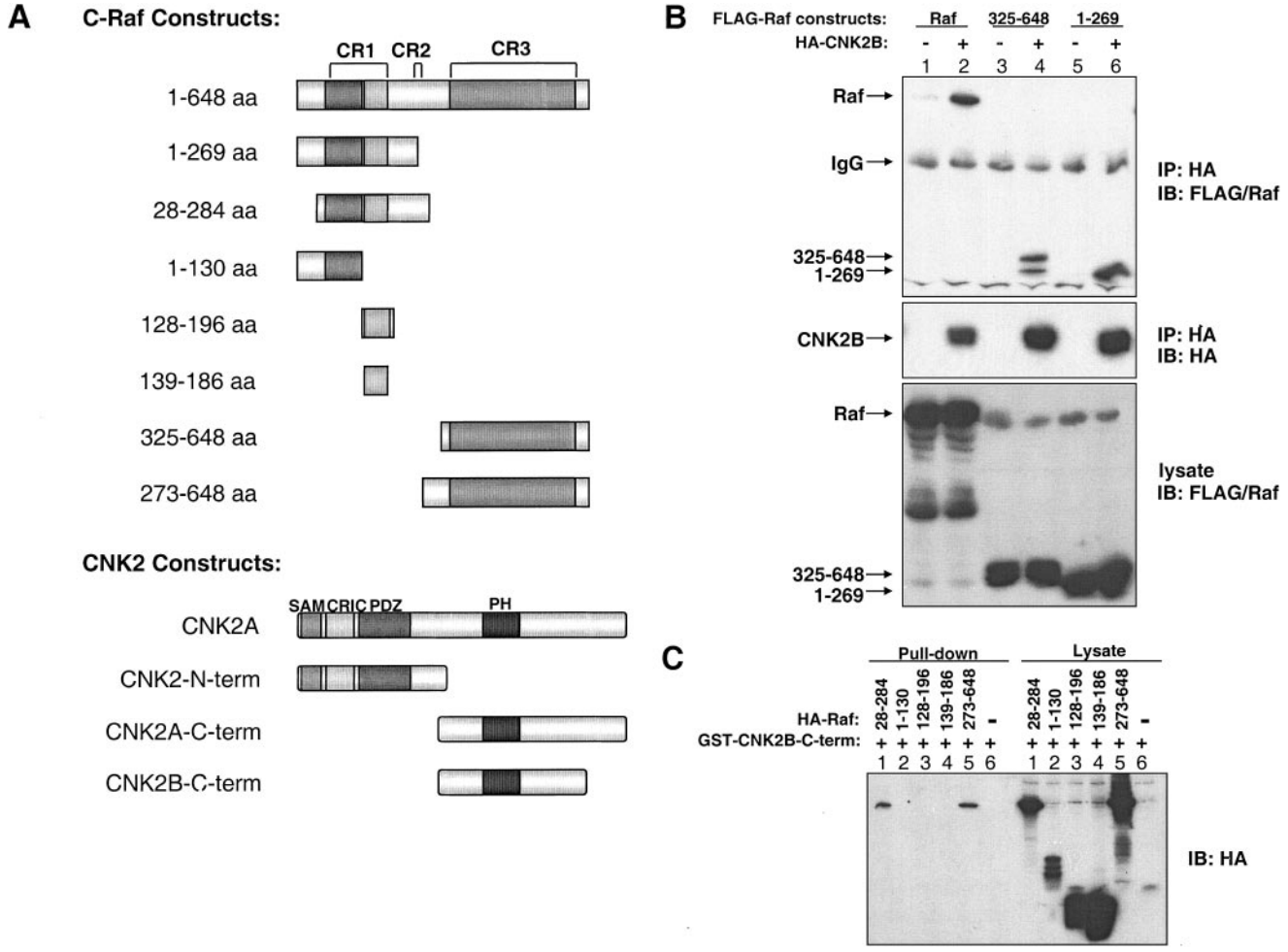

Figure 4. CNK2B-interacting domains of C-Raf. *A)* Schematic representation of the C-Raf and CNK2 deletion mutants. The conserved domains are labeled and indicated by brackets and shaded areas. The amino acids corresponding to each construct is shown on the left for the C-Raf constructs. *B)* Both FLAG-Raf-325-648 and FLAG-Raf-1-269 copurify HA-CNK2B. FLAG-C-Raf, FLAG-Raf-325-648, or FLAG-Raf-1-269 deletion mutants were expressed in the presence or absence of HA-CNK2B in HEK293 cells for 48 h. HA-CNK2B was immunoprecipitated by anti-HA. The precipitates and cell lysis were resolved by SDS polyacrylamide and immunoblotted with anti-FLAG and anti-Raf-1. FLAG-C-Raf and FLAG-Raf-1-269 were immunoblotted with anti-Raf-1 and FLAG-Raf-325-648 with anti-FLAG. The immunoblot was then stripped and probed again with anti-HA. *C)* GST fusion with the carboxyl-terminal construct of CNK2B (GST-CNK2B-C-term) was expressed with various HA-tagged C-Raf constructs as in panel *B*. GST-CNK2B-C-term was pulled down by glutathione-coupled Sepharose beads. The pulled down proteins and cell lysate were resolved and immunoblotted with anti-HA.

FLAG-CNK2B (**Fig. 5*A***). HA-ERK was immunoprecipitated and assayed for activity. Overexpression of CNK2B inhibited ERK activity in HEK293 cells. As an alternative approach, we used ELK phosphorylation and activation as a readout for MAPK pathway signaling. This was accomplished by cotransfecting the Gal4 DNA binding site enhancer fused to the thymidine kinase promoter-luciferase reporter gene (Gal4-luc) and ELK-Gal4 DNA binding domain fusion protein (ELK-Gal4) in the presence or absence of RasV12 and either HA-CNK2 or HA-smgGDS in HEK293 cells (Fig. 5*B*). RasV12-stimulated ELK-Gal4 induced luciferase activity by 12-fold. Overexpression of HA-CNK2A and HA-CNK2B reduced RasV12 stimulation threefold and twofold respectively. Overexpression of HA-CNK2-N-term and an unrelated protein, HA-smgGDS, had no effect on RasV12 stimulation of ELK-Gal4. As a control, a Western blot shows similar amounts of CNK2 proteins and smgGDS (Fig. 5*B*, inset).

To pursue these observations, we asked whether CNK2 could mediate ErbB4-stimulated MAPK signaling. ErbB4 receptor activity is regulated by PSD-95 via a direct interaction in neurons (32). Since MAGUIN-1 was shown to interact with PSD-95 and is expressed primarily in neurons, ErbB4 signaling may present a better model for testing CNK2 function. Therefore, FLAG-ERK1 was cotransfected with ErbB4, PSD-95, HA-CNK2A, and HA-CNK2B as indicated (Fig. 5*C*). ErbB4 receptor was stimulated with addition of neuregulin. Phosphorylation of immunoprecipitated ERK was determined using anti-phospho-ERK antibody. The amount of total ERK protein in the precipitates were determined by Western blotting (Fig. 5*C*, lower panel). Phosphorylated ERK was detected only upon stimula-

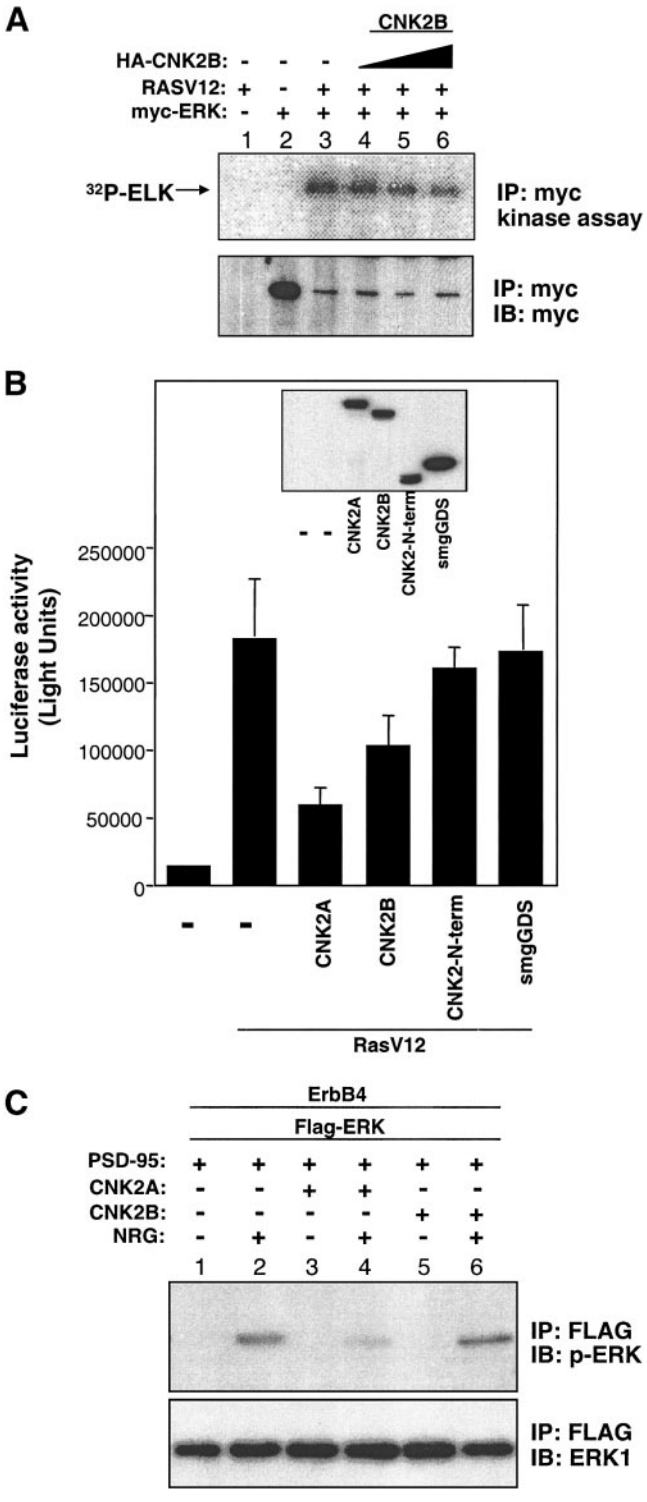

Figure 5. CNK2 inhibits the MAPK signaling pathway. *A)* RasV12-stimulated ERK activity is inhibited in the presence of overexpressed CNK2B. myc-ERK is expressed in the presence or absence of RasV12, increasing concentrations of FLAG-CNK2B (50, 100, and 250 ng) in HEK293 cells for 24 h, and serum starved in 0.1% FBS for 16 h. HA-ERK was immunoprecipitated by anti-HA and assayed for kinase activity using GST-ELK1 as a substrate. Phosphorylation of GST-ELK1 substrate is shown (top panel). The amount of myc-ERK in precipitates were immunoblotted with anti-myc (bottom panel). *B)* RasV12 induced luciferase activity is inhibited by overexpressed CNK2B. Gal4 DNA binding site-TK-luc reporter construct and Gal4 DNA binding domain–ELK fusion construct were cotransfected with RasV12, CNK2 constructs and smgGDS in HEK293 cells in serum-free media for 16 h. Cell extracts were assayed for luciferase and β-galactosidase activity. Transfection efficiencies were normalized with the β-galactosidase activity. Multiple experiments were compared by normalization to the unstimulated reporter. The amount of expression from the CNK2 constructs and smgGDS are shown (inset). *C)* CNK2A, but not CNK2B, inhibits neuregulin (NRG) stimulation of ERK. ErbB4 and FLAG-ERK1 are expressed with PSD-95, HA-CNK2A, or HA-CNK2B. 24 h after transfection, cells were starved with DMEM without serum for 6 h. Before cell lysis the cells were stimulated in the presence or absence of neuregulin (5 nM) for 10 min. FLAG-ERK was immunoprecipitated from cell lysate by anti-FLAG and immunoblotted with an anti-phospho-MAPK antibody. The amount of FLAG-ERK1 present in precipitates were immunoblotted with anti-ERK.

tion with neuregulin, not in the absence of neuregulin. Overexpression of CNK2A, but not CNK2B significantly inhibited ERK phosphorylation. This may be due to the selective ability of CNK2A to interact with PSD-95, as reported for MAGUIN-1 by Yao et al. (21).

To address the biological function of CNK2, NIH3T3 cells were transfected with RasV12 in the presence or absence of CNK2 and assayed for cell transformation (data not shown). Surprisingly, overexpression of CNK2A, CNK2B, or CNK2-N-term did not significantly affect RasV12-induced foci formation. This indicates that ERK activation in NIH3T3 cells is not significantly inhibited by CNK2.

CNK2 interacts with Ras effector proteins Rlf, but not PI3K

Genetic studies in *Drosophila* showed CNK mediates eye development upon stimulation with the Ras effector mutant RasV12G37 (17). It has been shown that RasV12G37 acts by binding to RalGEFs. To test whether CNK2 can interact with proteins in the Ral pathway, coimmunoprecipitation studies were performed. FLAG-CNK2B was expressed in HEK293 cells with HA-Ral in the presence or absence of Ral stimulators RasV12 and HA-Rlf. Immunoprecipitation of FLAG-CNK2B weakly copurified HA-Ral in the presence of RasV12 (**Fig. 6*A***). Due to the weak nature of the interaction, we are unable to determine whether the interaction is dependent on Ras stimulation (compare lanes 2 and 3). However, overexpression HA-Rlf does not appear to affect the amount of HA-Ral pulled down (compare lanes 3 and 4), suggesting that CNK2B-Ral interaction is not mediated by Rlf as a bridge. To further investigate the interaction between CNK2 and Ral, coimmunoprecipitation experiments using rat brain tissue were performed. Immunoprecipitation of endogenous CNK2 copurified endogenous Ral protein (Fig. 6*B*). As a control, anti-Tuberin, which does not immunoprecipitate CNK2 (compare lanes 1 and 3), does not copurify Ral. The anti-maguin N-term antibody should recognize both CNK2A and CNK2B. The

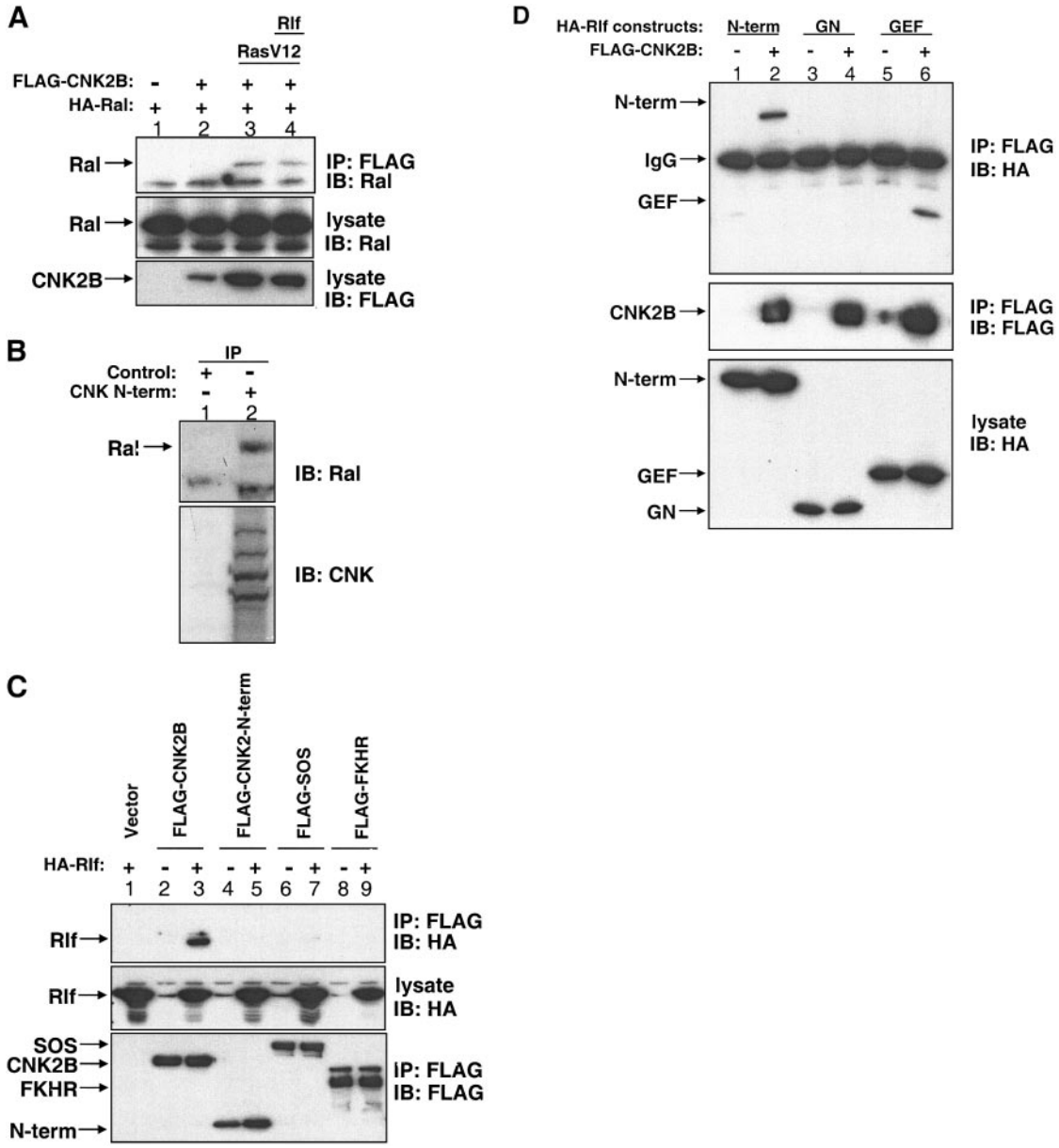

Figure 6. Ral signaling components coimmunoprecipitates with CNK2B and mapping of the CNK2B interaction domain of Rlf. *A)* HA-Ral weakly coimmunoprecipitates with FLAG-CNK2B. HA-Ral was expressed in the presence or absence of FLAG-CNK2B, RasV12, and HA-Rlf in HEK293 cells for 48 h. FLAG-CNK2B was immunoprecipitated with anti-FLAG. Precipitates and cell lysates were resolved by a 15% SDS polyacrylamide gel and immunoblotted with anti-Ral. Cell lysate was resolved on a 10% SDS polyacrylamide gel and immunoblotted with anti-FLAG. *B)* Endogenous CNK2 and Ral coimmunoprecipitate from rat brain lysate. CNK was immunoprecipitated with anti-Maguin N-term, an antibody generated against the mouse homologue of CNK2. The precipitates were resolved by a 15% SDS polyacrylamide gel and immunoblotted with either anti-Ral or anti-Maguin N-term. *C)* HA-Rlf coimmunoprecipitates with FLAG-CNK2B. HA-Rlf was expressed in the presence and absence of FLAG-tagged CNK2B, CNK2-amino-terminal construct (FLAG-N-term), SOS and FKHR as in panel *A*. The FLAG-tagged constructs were immunoprecipitated with anti-FLAG. The precipitates and cell lysates were resolved as in panel *A*, and immunoblotted with anti-HA. The blot was then stripped and reprobed with anti-FLAG. *C)* FLAG-CNK2B interacts with the GEF domain of Rlf. FLAG-CNK2B was expressed in the presence or absence of HA-tagged Rlf constructs as in panel *A*. FLAG-CNK2B was immunoprecipitated by anti-FLAG. The precipitates and cell lysates were resolved and immunoblotted as in panel *B*.

multiple bands of CNK2 immunoprecipitated from brain lysate with anti-Maguin N-term antibody are similar to data described previously for immunoprecipitation with maguin (21) and consistent with alternatively spliced form of CNK2A and CNK2B (Fig. 1). These data suggest the interaction between CNK2 and Ral is physiologically relevant.

Next we asked whether CNK2B interacts with Rlf by coimmunoprecipitation. FLAG-CNK2B was expressed in HEK293 cells in the presence or absence of HA-Rlf. HA-Rlf copurified with immunoprecipitated FLAG-CNK2B but not unrelated proteins FLAG-SOS or FLAG-FKHR (Fig. 6*C*). CNK2 interaction with Rlf was much stronger than with Ral. Neither FLAG-CNK2-N-term

(Fig. 6*C*) nor FLAG-CNK2B-C-term (data not shown) copurified HA-Rlf, suggesting that the full-length molecule may be required. Furthermore, HA-Rlf can interact with both FLAG-CNK2B and FLAG-CNK2A equally well (data not shown). Last, HA-RalGDS, an Rlf homologue, was also shown to copurify with FLAG-CNK2B, suggesting that CNK2 may be important for general Ral signaling (data not shown).

To map the CNK2 region of interaction within Rlf, coimmunoprecipitation studies were performed by expressing deletion mutants of Rlf in the presence or absence of FLAG-CNK2B in HEK293 cells. Immunoprecipitation of FLAG-CNK2B brought down the amino-terminal half of Rlf (HA-Rlf-N-term), which contains the GEF domain (Fig. 6*D*), but not the carboxyl-terminal half (HA-Rlf-C-term) consisting of the Ras binding domain (data not shown). HA-Rlf-N-term was further divided into the GEF domain (HA-Rlf-GEF) and the GEF amino-terminal-associating domain (HA-Rlf-GN). These Rlf constructs were then tested for the ability to interact with CNK2 by coimmunoprecipitation experiments. HA-Rlf-GEF, but not HA-Rlf-GN, copurifies with FLAG-CNK2B (Fig. 6*D*). These data suggest that CNK2B specifically interacts with the GEF domain of Rlf.

Since the Ras effector proteins Raf and Rlf both interact with CNK2B, we tested whether the PI3K subunits p110 and p85 could also interact with CNK2B in coimmunoprecipitation experiments. Neither subunit of PI3K, p85 ,or p110 could copurify with CNK2B (data not shown). Hence, CNK2 can interact with only a specific subset of Ras effector proteins.

CNK2 does not mediate Ral activity

Since CNK2 interacts with Ral signaling proteins, we asked whether CNK2 mediates Ral signaling. HA-Ral was transfected into HEK293 cells in the presence or absence of RasV12, HA-CNK2A, and HA-Rlf (**Fig. 7**). Ral-GTP was pulled out of the cell lysate with glutathione-coupled agarose beads bound to GST-RalBP Ral binding domain (RBD) fusion protein. RalBP-RBD has been shown to specifically interact with activated Ral (33). The amount of activated Ral pulled down was then immunoblotted with anti-Ral. From this assay, it appears that RasV12 can activate Ral only slightly, but that overexpression of HA-Rlf in the presence of RasV12 strongly activates Ral. Expression of HA-CNK2A appears to have little or no effect on Ral activation in the presence of RasV12 regardless of HA-Rlf expression (Fig. 7). Similar results were observed even when the expression level of Rlf was titrated down (data not shown). These results suggest that overexpression of CNK2A does not significantly affect Ral activation in HEK293 cells.

To identify a functional consequence for the CNK2-Rlf interaction, we asked whether CNK2B affected Rlf localization in the cell. Since Rlf translocates from the cytoplasm to the membrane upon activation, we used cell fractionation experiments to test whether CNK2B

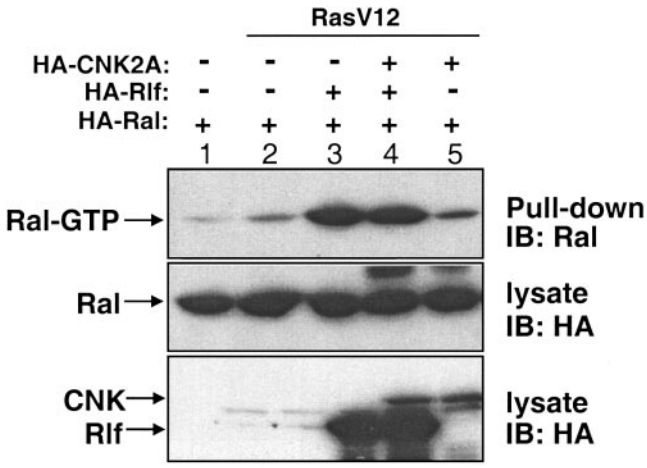

Figure 7. Overexpression of CNK2A does not affect Ral activation. HA-Ral was expressed in the presence or absence of RasV12, HA-Rlf and HA-CNK2A in HEK293 cells for 16 h, then grown-starved in 0.1% serum for 4 h prior to cell lysis. Ral-GTP was pulled down from cell lysate by GST-RalBP-RBD fusion protein immobilized to glutathione-coupled Sepharose. The amount of Ral-GTP in the pull downs were resolved by a 15% SDS polyacrylamide gel and immunoblotted with anti-Ral. Cell lysates were immunoblotted with anti-HA for the presence of expressed HA-Ral, HA-CNK2A, and HA-Rlf.

affects Rlf localization. HA-CNK2B and HA-Rlf were expressed in HEK293 cells both independently and together. The cells were lysed and membranes separated from the cytosol by ultracentrifugation. Cell cytosol and membranes were resolved and immunoblotted with anti-HA (**Fig. 8**). The amount of HA-Rlf in cytosol vs. membrane remains unchanged when coexpressed with HA-CNK2B. Similarly, immunofluorescence experiments suggest that CNK2B or CNK2A do not affect Rlf translocation in MDCK cells (data not shown). Membrane localized HA-CNK2B appears to increase in the presence of HA-Rlf. Overall, these data suggest that CNK2B does not affect Rlf translocation to the membrane but that Rlf may increase CNK2 membrane localization.

Lateral localization of CNK2 in MDCK cells

MAGUIN has been shown to be membrane localized in CHO and COS cells (20, 21). CNK was also membrane localized but with predominate staining at the apical portions of eye imaginal discs in *Drosophila* (15). To assess CNK2 localization in polarized cells, stable cell lines expressing relatively low levels of HA-CNK2 were generated in MDCK cells. MDCK cells are derived from kidney basement membrane cells that express several PDZ-containing proteins. Both HA-CNK2A (**Fig. 9*A***) and HA-CNK2B (data not shown) are localized to the lateral membrane but not at the apical portion of MDCK cells, as determined by immunofluorescence staining. Various HA-CNK2 constructs (see Fig. 1 and Fig. 4*A*) were expressed in MDCK cells to map the membrane localization region of CNK2. HA-CNK2B is membrane localized even though the carboxyl termi-

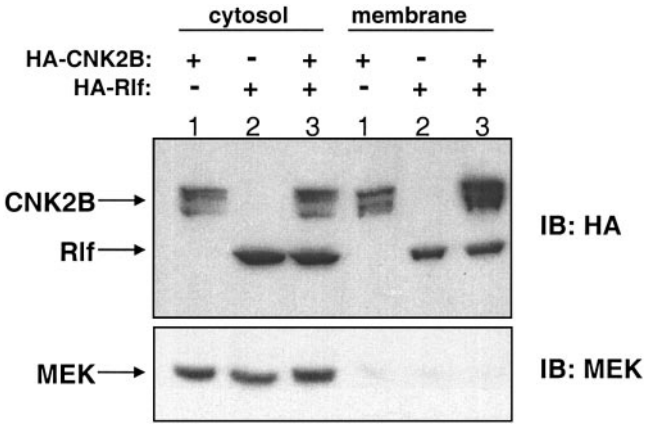

Figure 8. CNK2B does not affect Rlf localization. HA-CNK2B and HA-Rlf were expressed independently and together in HEK293 cells for 20 h. Cell lysates were fractionated by ultracentrifugation, resolved by SDS polyacrylamide gel, and immunoblotted with anti-HA (upper panel). As a control for membrane fractionation, the immunoblot was stripped and reprobed for MEK, a cytosolic localized protein (lower panel).

nus PDZ binding site is not present in HA-CNK2A (compare Fig. 9*B, E*). However, a deletion within the PDZ domain of CNK2 results in more mislocalization of HA-CNK2B than HA-CNK2A, although membrane localization still predominates with both (Fig. 9*C, F*). Surprisingly, the amino-terminal and carboxyl-terminal constructs of CNK2 are mislocalized from the lateral membrane (Fig. 9*D, G–I*). It has been reported that the carboxyl-terminal portion of MAGUIN is responsible for plasma membrane localization in CHO cells (20). Our data suggest that full-length CNK2 is required for proper localization to the membrane. The PDZ binding site and PDZ domain may play some role in membrane localization.

DISCUSSION

We found that CNK2 interacts with downstream effectors of Ras, including Raf and Rlf, but not PI3K. These observations support a model that CNK2 acts as a scaffold protein in Ras signaling. CNK2 interacts with the GEF domain of Rlf and with both the regulatory

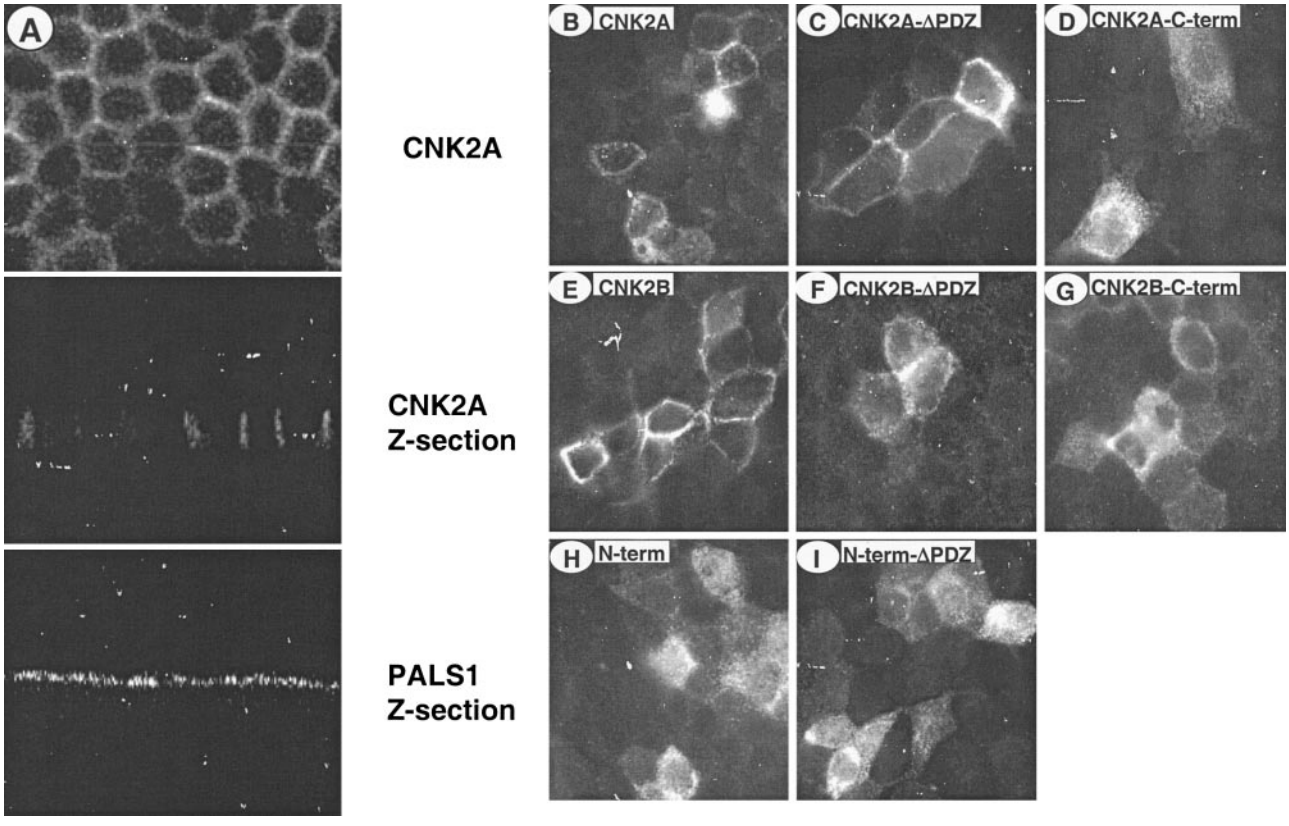

Figure 9. Localization of CNK2 and various constructs of CNK2 in MDCK cells. *A)* Confocal image of immunofluorescence staining of CNK2A. Stable cell line of HA-CNK2A was generated and stained using an anti-HA primary antibody and a Texas red-conjugated secondary antibody. Cross section view (Z-section) of the stained cells show lateral membrane staining (middle panel). Staining of myc-PALS 1 as a control sample showing apical membrane localization (lower panel). myc-PALS 1 was stained with anti-myc antibody (9E10) and an FITC-conjugated secondary antibody. *B–I)* Images of transient expression of HA-CNK2 and various constructs stained with an anti-HA primary antibody and a Texas red-conjugated secondary antibody. Shown are CNK2A (*B*); CNK2B (*E*); the CNK2-N-term (*H*); CNK2A, CNK2B, CNK2-N-term with a portion of the PDZ domain deleted by alternative splicing (*C, F,* and *I*, respectively); and the CNK2A-C-term and CNK2B-C-term (*D* and *G*, respectively). Images were taken with the 100× objective.

and catalytic domains of Raf. The Raf interaction was also mapped to the carboxyl-terminal half of CNK2. Overexpression of CNK2 results in inhibition of the MAPK signaling pathway. CNK2 is also phosphorylated via components of the MAPK signaling pathway. However, CNK2 does not appear to alter the localization of Raf (data not shown; see also ref 21) or Rlf in the cells. Characterization of CNK2 membrane localization, phosphorylation, and Raf interaction indicates that CNK2 is a functional homologue of *Drosophila* CNK and rat MAGUIN.

CNK2 is structurally conserved across species. CNK2 averages a 38% amino acid similarity to CNK across the conserved domains and is nearly identical to the rat MAGUIN-1 overall. The four putative protein–protein binding domains SAM, CRIC, PDZ, and PH are conserved in CNK2 and across species. The carboxyl-terminal region of CNK2, which includes the PH domain, interacts with Raf kinase as does CNK and MAGUIN. MAGUIN was shown to interact with the kinase domain of Raf (21). Similarly, CNK2 interacts with the Raf kinase domain. In addition, we observed that CNK2 interacts with the regulatory domain of Raf. The functional relevance for CNK2 to interact with both the regulatory and catalytic domains of Raf kinase is unclear. Unlike the tyrosine phosphorylation of CNK, tyrosine phosphorylation of CNK2 is detected only in the presence of tyrosine phosphatase inhibitors, suggesting that tyrosine phosphorylation is weak or rapidly turned over under physiological conditions. Furthermore, the MAPK signaling pathway appears to play a role in CNK2 phosphorylation, since MEK inhibitor reduces CNK2 phosphorylation in vivo. The role of phosphorylation on CNK2 function is unknown, but similar observations have been made with KSR, indicating a possible regulatory function. Phosphorylation of KSR by c-TAK results in membrane localization of KSR (34). A CNK loss-of-function allele was shown to contain a point mutation in a putative SH2 domain binding site, suggesting a possible role of phosphorylation in CNK function (15). Taken together, the structural and functional homology of CNK suggest a common role in Ras signaling across species.

Localization to the plasma membrane also appears to be an important characteristic of the CNK homologues. However, the region responsible for membrane localization of CNK2 and MAGUIN is unclear. It was reported that the carboxyl-terminal region (without the PH domain) of MAGUIN was sufficient for localization to the membrane. However, in this report we show that the carboxyl-terminal region (including the PH domain) of CNK2 is mislocalized from the lateral membrane. In fact, our data suggest that full-length CNK2 is required for proper lateral membrane localization, with minor contributions from the PDZ domain and the PDZ binding site in CNK2A. The differences in observations may be due to expression of CNK2 and MAGUIN constructs in different cell lines. The identity of the lateral membrane subcellular site remains to be determined. Based on the immunostaining in Fig. 9, it appears that some of the CNK2 is cytoplasmic and granular suggesting CNK2 may be localized to vesicles or insoluble aggregates. Fractionation and solubilization studies show that CNK2 is soluble, suggesting that CNK2 may be sublocalized to cytoplasmic vesicles (data not shown).

CNK2 interacts with the Ras effector protein Raf kinase. The CNK-Raf interaction appears to be evolutionarily conserved. Mammalian CNK2 interacts with both C-Raf and B-Raf and *Drosophila* CNK interacts with D-Raf. Although the interaction between CNK and Raf has been shown mostly by coimmunoprecipitation experiments, CNK and D-Raf were shown to interact in the yeast two-hybrid system, indicating a direct interaction between the proteins (15). MAGUIN was able to coimmunoprecipitate endogenous Raf from brain tissue, demonstrating the interaction in vivo (21). These observations suggest that CNK2 may play some role in Raf signaling. In agreement with this, overexpression of CNK2 in HEK293 cells inhibits MAPK signaling pathway. This may not reflect the physiological effects of CNK2, since inhibition is seen only with very high levels of CNK2 expression. A similar effect is seen with KSR, in which overexpression inhibits Ras signaling in *Drosophila*, *Xenopus* oocytes, and mammalian cells (22, 35–38), yet is proposed to act positively in the pathway. Similar results have been observed with other known scaffolding proteins. For example, overexpression of JIP, a scaffold in the JNK pathway, can inhibit JNK activation (39, 40), presumably by titrating out components from the pathway. Overexpression of MP1 had little effect on MAPK signaling except under specific conditions of MP1, MEK, and ERK expression (14). Knockdown of KSR or CNK in *Drosophila* S2 cells inhibits MAP kinase activation, indicating that these two scaffold proteins have a positive role in Ras signaling (16, 41). Taken together, these data suggest that CNK2 may play a positive role in MAPK signaling.

Genetic studies have suggested that CNK may modulate an additional Ras signaling pathway, possibly the Ral pathway (17). Biochemically, we showed that CNK2 interacts with Rlf and Ral of the Ral pathway, suggesting CNK2 may mediate both MAPK and Ral signaling in some capacity. Basal levels of MAPK activity have been shown to be required for Ral induction of F9 embryonic carcinoma cell differentiation (42). The mechanism by which MAPK modulates Ral signaling is not known. It is interesting to speculate that CNK2 may be a contributing factor since it appears to be phosphorylated by the MAPK pathway and interacts with Ral signaling components. Although CNK2 did not affect GTP binding of Ral in HEK293 cells, this does not necessarily rule out a role for CNK2 in Ral signaling. Similar to overexpression of scaffold-like proteins KSR, MP1, or CNK2 in MAPK signaling, overexpression of CNK2 may not reveal its physiological role in Ral signaling. Our finding that CNK2 interacts with components of the Ral pathway supports the genetic studies that CNK may positively regulate Ral signaling in *Drosophila* eye development (17). However, it has been

reported that the interplay between the Ras effector pathways in *Drosophila* eye development is more complex. For example, it has been reported that RasV12G37 may actually promote the PI3 kinase pathway (43). In another study, RasV12G37 interacts with RGL (*Drosophila* RalGEF) but does not stimulate the Ral pathway (44). Furthermore, genetic interactions between the Ras and Ral pathways in *Drosophila* do not support a simple linear pathway model (44). In light of these observations, it is interesting that CNK2 interacts with both Raf and Rlf. It is possible that CNK2 may integrate signals between MAPK and Ral pathways in a complex interplay of components. Also, neural-specific expression of CNK2, as determined with MAGUIN-1 (20), suggests a possible mechanism by which ubiquitous pathways elicit cell-specific responses. FJ

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