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TC21 Causes Transformation by Raf-Independent Signaling Pathways

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Although the Ras-related protein TC21/R-Ras2 has only 55% amino acid identity with Ras proteins, mutated forms of TC21 exhibit the same potent transforming activity as constitutively activated forms of Ras. Therefore, like Ras, TC21 may activate signaling pathways that control normal cell growth and differentiation. To address this possibility, we determined if regulators and effectors of Ras are also important for controlling TC21 activity. First, we determined that Ras guanine nucleotide exchange factors (SOS1 and RasGRF/CDC25) synergistically enhanced wild-type TC21 activity in vivo and that Ras GTPase-activating proteins (GAPs; p120-GAP and NF1-GAP) stimulated wild-type TC21 GTP hydrolysis in vitro. Thus, extracellular signals that activate Ras via SOS1 activation may cause coordinate activation of Ras and TC21. Second, we determined if Raf kinases were effectors for TC21 transformation. Unexpectedly, yeast two-hybrid binding analyses showed that although both Ras and TC21 could interact with the isolated Ras-binding domain of Raf-1, only Ras interacted with full-length Raf-1, A-Raf, or B-Raf. Consistent with this observation, we found that Ras- but not TC21-transformed NIH 3T3 cells possessed constitutively elevated Raf-1 and B-Raf kinase activity. Thus, Raf kinases are effectors for Ras, but not TC21, signaling and transformation. We conclude that common upstream signals cause activation of Ras and TC21, but activated TC21 controls cell growth via distinct Raf-independent downstream signaling pathways.

Ras is a member of a large superfamily of small GTPases that function as regulated molecular switches (6). Ras GDP/GTP cycling is controlled by two classes of regulatory proteins (3). Whereas guanine nucleotide exchange factors (GEFs; GRF/CDC25 and SOS1/2) function as positive regulators and promote formation of active Ras-GTP (3, 22, 61), GTPase-activating proteins (GAPs; p120-GAP and NF1-GAP) are negative regulators and greatly accelerate the intrinsic GTPase activity of Ras to promote formation of inactive Ras-GDP (4). Growth factors, cytokines, hormones, and other extracellular stimuli cause transient activation of Ras primarily through upregulation of Ras GEF activity (61). Mutations that activate Ras transforming potential (at residue 12, 13, or 61) render Ras insensitive to GAP stimulation, and consequently, these oncogenic proteins persist in a constitutively activated state (4, 6). Chronic upregulation of Ras, in the absence of mutations in Ras itself, can also be caused by the loss of GAP function. For example, the loss of NF1-GAP in some tumor cells leads to greatly elevated Ras-GTP levels, which are important for the tumorigenic growth of these cells (1, 19).

A well-defined and highly conserved signaling cascade whereby Ras functions as a critical intermediate relay switch between upstream receptor tyrosine kinases and a downstream cascade of serine/threonine kinases has been established in mammalian cells as well as in *Drosophila melanogaster* and *Caenorhabditis elegans* (21, 37, 54). For example, epidermal growth factor stimulation of the epidermal growth factor receptor promotes receptor autophosphorylation, which in turn recruits the Grb2-SOS1 complex to the plasma membrane, where SOS1 causes transient activation of Ras (22, 61, 66). Activated Ras in turn complexes with and activates the Raf-1 serine/threonine kinase (50). Activated Raf-1 phosphorylates and activates the mitogen-activated protein kinase (MAPK) kinase, designated MEK, which in turn activates the p42 and p44 MAPKs (18, 45). Activated MAPKs translocate from the cytoplasm to the nucleus, where they activate the Elk-1 transcription factor as well as other proteins (47). The essential role of Ras and Ras-mediated signaling activity in normal cellular function has been demonstrated by the use of dominant negative Ras proteins. The H-Ras(17N) and H-Ras(15A) mutant proteins exhibit impaired GDP/GTP-binding properties that cause them to form nonproductive complexes with Ras GEFs, thereby preventing GEF activation of endogenous Ras (12, 23, 36, 58, 67). The potent growth-inhibitory activities of Ras dominant negative proteins demonstrate that Ras-mediated signaling is essential for normal cellular proliferation.

Although there is substantial evidence that the Raf/MEK/MAPK pathway is essential for Ras function (16, 39, 43, 46), there is increasing evidence that Ras mediates its actions through interaction with multiple downstream effector targets (73). First, genetic studies of fission yeast *Schizosaccharomyces pombe* Ras (ras1) function have identified two distinct ras1 effector-mediated activities (11). One involves ras1 interaction with byr2 (a MEK kinase homolog), and the other involves ras1 interaction with scd1 (a putative Rho GEF) (11). scd1, in turn, may regulate the function of the cdc42sp Rho family protein. Oncogenic Ras transformation of mammalian cells has also been shown to be dependent on Rho protein function (38, 55–57). Second, the existence of Raf-independent Ras signaling pathways is suggested by the identification of additional candidate Ras effectors. These include GEFs for the Ras-related protein Ral (RalGDS and RGL) (33, 40, 69), phosphatidylinositol-3-OH kinase (63), the MEKK1 serine/

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threonine kinase (64), the two Ras GAPs (4), Rin-1 (29), and AF6 (72). Like Raf-1, these functionally diverse proteins show preferential binding to the active GTP-bound form of Ras, and this interaction requires an intact Ras core effector domain (amino acids 32 to 40) (48). Third, the determination that effector domain mutants of Ras, which have lost the ability to bind to and activate Raf-1, still retain potent transforming activity demonstrates that Ras interaction with other effectors contributes to Ras transformation (35, 39, 74). Finally, since Ras, but not Raf, causes transformation of RIE-1 and other epithelial cells (52), it is clear that Ras activation of the Raf/MAPK pathway alone is not sufficient for Ras transformation of some cells. Thus, Ras mediates transformation by activating both Raf-dependent and Raf-independent pathways.

Although Rap1A and TC21/R-Ras2 have 55% amino acid identity with Ras and 100% identity with the Ras core effector domain, only constitutively activated mutants of TC21 cause cellular transformation (10, 28). Instead, Rap1A antagonizes Ras signaling and transformation (15, 41, 42). The failure of Rap1A to cause transformation is surprising in light of the ability of Rap1A to interact with the Ras-binding domain of Raf-1 and other candidate Ras effectors (e.g., RalGDS) when analyzed in yeast two-hybrid or in vitro binding assays (69, 76). However, Rap1A fails to activate the same downstream signaling pathways as Ras, which suggests that Rap1A does not functionally interact with and activate the same effector targets as Ras. In contrast, we have shown that transforming mutants of TC21 cause constitutive activation of MAPKs and stimulate transcription from promoters containing Ras-responsive DNA elements (28). However, it is not known whether TC21 utilizes Ras effectors, or TC21-specific effectors, to mediate its signaling and transforming actions.

The morphologic and growth-transforming activity caused by aberrant TC21 function suggests that TC21 is involved in regulating signaling pathways that control normal cell growth (10, 28). However, the identities of extracellular stimuli that activate TC21 and TC21-dependent signaling pathways are not known. To identify a role for TC21 in normal cell function, we determined if TC21 function is controlled by the positive and negative regulators that control Ras GDP/GTP cycling and if activated TC21 utilizes Raf kinases to promote cellular transformation. First, we found that normal TC21 is sensitive to Ras GEFs and GAPs, which suggests that extracellular stimuli that activate Ras via these Ras regulators will also activate TC21. Second, we found that Ras, but not TC21, could interact with full length Raf-1, A-Raf, and B-Raf and that only Ras-transformed cells showed constitutive upregulation of Raf kinase activity. Thus, TC21 transformation is mediated by Raf-independent signaling pathways. Finally, TC21 protein expression showed a more restricted tissue distribution than Ras proteins. Taken together, these results show that TC21 and Ras have both common and distinct roles in normal cellular signaling activities.

MATERIALS AND METHODS

Generation of TC21 mutants and molecular constructs. Oligonucleotide-directed mutagenesis using the Chameleon mutagenesis protocol (Stratagene) was used to generate mutant *tc21* cDNA sequences encoding single amino acid substitutions at residues that correspond to analogous mutations in H-Ras(15A) and H-Ras(17N) dominant negative mutants, designated TC21(26A) and TC21(28N), respectively. All mutant cDNA sequences were generated in a 0.7-kb *Bam*HI fragment that contains the full-length human wild-type *tc21* cDNA sequence. TC21(26A) contains a Gly (GGC)-to-Ala (GCC) mutation that corresponds to Ras residue 15, whereas TC21(28N) contains a Ser (TCG)-to-Asn (AAT) mutation that corresponds to Ras residue 17. In the process of site-directed mutagenesis, the first *Kpn*I site in the 3′ noncoding region was converted to a *Bam*HI site. Each mutant *tc21* sequence was introduced into the unique *Bam*HI restriction site of a modified pZIP-NeoSV(x)1 retrovirus vector (9). All mutated sequences were verified by dideoxy sequencing with a Sequenase kit (U.S. Biochemical/Amersham) according to the manufacturer's instructions.

Cell culture and transformation/inhibition assays. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. DNA transfections were performed as described previously, using the calcium phosphate precipitation technique (13). Cells were transfected with, per dish, 0.1 to 1 μg (for Ras GEF assays) or 50 to 100 ng (dominant negative inhibition assays) of pZIP plasmid DNA encoding wild-type TC21 [TC21(WT)] or mutant TC21 protein. Focus inhibition assays were done by transfection of either pZIP-*ras*H(61L) or pZIP-*tc21*(72L) (10 ng) alone or together with pZIP-K*rev*-1(63E) plasmid DNA. Krev-1(63E) is a constitutively activated mutant which exhibited stronger inhibition of Ras transformation than its wild-type counterpart (41). The appearance of transformed foci was quantitated after 14 days. Growth inhibition was assessed by selecting transfected cells in growth medium supplemented with G418 (GIBCO/BRL) at 400 μg/ml. After approximately 14 days, cells were fixed and stained with 0.4% crystal violet.

In vitro GTP hydrolysis and GDP/GTP exchange assays. cDNA sequences encoding TC21(WT) or TC21(72L) were introduced into the pGEX-2T bacterial expression vector to generate glutathione *S*-transferase (GST)–TC21 chimeric proteins (8), which were purified as described previously (68) and quantitated by using Bradford reagents. Purified, bacterially expressed H-Ras (residues 1 to 166; obtained from S. Campbell, University of North Carolina at Chapel Hill) and GST-TC21 proteins were preloaded with [γ-^{32}P]GTP and incubated (400 or 420 ng, respectively), either alone or with purified full-length p120-GAP or the catalytic domain of NF1-GAP (designated NF1-GRD) (at a 1:4 molar ratio with H-Ras or TC21 obtained from S. Campbell). GTP hydrolysis within 30 min at room temperature was measured by the cleavage of the radiolabeled phosphate in an organic phosphate release assay as described previously (5).

Transcription activation assays. To determine the ability of Ras exchange factors to activate TC21(WT) transforming activity, NIH 3T3 cells were transiently cotransfected with 750 ng of pZIP-*tc21*(WT), either alone or together with either pZIP-cCDC25/CAAX or pZIP-cSOS/CAAX (150 ng or 1 μg, respectively). Activation of Elk-1 was determined by cotransfection with Gal4–Elk-1, which encodes a fusion protein containing the Gal4 DNA-binding domain together with the transactivation domain of Elk-1 (containing MAPK phosphorylation sites) and Gal4-Luc, which contains the luciferase gene driven by a minimal promoter containing tandem Gal4 DNA-binding sites (provided by R. Treisman) (47). Approximately 48 h after transfection, cell lysates were prepared, and the luciferase activity induced by each pZIP-*tc21* or pZIP-*ras*H construct was assayed in a luminometer, using procedures which we have described previously (32). To determine the ability of the Raf-N4 dominant negative mutant (17) to block TC21 signaling activity, NIH 3T3 cells were transiently cotransfected with 100 ng of pZIP-*tc21*(72L) plasmid DNA, either alone or together with 3 μg of pCGN-*raf*-N4 or pCGN-*raf*(WT) and 150 ng of Gal4–Elk-1 and 2.5 μg of Gal4-Luc. Raf-N4 is a COOH-terminally truncated, kinase-deficient mutant which retains the two NH_2-terminal Ras-binding domains of Raf-1 and functions as a dominant negative mutant by blocking Ras interaction with Raf-1 and other candidate effector proteins (17).

Raf kinase assays. Raf kinase activities were measured by using an in vitro coupled kinase assay in which the ability of immunoprecipitated Raf to activate MEK phosphorylation of MAPK was determined. Briefly, NIH 3T3 cells stably transfected with the empty pZIP-NeoSV(x)1 vector, or expressing TC21(72L) or H-Ras(61L) protein, were subjected to immunoprecipitation with Raf-specific antisera generated against c-Raf-1 and B-Raf [Raf-1(C-12) and Raf-B(C-19), respectively; Santa Cruz Biotechnology]. The immune complexes were collected on protein A-conjugated Sepharose beads. Beads with their attached immune complexes were incubated with [γ-^{32}P]ATP and purified recombinant wild-type MEK protein, a known substrate of Raf kinase (provided by Qiming Chen, University of North Carolina at Chapel Hill). After 15 min, these complexes were then incubated with recombinant kinase-deficient MAPK, a known substrate of MEK (provided by Qiming Chen), and the reaction mixture was incubated for a further 15 min. The reaction was stopped by the addition of 2× protein sample buffer, and the proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue to ensure that equal loading had taken place. The dried gel was then exposed to a Bio-Rad PhosphorImager to quantitate the amount of ^{32}P transferred onto MAPK.

Yeast two-hybrid analysis. Yeast two-hybrid binding analyses were done essentially as described previously (73). Briefly, we used a modification of the Fields two-hybrid system (24) wherein one hybrid is a fusion between the LexA DNA-binding domain (amino acids 1 to 211) and either H-Ras(12V) or TC21(WT) sequences. The second hybrid is a fusion between a nucleus-localized VP16 acidic activation domain and the full-length sequences of human c-Raf-1, A-Raf, and B-Raf or the NH_2-terminal Raf-1 sequences which contain a minimal Ras-binding domain. Individually, these hybrids are unable to activate transcription. However, when coexpressed in a *Saccharomyces cerevisiae* strain that contains two integrated reporter constructs (yeast *HIS3* and the bacterial *lacZ* gene) which contain binding sites for the LexA protein, successful interactions bring the DNA-binding domain of LexA into the proximity of the transactivation domain of VP16 and allow transcription. The yeast strain expressing both hybrid proteins that are capable of forming interactions are prototropic for histidine and contain detectable β-galactosidase activity. Interaction between the H-Ras or

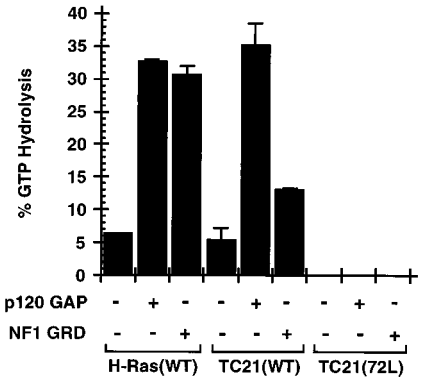

FIG. 1. Ras GAPs stimulated the intrinsic GTPase activity of TC21(WT), but not transforming TC21, in vitro. Purified H-Ras or GST-TC21 proteins were preloaded with [γ-^{32}P]GTP and incubated either alone or with recombinant p120-GAP and NF1-GRD, and GTP hydrolysis was determined after 30 min at room temperature. Data shown are the averages of duplicate samples and are representative of three independent assays.

TC21 fusions and Raf fusions were performed in the *S. cerevisiae* reporter strain YPB2 and assayed for β-galactosidase activity on filters.

Tissue expression of TC21 protein. Lysates of human adult tissues (Clontech) were resolved by SDS-PAGE (60 μg of total protein per lane) and Western blotted (immunoblotted) with an affinity-purified anti-TC21 rabbit polyclonal antibody, which was made against bacterially expressed recombinant TC21 protein (14). This antiserum does not recognize Ras proteins but shows very weak recognition of the related R-Ras protein.

RESULTS

TC21(WT), but not transforming TC21, is negatively regulated by p120-GAP and NF1-GAP. Ras GAPs require an intact Ras core effector domain (Ras residues 32 to 40) to bind to and stimulate the intrinsic GTPase activity of wild-type Ras proteins (48, 53). However, although Rap1A has complete amino acid identity with the Ras core effector domain, it is insensitive to Ras GAPs (25, 31). Thus, although TC21 also has complete identity with the Ras core effector domain, it was not obvious whether TC21 is sensitive to Ras GAPs. To determine if TC21(WT) is sensitive to this stimulation, we expressed recombinant GST fusion proteins containing TC21(WT) and TC21(72L) sequences and performed in vitro GTP hydrolysis assays. Bacterially expressed wild-type H-Ras [H-Ras(WT)] protein was used as a control for these studies.

Purified TC21 and H-Ras proteins were first preloaded with radiolabeled [γ-^{32}P]GTP and then incubated either with or without purified recombinant versions of p120-GAP or the NF1-GAP catalytic domain (NF1-GRD). Comparable to observations with H-Ras(WT), TC21(WT) displayed very weak intrinsic GTPase activity that could be greatly accelerated (sevenfold) by the addition of p120-GAP (Fig. 1). However, unlike H-Ras(WT), TC21(WT) GTPase activity was only weakly stimulated by the addition of NF1-GRD (2.5-fold stimulation). Thus, both Ras GAPs can stimulate GTP hydrolysis on TC21(WT) in vitro, although p120-GAP is more efficient. Finally, like oncogenic mutants of Ras (3), the transforming TC21(72L) mutant showed a very reduced intrinsic GTPase activity which was not further stimulated by either Ras GAP (Fig. 1). Therefore, Ras GAPs can act as negative regulators of TC21, and loss of GAP responsiveness may be responsible for activating the transforming potential of TC21(72L).

TC21 is positively regulated by Ras GEFs SOS1 and RasGRF/CDC25. We and others have shown that H-Ras residues in the region spanning amino acids 63 to 76 are important for H-Ras GEF binding and stimulation of GDP/GTP exchange (51, 58, 60). Among Ras-related proteins, TC21 and the closely related R-Ras protein (70% overall identity with TC21) show the strongest sequence identity with Ras in this region (27). However, since R-Ras is insensitive to Ras GEF stimulation (34), we anticipated that TC21 would not be activated by Ras GEFs. To address this possibility, we first examined whether purified Ras GEFs could stimulate TC21(WT) activity in vitro, by performing guanine nucleotide exchange assays on purified recombinant TC21(WT) protein. However, we found that in contrast to H-Ras(WT), TC21(WT) showed a very high intrinsic exchange activity in vitro (data not shown). Thus, we were unable to accurately measure any potential further stimulation of this activity by the addition of Ras GEFs. Therefore, we then used several in vivo assays that indirectly measure the abilities of Ras GEFs to activate Ras function to determine if SOS1 or RasGRF/CDC25 can also activate TC21(WT).

First, we examined whether overexpression of Ras GEFs would synergistically enhance TC21(WT) downstream signaling. For these assays, we determined the consequences of Ras GEF overexpression on the ability of TC21(WT) to activate Elk-1 transcriptional activity by using a transient-transfection luciferase assay. For this analysis, we used a plasmid expression vector encoding the catalytic domain of human RasGRF/CDC25 (cCDC25/CAAX) or mouse SOS1 (cSOS or cSOS/CAAX) (59). Transfection of pZIP-*tc21*(WT), pZIP-*ras*H(WT), pZIP-cSOS1, or pZIP-cCDC25/CAAX alone caused only limited stimulation of luciferase activity (2.2- to 5.5-fold) (Fig. 2A). As shown previously, coexpression of cCDC25/CAAX or cSOS1/CAAX synergistically enhanced H-Ras(WT) activity (2.1- and 2.9-fold, respectively, above the additive level). Similarly, coexpression of cSOS/CAAX with TC21(WT) caused a synergistic enhancement of Elk-1 activity (2.7-fold above the additive level). However, coexpression of cCDC25/CAAX did not cause significant enhancement of TC21(WT) activity.

Next, we determined if overexpression of Ras GEFs could synergistically enhance TC21(WT) transforming activity in NIH 3T3 focus formation assays. Transfection of 1 μg of pZIP-*ras*H(WT) or pZIP-*tc21*(WT) plasmid DNA alone caused very low focus-forming activity. However, cotransfection with the Ras GEF-expressing plasmids caused synergistic enhancement of H-Ras(WT) and TC21(WT) focus-forming activity (Fig. 2B). Whereas coexpression of either Ras GEF caused approximately 2-fold synergistic enhancement of H-Ras(WT) focus-forming activity, cSOS caused a greater enhancement than cCDC25/CAAX of TC21(WT) focus-forming activity (8.9- and 2.6-fold, respectively, above the additive level).

We also used an indirect assay to determine whether Ras GEFs can interact with TC21(WT). The H-Ras(15A) and H-Ras(17N) dominant negative proteins cause potent inhibition of cell proliferation as a result of their ability to antagonize Ras GEF activation of Ras (12, 36, 67). Therefore, we determined if analogous mutants of TC21(WT), designated TC21(26A) and TC21(28N), would also cause inhibition of cell growth. To examine this, we introduced both TC21(26A) and TC21(28N) into a mammalian expression vector that also contains the drug-selectable marker for G418 [Neo^r; pZIP-NeoSV(x)1]. NIH 3T3 cells transfected with these plasmids were grown in growth medium supplemented with G418 to select for drug-resistant colonies stably expressing TC21(26A) or TC21(28N) protein. Like the H-Ras dominant negative proteins, we found that cultures transfected with the expression plasmids encoding these two TC21 mutant proteins resulted in a significant reduction (greater than threefold) in the appearance of G418-resistant colonies (Fig. 3). Thus, like

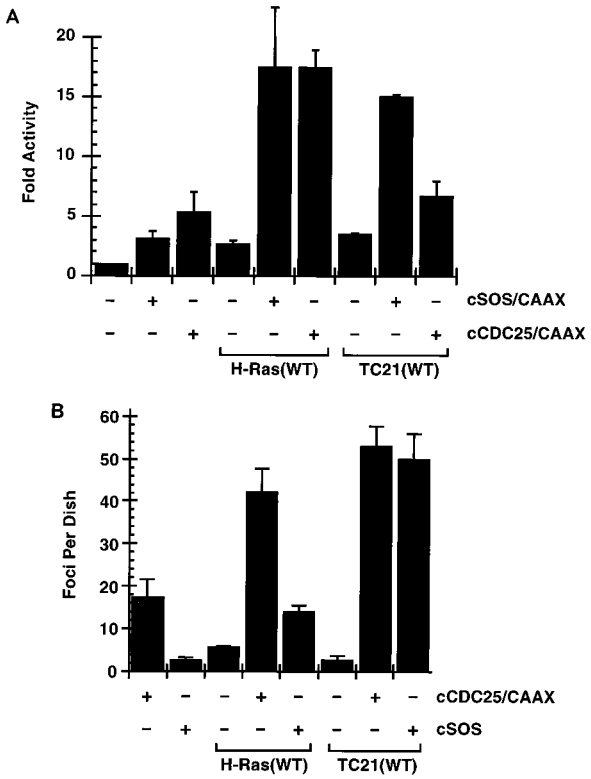

FIG. 2. Ras GEFs synergistically enhanced TC21(WT) transactivation and transforming activity in NIH 3T3 cells. (A) Coexpression of cCDC25/CAAX or cSOS1/CAAX synergistically enhanced H-Ras(WT) and TC21(WT) stimulation of Gal4–Elk-1 transcription activation of luciferase from the Gal4-Luc reporter plasmid in transient-transfection assays using NIH 3T3 cells. Data shown are the averages of duplicate samples and are representative of three independent assays. (B) Coexpression of cCDC25/CAAX or cSOS synergistically increased H-Ras(WT) and TC21(WT) focus formation in NIH 3T3 cells. The appearance of transformed foci was quantitated after 14 to 16 days. Data shown are the averages of four dishes and are representative of three independent assays.

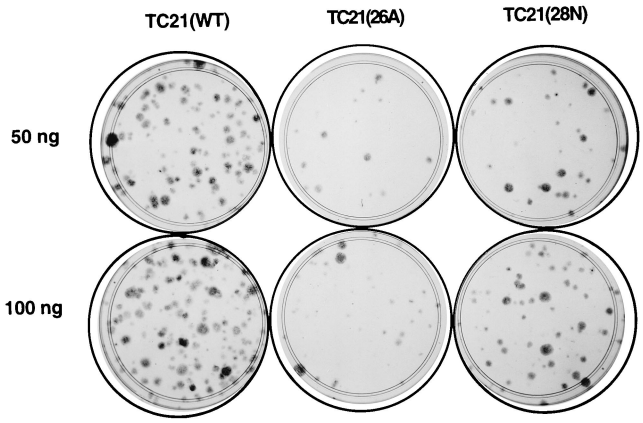

FIG. 3. TC21 mutants analogous to the H-Ras(15A) and H-Ras(17N) dominant negative mutant proteins inhibited the growth of NIH 3T3 cells. NIH 3T3 cells were transfected with 50 or 100 ng of pZIP-NeoSV(x)1 retrovirus expression vector plasmid DNA constructs (Neo^r) encoding wild-type or mutant forms of H-Ras or TC21. The appearance of G418-resistant colonies was visualized by staining with crystal violet after 14 to 16 days. A reduction in the appearance of G418-resistant colonies was used as a measure of growth inhibition. Cultures transfected with expression vectors of TC21(26A) and TC21(28N) caused a reduction in the appearance of G418-resistant colonies compared with cultures transfected with either empty vector (data not shown) or a vector encoding TC21(WT). Data are representative of three separate experiments.

H-Ras(15A) and H-Ras(17N), these mutants of TC21 may function as dominant negative proteins.

We next determined if coexpression of TC21(26A) and TC21(28N) could block Ras GEF stimulation of H-Ras(WT) or TC21(WT) transcription activation. For these assays, we cotransfected a plasmid encoding a chimeric Gal4–Elk-1 protein together with a reporter plasmid in which luciferase gene expression is controlled by a minimal promoter containing tandem Gal4 DNA-binding sequences (47). As we have shown previously, coexpression of the H-Ras(17N) mutant completely blocked cSOS1/CAAX stimulation of H-Ras(WT) activation of Elk-1 (Fig. 4A) (60). Similarly, we found that cotransfection of an expression plasmid encoding either TC21(26A) or TC21(28N) also caused a significant inhibition of cSOS1/CAAX enhancement of H-Ras(WT) transcription activation. A fivefold increase in the amount of cotransfected pZIP-cSOS1/CAAX was able to overcome this inhibition (data not shown). Coexpression of either TC21 mutant protein also inhibited cSOS1/CAAX stimulation of TC21(WT) activation of Elk-1 (Fig. 4B). Although these assays are indirect, when taken together with the ability of SOS1 to synergistically enhance TC21(WT) transactivation and transforming activities (Fig. 2), they provide strong evidence that Ras GEFs are activators of TC21.

TC21 fails to interact with and cause constitutive activation of Raf kinases in vivo. We previously showed that transforming mutants of TC21 caused constitutive activation of p42 and p44 MAPKs and activated transcription from both *ets*/AP-1 and NF-κB Ras-responsive promoter elements (28). Since TC21 has complete identity with the Ras core effector domain (27), we suspected that Raf-1 functioned as the critical effector for causing these activities. However, since Raf-independent mechanisms of MAPK activation have been described (75), it is also possible that TC21 activates these downstream events via other effectors. To address this possibility directly, we determined whether a Raf-1 dominant negative mutant could block TC21 transforming activity, whether TC21 could associate with the three Raf kinases in yeast two-hybrid binding assays, and whether Raf kinases are constitutively upregulated in TC21-transformed cells.

Consistent with the critical role of Raf-1 as an effector for Ras function, dominant negative mutants of Raf-1 have been shown to potently block oncogenic Ras signal transduction and transformation (7, 43). Therefore, we determined if a dominant negative mutant of Raf-1, which blocks oncogenic Ras transformation and signal transduction by forming nonproductive complexes with Ras, could also block TC21 function. For these analyses, we used the COOH-terminally truncated Raf-N4 mutant protein (Raf-1 residues 1 to 256), which contains the two NH_2-terminal Ras-binding sequences but lacks the COOH-terminal kinase domain (7). We previously showed that coexpression of Raf-N4 inhibited oncogenic Ras signaling and transformation (7). Whereas coexpression of wild-type Raf-1 (which alone is not transforming or transcriptionally active) synergistically enhanced TC21(WT) activity, coexpression of Raf-N4 blocked TC21(72L)-mediated stimulation of Elk-1 transcriptional activation (Fig. 5). We also observed that coexpression of Raf-N4 also greatly inhibited TC21(72L)-induced focus formation (data not shown). These results indicated that this truncated Raf-1 fragment can interact with the TC21 effector domain in vivo and block TC21 function.

Since Ras interaction with Raf proteins has also been demonstrated in the yeast two-hybrid assay (71, 73, 76), we used this assay to determine if TC21(WT) could interact with the

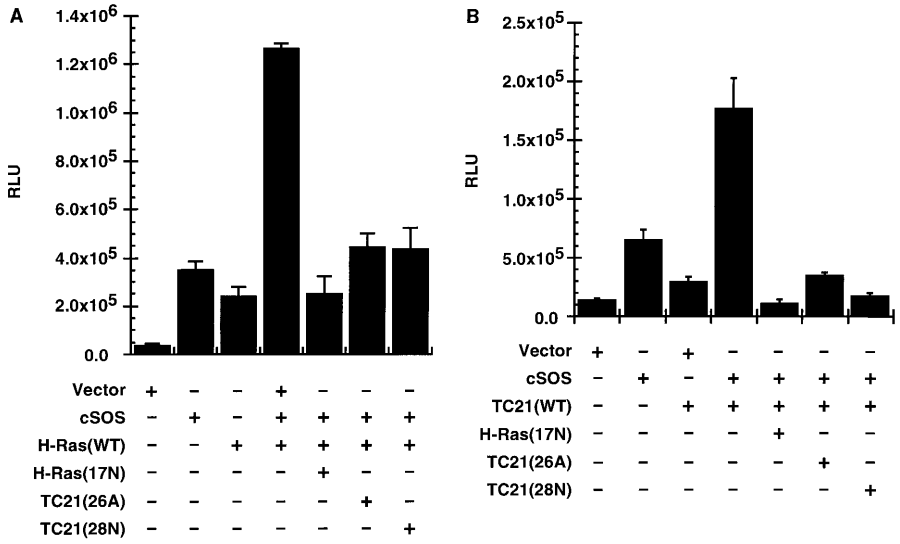

FIG. 4. Dominant negative mutants of TC21 inhibited SOS1 synergistic stimulation of H-Ras(WT) or TC21(WT) transcription activation. Transient transfection assays were done with NIH 3T3 cells to measure the ability of pZIP-cSOS1 (1 μg) to stimulate H-Ras(WT) (500 ng) (A) or TC21(WT) (750 ng) (B) transcription activation of a Gal4–Elk-1 chimeric protein to stimulate luciferase expression from the Gal4-Luc reporter plasmid. Coexpression of TC21(26A) or TC21(28N) (3 μg) was done to determine if these dominant negative mutant proteins could inhibit cSOS1 stimulation. H-Ras(17N) (3 μg) was used as a control for these assays. RLU, relative luciferase units. Data are averages of duplicate samples and are representative of three independent assays.

three Raf family proteins. Expression plasmids encoding the LexA DNA-binding domain (amino acids 1 to 211) fused to TC21 or H-Ras were coexpressed in *S. cerevisiae* with expression plasmids encoding the VP16 acidic activation domain fused to the full-length sequences of Raf-1, B-Raf, and A-Raf or to a truncated Raf-1 fragment which contains only a minimal Ras-binding domain (Raf-1 residues 51 to 130; designated Raf-RBD). Surprisingly, whereas both TC21 and H-Ras showed interaction with Raf-RBD, only H-Ras interacted with the full-length versions of the three Raf proteins (Fig. 6). While these results were consistent with the ability of a truncated Raf-1 dominant negative mutant protein to block TC21 function in NIH 3T3 cells, they suggested that H-Ras, but not TC21, interacted with authentic full-length Raf proteins in vivo.

In light of the yeast two-hybrid results, we addressed the possibility that Ras, but not TC21, transformation caused constitutive Raf kinase activation in NIH 3T3 cells. For these analyses, we immunoprecipitated c-Raf-1 or B-Raf from either untransformed control (vector-transfected) NIH 3T3 cells or H-Ras- or TC21-transformed cells (62). The immunoprecipitated Raf protein was then incubated with recombinant MEK and kinase-deficient MAPK to measure Raf activation of MEK to phosphorylate MAPK in an in vitro coupled assay. Whereas H-Ras(61L)-transformed cells showed constitutively elevated c-Raf-1 and B-Raf kinase activities, TC21(72L)-transformed cells showed the same low kinase activity as seen in untransformed NIH 3T3 cells (Fig. 7). These results, taken together with those of our yeast two-hybrid binding analysis, show that TC21 neither interacts with nor activate Raf kinases in vivo.

Krev-1/Rap1A antagonizes TC21 focus-forming activity. The Ras-related protein Krev-1/Rap1A has been shown to antagonize Ras signaling and transformation, presumably because Krev-1 forms inactive complexes with critical Ras effec-

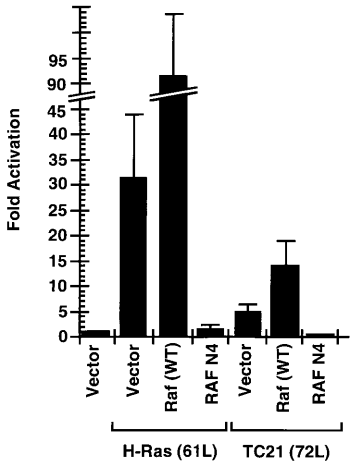

FIG. 5. Coexpression of the Raf-N4 dominant negative mutant inhibited H-Ras(61L) and TC21(72L) transcription activation of Elk-1. Transient transfection assays were done in NIH 3T3 cells to measure the ability of the Raf-N4 dominant negative protein, which contains only the Ras-binding domains of human Raf-1, to block the ability of transforming mutants of H-Ras and TC21 to stimulate Gal4–Elk-1 transcription activation of luciferase from the Gal4-Luc reporter plasmid. Data shown are the averages of two samples, are relative to the activity seen with cells transfected with the empty vector, and are representative of three independent assays.

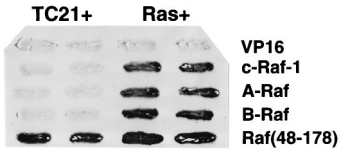

FIG. 6. TC21(WT) interacted with truncated but not full-length Raf proteins. Yeast two-hybrid analysis was done to compare the ability of H-Ras and TC21 to bind full-length Raf-1, B-Raf, and A-Raf or the isolated Ras-binding domain of Raf-1. β-Galactosidase activity was determined by filter assay. Yeast patches containing interacting protein pairs are dark as a result of β-galactosidase activity producing a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) cleavage product. Yeast patches containing noninteracting protein pairs remain white as a result of the absence of detectable β-galactosidase activity.

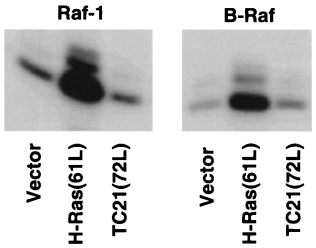

FIG. 7. Oncogenic H-Ras(61L)- but not TC21(72L)-transformed NIH 3T3 cells showed elevated Raf-1 and B-Raf kinase activities. Raf-1 or B-Raf was immunoprecipitated from cell lysates of equivalent cell numbers of NIH 3T3 cells stably expressing H-Ras(61L) or TC21(72L). In vitro Raf kinase assays were done on immunoprecipitated Raf-1 or B-Raf, using a Raf-immunocomplex coupled kinase assay as described in Materials and Methods. Data are representative of three independent assays.

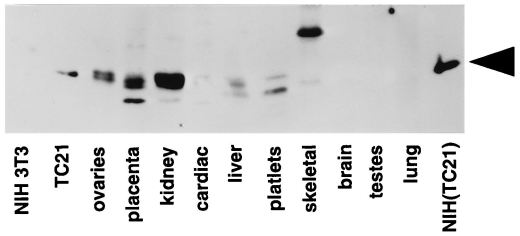

FIG. 9. TC21 protein is preferentially expressed in adult human kidney, ovary, and placental tissues. Detergent lysates of tissue from the indicated human organs (provided by Gil White, University of North Carolina at Chapel Hill) were resolved by SDS-PAGE (equivalent total protein amounts were loaded in all lanes), transferred to membranes, and Western blotted with affinity-purified anti-TC21 antiserum. A lysate of NIH 3T3 cells stably transfected with pZIP-*tc21*(WT) was used as a positive control for the mobility of TC21(WT) (indicated by arrow). Antibody binding was detected by enhanced chemiluminescence (Amersham).

tor targets such as Raf-1. However, since our data suggest that TC21 causes transformation via a Raf-independent mechanism, we determined if Krev-1 was capable of blocking TC21 transforming activity. To evaluate this, we transfected TC21(72L) or H-Ras(61L), either alone or together with the Krev-1(63E) mutant (41), into NIH 3T3 cells and assayed for inhibition of focus-forming activity. Consistent with previous studies (41), we found that this Krev-1 mutant caused a greater than 50% reduction in H-Ras(61L) focus-forming activity (Fig. 8). Unexpectedly, Krev-1(63E) also caused a significant (>50%) reduction in TC21(72L) focus-forming activity. Since Raf kinases are not critical effectors for TC21 transformation, these results suggest that Krev-1 antagonizes Ras and TC21 by competing with effectors in addition to, or other than, Raf.

TC21 protein is differentially expressed in human tissues. The three Ras proteins are expressed ubiquitously in all tissues, although higher expression is seen in some tissues (26). For example, H-Ras expression is highest in the brain (26). Since our data suggest that Ras and TC21 proteins have overlapping functions, we wanted to determine if TC21 is also ubiquitously expressed. For these analyses, we used an affinity-purified anti-TC21 antiserum for Western blot analyses of cell lysates derived from a spectrum of human tissues. NIH 3T3 cells stably expressing high levels of TC21(WT) from an exogenously introduced expression vector were used as a control for the electrophoretic mobility of human TC21(WT). We observed that the level of TC21 protein expression varied widely in the different tissues (Fig. 9). Whereas high TC21 protein expression was seen in kidney, placenta, and ovaries, no significant expression was seen in brain, testes, or lung tissue. Low levels were seen in cardiac and skeletal muscle as well as in liver tissue. The identity of the additional bands that were detected by this antiserum in some samples (e.g., skeletal muscle) is not known. These results suggest that in contrast to Ras proteins, TC21 protein function may be restricted to certain tissues.

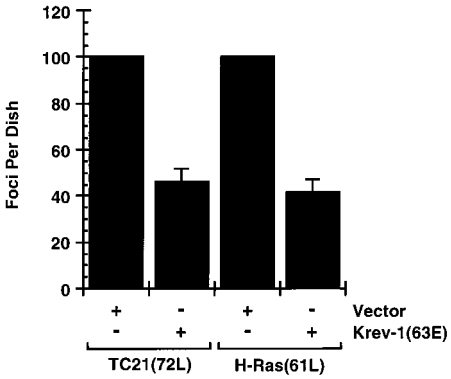

FIG. 8. Coexpression of Krev-1/Rap1a inhibited TC21(72L) focus-forming activity. Cotransfection focus formation assays were done to determine if activated Krev-1(63E) could block TC21(72L) focus-forming activity in NIH 3T3 cells. Ten nanograms of pZIP-NeoSV(x)1 retrovirus expression plasmid DNA construct encoding TC21(72L) or H-Ras(61L) was transfected per 60-mm-diameter dish, together with either 2 μg of pZIP-K*rev*-1(63E) or pZIP-NeoSV(x)1 (empty vector) plasmid DNA. The appearance of transformed foci was quantitated after 14 to 16 days. Data are the averages of four plates and are representative of two independent assays.

DISCUSSION

We and others have observed that mutant forms of TC21 show transforming potencies which are comparable to those seen with oncogenic forms of the three Ras proteins (10, 28). Thus, like Ras, TC21 is likely to regulate signaling pathways that influence cell growth and differentiation. However, the extracellular signal-mediated signaling pathways that cause TC21 activation, and the effectors which are stimulated by activated TC21, have not been identified. To begin to define these pathways, we determined if TC21 function is controlled by Ras regulators and effectors. First, we found that Ras GEFs and GAPs can function as positive and negative regulators of TC21. These results suggest that extracellular signals that activate Ras via upregulation of Ras GEF, or downregulation of Ras GAP, function are likely to cause a coordinate activation of TC21. Second, we observed that H-Ras, but not TC21, interacted with and activated Raf-1 and B-Raf kinases. Therefore, whereas Raf kinases are clearly important effectors for Ras transformation, TC21 causes transformation via Raf-independent signaling events. Finally, we found high TC21 protein expression in kidney, placenta, and ovaries, but no detectable expression was seen in brain, testes, or lung tissue. This expression pattern is distinct from those described for the three Ras proteins and suggests that TC21 function is more restricted than that of Ras proteins. Taken together, these observations show that TC21 possesses functions which are both common to and distinct from those of Ras proteins.

While we were not able to determine directly that Ras GEFs could stimulate GDP/GTP exchange in vitro, we used two in vivo assays to demonstrate that Ras GEFs could regulate TC21 function. First, we showed that SOS1 overexpression synergistically enhanced TC21(WT) stimulation of Elk-1 transcriptional activity and focus-forming activity. Second, we showed

that dominant negative mutants of TC21, like H-Ras mutants which block Ras GEF function, also blocked SOS1 activation of H-Ras(WT) and TC21(WT). While these assays are indirect, and do not exclude the possibility that Ras GEF activation of Ras then enhances TC21 function, our observations clearly show that Ras GEFs can enhance TC21 function. The ability of SOS1 to stimulate TC21 was unexpected in light of our recent observation that the closely related R-Ras protein (70% identity) was not responsive to either SOS1 or RasGRF/CDC25 (34). However, these results suggest that extracellular stimuli that transiently activate Ras, either via upregulation of GEFs or downregulation of GAPs, will also activate TC21. Final confirmation of this possibility will require a demonstration that TC21-GTP levels are upregulated by the same stimuli that activate Ras. Since our preliminary analyses have found that the currently available TC21 antisera are not useful for assaying GDP/GTP loading in vivo, we have been unable to directly address this possibility.

The ability of Ras GEFs to activate TC21 also suggests that the H-Ras(15A) and H-Ras(17N) dominant negative proteins, which inhibit Ras function by forming inactive complexes with Ras GEFs (36, 58, 67), are also inhibitors of TC21 function. Since these dominant negative proteins have been used widely as reagents to establish the role of Ras in specific signaling activities (61), it is important to consider the possibility that TC21 function is also involved in biochemical or biological activities that are antagonized by dominant negative H-Ras. Whether TC21 is also activated by additional TC21-specific GEFs will be important to determine since it is possible that extracellular stimuli that activate such GEFs will specifically activate TC21 and not Ras.

We also observed that Ras GAPs can stimulate the low intrinsic GTPase activity of TC21(WT) and that the biochemical defect in the TC21(72L) transforming mutant is a loss of GAP sensitivity. Since TC21 is sensitive to NF1-GAP, it is possible that TC21 activity, like that of Ras, is upregulated in malignant schwannomas that have lost NF1-GAP activity (1, 19). Hence, the deregulated function of not only Ras but also TC21 may contribute to the tumorigenic growth properties of these cells. Alternatively, since the NF1-GRD activity was weak, it is possible that NF1 is not a physiologically relevant negative regulator of TC21 function. Finally, it remains possible that TC21 is also regulated by TC21-specific GAPs which are not active on Ras proteins.

Although TC21 has complete identity with the Ras core effector domain, which is required for Ras interaction with Raf-1, our results showed that TC21 neither binds to nor promotes the activation of Raf kinases. First, we observed that TC21 failed to bind to full-length Raf kinases in yeast two-hybrid binding assays. Second, we found that Raf-1 and B-Raf kinase activities were constitutively elevated in H-Ras- but not TC21-transformed cells. The ability of TC21 to bind to an isolated Ras-binding sequence of Raf-1 but not to full-length versions of the three Raf kinases is not surprising in light of our recent observations that H-Ras complexes with Raf-1 via two distinct binding sequences on each protein (7). The conversion from inactive Ras-GDP to active Ras-GTP causes limited conformation changes which are restricted to two regions of Ras, designated switch I (residues 30 to 38 in the Ras core effector domain) and switch II (residues 59 to 76) (44, 49). Whereas Ras switch I residues interact with a Ras-binding domain within Raf-1 residues 51 to 130, Ras switch II residues are required for interaction with residues in the Raf-1 cysteine-rich domain (20). Since mutations in either switch I or II abolish Ras transforming activity, Ras interactions with both Raf sequences are probably required for Ras-mediated activation of Raf-1 and for Ras transformation (20). Thus, we suspect that TC21 fails to interact with both Raf-1 domains and consequently fails either to promote Raf translocation to the plasma membrane or to allow the as yet unidentified events required to activate Raf kinase at the plasma membrane. Finally, the ability of an isolated Ras-binding sequence of Raf-1, but not full length Raf-1, to interact with TC21 shows that binding analyses done with isolated Ras-binding sequences may not accurately predict whether interactions will occur with the full-length counterparts.

We had determined previously that transforming mutants of TC21 caused constitutive upregulation of p42 and p44 MAPKs and activated the Elk-1 transcription factor (28). Therefore, we had anticipated that TC21 would activate these downstream events by activation of Raf kinases. However, our observations suggest that TC21 must activate MAPKs via a non-Raf effector. Since there is evidence for Raf-independent signaling pathways that cause activation of MAPKs (75), it is likely that TC21 utilizes other MEK activators, such as MEK kinases, to activate this pathway.

The ability of TC21 to cause transformation via Raf-independent effectors is not surprising in light of recent observations that Raf-independent pathways are also important for Ras transforming activity (35, 39, 74). In particular, we found that two H-Ras effector domain mutants [designated H-Ras(12V, 37G) and H-Ras(12V, 40C)], which failed to bind to or activate Raf-1, still retained the ability to cause full tumorigenic transformation of NIH 3T3 cells. While the effectors used by these two mutants to cause transformation are not known, they may also represent the key effectors that mediate TC21 transformation. Thus, it is likely that at least some of the recently identified candidate Ras effectors, such as phosphatidylinositol-3-OH kinase or RalGDS proteins, will be important effectors of TC21 and Ras transformation.

How Rap1A/Krev-1 antagonizes Ras signaling and transformation is presently still unresolved. In vitro and yeast two-hybrid binding analyses have shown that Rap1A can bind to the Ras-binding domain of Raf and other candidate Ras effectors. Since Rap1A has been found to exhibit a subcellular localization to the endoplasmic reticulum and Golgi complex (2, 65), rather than to the inner surface of the plasma membrane where Ras is found, it has been speculated that Rap1A may compete for key Ras effectors which are essential for Ras transforming activity. In the present study, we found that TC21 transforming activity was not mediated through interaction with Raf kinases, yet TC21 transforming activity is blocked by Rap1A. Thus, our results suggest that Krev-1 antagonizes both TC21 and Ras transforming activity by sequestering non-Raf proteins and provide further support for the importance of other candidate Ras effectors in Ras transformation.

It is still unclear whether the three Ras proteins (H-, K-, and N-Ras) exhibit functionally distinct roles in cell physiology. Since gene knockout studies showed that H-*ras*- or N-*ras*-deficient mice exhibited essentially normal growth and development (70), it seems likely that Ras proteins are functionally redundant. In this study, we show that TC21 is distinct from Ras and causes transformation by Raf-independent downstream signaling. Since a TC21 homolog is also found in *D. melanogaster* (30), we suggest that TC21 is likely to have functions which are distinct from those of the three Ras proteins. Confirmation of this possibility will require the generation of TC21-deficient mice. Finally, whereas Ras proteins are expressed ubiquitously, TC21 expression is more restricted. Interestingly, TC21 is expressed in tissues (ovaries, kidney, and liver) which give rise to tumors which typically lack mutated *ras* genes. Therefore, it is possible that mutation (10) or overex-

pression of TC21 is important in the development of neoplasms that arise from these tissues. Support for this possibility is provided by the detection of a mutated and transforming form of TC21 in an ovarian carcinoma cell line (10). In summary, it is possible that TC21 controls normal cellular processes which are distinct from those that are controlled by Ras proteins and that aberrant TC21 function causes neoplasms which are distinct from those associated with mutated Ras.

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