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Two Distinct Raf Domains Mediate Interaction with Ras*

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A key event for Ras transformation involves the direct physical association between Ras and the Raf-1 kinase. This interaction promotes both Raf translocation to the plasma membrane and activation of Raf kinase activity. Although substantial experimental evidence has demonstrated that Raf residues 51–131 alone are sufficient for Ras binding, conflicting observations have suggested that the Raf cysteine-rich domain (residues 139–184) may also be important for interaction with Ras. To clarify the role of the Raf cysteine-rich domain in Ras-Raf binding, we have compared the ability of two distinct Raf fragments to interact with Ras using both *in vitro* Ras binding and *in vivo* Ras inhibition assays. First, we determined that both Raf sequences 2–140 and 139–186 (designated Raf-Cys) showed preferential binding to active, GTP-bound Ras *in vitro*. Second, we observed that Raf-Cys antagonized oncogenic Ras(Q61L)-mediated transactivation of Ras-responsive elements and focus-forming activity in NIH 3T3 cells and insulin-induced germinal vesicle breakdown in *Xenopus laevis* oocytes *in vivo*. This inhibitory activity suggests that Raf-Cys can interact with Ras *in vivo*. Taken together, these results suggest that Ras interaction with two distinct domains of Raf-1 may be important in Ras-mediated activation of Raf kinase activity.

Numerous biochemical and genetic studies have positioned Raf downstream of Ras in Ras-dependent signal transduction pathways (1) that lead to the activation of the mitogen-activated protein kinases (2). Furthermore, several recent investigations have used *in vitro* protein binding assays and the *in vivo* yeast two-hybrid system to demonstrate a direct physical association between the Ras and Raf proteins, thus identifying Raf as a key downstream target, or effector, of Ras-mediated signal transduction (3–8). This interaction requires the effector domain of Ras (residues 32–40) and a region in the amino-terminal regulatory domain of Raf. The region encompassing Raf-1 amino acids 51–131 has been shown to be sufficient for interaction with Ras (6, 9, 10). In addition, a single amino acid mutation (R89L) in this domain of Raf disrupts the interaction with Ras *in vitro* and prevents Ras-mediated activation of Raf in Sf9 insect cells (11).

While Raf residues 51–131 clearly define a minimal Ras binding domain, other evidence has suggested that the Raf cysteine-rich domain (residues 139–184) may also be involved in Ras-Raf binding. First, a single point mutation (C168S) in this domain was found to reduce Raf (residues 1–257) binding to Ras in both two-hybrid and *in vitro* binding assays (5). Second, it was observed that renaturation of Raf-(1–257) in the presence of zinc, which is required for folding of the cysteine-rich domain, led to greater restoration of Ras binding activity (4). Finally, we and others observed that Raf residues 131–147, which are adjacent to and extend partially into the cysteine-rich domain, are critically important for conferring high affinity binding to Ras *in vitro* (9, 10). However, it remains to be clarified whether the Raf cysteine-rich domain enhances Ras association with Raf residues 51–131 or independently interacts with Ras. To address this question, we have utilized both *in vitro* and *in vivo* analyses to characterize Ras interaction with the Raf cysteine-rich domain. We observe that the isolated Raf cysteine-rich domain shows high affinity, guanine nucleotide-dependent binding to Ras *in vitro* and can function as a dominant inhibitor of Ras signaling and transformation *in vivo*. Thus, the Raf NH_2 terminus contains two distinct Ras binding domains that may be important for Ras-mediated activation of Raf kinase activity.

EXPERIMENTAL PROCEDURES

Molecular Constructs—To generate mammalian expression vector constructs encoding different Raf fragments, we isolated *Bam*HI-*Eco*RI DNA fragments from pGEX-*raf* constructs that encoded the different Raf sequences indicated in Fig. 1 (12) and introduced each into the *Bam*HI site of the pCGN-hyg mammalian expression vector (13) (generously provided by M. Ostrowski, Duke). Raf301 encodes a full-length mutant human Raf-1 sequence that contains a single amino acid substitution (K to W) in the ATP binding site, which inactivates its kinase activity (14).

Expression and Purification of Ras and Glutathione S-Transferase-Raf Proteins—The pAT-*ras*H bacterial expression plasmid and procedures for expression and purification have been described previously (15). Ras protein complexed to GMPPCP,[1] a nonhydrolyzable GTP analog (Boehringer Mannheim), was prepared as described elsewhere (16) by replacement of GDP bound to Ras. Glutathione *S*-transferase-Raf proteins were purified as described previously (12).

Enzyme-linked Immunosorbent Assay (ELISA) for Measuring Ras-Raf Interaction in Vitro—Purified glutathione *S*-transferase and glutathione *S*-transferase-Raf proteins were plated onto 96-well microtiter plates (Costar) coated with 0.025 mg/ml poly-L-lysine and allowed to bind overnight. Wells were blocked for 1 h at room temperature with a phosphate-buffered saline solution containing 130 mM NaCl, 10 mM Na_2HPO_4, and 3 mM KCl, pH 7.40 (PBS), which was supplemented with 0.5% gelatin, 0.05% Tween 20, and 0.2% sheep serum (PBSGTS). H-Ras complexed to either GDP or GMPPCP was captured in PBSGTS for 1 h at concentrations ranging from 31 nM to 2 μM. The plates were washed

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[1] The abbreviations used are: GMPPCP, guanosine 5′-(β,γ-methyleneriphosphate); CAT, chloramphenicol acetyltransferase; ELISA, enzyme-linked immunosorbent assay; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

3 times in PBS with 0.1% Tween 20 and then incubated for 1 h with an anti-H-Ras antibody (LAO69) (Quality Biotech) diluted 2000-fold in PBSGTS. The wash step was repeated, and the plates were incubated with a 1:1000 dilution of sheep anti-mouse IgG-alkaline phosphatase conjugate (Sigma) in PBSGTS for 30 min, followed by development with the chromogen, *p*-nitrophenyl phosphate (Sigma). Optical densities (405 nm) were read after 30 min in a Biotech microtiter plate reader. The intensity of the absorbance was directly related to the amount of Ras bound. The concentration of Ras at half-maximal binding was determined as described previously (12).

NIH 3T3 Transcription Activation and Transformation Assays—NIH 3T3 cells were grown 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 (17). The pB4X-CAT reporter plasmid contains the CAT gene, and CAT is driven by a minimal promoter that contains the *ets*/AP-1 Ras-responsive promoter element (18). Cells were co-transfected with the pZIP-*ras*H(Q61L) plasmid DNA encoding H-Ras(Q61L) (50 ng/dish) and either the empty pCGN-hyg vector or the appropriate pCGN-*raf* construct (5 μg/dish) along with 1 μg of the pB4X-CAT reporter. Forty-eight h after transfection, total cell lysates were prepared, and CAT activity was determined as described previously (19). A similar co-transfection analysis was done to assess the ability of each Raf mutant (5 μg/dish) to block Ras(Q61L) (10 ng/dish) transforming activity. Transfections were performed in triplicate, and transformed foci were quantitated after 14–16 days.

Preparation and Experimental Manipulation of Oocytes—Oocytes were isolated from primed *Xenopus laevis* ovaries by procedures described previously (20). Isolated stage VI oocytes were injected with 200 μg of the indicated glutathione *S*-transferase-Raf protein (20 oocytes/Raf protein) in a volume of 40 nl. Subsequent to the injection, the oocytes were treated with 1 μM insulin and maintained in modified Barth's solution with Ca^{2+}, between 18 and 20 °C, for maturation. Insulin-induced maturation was scored at definite time intervals by counting the number of oocytes undergoing germinal vesicle breakdown manifested by the appearance of a whitish spot at the pigmented animal pole of the oocytes.

RESULTS AND DISCUSSION

The Raf Cysteine-rich Domain Shows High Affinity, GTP-dependent Association with Ras in Vitro—Although extensive analyses have clearly shown that Raf residues 51–131 alone are sufficient for interaction with Ras, it is presently unclear what the precise role of the cysteine-rich domain (residues 139–184) is in mediating Ras-Raf binding. To address the possibility that this domain can bind Ras, we have conducted both ELISA and direct binding assays (12) to determine if Raf-Cys binds directly to Ras *in vitro* and to assess the dependence of this association on the guanine nucleotide-bound state of Ras. For these assays, we compared the binding activities of a glutathione *S*-transferase fusion protein containing Raf-Cys with the binding properties of glutathione *S*-transferase-Raf-(2–140) (designated Raf-N2), which contains the minimal Raf sequence required for Ras binding (51–131) and includes only the first two residues from the cysteine-rich domain (Fig. 1).

Fig. 2 shows data obtained from an ELISA using various purified recombinant glutathione *S*-transferase-Raf fragments and Ras. For these experiments we prepared stoichiometric complexes of Ras bound to GMPPCP. This nonhydrolyzable GTP analog eliminates GTP hydrolysis during the course of the experiment and is useful for determining whether various Raf fragments bind to Ras in a GTP-dependent fashion. Binding curves were generated by serially diluting Ras complexed to either GMPPCP or GDP into wells containing 100 pmol of each glutathione *S*-transferase-Raf protein. We observed that both Raf-Cys and Raf-N2 showed high affinity binding to Ras. In contrast, a Raf fragment lacking the NH_2-terminal regulatory domain (containing Raf residues 273–648; designated Raf-C) did not bind to Ras. Furthermore, the concentration dependence of the binding curves demonstrates that much higher concentrations of Ras-GDP relative to Ras-GMPPCP were required to saturate binding to either Raf-N2 or Raf-Cys, and

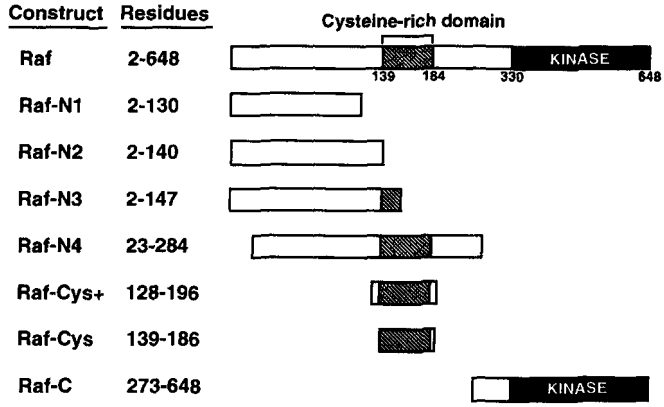

FIG. 1. **Molecular constructs of Raf-1.** The amino acid residues encoded by the various human Raf constructs are indicated as well as the locations of the Raf cysteine-rich and kinase domains. The pGEX-*raf* bacterial expression and pCGN-*raf* mammalian expression constructs were generated as described under "Experimental Procedures."

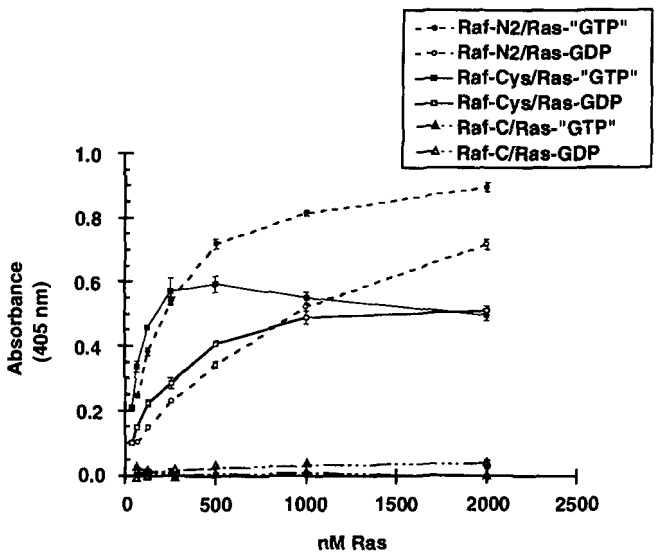

FIG. 2. **The Ras cysteine-rich domain preferentially binds to Ras-GTP.** Three distinct Raf fragments, prepared as glutathione *S*-transferase-Raf fusion proteins, were tested for their ability to bind Ras-GDP and Ras-GMPPCP by antigen capture ELISA as described under "Experimental Procedures." Both Raf-N2 and Raf-Cys exhibited preferential binding to Ras-"GTP," whereas Raf-C did not bind to either form of Ras. Raf-N2 and Raf-Cys bound to Ras-GTP with 130 and 48 nM C_{50} values compared with 870 and 310 nM for Ras-GDP, respectively. All experiments were performed in triplicate with glutathione *S*-transferase (control) absorbance values subtracted from the absorbance values of glutathione *S*-transferase-Raf proteins.

both fragments showed ~7-fold preferential binding to Ras-GTP relative to Ras-GDP. Therefore, these results indicate that two distinct regions within the NH_2-terminal regulatory domain of Raf-1 are capable of specific interaction with GTP-Ras *in vitro*.

Two Independent Amino-terminal Domains of Raf Block Oncogenic Ras Signaling and Transformation in Vivo—Previous studies have yielded discrepancies between the ability of different Ras and Raf mutants to bind when performed by *in vitro versus in vivo* two-hybrid analyses (21). Therefore, we employed three biological assays to determine if Ras-Cys could associate with Ras *in vivo* and consequently block Ras signaling and transforming activity. We and others have previously shown that kinase-deficient mutants of Raf can block Ras function by antagonizing Ras interaction with its downstream effectors (14, 22, 23). Thus, an inhibitory activity of a Raf mutant

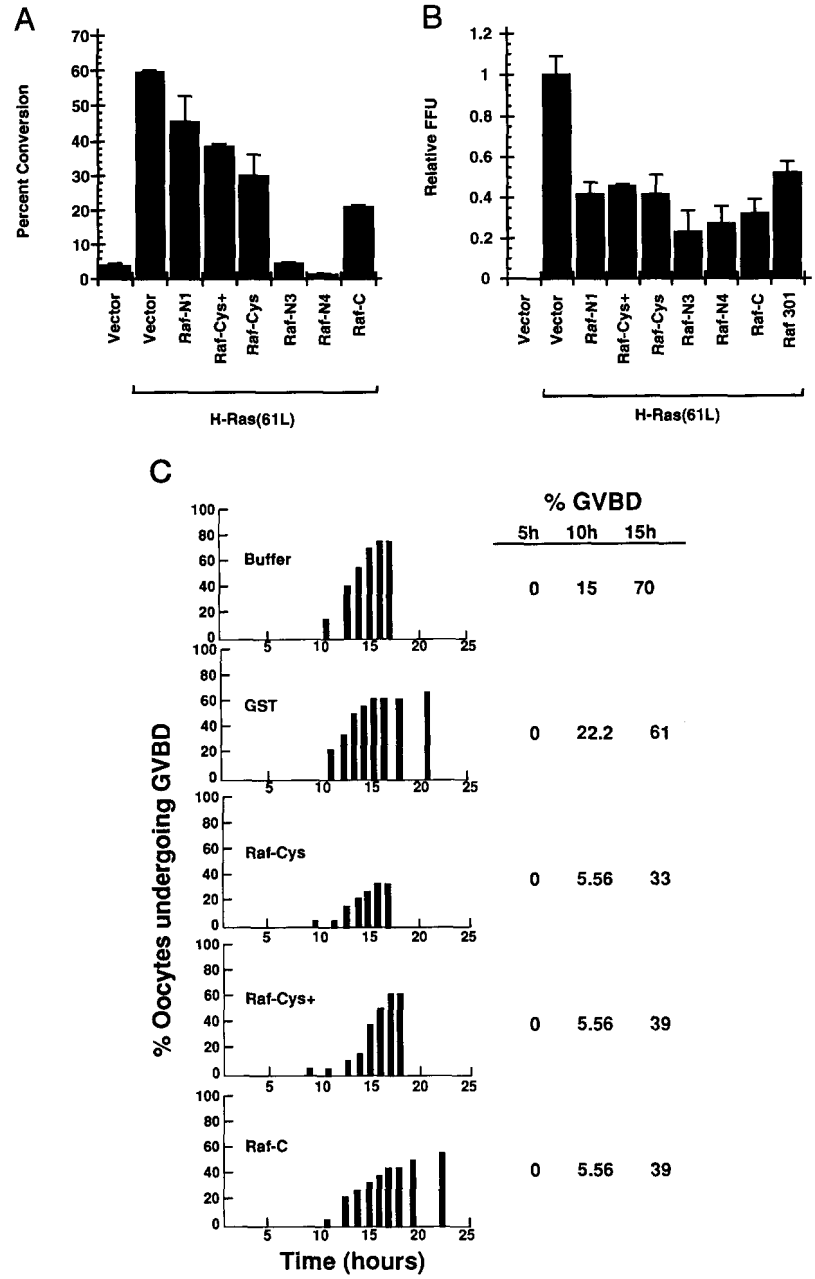

FIG. 3. **Multiple, independent domains of Raf-1 inhibit Ras-dependent signaling *in vivo*.** *Panel A*, Raf fragments block Ras(Q61L) stimulation of transcriptional activation of the *ets*/AP-1 Ras-responsive element. Results from one of four experiments performed in duplicate are shown. CAT assays were performed as described under "Experimental Procedures." *Panel B*, co-transfection of various Raf fragments significantly reduces Ras(Q61L) focus-forming activity. Two experiments were performed in triplicate, and results of one are presented. Relative focus-forming units (*FFU*) shown are normalized to the activity of Ras(Q61L) (8.97×10^3 foci/μg of transfected DNA). *Panel C*, stage VI oocytes from *X. laevis* were isolated and injected with 200 μg each of the indicated proteins. The oocytes were then treated with insulin and scored for germinal vesicle breakdown (*GVBD*), as described under "Experimental Procedures." The data are presented as the percent of oocytes undergoing germinal vesicle breakdown as a function of time after treatment with insulin (compare 15-h time point). GST, glutathione *S*-transferase.

is a strong indication of an *in vivo* interaction with Ras that prevents Ras association with full-length, endogenous Ras effectors. We included the well characterized, kinase-deficient Raf301 dominant inhibitory protein as a control for these studies (14).

We first evaluated the ability of each Raf-1 fragment to inhibit oncogenic Ras-mediated stimulation of transcription from a Ras-responsive reporter plasmid (Fig. 3*A*). As shown previously (22, 23), Raf301 reduced Ras-induced transcriptional activation, whereas wild type Raf further stimulated Ras transcriptional activation (data not shown). Additionally, we observed that both Raf-Cys and Raf-N1 reduced activation by oncogenic Ras *in vivo*. Furthermore, Raf constructs containing both the minimal Ras binding sequence (residues 51–131) and sequences from the cysteine-rich domain (residues 139–184) showed the strongest inhibition of transcriptional activity, possibly due to the cooperative binding of the two independent binding sites.

We next determined whether Raf-Cys could also inhibit oncogenic Ras(Q61L) focus-forming activity. Co-transfection of Raf-Cys as well as other Raf fragments that contained overlapping sequences showed >50% inhibition of oncogenic Ras(Q61L) focus-forming activity (Fig. 3*B*). One possible explanation for the inhibitory action of these Raf fragments is that they inhibit cell growth in a nonspecific manner. However, we observed that for NIH 3T3 cells transfected with each *raf* construct, equivalent numbers of hygromycin-resistant colonies are obtained following drug selection of cells transfected with vector only or with vector constructs encoding each of the Raf fragments (data not shown), thus arguing against this possibility. Additionally, we have isolated stably transfected cells that co-express both oncogenic Ras(Q61L) and certain Raf fragments, and these cells show a flatter morphology, which is more characteristic of untransformed NIH 3T3 cells (data not shown).

Finally, we observed that Raf-Cys and Raf-Cys+ could block insulin-induced germinal vesicle breakdown in *X. laevis* oocytes (Fig. 3*C*). Previous studies have shown that this insulin response is dependent on Ras activity (24–26). This inhibitory activity could be reversed by co-injection of excess oncogenic

Ras protein (data not shown). Thus, we have observed that, like the truncated Raf fragment containing the residues corresponding to the minimal Ras binding sequence (residues 51–131), the Raf cysteine-rich domain can inhibit oncogenic Ras transcriptional activation, transforming activity, and oocyte maturation. Taken together, these observations *in vivo* are consistent with our demonstration that Raf-Cys can bind directly to Ras *in vitro* and support the possibility that Raf contains a second distinct Ras binding site, which can promote Ras-Raf interaction *in vivo*.

A Noncatalytic Carboxyl-terminal Raf Mutant Blocks Ras Signaling in Vivo—Although Raf-C showed no Ras binding activity *in vitro* (Fig. 2*A*), we did observe that this Raf fragment could inhibit oncogenic Ras-mediated transcription activation (Fig. 3*A*), focus-forming activity (Fig. 3*B*), and insulin-induced oocyte maturation (Fig. 3*C*). Raf-C (residues 273–648) lacks both NH_2-terminal Ras binding domains and contains the serine-threonine kinase domain (residues 333–625). However, a previous study has demonstrated that Raf-C lacks transforming activity (27). Thus, we suggest that the inhibitory activity of Raf-C is not a consequence of complex formation with Ras but instead that it inhibits Ras transforming activity by complex formation with the Raf substrate, MEK. MEK has been shown to bind to Raf COOH-terminal sequences that include the kinase domain (7). Consistent with this possibility, we found that exogenously introduced wild type MEK reversed the inhibitory action of Raf-C but not of Raf-N1 (data not shown). Therefore, Raf-C may function as a dominant inhibitor of MEK activity, thereby inhibiting signaling downstream of Ras.

Although previous studies have implicated the Raf cysteine-rich domain in facilitating Ras binding, the precise nature of this role was unclear. Our observations that Raf-Cys shows high affinity, GTP-dependent binding to Ras *in vitro* and can antagonize Ras function *in vivo* provide evidence that the cysteine-rich domain constitutes a second Ras binding site in the Raf NH_2-terminal regulatory domain. These results provide an explanation for the reduction in Ras binding, observed both *in vitro* and *in vivo*, to an amino-terminal Raf fragment, Raf-(1–257), containing a mutation (C168S) that disrupts the integrity of the cysteine-rich domain (5). Our finding is also consistent with the observation that Raf NH_2-terminal fragments containing residues 131–147 (which includes residues 139–147 of the Raf cysteine-rich domain) display increased affinity for binding Ras (9, 10). A recent investigation has demonstrated partially reduced coprecipitation and *in vitro* binding between Ras and a Raf mutant lacking the cysteine-rich domain (28), supporting our finding of two Ras binding sites in Raf.

Our observation that the cysteine-rich domain can bind Ras can be reconciled with the report that a single amino acid substitution at Raf residue 89 is sufficient to abolish Ras-Raf interaction (11). While it is possible that expression of the isolated cysteine-rich domain may have unmasked a nonspecific binding activity for Ras that is not a property of full-length Raf, our observation that this domain preferentially interacts with the active, GTP-complexed form of Ras argues that this interaction is specific. Instead, we propose that the Ras binding site in the cysteine-rich domain is protected in the intact, unstimulated protein, possibly due to negative regulatory contacts with the COOH-terminal domain. We propose that Ras may initially interact with Raf via contacts with residues between positions 51 and 131. The initial Ras-Raf interaction and/or the resultant membrane translocation of Raf may promote exposure of the cysteine-rich domain for interaction with Ras and possibly other activating molecules. Thus, Ras interaction with these two binding sequences may be necessary to induce the removal of the negative regulatory action of the Raf NH_2 terminus and consequently may facilitate Raf activation by additional events. These may include interaction with 14–3-3 proteins (29), tyrosine kinases (30), or lipids (12).

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REFERENCES

1. Williams, N. G., and Roberts, T. M. (1994) *Cancer Metastasis Rev.* **13,** 105–116
2. Davis, R. J. (1993) *J. Biol. Chem.* **268,** 14553–14556
3. Moodie, S. A., Willumsen, B. M., Weber, M. J., and Wolfman, A. (1993) *Science* **260,** 1658–1661
4. Warne, P. H., Viciana, P. R., and Downward, J. (1993) *Nature* **364,** 352–355
5. Zhang, X., Settleman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. (1993) *Nature* **364,** 308–313
6. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) *Cell* **74,** 205–214
7. Van Aelst, L., Barr, M., Marcus, S., Polverino, A., and Wigler, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6213–6217
8. Finney, R. E., Robbins, S. M., and Bishop, J. M. (1993) *Curr. Biol.* **3,** 805–812
9. Chuang, E., Barnard, D., Hettich, L., Zhang, X.-f., Avruch, J., and Marshall, M. S. (1994) *Mol. Cell. Biol.* **14,** 5318–5325
10. Ghosh, S., and Bell, R. M. (1994) *J. Biol. Chem.* **269,** 30785–30788
11. Fabian, J. R., Vojtek, A. B., Cooper, J. A., and Morrison, D. K. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 5982–5986
12. Ghosh, S., Xie, W. Q., Quest, A. F. G., Mabrouk, G. M., Strum, J. C., and Bell, R. M. (1994) *J. Biol. Chem.* **269,** 10000–10007
13. Tanaka, M., and Herr, W. (1990) *Cell* **60,** 375–386
14. Kolch, W., Heidecker, G., Lloyd, P., and Rapp, U. R. (1991) *Nature* **349,** 426–428
15. DeLoskey, R. J., Van Dyk, D. E., Van Aken, T. E., and Campbell-Burk, S. (1994) *Arch. Biochem. Biophys.* **311,** 72–78
16. Miller, A.-F., Halkides, C. J., and Redfield, A. G. (1993) *Biochemistry* **32,** 7367–7376
17. Cox, A. D., and Der, C. J. (1994) *Methods Enzymol.* **238,** 277–294
18. Wasylyk, C., Imler, J. L., and Wasylyk, B. (1988) *EMBO J.* **7,** 2475–2483
19. Hauser, C. A., Der, C. J., and Cox, A. D. (1994) *Methods Enzymol.* **238,** 271–276
20. Wu, M., Kim, R., and Kim, S.-H. (1990) in *Methods: A Companion to Methods in Enzymology* (Casey, P., ed) pp. 315–318, Academic Press, Inc., New York
21. Avruch, J., Zhang, X., and Kyriakis, J. M. (1994) *Trends Biochem. Sci.* **19,** 279–283
22. Cox, A. D., Brtva, T. R., Lowe, D. G., and Der, C. J. (1994) *Oncogene* **9,** 3281–3288
23. Bruder, J. T., Heidecker, G., and Rapp, U. R. (1992) *Genes & Dev.* **6,** 545–556
24. Deshpande, A. K., and Kung, H. F. (1987) *Mol. Cell. Biol.* **7,** 1285–1288
25. Korn, L. J., Siebel, C. W., McCormick, F., and Roth, R. A. (1987) *Science* **236,** 840–843
26. Garcia, A. M., Rowell, C., Ackermann, K., Kowalczyk, J. J., and Lewis, M. D. (1993) *J. Biol. Chem.* **268,** 18415–18418
27. Stanton, V. P., Jr., Nichols, D. W., Laudano, A. P., and Cooper, G. M. (1989) *Mol. Cell. Biol.* **9,** 639–647
28. Pumiglia, K., Chow, Y.-H., Fabian, J., Morrison, D., Decker, S., and Jove, R. (1995) *Mol. Cell. Biol.* **270,** 398–406
29. Morrison, D. K. (1994) *Science* **266,** 56–57
30. Fabian, J. R., Daar, I. O., and Morrison, D. K. (1993) *Mol. Cell. Biol.* **13,** 7170–7179