Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 1577–1581, February 1996
Biochemistry

Peptides containing a consensus Ras binding sequence from Raf-1 and the GTPase activating protein NF1 inhibit Ras function

(mitogen-activated protein kinase/signal transduction/transformation)

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Communicated by Alfred G. Redfield, Brandeis University, Waltham, MA, October 30, 1995 (received for review August 28, 1995)

ABSTRACT **A key event in Ras-mediated signal transduction and transformation involves Ras interaction with its downstream effector targets. Although substantial evidence has established that the Raf-1 serine/threonine kinase is a critical effector of Ras function, there is increasing evidence that Ras function is mediated through interaction with multiple effectors to trigger Raf-independent signaling pathways. In addition to the two Ras GTPase activating proteins (GAPs; p120- and NF1-GAP), other candidate effectors include activators of the Ras-related Ral proteins (RalGDS and RGL) and phosphatidylinositol 3-kinase. Interaction between Ras and its effectors requires an intact Ras effector domain and involves preferential recognition of active Ras–GTP. Surprisingly, these functionally diverse effectors lack significant sequence homology and no consensus Ras binding sequence has been described. We have now identified a consensus Ras binding sequence shared among a subset of Ras effectors. We have also shown that peptides containing this sequence from Raf-1 (RKTFLKLA) and NF1-GAP (RRFFLDIA) block NF1-GAP stimulation of Ras GTPase activity and Ras-mediated activation of mitogen-activated protein kinases. In summary, the identification of a consensus Ras–GTP binding sequence establishes a structural basis for the ability of diverse effector proteins to interact with Ras–GTP. Furthermore, our demonstration that peptides that contain Ras–GTP binding sequences can block Ras function provides a step toward the development of anti-Ras agents.**

Ras proteins are GDP/GTP binding proteins that function as molecular switches by relaying signaling events from the cell surface to the nucleus, thus regulating cell growth and differentiation (1, 2). Ras function is regulated by guanine nucleotide exchange factors that promote formation of active Ras–GTP and by GTPase activating proteins (GAPs; p120- and NF1-GAP) that promote formation of inactive Ras–GDP (3–5). Oncogenic Ras mutants are defective in GAP responsiveness and are chronically GTP-bound, resulting in constitutive activation of Ras-mediated signaling events that promote the aberrant growth of tumor cells.

It is now clear that the c-Raf-1 serine/threonine kinase functions as a downstream effector for promoting Ras-triggered activation of the mitogen-activated protein kinase (MAP kinase) pathway (1, 2). However, there is increasing evidence that Ras function is also mediated by Raf-independent signaling pathways (6). For example, Wigler and colleagues (7) have recently identified an effector domain mutant of oncogenic Ras that does not bind Raf-1 yet retains a signaling activity required for Ras transformation. Furthermore, the increasing number of putative Ras effectors also provides support for the existence of Raf-independent Ras signaling pathways. These include p120- and NF1-GAP, two guanine nucleotide exchange factors and activators of Ral proteins (RalGDS and RGL) (8–10), phosphatidylinositol 3-kinase (11), MAP kinase/ERK kinase kinase 1 (MEKK1) (12), Rin1 (13), and the *Schizosaccharomyces pombe* proteins Scd1 (a Rho guanine nucleotide exchange factor) and Byr2 (a serine/threonine kinase) (14). Like Raf-1, these structurally and functionally diverse proteins exhibit preferential binding to Ras–GTP and these interactions are dependent on an intact Ras effector domain (residues 32–40). Mutations in the Ras effector domain impair both Ras transforming activity and interaction with these effector proteins. While the importance of Raf-1 in mediating Ras function is well-established, the precise roles of these other putative Ras effectors in Ras function remain to be determined.

Since high-affinity binding to Ras–GTP requires a small region surrounding the Ras effector domain (residues 26–48) (15), it is plausible that the complementary effector contacts also employ a compact region. Surprisingly, many putative Ras effectors lack significant sequence homology, and attempts to identify a common framework containing recognition elements for binding Ras–GTP have been unsuccessful. However, the inability to identify a consensus binding sequence shared among dissimilar Ras effectors may instead reflect the inability of sequence comparison algorithms to correctly identify a small consensus motif in the context of large input sequences. Consequently, we initiated studies to identify more restricted Ras–GTP binding sequences that could be more effectively analyzed for sequence homology. In this study, we describe the identification of a consensus Ras-binding sequence that is shared among a subset of Ras effectors. Furthermore, we show that peptides containing this sequence from Raf-1 and NF1-GAP can inhibit Ras interaction with NF1-GAP and Raf-1.

MATERIALS AND METHODS

Cell Culture and Transformation Analysis. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) calf serum. DNA transfections were performed by using the calcium phosphate precipitation technique. Cells were transfected with pZIP-*ras*H plasmid DNAs encoding oncogenic H-Ras (12V or 61L) (10 or 50 ng per dish), either alone or with 2 μg of the empty pZIP-NeoSV(x)1 retrovirus vector plasmid or plasmid constructs encoding Raf-Cys (pCGN-*raf*-Cys) (16) or NF1-56 (pZIP-NF1-56). Transfections were performed in triplicate, and transformed foci were quantitated after 14–16 days. Relative focus-forming units shown are normalized to the activities of

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Abbreviations: MAP kinase, mitogen activated protein kinase; MEKK1, MAP kinase/ERK kinase kinase 1; GAP, GTPase activating protein; GST, glutathione *S*-transferase.

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oncogenic Ras ($3–6 \times 10^3$ foci per μg of transfected plasmid DNA).

Expression and Purification of Ras and Glutathione *S*-Transferase (GST)–Raf Fusion Proteins. Expression and purification of bacterially expressed H-Ras protein was performed as described (17). Complex formation of Ras and guanosine 5′[β,γ-methylene]triphosphate, a nonhydrolyzable GTP analog (Boehringer Mannheim), has been described (18). Both NF1-56 and Raf-Cys were prepared as GST fusion proteins, as described (19).

Enzyme-Linked Immunosorbent Assay (ELISA). *In vitro* Ras binding interactions were measured by incubating 100 pmol of GST fusion protein and corresponding amounts of GST bound to 96-well microtiter plates with H-Ras complexed to guanosine 5′-[β,γ-methylene]triphosphate or GDP at concentrations from 31 nM to 2 μM as described (16).

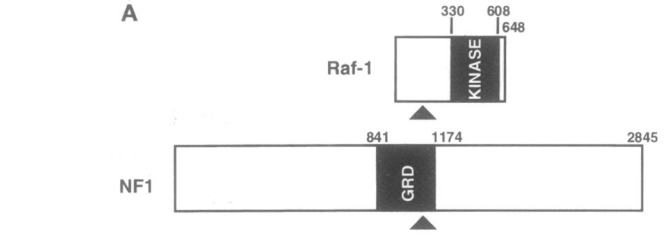
Synthesis of Peptides. Peptides were synthesized by the UNC/PMBB Facility by using standard solid-phase methods and purified by reverse-phase HPLC. All amides were acetylated at their NH_2 termini. Peptide sequences are as follows (consensus sequence residues are underlined): Raf-L, CNFARKTFLKLAFC; Raf-L-Scram, FFNCACLKAFRHATK; Raf-S, FARKTFLKLAF; Raf-S-Scram, AKFLRLFKFAT; NF1-L, CNFDAARRFFLDIASC; NF1-S, ARRFFLDIAD; NF1-S-Scram, DFLRIFRADA. The Raf-L and NF1-L peptides were synthesized with one additional cysteine at the NH_2 terminus so that constrained peptides could be generated.

NF1-GRD GAP Inhibition Assay. GTPase activity was determined using 4 nM [γ-^{32}P]GTP-labeled Ras, 0.2 nM NF1-GRD, and various concentrations of peptides as described (20). The amount of phosphate released was quantitated by scintillation counting, after an organic extraction in the presence of ammonium molybdate. Assays were performed three times in duplicate.

Oocyte Lysate MAP Kinase Assay. Preparation of *Xenopus* oocyte lysate and the Ras-mediated activation of MAP kinase assay were done as described (21–23). Assays were performed with H-Ras(61L) (5 μg/ml) and 35 μM synthesized peptide. Peptide concentrations were determined by amino acid composition analysis of peptide stock solutions by the Protein Chemistry Laboratory (Univ. of North Carolina, Chapel Hill-National Institute of Environmental Health Sciences). Briefly, oocyte lysate, an ATP regeneration system, Ras, and the appropriate peptide were incubated at 20°C for 2 hr. The reaction was then stopped and frozen. One-third of the reaction mixture was then added to a kinase assay system with an excess of myelin basic protein as the substrate. After incubation for 20 min at 20°C, the reaction was stopped and resolved by SDS/polyacrylamide gel electrophoresis. The degree of activation of MAP kinase at a given time point for a given sample was measured by the relative incorporation of ^{32}P by the myelin basic protein substrate and was quantitated by autoradiography and AMBIS β scanning.

RESULTS

Identification of a Consensus Ras Binding Sequence in Raf-Cys and NF1-56. To determine whether Raf-1 and other Ras effectors shared a consensus Ras-binding sequence, we first wanted to more precisely determine and characterize the sequences of NF1-GAP and Raf-1 that were involved in Ras binding. We concentrated our analyses on 48- and 56-amino acid fragments from Raf-1 and NF1-GAP, designated Raf-Cys and NF1-56, respectively (Fig. 1*A*). We have recently shown that Raf-Cys, corresponding to the cysteine-rich domain (Raf-1 residues 139–184), shows high-affinity GTP-dependent binding to Ras *in vitro* and antagonizes Ras function *in vivo* (16). NF1-56 is derived from the 334-amino acid GAP-related domain of NF1-GAP, designated NF1-GRD. NF1-GRD exhibits the same abilities as NF1-GAP to bind to Ras and stimulate its GTPase activity (4). Although the interaction of NF1-56 and Ras has not been observed previously, the expression of this fragment in Ras-transformed cells reduced their ability to form colonies in soft agar (24), suggesting that it may directly complex with Ras to block transformation.



											Accession #
c-Raf-1	143	R	K	T	F	L	K	L	A	150	P04049
A-Raf	103	R	K	T	F	F	S	L	A	110	P10398
B-Raf	123	R	K	T	F	F	T	L	A	130	P15056
D-Raf	269	R	K	T	F	F	S	L	V	276	P11346
NF1-GAP	1121	R	R	F	F	L	D	I	A	1128	P21359
p120-GAP	927	A	R	T	L	I	L	V	A	934	P20936
Ira1	1729	R	K	P	F	I	T	L	A	1736	P18963
Ira2	1875	K	R	T	F	I	S	L	A	1882	P19158
SarGap	364	R	K	T	L	A	T	I	A	371	A40258
		1	2	3	4	5	6	7	8		

FIG. 1. Diagram and sequences of Ras-binding fragments and peptides from Raf-1 and NF1-GAP. (*A*) Arrow indicates location of Raf-Cys and NF1-56 in Raf-1 and NF1-GAP, respectively. (*B*) Regions of Raf-Cys and NF1-56 sharing high sequence similarity and a comparison of Ras–GTP recognition sequences contained in Raf and GAP family members. Sequence alignment and data base searches were performed by using the Sequence Analysis Software Package from Genetics Computer, Inc. (the GCG Package). Homologous sequences are boxed. Numbers indicate residue positions in the full-length proteins. GenBank accession numbers are given.

Therefore, we utilized two approaches to evaluate NF1-56 interaction with Ras (16).

First, as we have described (16) for Raf-Cys, we observed that cotransfection of NF1-56 with oncogenic Ras resulted in efficient (≈70%) inhibition of Ras focus-forming activity (Fig. 2*A*). To demonstrate that this inhibitory activity results from an inactive complex between NF1-56 and Ras, we utilized an *in vitro* ELISA as described (16). NF1-56, like Raf-Cys, showed high-affinity preferential binding to GTP-complexed Ras (Fig. 2*B*). Therefore, the NF1-GAP and Raf-1 fragments employed here contain the shortest sequences that have been shown to be capable of interacting with active Ras–GTP and blocking its transforming activity in mammalian cells.

Our demonstration that Raf-Cys and NF1-56 share common Ras binding properties provided us with two small fragments with which to search for a common Ras-binding sequence. Using the GCG BESTFIT algorithm, we identified a contiguous stretch of 8 amino acids shared between Raf-Cys and NF1-56 displaying 75% amino acid similarity (Fig. 1*B*). Since no additional sequence homologies were detected, we anticipated that this represented a consensus Ras-binding sequence. To address this possibility, we synthesized short amino acid peptides corresponding to these sequences from NF1-GAP and Raf-1 and then determined their abilities to antagonize Ras–effector interactions.

Peptides Containing the Consensus Ras Binding Sequence from Raf-1 and NF1-GAP Inhibit Ras–Effector Interactions. To determine whether these peptides could inhibit the ability of NF1-GAP to stimulate the intrinsic GTPase activity of Ras, we utilized NF1-GRD, which contains the catalytic domain responsible for Ras binding and Ras GTPase stimulation. We observed that 14- to 16-amino acid peptides corresponding to the putative Ras binding sequences of both Raf-1 (designated Raf-L; Fig. 3) and NF1-GAP (NF1-L; data not shown) were potent inhibitors of NF1-GRD stimulation. In contrast, a control peptide containing the identical amino acid content of Raf-L but scrambled in sequence (Raf-L-Scram) showed no inhibition of NF1-GRD. Comparable inhibition was also observed with shorter 10- to 11-amino acid peptides containing the consensus sequence (Raf-S and NF1-S peptides; data not shown). The concentrations required for 50% inhibition of NF1-stimulated Ras GTPase activity were found to be 86.5 ± 8.9 μM and 44.4 ± 6.6 μM, for NF1-S and Raf-S, respectively. Since NF1-GRD interaction with Ras is dependent on an intact Ras effector domain, these results suggest that both Raf-1 and NF1-GAP peptides could block Ras–NF1-GAP interaction.

We next determined whether these peptides could antagonize oncogenic Ras activation of p42 and p44 MAP kinases by using an *in vitro Xenopus* oocyte lysate assay (3). It has been shown that addition of oncogenic Ras protein to oocyte lysates results in a Raf-dependent activation of MEK and MAP kinases (21–23). The addition of either Raf-1 or NF1-GAP peptide showed potent inhibition of MAP kinase activation at concentrations of 35 μM (>75%), whereas the control peptide showed no inhibition (Fig. 4). These results suggest that peptides derived from the consensus sequence of Raf-Cys and NF1-56 prevented Ras-mediated activation of Raf-1, thus preventing activation of MAP kinases.

Having identified a putative functional Ras-GTP recognition motif, we performed data base searching to identify this consensus sequence in other mammalian and yeast Ras GAPs (IRA1, IRA2, and SarGAP) and in other Raf kinase family members (A-Raf, B-Raf, and D-Raf) (Fig. 1*B*). With the exception of p120-GAP, the first 2 residues in the consensus sequence contain either lysine or arginine. The absence of a basic residue at position 1 may explain why p120-GAP possesses a lower affinity for Ras–GTP relative to other Ras effectors. Position 4 requires phenylalanine or leucine, whereas hydrophobic amino acids are preferred at positions 5 and 7. Conservation of alanine at position 8 suggests this residue is important for Ras binding.

DISCUSSION

Although substantial experimental evidence has established a critical role of the Raf-1 serine/threonine kinase as a downstream effector for Ras signal transduction and transformation, there is increasing evidence that Ras function may also require stimulation of Raf-independent signaling pathways. The possibility that Ras function is triggered via its interaction with multiple effectors is supported by the identification of increasing numbers of candidate downstream effectors for Ras. Included among these are the two Ras GAPs, phosphatidylinositol 3-kinase, the MEKK1 serine/threonine kinase, and two Ral guanine nucleotide exchange factors (4, 8–12). Like Raf-1, each of these proteins show preferential association with active Ras–GTP and this binding requires an intact Ras effector domain. Surprisingly, sequence homology analyses showed no common sequences in these structurally and functionally diverse proteins that would mediate interaction with activated Ras–GTP. Therefore, it has been assumed that Ras effectors do not share a common sequence motif involved in Ras binding. However, in the present study, we have identified a consensus Ras binding sequence that is present in a subset

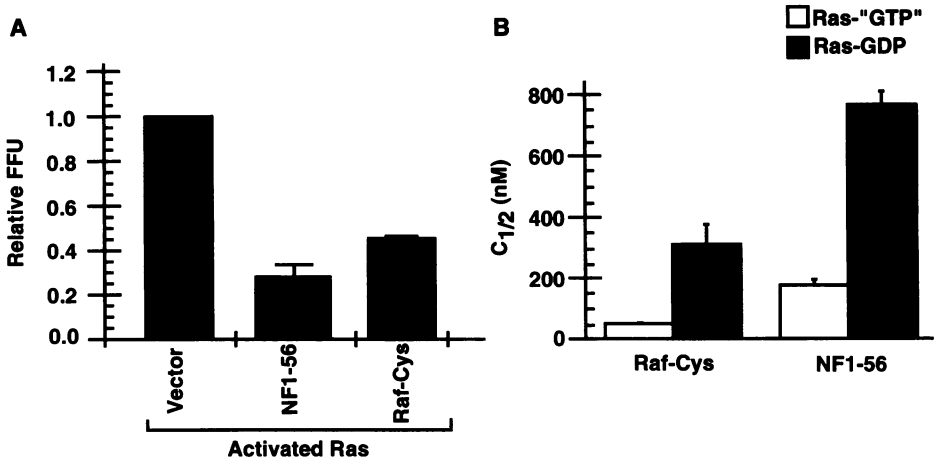


FIG. 2. Interaction of Raf-Cys and NF1-56 with Ras *in vivo* and *in vitro*. (*A*) Cotransfection of Raf-Cys or NF1-56 significantly reduces Ras(61L) focus-forming activity. FFU, focus-forming units. (*B*) NF1-56 preferentially binds to Ras–GTP. Ras concentrations required for half-maximal binding ($C_{1/2}$) to 100 pmol of plated Raf-Cys and NF1-56 were compared. All experiments were performed in triplicate with GST (control) absorbance values subtracted from the absorbance of GST–Raf and GST–NF1 proteins.

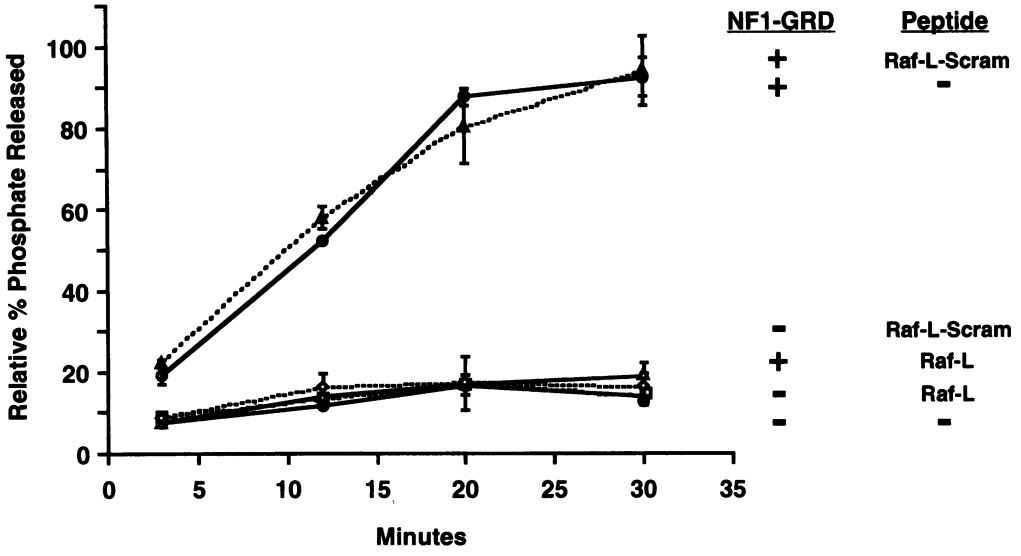


FIG. 3. Consensus Ras-binding sequence peptides inhibit NF1-GRD-stimulated Ras GTPase activity. Raf-L, but not the scrambled peptide control at 250 μM, shows complete inhibition of NF1-GRD stimulation of H-Ras GTPase activity. The results represent two assays that were performed in triplicate. NF1-GRD competition experiments were also conducted on the following peptides: NF1-L, Raf-S, Raf-S-Scram, NF1-S, and NF1-S-Scram. All reactions contained Ras and the following additions: ●, no addition, ○, NF1-GRD only; △, Raf-L-Scram only; ▲, NF1-GRD + Raf-L-Scram; □, Raf-L only; ◇, NF1-GRD + Raf-L.

of Ras effectors. The importance of this sequence is demonstrated by the ability of peptides that contain the consensus sequence from Raf-1 and NF1-GAP to block functional interaction with these two effectors.

Although the region encompassing Raf-1 amino acids 51–131 clearly defines a minimal Ras binding sequence (25), we recently determined that the Raf-1 cysteine-rich domain (residues 139–184) represents a second Ras binding sequence in the NH_2 terminus of Raf-1 (16). The existence of a second Ras binding domain was unexpected since a single amino acid substitution at Raf-1 residue 89 is sufficient to abolish Ras–Raf interaction (26). Furthermore, the recent determination of the crystal structure of Raf-1 residues 51–131 with the Ras-related protein Rap1A (which shares complete identity with Ras residues 32–40) implicated a direct interaction between these Raf-1 residues and the Ras effector domain (27). However, we have recently established that Raf-Cys contains a cryptic Ras binding site in unstimulated Raf-1 and that Ras interaction with both Ras binding sites are required for Ras transformation (28, 30). Furthermore, our demonstration that peptides containing the consensus Ras binding sequence can block Ras activation of the MAP kinase cascade *in vitro* provides additional evidence that Ras interaction with Raf-Cys is required for Ras-mediated activation of Raf. Thus, inhibition of Ras interaction with either Ras binding domains of Raf-1 is likely to be sufficient to impair the ability of Ras to mediate activation of the Raf-1 kinase.

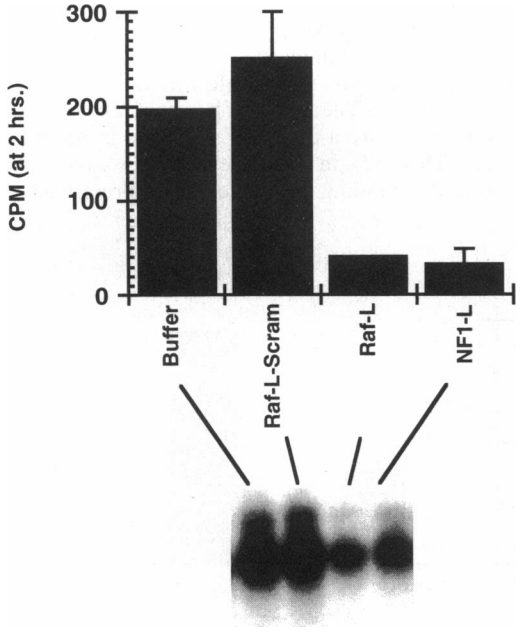


FIG. 4. Consensus Ras binding sequence peptides inhibit oncogenic Ras activation of MAP kinases *in vitro*. MAP kinase activity was measured by phosphorylation of exogenous myelin basic protein. Data from three experiments were averaged, with each condition done in triplicate. Representative data from one experiment are shown below. Background counts were subtracted from cpm determinations at 2 hr.

We have searched for this motif in other candidate Ras effectors and identified similar sequences in mammalian MEKK1 (GenBank accession no. A46212; residues 43–50), RalGDS (GenBank accession no. 528415; residues 820–827), as well as in yeast adenylate cyclase (GenBank accession no. P08678; residues 951–958). MEKK1 has been shown to complex with Ras and to be activated by a Ras-dependent pathway (12, 28). In addition, both yeast adenylate cyclase and RalGDS can associate with Ras and the consensus sequence is present in RalGDS fragments that were identified in yeast two-hybrid binding assays (8–10). However, the absence of this consensus sequence in Rin1 suggests that there are multiple Ras-binding sequences in different Ras effectors. For example, we have shown that two nonhomologous sequences in Raf-1 are capable of discriminating between active and inactive forms of Ras (16). In summary, the identification of consensus Ras–GTP recognition sequences establishes a structural basis for the ability of functionally diverse proteins to recognize Ras–GTP.

The frequent association of mutated Ras with human tumors has prompted considerable interest in identifying approaches to blocking Ras function for cancer treatment. For example, peptide-based inhibitors of protein farnesyltransferase, which catalyzes the addition of a farnesyl isoprenoid to Ras, have been developed and shown to be potent inhibitors of Ras function (29). However, since such inhibitors also affect other farnesylated proteins and since farnesylation is critical for both normal and oncogenic Ras function, such compounds may not preferentially antagonize oncogenic Ras. In contrast,

since oncogenic, but not normal, Ras proteins persist in a chronic GTP-complexed state, agents that show preferential recognition of Ras–GTP may provide more effective approaches for selective blocking of oncogenic Ras proteins. Thus, our demonstration that peptides containing a consensus Ras–GTP binding sequence can block Ras function may provide a step toward the development of anti-Ras agents.

We thank Katherine Swenson for advice on the oocyte lysate assays, Fuyu Tamanoi for the GST–NF1-GRD expression vector and Hiroshi Maruta for NF1-56. We thank Teresa Brtva, Adrienne Cox, Roya Khosravi-Far, John O'Bryan, and Lawrence Quilliam for critical comments and Ashley Overbeck for excellent assistance in the preparation of figures and the manuscript. This work was supported by grants from the National Institutes of Health to C.J.D. (CA42978, CA55008, and CA63071), R.M.B. (GM38737 and DK20205), and S.C. (CA64569).

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