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Shahrooz Rabizadeh,[a,b,c,d,e] Ramnik J. Xavier,[a,b,d,f] Kazuhiro Ishiguro,[f] Juliocesar Bernabeortiz,[f] Marco Lopez-Ilasaca,[g] Andrei Khokhlatchev,[a] Pamela Mollahan,[c,h] Gerd P. Pfeifer,[i] Joseph Avruch,[a] and Brian Seed,[a,b,j]

From the [a]Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114, [b]Department of Genetics, Harvard Medical School, Boston, Massachusetts 02215, [c]Discovery Translation Unit, The Buck Institute for Age Research, Novato, California 94945, [f]Gastrointestinal Unit, Massachusetts General Hospital, Boston, Massachusetts 02114, [g]Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02115, and [i]Department of Biology, City of Hope Medical Center, Duarte, California 91010

The connector enhancer of KSR (CNK) is a multidomain scaffold protein discovered in *Drosophila*, where it is necessary for Ras activation of the Raf kinase. Recent studies have shown that CNK1 also interacts with RalA and Rho and participates in some aspects of signaling by these GTPases. Herein we demonstrate a novel aspect of CNK1 function, *i.e.* reexpression of CNK1 suppresses tumor cell growth and promotes apoptosis. As shown previously for apoptosis induced by Ki-Ras(G12V), CNK1-induced apoptosis is suppressed by a dominant inhibitor of the mammalian sterile 20 kinases 1 and (MST1/MST2). Immunoprecipitates of MST1 endogenous to LoVo colon cancer cells contain endogenous CNK1; however, no association of these two polypeptides can be detected in a yeast two-hybrid assay. CNK1 does, however, bind directly to the RASSF1A and RASSF1C polypeptides, constitutive binding partners of the MST1/2 kinases. Deletion of the MST1 carboxyl-terminal segment that mediates its binding to RASSF1A/C eliminates the association of MST1 with CNK1. Coexpression of CNK1 with the tumor suppressive isoform, RASSF1A, greatly augments CNK1-induced apoptosis, whereas the nonsuppressive RASSF1C isoform is without effect on CNK1-induced apoptosis. Overexpression of CNK1-(1–282), a fragment that binds RASSF1A but is not proapoptotic, blocks the apoptosis induced by CNK1 and by Ki-Ras(G12V). Thus, in addition to its positive role in the proliferative outputs of active Ras, the CNK1 scaffold protein, through its binding of a RASSF1A·MST complex, also participates in the proapoptotic signaling initiated by active Ras.

CNK was discovered in a screen for modifiers of Ras-dependent photoreceptor development in *Drosophila*. CNK loss-of-function enhances the ability of a dominant-interfering allele of kinase suppressor of Ras to inhibit photoreceptor development and also suppresses the effects of activated alleles of Sevenless and Ras but not Raf (1). Conversely, overexpression of CNK greatly enhances the effect of Ras(G12V) on eye development but suppresses the effect of activated Raf. The latter phenomenon was attributed to sequestration of Raf, which binds directly to a carboxyl-terminal segment of CNK. On this basis, CNK was proposed to function as a positive element in Ras activation of Raf, and subsequent work established that CNK expression is required for Ras activation of Raf kinase in *Drosophila* S2 and mammalian cells (2, 3). Surprisingly, however, the ability of CNK to enhance Ras(G12V) action in eye development was shown to reside in a CNK amino-terminal fragment that lacks the ability to bind Raf and which does not alter Ras-induced mitogen-activated protein kinase activation (4). Moreover, this amino-terminal CNK fragment enhances the action of Ras(G12V,E37G), a Ras effector loop mutant that lacks the ability to activate Raf and mitogen-activated protein kinase (MAPK), whereas CNK does not cooperate with Ras(G12V,T35S), an effector loop mutant that retains the ability to signal via the Raf/MAPK pathway. These findings indicated that the role of CNK in Ras signaling is more complex than simply coupling Ras to Raf and strongly suggested that CNK functions in more than one pathway downstream of Ras.

Drosophila CNK is a 1557-amino acid polypeptide that contains SAM, CRIC, and PDZ domains in its Ras-cooperating amino-terminal segment and a PH domain and several proline-rich motifs in its Raf binding carboxyl-terminal segment. The human genome encodes three CNK homologs (each with splice variants); CNK1 (chromosome 1) was described by Therrien *et al.* (1), whereas CNK2 (also called MAGUIN-1, chromosome X) and CNK3 (chromosome 6) were revealed by SAM and CRIC domain Blast searches of sequences deposited in NCBI databases. Although much shorter than *Drosophila* CNK, human CNK1 (713 amino acids) bears the closest overall similarity to *Drosophila* CNK in domain structure and amino acid sequence; CNK1 also binds c-Raf1 through its kinase domain (data not shown).

While attempting to define the functions of CNK1 in mammalian cells we made the unexpected observation that CNK1 overexpression suppresses tumor cell proliferation by initiating cell death. In view of the participation of *Drosophila* CNK in Ras signaling, we inquired whether the mechanism by which CNK1 promotes apoptosis reflects an interference with an antiapoptotic output of Ras or the recruitment of elements

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[j] To whom correspondence should be addressed: Dept. of Molecular Biology, Massachusetts General Hospital, 50 Blossom St., Boston, MA 02114. Tel.: 617-726-5975; Fax: 617-726-5962; E-mail: seed@molbio.mgh.harvard.edu.

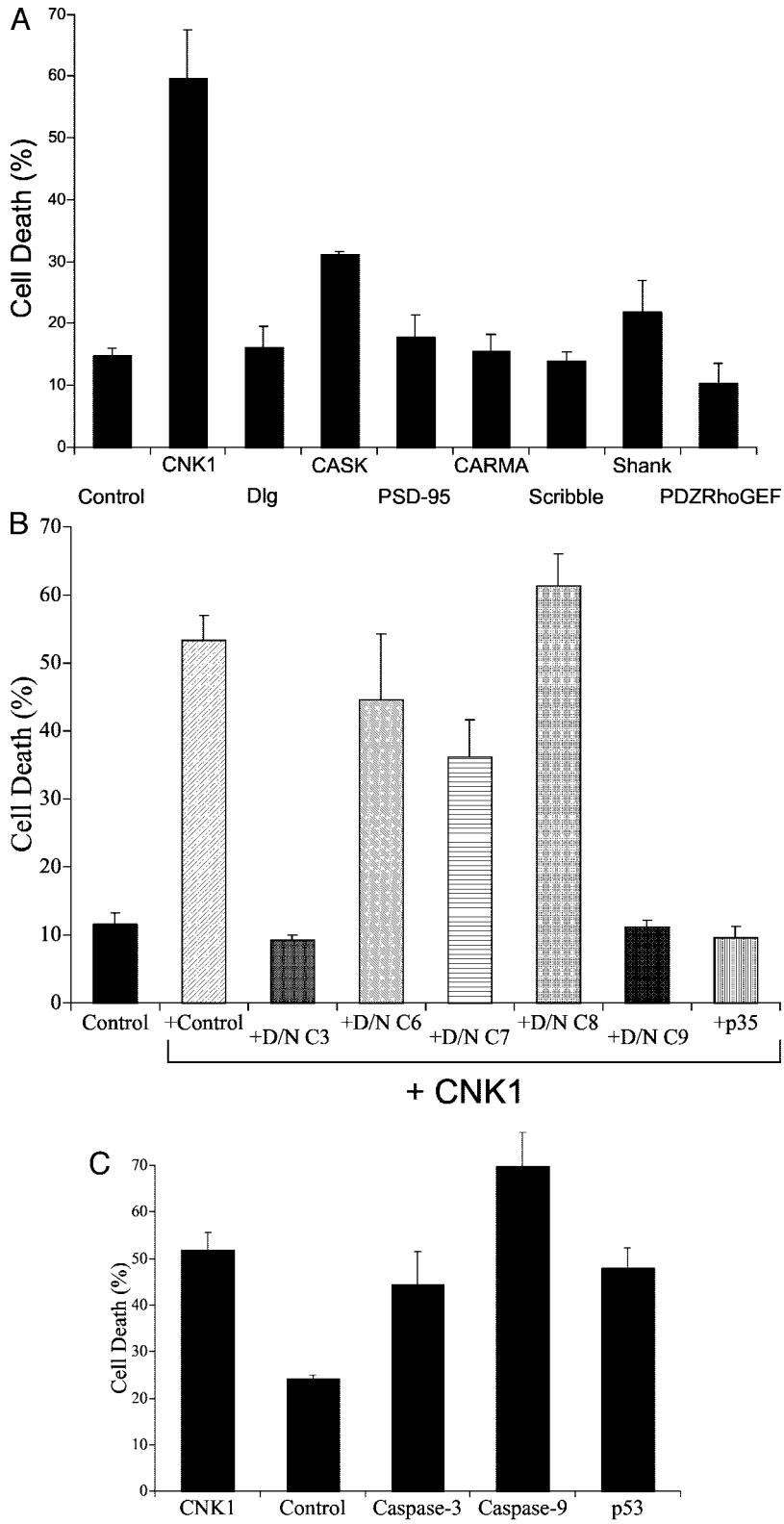


FIG. 1. **Transient expression of CNK1 induces cell death in 293 cells.** *A*, proteins bearing PDZ domains were transiently expressed in 293 cells, and their ability to induce cell death was evaluated by trypan blue exclusion as previously described (7, 24, 25). 293 cells transfected with constructs encoding the PDZ proteins shown were incubated for 4 days before quantitation of cell death by trypan blue exclusion or by determination of free lactate dehydrogenase activity (data not shown). *B*, dominant negative caspases selectively block CNK1-induced death. Coexpression of CNK1 and vector control resulted in cell death after a 4-day incubation. Of the initiator (C8 and C9) and executioner (C3, C6, and C7) caspases, only dominant negative forms of caspases 3 and 9 (and the general caspase inhibitor p35) block CNK1-induced death. Control represents the addition of a vector to compensate for total DNA load. *C*, extent of death induced by CNK1 is comparable in magnitude to that induced by expression of p53 and caspase-3 zymogen. 293 cells transfected with constructs encoding CNK1, caspases 3 and 9, GFP (as control), and p53 were incubated for 4 days after transfection.

that participate in Ras-induced apoptosis. The mechanisms through which Ras induces apoptosis are incompletely understood (for review, see Refs. 5 and 6). We recently described a pathway through which Ki-Ras(G12V) promotes apoptosis by recruitment of the proapoptotic kinase MST1 (7). A fraction of this kinase is found in complexes with the proteins NORE1 and RASSF1. The latter are noncatalytic polypeptides, each expressed as several isoforms that contain variable amino termini but a homologous carboxyl-terminal segment encompassing a Ras association (RA)[1] domain followed by a conserved carboxyl terminus (7–10), recently designated as a SARAH domain (11); the NORE RA domain

[1] The abbreviations used are: RA, Ras association; CMV, cytomegalovirus; PBS, phosphate-buffered saline; RIPA, radioimmune precipitation assay buffer; GFP, green fluorescent protein; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; CNK, connector enhancer of KSR; MST, mammalian sterile 20 kinases.

binds specifically and with high affinity to Ras-GTP (7, 8), whereas the SARAH domain serves as the binding site for MST1 or MST2 (7). As compared with NORE, RASSF1A/C binds with much lower affinity, if at all, to Ras-GTP, but RASSF1A (but not 1C) can heterodimerize with NORE1A (12). The longer splice variants, *i.e.* RASSF1A and NORE1A, function as tumor suppressors. RASSF1 is encoded on chromosome 3p21.3 in a region that is frequently deleted in lung, breast, renal, and other tumors (13–15). Moreover, expression of RASSF1A from the other allele is commonly extinguished by selective hypermethylation of its promoter, whereas expression of the shorter RASSF1C isoform is unimpaired. NORE1 has been identified as the gene inactivated by translocation in a familial renal cell cancer syndrome (16); expression of the longer NORE1A isoform is also extinguished by promoter methylation in many cancers although at a lesser frequency than RASSF1A (17). Reexpression of NORE1 (17, 18) or RASSF1A (9, 20–22) in tumor cells lacking expression inhibits proliferation either through cell cycle delay in G_1 (18, 22) or M (23) or by apoptosis (19). The biochemical mechanisms that underlie the tumor suppressive actions of NORE1 and RASSF1A are incompletely understood; inhibition of anaphase-promoting complex function may be responsible for RASSF1A-induced mitotic delay (23).

The ability of overexpressed Ki-Ras(G12V) to induce apoptosis is strongly suppressed by concomitant overexpression of the MST1 binding segment of NORE1 or the carboxyl-terminal segment of MST1 that binds to NORE1 or RASSF1 (7); thus, Ki-Ras(G12V)-induced apoptosis requires the recruitment of the MST1 (or MST2) kinase. The present data indicate that CNK-induced apoptosis reflects its intersection with this pathway. CNK1 binds RASSF1A (but not NORE1); RASSF1A relocalizes CNK1 to the cell periphery, and although RASSF1A itself provokes little or no apoptosis in human embryonic kidney 293 cells, coexpression with CNK1 substantially augments CNK1-induced cell death. The RASSF1C splice variant also binds both CNK1 and MST1; however, RASSF1C, which lacks tumor suppressive activity, does not augment CNK1-induced cell death. As with Ki-Ras(G12V), CNK1-induced apoptosis is suppressed by concomitant expression of the MST carboxyl-terminal segment. Moreover, an amino-terminal fragment of CNK1 that binds RASSF1A but is not itself proapototic inhibits cell death induced by both CNK1 and by Ki-Ras(G12V). Thus, in addition to its role in Ras activation of Raf, CNK1 participates in an antiproliferative output mediated by the tumor suppressor RASSF1A and the protein kinase MST1 (or MST2).

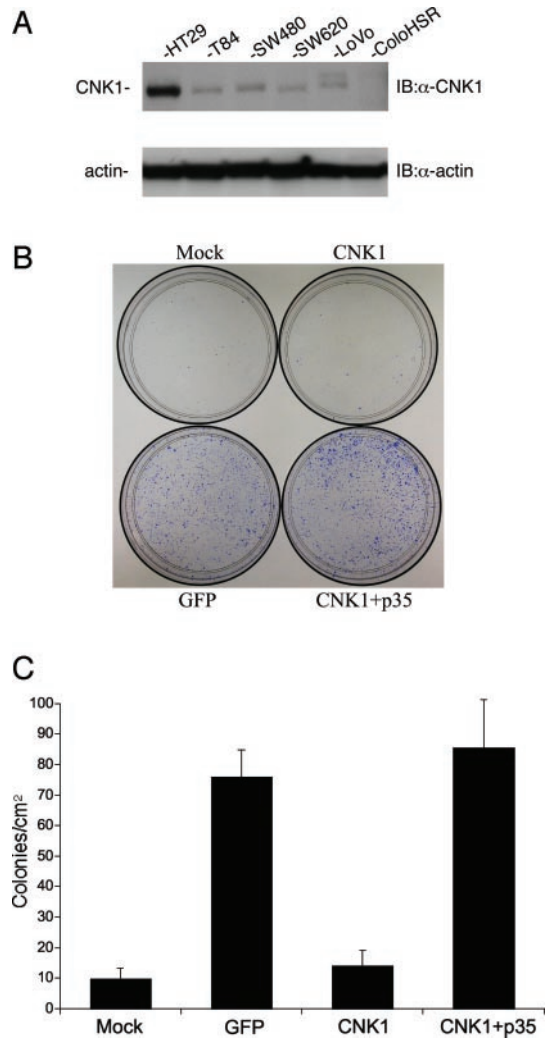


FIG. 2. **Antiproliferative activity of stably expressed CNK1 in human colon carcinoma ColoHSR cells.** *A*, immunoblot analysis of colon carcinoma cell lines shows that ColoHSR cells lack CNK1 expression (*top*). To determine the presence of CNK1 in various colon cancer cell lines, 10^7 cells were lysed in RIPA buffer, resolved by SDS-PAGE, and subjected to immunoblotting (*IB*) with anti-CNK1 antibody (Transduction Laboratories). The *bottom panel* is a lane-loading control. *B*, colony formation by ColoHSR cells transfected with either no DNA (*Mock*), pEAK8-CNK1 (*CNK1*), pEAK8-GFP (*GFP*), or pEAK8-CNK1 plus pCDNA3-p35 (*CNK1+p35*). Selection for CNK1 expression was initiated by exposure of transfected cells to 2 μg/ml puromycin and completed by prolonged exposure to 5 μg/ml puromycin. Established colonies were fixed with 50% methanol, 10% acetic acid, stained with Coomassie Blue, and quantitated as previously described (24). *C*, quantitation of transfected ColoHSR colonies for each culture in *panel B*.

EXPERIMENTAL PROCEDURES

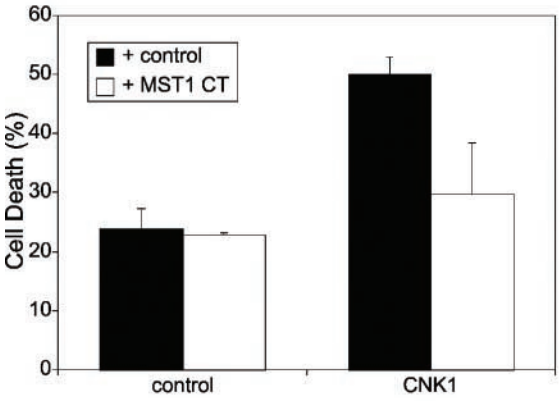
Cell Culture—Human embryonic kidney 293 and colon cancer SW480 cells were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Colon epithelial cancer ColoHSR and LoVo cells were maintained in RPMI 1640 and Ham's F-10, respectively, supplemented with 10% fetal bovine serum.

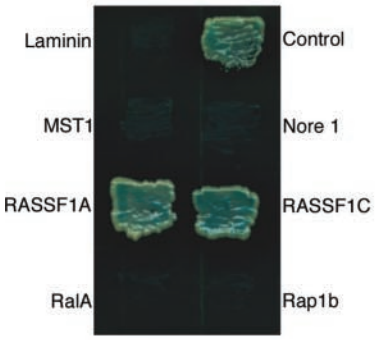
Expression Constructs—CNK1 was isolated via PCR amplification from an EST library (I.M.A.G.E.) as described earlier (1) and was subcloned between NcoI and NotI sites downstream of a Myc epitope tag in the pEAK8 vector. CNK1 was also independently cloned from human breast and prostate cDNA libraries and similarly subcloned into pEAK8 (EF1α promoter) and pcDNA3 (CMV promoter) vectors. CNK1-(1–282) and CNK1-(199–713) were PCR-amplified using CNK1 as a template and similarly cloned into the pEAK8-Myc vector. RASSF1A, RASSF1C, MST1 (and fragments), and MST2 were cloned in pCMV-FLAG as described earlier (10, 15).

Cell Death Assay—Cell death assays were performed as previously described (7, 24, 25). Briefly, 2×10^5 293 cells were seeded in 24-well plates 1 day before transfection. Transfections were performed with 0.5 μg of DNA and 3.5 μl of LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Dead cells were counted via trypan blue exclusion at 96 h after treatment.

ColoHSR cells were seeded at 80% density in 10-cm plates and transfected with 1.5 μg of pEAK8-CNK1, 3 μg of pcDNA3-p35 and 15 μl of LipofectAMINE 2000 (Invitrogen). Upon an initial selection with 2 μg/ml puromycin and prolonged selection with 5 μg/ml puromycin, established colonies were fixed with 50% methanol, 10% acetic acid, stained with Coomassie Blue, and quantitated as previously described (24).

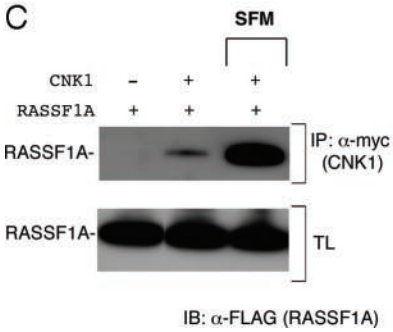
Immunoprecipitations—293 cells were seeded onto 6-well plates and transfected with 2 μg of DNA (FLAG and Myc-tagged) and 15 μl of LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Activation of caspases was inhibited by the addition of 50 μM 1–3-Boc-aspartyl(OMe)-fluoromethyl ketone (Calbiochem). After incubating for 20–24 h, the cells were washed in cold PBS and lysed in 750 μl of RIPA lysis buffer containing 1% Nonidet P-40, 50 mM Tris, pH 7.4, 0.5% deoxycholate, 150 mM NaCl, 0.1% SDS and supplemented with the following protease inhibitors: 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml phenylmethylsulfonyl fluoride. For RASSF1A/CNK1/serum withdrawal studies, transfected cells were washed with serum-free medium 20–24 h after transfection and placed on either Dulbecco's modified Eagle's medium supplemented with 3% fetal bovine serum or serum-free Dulbecco's modified Eagle's medium for 18 h. To inhibit serum

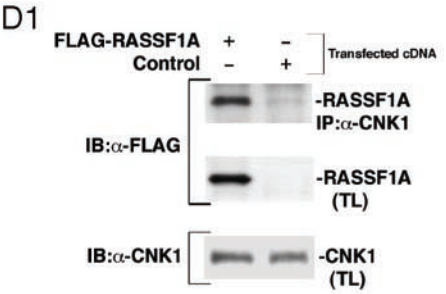


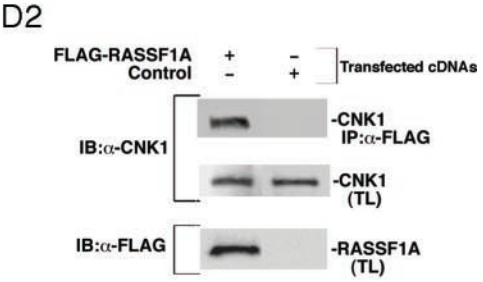


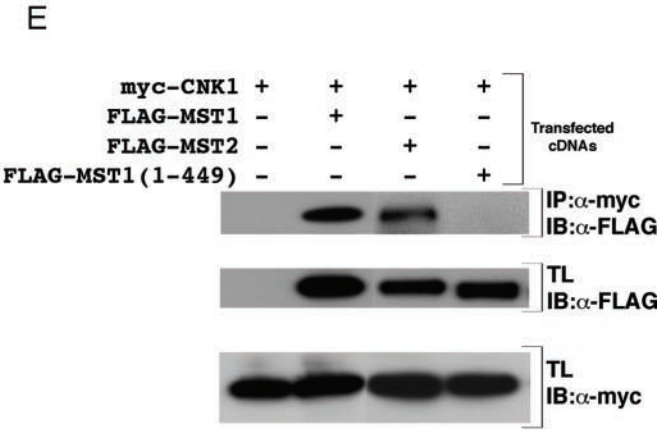
B2

pACT-CNK +	-Trp/-Leu	QDO/α-Gal Assay
pGBKT7 (Gal4BD)-Lam	+	-
pAS-RASSF1A	+	++
pAS-RASSF1C	+	+++
pAS-Nore1 (WT, 1-413)	+	-
pAS-Nore1 (192-413)	+	-
pAS-Rap1b WT	+	-
pAS-RalA WT	+	-









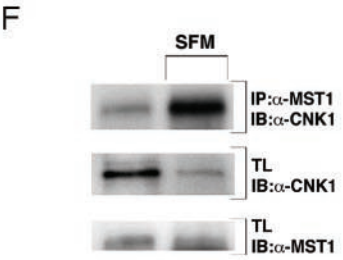


FIG. 3. **CNK1 interacts with RASSF1A/C and the MST kinase, and CNK1-induced cell death requires the MST kinase.** *A*, CNK1-induced cell death is suppressed by coexpression with a dominant negative inhibitor of MST1. Trypan blue exclusion was employed to quantify viability of 293 cells transfected with constructs encoding CNK1 and an inhibitory fragment of MST1 and incubated for 4 days post-transfection. *B*, CNK1 and members of the RASSF1 family directly interact as shown by a yeast two-hybrid analysis. cDNA coding the full-length CNK1 was

withdrawal-induced caspase activation, 50 μM 1–3-Boc-aspartyl(OMe)-fluoromethyl ketone was included in all cultures. Lysates were produced as above. The lysates were subjected to interaction with anti-Myc antibody (Calbiochem), resolved by SDS-PAGE, and immunoblotted with anti-FLAG antibody (Sigma).

The interactions between CNK1 and MST1 in LoVo cells were demonstrated by immunoprecipitating MST1 with anti-MST1 antibody (Zymed Laboratories Inc.). Briefly, LoVo cells were placed in serum-free medium for 24 h and lysed in RIPA buffer as above. Upon immunoprecipitation with anti-MST, the interaction was resolved by SDS-PAGE and immunoblotted with anti-CNK1 antibody (Transduction Laboratories).

To demonstrate the interaction between endogenous CNK1 and transfected RASSF1A, SW480 cells were transfected with FLAG-RASSF1A (1 μg) cDNA using LipofectAMINE 2000 reagent (Invitrogen) as above. Cells were lysed 30 h after transfection in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM, 1% Triton X-100, 2 mM EDTA, 1 mM orthovanadate, protease inhibitor mixture tablet). Lysates were centrifuged at 13,200 rpm for 10 min. Supernatants were incubated with 1 μg of anti-CNK1 monoclonal antibody or anti-FLAG for 2 h at 4 °C. The immune complexes were recovered using goat anti-mouse beads followed by four washes and resolved on SDS-PAGE. To determine the presence of CNK1 in various colon cancer cell lines, 10^7 cells were lysed in RIPA buffer, resolved by SDS-PAGE, and subjected to immunoblotting with anti-CNK1 antibody (Transduction Labs).

Yeast Two-hybrid Interaction Analysis—We used the Matchmaker two-hybrid system (BD Biosciences Clontech, Palo Alto, CA). cDNA coding the full-length CNK1 was cloned in the pACTII plasmid; this recombinant plasmid was transformed into AH109 yeast reporter strain, which was then used as a host cell for sequential co-transformation of the following cDNAs cloned in the pAS vector: NORE1, RASSF1A, RASSF1C, MST1, Rap1b, or RalA. Cotransformants were selected in SD medium lacking leucine, tryptophan, histidine, and adenine (QDO). Yeast α-galactosidase activity, expressed from the *MEL1* gene in response to GAL4 activation, was determined in plates containing X-Gal (2 mg/ml) as a chromogenic substrate.

Immunocytochemistry—Human embryonic kidney 293 cells were transfected with LipofectAMINE 2000 (Invitrogen) and expression constructs as above on cover slips coated with 0.01% w/v poly-L-lysine solution (Sigma-Aldrich) in each well of a 6-well plate. At the time points indicated in the figure legend, the cells were fixed for 10 min at room temperature with 3.5% paraformaldehyde and 0.1% Tween 20 in PBS. The fixed cells were washed with PBS, quenched with 0.1 M NH_4Cl, and washed with PBS again. After blocking with 1% bovine serum albumin-supplemented PBS, the cells were incubated with the primary antibodies; after incubation with fluorescent agent-conjugated secondary antibodies, the cells were exposed to 4,6-diamidino-2-phenylindole HCl to label nuclei. The cover slips were mounted on slides with Aqua Poly/Mount (Polysciences). The samples were then examined by fluorescence microscopy (Olympus).

RESULTS

Transient overexpression of CNK1 in human embryonic kidney 293 cells induces cell death, a response that is not seen with transient expression of a wide selection of other multidomain scaffold proteins (Fig. 1*A*). Cell death induced by CNK1 is comparable in magnitude to that seen with overexpression of p53 or caspase 3 and can be suppressed by coexpression with the baculoviral general caspase inhibitor, p35, or with dominant-interfering mutants of caspase 9 or caspase 3, whereas similar mutants of caspases 6, 7, and 8 are without effect (Fig. 1, *B* and *C*).

Antiproliferative activity of CNK1 can also be seen by inhibition of tumor cell colony formation after stable transfection. Although normally present in the human colon (data not shown), CNK1 is variably expressed by human colon carcinoma cell lines. In one line, ColoHSR, CNK1 was not detectable by immunoblotting (Fig. 2*A*). Transfection of ColoHSR with an expression construct encoding GFP and a selectable marker yielded a large number of stably expressing colonies, whereas transfection of an expression construct encoding CNK1 and the selectable marker yielded a dramatically reduced number of colonies; cotransfection of a gene encoding the baculoviral caspase-inhibitor p35 resulted in a colony density comparable with that found in cells transfected with GFP instead of CNK1 (Fig. 2, *B* and *C*).

The participation of *Drosophila* CNK in Ras signal transduction led us to inquire whether the mechanism of CNK1-induced apoptosis is related to that responsible for Ras(G12V)-induced cell death. We recently showed that Ki-Ras(G12V)-induced apoptosis in 293 cells requires the recruitment of the proapoptotic kinase, MST1 (or MST2) (7, 26). MST1 and MST2 bind through their carboxyl terminus to the proteins of the NORE/RASSF1 family. The latter all share a homologous Ras association domain followed by a conserved carboxyl-terminal tail; MST1 and MST2 bind to this conserved NORE/RASSF1 carboxyl-terminal segment. Ki-Ras(G12V)-induced cell death can be strongly inhibited by coexpression with a carboxyl-terminal noncatalytic fragment of MST1, which binds endogenous NORE/RASSF1 polypeptides (GST-MST1-(456–487)) (7). As shown in Fig. 3*A*, CNK1-induced apoptosis is also strongly inhibited by coexpression with this carboxyl-terminal fragment of MST1, indicating that the recruitment of MST1 or -2 is involved in CNK1-induced apoptosis. We, therefore, examined the ability of CNK1 to interact with MST1/2, NORE, RASSF1, and related polypeptides. In a yeast two-hybrid assay, CNK1 associated with RASSF1A and RASSF1C but not with NORE or MST1(K/R) (Fig. 3*B*). The ability of CNK1 to bind RASSF1A was confirmed by coexpression in 293 cells (Fig. 3*C*); coprecipitation of recombinant CNK1 and RASSF1A is readily observed and is strikingly enhanced after 24 h of serum deprivation (Fig. 3*C*). Similarly, CNK1 endogenous to SW480 colon cancer cells can be coprecipitated with recombinant RASSF1A (Fig. 3*D*). Moreover, although CNK1 does not associate with MST1 in the two-hybrid assay, recombinant CNK1 coprecipitates both

cloned in the pACTII plasmid (Matchmaker two-hybrid system, BD Biosciences); this recombinant plasmid was transformed into AH109 yeast reporter strain, which was then used as a host cell for sequential co-transformation of the following cDNAs cloned in the pAS vector: NORE1, MST1, RASSF1A, RASSF1C, Rap1b, or RalA. Cotransformants were selected in SD medium lacking leucine, tryptophan, histidine, and adenine (QDO). Yeast α-galactosidase activity, expressed from the *MEL1* gene in response to GAL4 activation, was determined in plates containing X-Gal (2 mg/ml) as a chromogenic substrate. The strength of the interactions was evaluated qualitatively as the intensity of the blue color of yeast colonies growing in the selective medium. A semiquantitative estimate of the yeast two-hybrid interactions is shown in tabular form; the intensity of blue color of growing yeast colonies is assessed relative to the interaction strength between p53 and SV40 T antigen, scored as 1+ in this assay. *WT*, wild type. *C*, CNK1 coimmunoprecipitates with RASSF1A. 293 cells were transfected with Myc-CNK1 and FLAG vector or FLAG-RASSF1A; after 24 h some of the cells were transferred to serum-free Dulbecco's modified Eagle's medium for 18 h. Thereafter, the cells were extracted in RIPA buffer, and the lysates were subjected to anti-Myc immunoprecipitation. An anti-FLAG immunoblot (*IB*) of the Myc immunoprecipitation (*IP*) is shown in the *upper panel*, and that of the cell lysates (total lysate (*TL*)) is shown in the *lower panel*. *D*, endogenous CNK1 interacts with recombinant RASSF1A. SW480 cells were transfected with FLAG vector or FLAG-RASSF1A. In the *upper half*, lysates were immunoprecipitated with an antibody raised against endogenous CNK1, and the CNK1 immunoprecipitate was blotted for FLAG; in the *lower half*, an anti-FLAG immunoprecipitate was probed for CNK1. *Upper panel*, proteins retained on goat anti-mouse beads were probed with anti-FLAG antibody. *E*, human embryonic kidney 293 cells were transfected with vector encoding Myc-CNK1 and empty FLAG vector or FLAG-MST1, FLAG-MST2, or FLAG-MST1 (1–449), a mutant lacking the carboxyl-terminal segment 450–487; the latter has been previously shown to be deficient in homodimerization and in binding to NORE1 and RASSF1 (7). A FLAG immunoblot of the Myc immunoprecipitation is shown in the *upper panel*. *F*, endogenous MST1 binds endogenous CNK1. LoVo colon carcinoma cells were extracted after incubation in serum-replete medium (*left*) or after overnight incubation in serum-deficient medium (*right*). Endogenous MST1 was immunoprecipitated from the lysates and probed for the presence of endogenous CNK1 (*upper panel*); immunoblots of the lysates for CNK1 (*middle panel*) and MST1 (*lowest panel*) are shown.

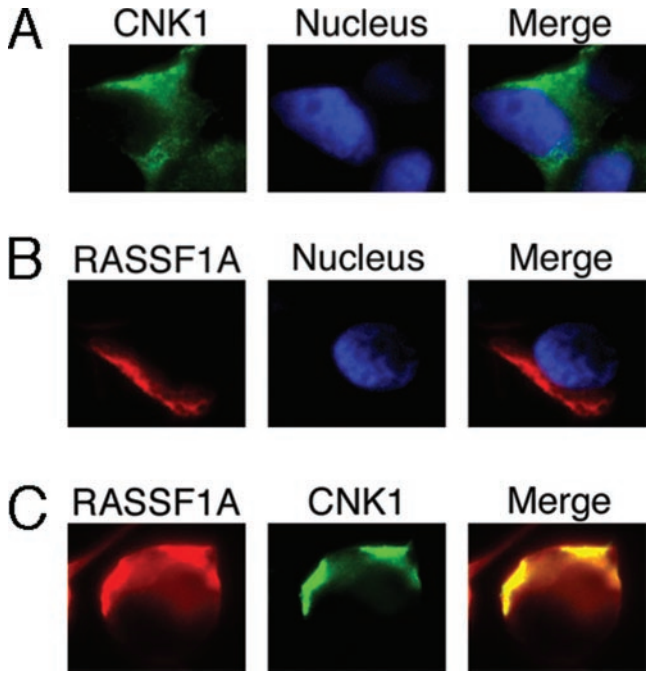


FIG. 4. **CNK1 colocalizes with RASSF1A.** *A*, transfection of CNK1 (*green*) in 293 cells results in a diffuse nonuniform cytosolic expression pattern. *B*, transfection of RASSF1A (*red*) in 293 cells results in a nonuniform diffuse expression pattern. *C*, coexpression of CNK1 (*red*) and RASSF1A (*green*) results in complete colocalization and membrane recruitment.

MST1 and MST2 during transient expression, and deletion of the MST1 carboxyl-terminal coiled-coil (so called SARAH) domain, which is responsible both for MST dimerization and association with the RASSF1 polypeptides, prevents MST coprecipitation with CNK1 (Fig. 3*E*). In addition, endogenous MST1 immunoprecipitated from the human colon carcinoma line LoVo contains endogenous CNK1, and the abundance of CNK1 in the MST1 immunoprecipitate is greatly increased after overnight serum withdrawal (Fig. 3*F*).

Recombinant CNK1 exhibits a diffuse cytoplasmic distribution when expressed alone (Fig. 4*A*), whereas recombinant RASSF1A exhibits an asymmetric peripheral localization (Fig. 4*B*). When coexpressed with RASSF1A, CNK1 redistributes to a submembranous region and exhibits essentially complete colocalization with RASSF1A (Fig. 4*C*). The results shown in Figs. 3 and 4 strongly support the conclusion that RASSF1 polypeptides form a complex with CNK1 *in vivo* and couple the proapoptotic kinase MST1 to CNK1. As regards the site of RASSF1A binding on CNK1, an amino terminal CNK1-(1–282) fragment and the fragment CNK1-(77–279) encompassing the CRIC and PDZ domains bind RASSF1A as well as full-length CNK1; CNK1-(199–713) also exhibits modest binding of RASSF1A, whereas the carboxyl-terminal fragment CNK1-(279–713), which lacks the PDZ domain, shows no binding of RASSF1A (Fig. 5*A*). Thus robust binding of RASSF1A requires the CNK1 sequences 77–199, encompassing the CRIC and PDZ domains. Moreover, despite the modest binding of RASSF1A to CNK1-(199–713), which contains the PDZ but not the CRIC domain, neither the PDZ nor CRIC domains expressed individually show any binding of RASSF1A (data not shown). As shown previously (3), c-Raf1 binds to a carboxyl-terminal region of CNK1 (Fig. 5*B*), entirely distinct from RASSF1A.

We next inquired as to whether RASSF1A or -C alter the ability of CNK1 to promote apoptosis. Whereas transient expression of CNK1 in human embryonic kidney 293 cells gives a reproducible increase in cell death, neither RASSF1A nor RASSF1C causes cell death beyond that seen with vector alone (Fig. 6*A*); nevertheless, coexpression of CNK1 with RASSF1A results in an increase in cell death far beyond that seen with CNK1 alone, whereas RASSF1C does not alter the extent of CNK1-induced apoptosis (Fig. 6*A*). Thus, although both RASSF1A and RASSF1C bind to CNK1 (and to MST1/2), only RASSF1A, the isoform that exhibits tumor suppressor activity, collaborates with CNK1 to promote apoptosis. The ability of CNK1 to bind RASSF1A is not sufficient to enable CNK1 to induce apoptosis inasmuch as the amino terminal CNK1 fragment (1–282), which binds RASSF1A, is not proapoptotic (Fig. 6*B*). By contrast, the CNK1 fragment (199–713), which exhibits a modest ability to bind RASSF1A (Fig. 5*A*) is proapototic. It is likely that the ability of CNK1-(199–713) to induce apoptosis is dependent on its ability to bind RASSF1A inasmuch as concomitant overexpression of CNK1-(1–282) with CNK1-(199–713) entirely blocks CNK1-(199–713)-induced apoptosis. Moreover, CNK1-(1–282) also blocks Ki-Ras(G12V)-induced apoptosis (Fig. 6*B*). Thus, the ability of CNK1 to induce apoptosis appears to require the recruitment of an RASSF1A·MST complex through its amino terminal region together with an as yet unidentified effector that binds to its carboxyl-terminal segment.

DISCUSSION

This report demonstrates that the human scaffold protein CNK1, a c-Raf1 binding protein homologous to a *Drosophila* protein necessary for Ras-induced Raf activation, binds the human tumor suppressor RASSF1A and activates cell death during transient overexpression and when stably reintroduced into human tumor cell lines. The CNK1-induced cell death requires the recruitment of the kinase MST1 or MST2, which is achieved because of the stable association of these kinases with RASSF1 polypeptides. The mechanism by which MST1/2 recruitment initiates cell death is not known, although previous work indicated that membrane localization of MST1 enhances its proapoptotic efficacy (7). The requirement for MST1 in the induction of cell death in 293 cells is also shared by Ki-Ras(G12V), which can recruit MST1 directly in a complex with the Ras-GTP-binding protein, NORE. RASSF1A/C, although possessed of a canonical RA domain, exhibits little or no ability to bind Ras-GTP directly, certainly in comparison to NORE (12). Thus, it is possible that CNK1 provides another mechanism by which MST1 can be functionally coupled to Ras through a RASSF1A/CNK1 adaptor. It should be emphasized, however, that our ability to demonstrate a physical association between Ras and CNK1 has been inconsistent. Coprecipitation of recombinant CNK1 with Ki-Ras(G12V) has been observed repeatedly but not reproducibly; conversely, we have never detected an association of CNK1 with Ha-Ras(G12V) or other small GTPases in the Ras subfamily. In addition, the extent to which the association of RASSF1A with CNK1 or MST1 contributes to the RASSF1A tumor suppressive function remains to be established.

The present demonstration that CNK1 binds the tumor suppressor protein RASSF1A and promotes apoptosis when overexpressed through a pathway that depends on RASSF1A and the MST1 or MST2 kinases represents the fourth signal transduction pathway with which CNK1 has been shown to participate. In addition to its necessary role in the Ras-dependent activation of c-Raf1 (2, 3), the PH domain of human CNK1 has recently been shown to bind specifically to the GTP-liganded form of Rho and to be necessary for Rho activation of transcription through the serum response element, although dispensable for Rho recruitment of the actin cytoskeleton (27). Human CNK1 also binds RalGDS, the guanyl nucleotide exchange factor (GEF) for the RalA/B GTPases (27, 28); similarly, CNK2 has been shown to interact with Rlf, another GEF for RalA/B,

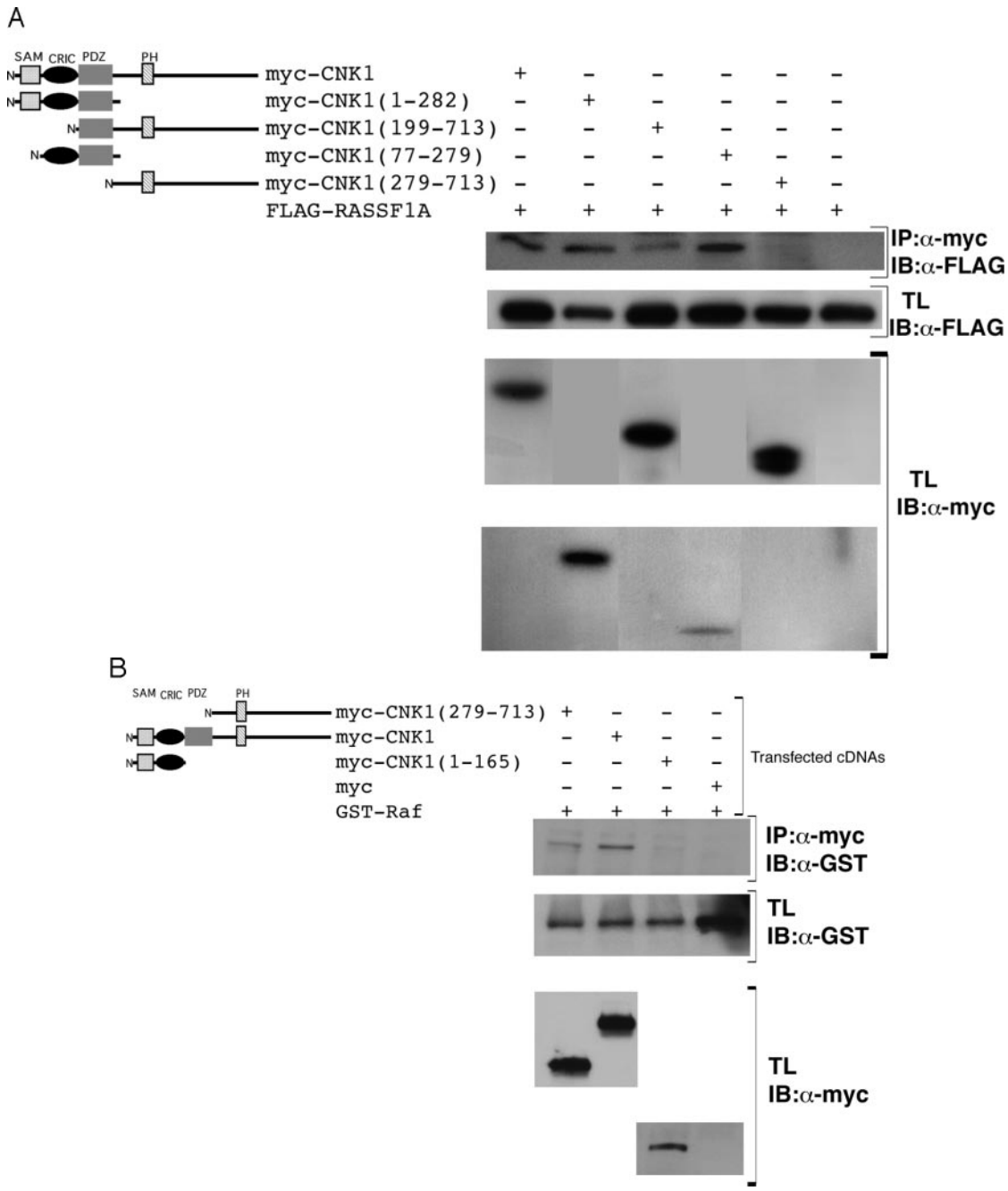


FIG. 5. **Localization of the RASSF1A and c-Raf1 binding regions on CNK1.** *A*, RASSF1A binds the CNK1 amino terminal segment between amino acids 77–199. Myc-tagged CNK1 and CNK1 fragments were co-expressed with FLAG-tagged RASSF1A in 293 cells, which were lysed in RIPA buffer 24 h after transfection. The lysates were immunoprecipitated with anti-Myc antibody and immunoblotted (*IB*) with anti-FLAG antibody. The *lower panels* represent lane loading controls. *IP*, immunoprecipitation; *TL*, total lysate. *B*, c-Raf1 binds the CNK1 carboxyl-terminal. GST-c-Raf1 was coexpressed in 293 cells with Myc vector or with the Myc-CNK1 constructs indicated. The GSH-agarose isolates were blotted for Myc (*upper panel*), and lysate expression of the GST-Raf and Myc-CNK1 polypeptides is shown in the *middle* and *lower panels*.

as well as to RalA itself. Both RalGDS and Rlf bind preferentially to the GTP-liganded forms of Ras and are considered potential Ras effectors (29, 30); however, the significance of CNK1/2 to RalA/B signaling remains to be established.

Scaffold proteins are thought to facilitate rapid and accurate transmission of intracellular signals, and two primary mechanisms are recognized. Proteins such as Ste5p and Pbs2p in *Saccharomyces cerevisiae* or kinase suppressor of Ras, MP1, and JIP-1 in mammalian cells assemble functionally interdependent and/or sequential elements in a single signaling pathway (31–35). In contrast, INAD assembles a set of diverse effectors that signal downstream in a parallel and largely independent way that in aggregate produces a major part of the characteristic features of the cellular program of photoreceptor activation (36). The ability of CNK1 to positively regulate c-Raf1 and to bind RalGDS and RhoGTP may reflect a similar example of the integration of proliferative signals downstream of Ras-GTP. The present results unveil a third mode of scaffold function through the ability of CNK1 to independently bind fundamentally opposing effectors Raf and RASSF1A. Raf is a major proliferative and anti-apoptotic effector of Ras; the ability of CNK1-(1–282) to suppress Ki-Ras(G12V)-induced apoptosis indicates that CNK1, probably through its ability to bind RASSF1A, can mediate Ki-Ras-induced apoptosis. Nevertheless, the physiologic context wherein CNK1 acts as a proapoptotic effector is not known; specifically, it is unclear whether

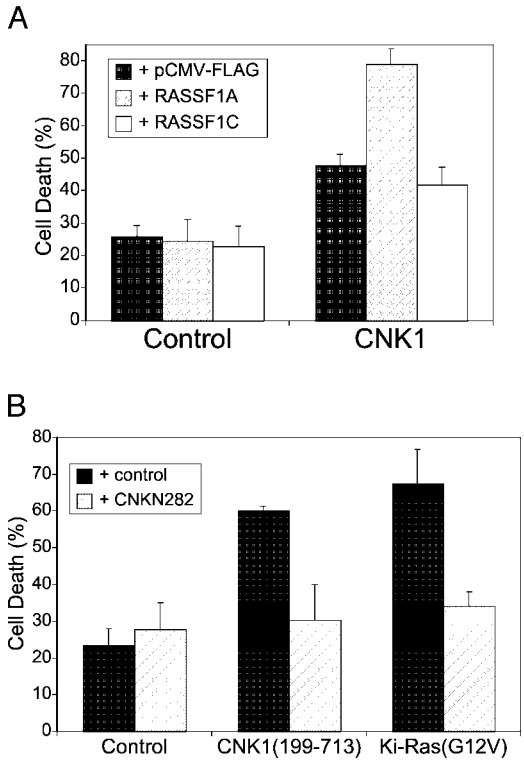


FIG. 6. **An RASSF1A/CNK1 complex participates in Ki-Ras(G12V)-induced cell death.** *A*, RASSF1A but not RASSF1C potentiates CNK1-induced cell death. 293 cells were transiently co-transfected with pEAK8-Myc-CNK1 and either pCMV-FLAG (*Control*), pCMV-FLAG-RASSF1A, or pCMV-FLAG-RASSF1C and incubated for 4 days. Trypan blue exclusion was employed to quantitate cell death. Control represents the addition of a vector to compensate for total DNA load. *B*, CNK1-(1–282), a nonapoptotic RASSF1A-binding fragment of CNK1, inhibits Ki-Ras(G12V)-induced cell death. Coexpression of CNK1-(1–282), which is not proapoptotic despite its ability to bind RASSF1A, suppresses the apoptosis induced by the carboxyl-terminal fragment of CNK1-(199–713) and by Ki-Ras(G12V). In experiments not shown, CNK1-(279–713) was shown to lack apoptotic activity.

Ki-Ras(G12V)- or CNK1-induced apoptosis reflects the direct recruitment of an apoptotic signaling pathway or the interference by these elements when overexpressed with a critical cell function resulting in the secondary activation of a proapoptotic checkpoint response. Similarly, the mode of action of RASSF1A as a tumor suppressor is not entirely clear. RASSF1A, in addition to its ability to delay progression through G_1 (22), has recently been shown to be an inhibitor of mitotic progression in mammalian cells, apparently acting as a direct inhibitor of anaphase-promoting complex function (23). The protein kinase(s) MST1/2, constitutive partners of the RASSF1 polypeptides in mammalian cells, are also antiproliferative, at least in *Drosophila*, and proapototic during both *Drosophila* development (37–41) and when overexpressed in mammalian cells (7, 42). Inasmuch as the *Drosophila* genome does not encode a structural homolog of RASSF1, it will be of interest to determine whether *Drosophila* CNK interacts with *Drosophila* MST (*hippo*) through another polypeptide, such as the *hippo* partner, *Salvador* (43). Nevertheless, whether the CNK1/RASSF1A interaction acts physiologically to delay cell cycle progression, initiate apoptosis, or both, such actions are clearly opposing and essentially incompatible with the proliferative actions directed by activated Ki-Ras. It is likely that this dual function of CNK1 is essential to ensuring the transmission of a single output, appropriate to the overall physiologic program. An understanding of the biochemical mechanisms that enable CNK to function in both proliferative and antiproliferative pathways will clarify the operation of Ras in normal development as well as in neoplastic transformation.

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The Scaffold Protein CNK1 Interacts with the Tumor Suppressor RASSF1A and Augments RASSF1A-induced Cell Death

Shahrooz Rabizadeh, Ramnik J. Xavier, Kazuhiro Ishiguro, Juliocesar Bernabeortiz, Marco Lopez-Ilasaca, Andrei Khokhlatchev, Pamela Mollahan, Gerd P. Pfeifer, Joseph Avruch and Brian Seed

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