A mutational analysis of the Ras-binding domain (RBD) of c-Raf-1 identified three amino acid positions (Asn\(^{64}\), Ala\(^{85}\), and Val\(^{88}\)) where amino acid substitution with basic residues increases the binding of RBD to recombinant v-Ha-Ras. The greatest increase in binding (6–9-fold) was observed with the A85K-RBD mutant. The elevated binding for the A85K-RBD and V88R-RBD mutants was also detected with Ras expressed in cultured mammalian cells, namely NIH-3T3 and BAF cells. None of the wild type residues in RBD positions Asn\(^{64}\), Ala\(^{85}\), and Val\(^{88}\) have been previously implicated in the interaction with Ras (Block, C., Janknecht, R., Herrmann, C., Nassar, N., and Wittinghofer, A. (1996) *Nat. Struct. Biol.* 3, 244–251; Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F., and Wittinghofer, A. (1995) *Nature* 375, 554–560). The discovery of elevated binding among the mutants in these positions implies that additional RBD residues in these mutants can be used to generate the Ras-RBD complex. These findings are of particular significance in the design of Ras antagonists based on the RBD prototype.

The A85K-RBD mutant can be used to develop an assay for measuring the level of activated Ras in cultured cells; Sepharose-linked A85K-RBD-GST fusion protein served as an activation-specific probe to precipitate Ras-GTP but not Ras-GDP from epidermal growth factor-stimulated cells. A85K-RBD precipitates up to 5-fold more Ras-GTP from mammalian cells than wild type RBD.

Ras is a small G protein that functions as a molecular switch for the transduction of signals generated by a variety of activated cell surface receptors. In resting cells Ras exists in its inactive GDP-bound form. Upon receptor activation, guanine nucleotide exchange factor proteins catalyze the replacement of GDP by GTP (3–5). The association with GTP converts Ras to an active form capable of relaying signals to an array of downstream effectors (6, 7). c-Raf-1 is an effector of Ras (6, 8–10). Raf is a serine-threonine kinase (11), which transmits incoming stream effectors (6, 7). c-Raf-1 is an effector of Ras (6, 8–10). c-Raf-1 stimulates the Raf-RBD (12–14). This pathway has been implicated in a variety of cellular responses, including cell proliferation, survival, and differentiation (6, 7). The N-terminal Ras-binding domain (RBD) of Raf has been narrowed to a region encompassed by Raf residues 51–131 (15, 16). Ras-GTP binds to the Ras-GTP complex directly in vitro (15, 16). Bacterially expressed recombinant RBD adopts a stable folded conformation in vitro (16). Ras expression in v-Ha-Ras-transformed NIH-3T3 fibroblasts leads to the suppression of the transformed phenotype (17). The ability of RBD to reverse v-Ha-Ras-induced cell transformation makes the Ras-RBD interface a target for the development of Ras antagonists.

The details of the RBD interaction with Ras revealed in part by a high resolution x-ray crystal structure of the complex between the RBD and another Ras family member, Rap1A (2). A computer model of the RBD-Ras complex has been generated on the basis of the Rap1A-RBD crystal complex (18). Several RBD residues that are in direct contact with Ras have been inferred from the crystal structure (2) as well as from a mutational analysis of the proposed binding surface of RBD (1, 19). RBD residues Gln\(^{86}\), Arg\(^{89}\), Lys\(^{84}\), Arg\(^{84}\), Arg\(^{89}\), Gln\(^{76}\), and Thr\(^{86}\) are in direct contact with Ras (1, 2). A mutational analysis of the RBD binding site showed that Gln\(^{86}\), Lys\(^{84}\), and Arg\(^{89}\) are the major contributors to the binding affinity between Ras and Raf (1). Genetic studies on *Drosophila melanogaster* have also demonstrated that Arg\(^{89}\) is important to the interaction with Ras both in vivo and in vitro (20). Even a highly conservative substitution of Arg\(^{89}\)-RBD with lysine abolished the binding between v-Ha-Ras and RBD (21). Lys\(^{84}\) is thought to be responsible for effector specificity, favoring the formation of the Ras-Raf complex in preference to the Rap1A-Raf complex (1, 22, 23). Arg\(^{89}\) represents a point of A-Raf and c-Raf-1 isozyme discrimination; it is one of the residues that determine the higher affinity of binding for the c-Raf-1-v-Ha-Ras complex compared with the A-Raf-v-Ha-Ras complex (24).

The measurement Ras activation is critical to the analysis of Ras function. If a significant change in the Ras-GDP:Ras-GTP ratio is detected between unstimulated and stimulated cells, it indicates that, at least in part, the signal from the ligand of interest is being mediated by Ras (25). The binding domains from effector proteins of small GTPases have been used previously as activation-specific probes to discriminate between GDP- and GTP-bound forms of G-proteins, with differences in binding affinities of several orders of magnitude (26–28). The binding domain of RapGDS was used to measure Rap1 activation in platelets (26), the Rap-binding domain of the putative Rap effector (RLIP76) isolated GTP-bound Rap (27), and the minimal RBD of c-Raf-1 was used as an activation-specific probe for Ras (28).

The affinity of wild-type (wt) RBD for Ras-GDP is 100-fold lower than for Ras-GTP (29). GST-RBD fusion protein linked to Sepharose beads has been used to precipitate oncogenic Lyr\(^{61}\)-Ha-Ras, which is bound constitutively with GTP (28). The dominant negative Asn\(^{37}\)-Ha-Ras, which is in the constitutively GDP-bound form (30), does not associate with the RBD-GST

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‡The abbreviations used are: RBD, Ras-binding domain; wt, wild type; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; EGFR, epidermal growth factor receptor; GDP:Ras, guanosine 5’:O-(thiotriphosphate); EGF, epidermal growth factor.
fusin protein (28). Recently, another activation-specific probe for Ras has been described, in the form of a Fab antibody fragment that exclusively binds Ras-GTP but not Ras-GDP (31).

Previous mutational studies of RBD described a reduced or unaltered affinity for Ras (1, 19, 24). The present work has identified several RBD mutants with increased binding to Ras. These mutants provide new tools to analyze the Ras-Raf interaction and can be used as more sensitive probes than wt-RBD for GTP-bound Ras.

EXPERIMENTAL PROCEDURES

Construction of RBD (Residues 51–131) Mutants—The mutants were constructed by polymerase chain reaction site-directed mutagenesis (33), using c-Raf-1 cDNA (17) as a template. Oligonucleotides were synthesized incorporating the desired mutations as well as EcoRI restriction sites at the 5'-and 3'-ends to facilitate subcloning into the pGEX-2TH vector linearized with EcoRI. Mutations were verified by nucleotide sequencing.

Protein Expression and Purification—The procedure used for the expression and affinity purification of the glutathione S-transferase-RBD fusion proteins, using glutathione-coated Sepharose beads (Center for Protein and Enzyme Technology, LaTrobe University), was described by J. Frangioni and B. Neel (34). Sepharose-linked GST fusion proteins were suspended in storage buffer (34) as a 1:1 suspension and then stored in 1-ml aliquots at −20 °C. v-Ha-Ras was expressed in NM522 cells (17) using the pUC8 expression vector (35), as described previously (36). v-Ha-Ras was isolated using the procedure of Johnson and Hecht (37). The proteins released from bacterial cells were resolved on a 15 × 3 cm DEAE-Sephalch (Amersham Pharmacia Biotech) column with a 120-ml linear gradient of 0–0.4 M NaCl in buffer A (38). Fractions containing v-Ha-Ras (determined by SDS-PAGE) were pooled and stored at −20 °C.

Measurement of Protein Concentrations—The concentrations of proteins linked to Sepharose beads (GST-RBD as well as RBD-bound Ras) were determined after scanning Coomassie Blue-stained SDS-PAGE by densitometry (Molecular Dynamics 300 Series computing densitometer) and measuring band densities relative to bovine serum albumin standards with ImageQuant 3.3 (Molecular Dynamics).

In Vitro Ras-binding Assays—[γ-32P]GTP or GTP-S labeling of v-Ha-Ras and its binding to GST-RBD fusion proteins were performed as described previously (17). The labeled v-Ha-Ras was added to GST-RBD constructs linked to glutathione-coated Sepharose beads (Center for Protein and Enzyme Technology, LaTrobe University). Dilutions of the beads were carried out with 200-μl pipette tips that had been cut with a scalpel to allow unobstructed movement of the beads. Each sample contained 0.9 μM RBD-GST and 1 μM [γ-32P]GTP labeled v-Ha-Ras. The reactions were carried out in 200 μl of binding buffer (50 mm Tris-HCl, pH 7.5, 10 mm MgCl2, 0.5 mg/ml bovine serum albumin, 0.5 mm diithiothreitol, 0.1 μM NaCl), on a rotating wheel, at room temperature for 15–30 min. The beads were washed four times in binding buffer by centrifugation. The radioactivity associated with the beads was measured using a Tri-Carb liquid scintillation analyzer (Packard United Technologies). The binding between v-Ha-Ras and the different RBD mutants was expressed as a percentage of the radioactivity associated with wt-RBD.

Conditions for the Ras-binding Assay Visualized by SDS-PAGE—Reaction mixtures contained 6.0 μM GST-RBD linked to Sepharose beads as well as 1.2 μM v-Ha-Ras-GST or v-Ha-Ras-GDP in 200 μl of binding buffer. The reactions were shaken for 15 min at room temperature. The beads were washed three times with ice-cold binding buffer, suspended in 100 μl of sample buffer, and heated (95 °C for 5 min). Aliquots (50 μl) of the supernatants were resolved by 12% SDS-PAGE and stained with Coomassie Blue.

Nucleotide Dissociation Assays—GTP-S-labeled v-Ha-Ras (2.3 μM) was incubated with 1.1 μM wt-RBD-GST or As85K-RBD-GST linked to glutathione-coated Sepharose beads. After a 75-min incubation period, the Sepharose beads were washed three times by centrifugation. Dissociation of the GST-RBD complexes was facilitated by adding 287 μM GTP-S. At given intervals, aliquots (60 μl) were taken from the dissociation mixture (500 μl starting volume) and washed four times by centrifugation. The radioactivity associated with the beads was determined using a Tri-Carb liquid scintillation analyzer (Packard United Technologies).

Ras Activation in BAF/EGFR Cells—BAF cells, which had been transfected with a human EGF receptor or a kinase negative EGF receptor mutant expression vector (39), were starved of serum and interleukin-3 for 5 h in minimal medium (RPMI). The cells (15 cells/sample) were collected as a pellet after centrifugation (1000 rpm, 5 min, Sigma 4K15 centrifuge), transferred to a 1.5-ml Eppendorf tube, and suspended in 1 ml of serum-free medium (31). Ras activation was measured with 100 nm/ml EGF (40, 41) at room temperature for 3 min. The cells were centrifuged, and the cell pellets were snap frozen on dry ice and stored at −70 °C overnight. The freezing step facilitated cell lysis.

Precipitation of Cellular Ras-GTP—A modification of the method described by de Rooij and Bos (28) was used. Frozen NIH-3T3 cells, which had been transfected with v-Ha-Ras (17), or activated BAF/EGFR cells were lysed in 200 μl/sample of ice-cold lysis buffer (0.5% Triton X-100, 0.5% deoxycholate, 20 μM Tris-HCl, pH 7.5, 150 μM NaCl, 1% Trasylol, and 1 μM phenylmethylsulfonyl fluoride). Insoluble cellular debris were removed after centrifugation (5 min, 4 °C). The supernatant was then added to tubes containing 1 ml of cold binding buffer and 100 μg of GST-RBD fusion protein linked to Sepharose beads. In positive control samples, total cellular Ras was immunoprecipitated with the anti-Ras Y13–259 antibody (American Type Culture Collection) prebound to protein-G-coated Sepharose beads (20 μl). All assay samples were incubated on a rotating wheel (4 °C, 30 min). The beads were then washed three times in binding buffer by centrifugation. The samples were subjected to Western blot analysis. Proteins resolved by 15% SDS-PAGE were transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon, Millipore). RBD-bound Ras was visualized using the Pan-ras (AB-2) antibody (Calbiochem), horseradish peroxidase-linked goat anti-mouse IgG (Bio-Rad) antibody, the enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech) and Eastman Kodak Co. X-AR x-ray film. Ras signals were quantified by scanning densitometry (Molecular Dynamics 300 Series Computing Densitometer) with ImageQuant 3.3 software (Molecular Dynamics).

Computer Modeling of the Complexes between the c-Raf-1 RBD Mutants and Ras—The structure of the wild type Ras-RBD complex (18) was used to model the complexes between RBD mutants and Ras. The procedure involved two steps. First, Ala85 was replaced with Lys85, Val91 with Arg91, and Asn64 with Lys64. Second, the complexes were energy minimized with the CVFF force field, as implemented in DISCOVER (INSIGHTII, 1999). A 500-step conjugate gradient energy minimization was carried out to optimize position and orientation if side chains of the mutants with the rest of Raf and Ras protein fixed. The nonbonded (electrostatic and van der Waals) interactions were truncated at 10 and a distance-dependent dielectric constant (4r) was used to mimic solvent.

RESULTS

A mutational scan of the c-Raf-1 RBD involved the measurement of in vitro v-Ha-Ras binding to the point mutants of RBD, which were expressed as GST fusion proteins and linked to Sepharose beads. The results of the scan are summarized in Fig. 1. The substitution of alanine in RBD position 85 with arginine resulted in a 2.5-fold increase in binding to v-Ha-Ras (Fig. 1B). Ala85 is part of an α-helix in RBD (Fig. 2). Residues Arg44 and Arg59, which are adjacent to Ala85 within the helix (Fig. 4A), are part of the Ras/Raf binding interface (1); however, Ala85 had not been previously implicated in the formation of the Ras-Raf complex.

The purity of GST-RBD fusion proteins was analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 1A). In some instances the mutations destabilized the structure of RBD making it more susceptible to degradation by Escherichia coli proteases. The degradation products are clearly visible in some of the mutant preparations and are most prominent in the R89V-RBD preparation. The degradation products were excluded from the estimates of GST-RBD concentrations, because the protein estimates were based on the densities of the discrete GST-RBD bands (see “Experimental Procedures”).

The remaining RBD mutants in the binding scan showed reduced binding to v-Ha-Ras (Fig. 1B). Residues Arg59, Arg67, Lys64, and Arg69 are part of the binding interface between Raf and Ras (1). In previous investigations, substitution of the basic residues in these positions with neutral amino acids
similarly reduced the affinity of the RBD for Ras (1, 2, 42). The substitution of arginine with leucine in position 89 abolished
the interaction between Ras and Raf both in vivo and in vitro (see Fig. 1B (1, 20)). The affinity of the K84A mutant was
reduced by more than 100-fold (1), the binding of R59A-RBD was 3.4% relative to wt-RBD, whereas the binding of R67A was
6.2%. In our hands, mutation of the known interactive RBD residues led to less dramatic reductions in binding than in
previous studies (1, 19). In Fig. 1B, the binding relative to
wt-RBD was 8.6% for K84L-RBD, 43% for R59L-RBD, and 42%
for R67L-RBD. The discrepancy in the binding ratios could
arise from the differences in the particular neutral residues
used. Leu has a longer hydrophobic side chain than Ala, which
can alter the RBD-solvent interactions. Furthermore, in the
present investigation, the assays were carried out at high protein
concentrations, which should minimize the difference be-
tween high and low affinity constructs.

Arginine substitutions at positions 91 and 98, which appear
to be adjacent to the binding interface, lead to a dramatic loss
of binding (Fig. 1B). According to a computer simulation of the
Ras-RBD complex (18), RBD Leu91-RBD forms hydrogen bonds with Arg41-Ras and Lys42-Ras. The L91R-RBD mutant dis-
rupts this interaction. The side chain of Val98-RBD is located
within the hydrophobic core of the RBD molecule (43). The
V98R-RBD mutant is likely to abolish RBD/Ras binding as a
consequence of distorted RBD folding. The Ala103–111 cluster
mutant, in which a 9-residue stretch of random coil was re-
placed with alanines, also displayed reduced binding to v-Ha-
Ras. It must be noted that no steps were taken to monitor
correct folding of the RBD mutants. Therefore, misfolding can-
not be ruled out as the cause of reduced binding. An attempt
was made to purify other arginine RBD mutants, V72R, C81R,
L86R, L121R, and V128R, but the protein yields of these con-
structs were too low to detect in our binding assay.

The Importance of Charge and Molecular Packing in Position
85 of RBD—The replacement of the small, nonpolar alanine
side chain in position 85 of RBD with the positively charged,
large arginine side chain leads to an increase in binding to the
v-Ha-Ras·GTP complex (Fig. 1B). Residue Ala85 is part of the
A1 a-helix within wt-RBD (Fig. 2 and 4A). There are no
charged residues in this position in the native Raf primary
sequences (Fig. 2). No interaction between Ala85 and Ras res-
ides has been reported to date, but the neighboring residues
Lys84 and Arg89 appear to be major contributors to the high
affinity binding between Ras and RBD (23). It is plausible that an
additional positive charge in this critical binding region would
enhance the strength of its coulombic interaction with Ras.

Several point mutants in RBD were expressed to test the
importance of charge and molecular packing in position 85 to
the binding of RBD to Ras. Isoleucine, lysine, arginine, and
aspartic acid were used to make a set of position 85 mutants.
Again, 12.5% SDS-PAGE and Coomassie Blue staining (Fig.
3A) were used to measure the protein concentrations of the

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**Fig. 1. Mutational analysis of RBD.** A. RBD protein preparations
resolved on a reducing 12.5% SDS-PAGE and stained with Coomassie
Blue. Positions for RBD-GST and GST are marked. B. the binding of
Raf-RBD mutants to v-Ha-Ras·GTP, expressed as a percentage of wt-
RBD binding. The reactions were performed in duplicate. The sloping
top of each bar joins the higher and lower replicate values. The mutants
with increased binding to Ras were assayed at least three times.

**Fig. 2. Sequence alignment and secondary structure assignment for
**c-Raf-1 RBD (2, 19). Raf-RBD contact residues (44) at the Ras binding site are
emphasized in bold letters. The positions of the mutated c-Raf-1 RBD residues are
numbered above the sequences. The resi-
dues, aligned with the c-Raf-1 positions,
where mutagenesis led to increased bind-
ing between c-Raf-1 and v-Ha-Ras, are
highlighted in blue. Secondary structure
elements are shown below the sequences;
the a-helices (A1 and A2) are indicated by
the green bars and the b-sheet folds (B1-
B5) are indicated by the yellow bars.
could bring the positive charges from Arg\textsuperscript{85} in the vicinity of Arg\textsuperscript{89}. If this were to happen, the repulsion between the two adjacent positive charges would distort the orientation of the Arg\textsuperscript{89} side chain and, hence, reduce the strength of the critical bond between Arg\textsuperscript{89}-RBD and Asp\textsuperscript{38}-Ras. The reduction in binding between v-Ha-Ras and A85I-RBD (Fig. 3B) indicates that a large side chain is of itself disruptive in position 85. The loss of binding in the A85D-RBD mutant (Fig. 3B) is also consistent with the computer model of the RBD-Ras complex (18), which suggests that a negatively charged residue in position 85 would undermine the bond between Arg\textsuperscript{89}-RBD and Asp\textsuperscript{38}-Ras through the coulombic repulsion of Asp\textsuperscript{38}-Ras.

**Point Mutations in the Region of the RBD α-Helix—Mutation of Ala\textsuperscript{85} in RBD to a basic residue led to increased binding to Ras (Fig. 3A). We examined whether a similar trend could extend to other amino acids in this region of RBD. Ala\textsuperscript{85} is part of the A1 α-helix (Fig. 2), which is illustrated schematically in Fig. 4A. The spiraling structure of the helix brings together residues that are separated within the primary sequence. Thus Cys\textsuperscript{81}, Leu\textsuperscript{82}, Lys\textsuperscript{84}, Leu\textsuperscript{86}, and Val\textsuperscript{88} are adjacent to Ala\textsuperscript{85}. The diagram of the RBD α-helix (Fig. 4A) suggests that Val\textsuperscript{88} is ideally placed to mimic the increased Ras binding of Ala\textsuperscript{85} mutants. Just like Ala\textsuperscript{85}, Val\textsuperscript{88} is a small nonpolar residue and does not appear to be directly involved in binding Ras. Its closest neighbors are in positions 85 and 84, which, in mutated and native forms, respectively, formed part of the RBD binding interface. The substitution of valine at position 88 with arginine results in a 4-fold increase in binding to v-Ha-Ras (Fig. 4C). Other basic residues at position 88 produce a lower, though still significant increases of 71% for V88K and 166% for V88H. As expected, a negative charge in this position interferes with complex formation, just as it does at position 85 (Fig. 3C); the binding of V88D-RBD is less than 10% the wt-RBD binding (Fig. 4C). A computer simulation of the RBD-Ras complex (18), based on the Rap1A-RBD crystal structure (2), suggested that the side chains of residues 88 and 85 point in different directions. Asp\textsuperscript{30} and Glu\textsuperscript{31} in Ras are the most likely candidates for binding Arg\textsuperscript{88}-RBD (Fig. 4D); the same residues that may form salt bridges with Lys\textsuperscript{84} in wt-RBD (23).

We also constructed, expressed, and purified a double mutant, A85K/V88R-RBD. The double mutant incorporated both mutations, which individually elevate RBD binding to Ras. Fig. 4C shows that the effect of the two mutations was not cumulative. The increase in binding was greater than for the V88R mutation alone, but less than for the A85K mutation (Fig. 4C). The effect of simultaneous substitutions in positions 88 and 85 is difficult to interpret from the computer model of the Ras-RBD complex (18).

Mutation of other positions of the α-helix to basic residues either reduced or had no effect on the binding of RBD to Ras (Fig. 4C). Lys\textsuperscript{84} is one of three RBD residues responsible for high affinity binding with Ras (1). In this position, not only the positive charge, but also the lysine side chain per se is important to the interaction with Ras. The ε-amino group forms salt bridges with two Ras residues, Glu\textsuperscript{31} and Asp\textsuperscript{33} (44). A conservative substitution to arginine led to a 2-fold decline in binding (49%). In contrast, the same change in position 87 had no effect; no difference was detected in the binding of Lys\textsuperscript{87}-RBD and K87R-RBD. Deletion of the positive charge from position 87 reduced binding to 30% in K87Q-RBD. Reversing the charge in the D80K-RBD mutant had a relatively small impact, retaining 57% of binding. This residue is exposed to the solvent and it forms no direct contacts with Ras (18).

Arginine mutations in two positions adjoining Ala\textsuperscript{85}, Cys\textsuperscript{81}, and Leu\textsuperscript{86}, resulted in low protein yields, indicating that the mutant proteins were either unstable or misfolded, presumably...
making them more sensitive to bacterial proteolytic enzymes. The side chains of both Cys81 and Leu86 are buried in the hydrophobic interior of RBD (18), and charged substitutions in these positions are likely to interfere with the folding of RBD.

Positive charges just outside the helix, at position 90, also interfered with binding. The activity of the G90K mutant was 29% of wt-RBD (Figs. 3 and 4). In the Ras-RBD complex, Gly90 is located in the vicinity of three basic Ras residues Arg41-Ras, Lys42-Ras, and His57-Ras. The insertion of another positive charge into this environment from G90K-RBD would be expected to repel the Ras residues and distort their orientation.

Negatively charged G90D-RBD had the least impact (80%), whereas the binding of G90Q-RBD was 40% of wt-RBD.

Mutations in B1 and B2 β-Sheets of RBD—The residues of RBD that interact with Ras (see Fig. 2) are located in the A1 α-helix and the B1 and B2 β-sheets (2). Our results in Figs. 1, 3, and 4 show that it is possible to increase the strength of interaction between Ras and RBD by mutating amino acids in the vicinity of the interactive α-helical RBD residues, Lys84 and Arg89. Consequently, we have searched for further examples of increased binding to Ras among the amino acids in the vicinity of other interactive residues, i.e. within the β-sheets B1 and B2. A series of RBD point mutants in positions 64–71 were made.

A series of RBD point mutants in positions 64–71 were made. The SDS-PAGE analysis of purified mutant and native RBD-GST fusion proteins are shown in Fig. 5A. The substitution of asparagine in position 64 by lysine increased binding to Ras 3.8-fold (Fig. 5B). Asn64-RBD is part of the Ras binding site, forming three hydrogen bonds with Arg41-Ras (43). One bond is formed between the carbonyl oxygen of Asn65-RBD and the Ne of the Arg41-Ras, the second bond is between the same Ras nitrogen and the Od of Asn64-RBD, and the third is between the Od of Asn64-RBD and the guanidium group of Arg41-Ras (2, 23, 44). However, the association is relatively weak, as these hydrogen bonds are solvent exposed (23). A mutation to N64A-RBD was reported to have no effect on the Ras-RBD interaction (19). Another study (1) reported that under physiological ionic conditions over 20% of wt-RBD binding was retained by the N64A-RBD mutant. The N64K-RBD mutant might be expected to disrupt the interaction with Arg41-Ras. However, the binding between v-Ha-Ras and N64K-RBD is increased (Fig. 5B). Other Ras residues, such as Glu3 and Asp54 of Ras (18), could form a direct salt bridge N64K-RBD (Fig. 5C).

The insertion of the negatively charged aspartic acid into position 64 reduced binding by 50% in our scan (Fig. 3 and 6B). The same mutation has been reported to reduce the affinity between Ras and RBD by a factor of 14 (1). The results from the two studies are not directly comparable because of the difference in the experimental conditions.

Several other RBD mutants were included in the binding scan in Fig. 5. Their association with v-Ha-Ras was as follows: 9% for Q66K, 11% for T68K, 29% for V69R, 34% for N71E, and 60% for S77R. Residues Glu33, Thr68, and Val88 are part of the Ras binding site (1, 2). Alanine point mutants of these residues were also made in an earlier study (1), which reported reductions in affinity by factors of 55, 10, and 7, respectively. Once again, the figures from the two studies are not directly comparable because of the difference in the experimental conditions likely salt bridge formed between the V88-RBD mutant and Glu31-Ras. The mutant side chain is shown in yellow.

FIG. 4. The effect of point mutations in the region of the RBD A1 α-helix. A, a schematic diagram of the RBD A1 α-helix. A filled black circle represents each residue. The lines represent the bonds between adjoining residues. Arg89 is shown in the helical conformation (43). B, RBD protein preparations resolved on a 12.5% SDS-PAGE and stained with Coomassie Blue. The position for RBD-GST is marked. C, the binding of Raf-RBD mutants to v-Ha-Ras-GTP, expressed as a percentage of wt-RBD binding. GST was used as a negative control, and A85K-RBD set an upper limit for Ras binding, ensuring that bindable Ras-GTP was not depleted below that level. The mutants with increased binding to Ras were assayed at least three times. D, illustration of the
during the measurements of relative binding and affinity.

**Ras-GDP Binding and GTP Exchange—**Wt-RBD preferentially binds active, GTP-bound Ras (29). We tested whether two of the mutants with elevated binding to Ras, V88R-RBD and A85K-RBD, retained the preference for GTP-bound Ras. The association between RBD and Ras-GDP or Ras-GTP was visualized by SDS-PAGE (Fig. 6A). GTP or GDP-bound v-Ha-Ras was prepared in a nucleotide exchange reaction (see “Experimental Procedures”) containing 4.8 mM GTP or GDP in 1.2 ml of exchange buffer. The results (Fig. 6A) clearly show that wt-RBD, V88R-RBD, and A85K-RBD show preferential binding to GTP-bound v-Ha-Ras. No v-Ha-Ras-GDP binding was detected. The highest amount of v-Ha-Ras-GTP was precipitated with the Ala85-RBD construct, less with V88R-RBD, less still with wt-RBD, and none with R89L-RBD. The order of binding corresponds to the results of the binding scan (Fig. 3B).

To allay concerns about different rates of GTP dissociation from v-Ha-Ras in the presence of wt-RBD or RBD mutants with elevated binding, we compared the effect of wt-RBD and A85K-RBD on the dissociation of GTP\(^\gamma\)S (a nonhydrolyzable analogue of GTP) from v-Ha-Ras. GTP\(^\gamma\)S dissociation from the v-Ha-Ras\(^\gamma\)S complex in the presence of excess unlabeled GTP\(^\gamma\)S is shown in Fig. 6B. There is no difference in GTP\(^\gamma\)S exchange between the samples containing wt-RBD or A85K-RBD, indicating that the increased binding of the A85K-RBD mutant to Ras is not a consequence of reduced GTP dissociation.

**Binding of RBD Mutants to Eukaryotically Expressed v-Ha-Ras—**One concern about the binding scan, which uses recombinant Ras, is its relevance to physiological binding. In bacteria, Ras proteins are not subjected to post-translational processing, whereas in mammalian cells post-translational modifications appear to be essential for the biological activity of Ras (45–47). These modifications involve farnesylation (48), palmitoylation (49, 50), cysteine methylation (51, 52), and proteolysis of the AA\(^\times\)X amino acids from the C-terminal CAX motif (51, 53) (C, cysteine; A, aliphatic; X, any amino acid). The post-translational modifications are responsible for the localization of Ras at the plasma membrane (51, 54), where it associates with an array of downstream effectors (6, 7). We tested the binding of some of the RBD mutants to Ras produced in mammalian cells. RBD-GST fusion protein Sepharose beads were used to capture Ras from the lysates of v-Ha-Ras-transfected NIH-3T3 cells. v-Ha-Ras carries two oncogenic mutations, G12V and A59T, which reduce the GTPase activity of v-Ha-Ras (55) and render it resistant to stimulation by FIG.5. 

**Mutations in B1 and B2 \(\beta\)-sheets of RBD.** A, RBD protein preparations resolved on a reducing 12.5% SDS-PAGE and stained with Coomassie Blue. The positions for RBD-GST and GST are marked. Protein concentrations were estimated by densitometry relative to standard bovine serum albumin concentrations. B, the binding of RBD mutants to v-Ha-Ras-GTP, expressed as a percentage of wt-RBD binding. GST was used as a negative control, and A85K-RBD set an upper limit for Ras binding, ensuring that bindable Ras-GTP was not depleted below that level. The mutants with increased binding to Ras were assayed at least twice. C, illustration of the likely salt bridge formed between the N64K-RBD mutant and Glu\(^3\)-Ras. The mutant side chain is shown in blue. Arg\(^\alpha\)-Ras also forms salt bridges to Glu\(^3\)-Ras and Asp\(^\alpha\)-Ras. See “Results” for details.

**FIG.6.** The role of GTP hydrolysis and GDP binding. A, reaction mixtures contained GST-RBD constructs together with v-Ha-Ras, which was in complex with either GTP or GDP, as shown. The position of Ras is marked. B, the dissociation of labeled v-Ha-Ras/GTP\(^\gamma\)S from wt-RBD or A85K-RBD in the presence of excess unlabeled GTP\(^\gamma\)S was carried out.
GTPase-activating protein (56). In addition, the A59T mutation increases the exchange of GDP for GTP (57, 58). As a result, v-Ha-Ras exists predominantly in the active GTP-bound form, which is able to bind RBD. The binding of RBD mutants to v-Ha-Ras expressed in NIH-3T3 cells is shown in Fig. 7A. Following incubation of cell lysates with the Sepharose-linked GST-RBD fusion proteins, bound Ras was visualized by Western blotting. A85K-RBD bound nearly 6-fold more v-Ha-Ras than wt-RBD, whereas the increase for V88R-RBD was over 4-fold. No Ras associated with the inactive form of RBD, R89L-RBD (20). Fig. 7B shows the relative amounts of v-Ha-Ras associated with RBD-coated Sepharose beads or remaining in the cell extracts after incubation with the beads. In comparison with the negative control (R89L-RBD), most of the Ras is removed from the samples after incubation with A85K-RBD; less is removed from the extracts by wt-RBD-coated beads. The total amounts of v-Ha-Ras recovered with the wt- and A85K-RBD-coated beads are less than the amounts of Ras lost from the corresponding extracts. It is likely that some of the v-Ha-Ras dissociated from RBD during the washing steps.

The Use of A85K-RBD as an Activation Specific Probe for Ras—The elevated binding between Ras and A85K-RBD suggested that the mutant protein would be more effective than wt-RBD as a probe for detecting and quantifying Ras activation in cells (28). The effectiveness of A85K-RBD as an activation specific probe for Ras-GTP was tested in BAF/EGFR cells that had been transfected with the wild type EGF receptor (wt) or an inactive, kinase negative mutant of the EGF receptor (K721R) (39). Acting through a cascade of intermediary signaling proteins, the binding between EGF and its receptors indirectly leads to a transient association of Ras with GTP (59–61). Serum-starved cells were stimulated with EGF, lysed, and incubated with the different forms of the RBD-GST fusion protein linked to Sepharose beads. Association of Ras with the beads was analyzed by Western blotting. The results in Fig. 8 show that A85K-RBD was 5-fold more effective than wt-RBD for detecting Ras activation. (lanes 3 and 4). No bands corresponding to activated Ras were detected in the serum-starved cells, in the absence of EGF stimulation (lanes 1 and 2), indicating that neither wt-RBD nor K85A-RBD bind to Ras in its inactive GDP form. In quiescent cells Ras remains in a complex with GDP (41), incapable of binding wt-RBD (8, 9, 15, 62). Activated Ras was barely detectable in control BAF/EGFR cells that had been transfected with K721R-EGFR, the kinase negative EGF receptor (lanes 5–8). The difference in the signals from wt and K721R-transfected cells served to confirm that Ras activation was largely the result of stimulating the EGF receptor tyrosine kinase.

DISCUSSION

A mutational analysis of the Ras-binding domain of c-Raf-1 identified three amino acid positions (Asn64, Ala85, and Val88) where substitution with basic residues increases the binding between RBD and bacterial recombinant v-Ha-Ras. The greatest increase in binding was observed with the A85K-RBD mutant (Figs. 3–5). The elevated binding to the A85K-RBD and V88R-RBD mutants occurs with Ras expressed in mammalian cells, namely NIH-3T3 (Fig. 7) or BAF cells (Fig. 8).

Like wt-RBD, the mutants associate preferentially with GTP-bound Ras. There was no binding to Ras/GDP for any of the RBD constructs (Fig. 6A) nor was any Ras precipitated from unstimulated BAF cells (Fig. 8). Whereas Moodie et al. (63) described a mutation of Ras, Q61L, which rendered the Q61L-Ras/GDP complex capable of binding c-Raf-1, B-Raf, and phosphatidylinositol 3-kinase, there have been no previous reports of Ras effector mutants associating with the Ras/GDP complex. The increase in the binding of RBD mutants to v-Ha-Ras is not related to GTP exchange or hydrolysis. The rate of GTP exchange is the same in the presence of wt-RBD or A85K-RBD (Fig. 6B). There is contradictory evidence in the literature about the GTPase activity of c-Raf-1. Warne et al. (9) reported a weak increase of about 20% in the GTPase stimulating activity of Ras in the presence of an N-terminal Raf fragment (residues 1–257). Subsequent studies showed that c-Raf-1 N-terminal truncation mutants (including wt-RBD) have no impact on the GTPase activity of Ras (8, 15, 29, 64). In our assays, a 20% reduction in GTP hydrolysis would not account for the 200–900% increases in the binding of RBD mutants to Ras. Furthermore, because v-Ha-Ras is an oncogenic variant of Ras that is resistant to GTPase-activating protein activation (65), GTP hydrolysis is unlikely to play a role in the binding scans or in Ras precipitation from v-Ha-Ras-transfected NIH-3T3 cells. In addition, the increased binding of the A85K-RBD mutant was subsequently confirmed using a nonhydrolyzable GTP analogue, GTPγS.2

The binding between the remaining RBD mutants and v-Ha-Ras was reduced or unaltered. Mutation of residues

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that form the binding interface with Ras resulted in reduced binding, in agreement with previous observations (1, 2, 42). In the Ala103–111 cluster mutant a random coil segment was replaced with alanines. A shorter stretch of sequence was previously mutated in the same region (19); the substitution with alanines of amino acids 104–106 and 108–111 resulted in 76 and 56% binding, respectively. Our cluster mutant covered two additional amino acids, His105 and Gly107. His103 is not likely to be of significance because it is present in only two known Raf sequences (Fig. 2). In contrast, Gly107 is highly conserved. Glycine can adopt a wider range of conformations than other residues allowing the main chain of proteins to bend more freely. The twist in the loop joining RBD β-sheets 3 and 4 on the computer that generated a model of the RBD-Ras complex (18) suggests that Gly107 may form a glycine turn.

It is likely that more RBD positions could be found where the substitution of wild type residues would enhance the binding to Ras. A random mutagenesis approach may help in identifying such positions. A combination of such mutations in a single RBD molecule could possibly lead to further increases in Ras binding. The binding of one double mutant, A85K/V88R-RBD, is shown in Fig. 4C. In this instance, no cumulative increase in binding was evident. The lack of cumulative binding in the A85K/V88R-RBD protein could be because of the repulsion between the adjacent positively charged mutant residues destabilizing the α-helix or because of unfavorable interactions between the combination of these residues and Ras. It would be interesting to try a combination of mutants where the positively charged side chains are separated by a greater distance e.g. N64K/A85K-RBD or N64K/V88R-RBD.

It is interesting to note that in every RBD mutant with elevated binding to Ras, the residues responsible for the increases in binding are absent from the native Raf orthologues (Fig. 2). The RBD sequence alignment of in Fig. 2 shows only neutral and predominantly small amino acids corresponding with position 85 of c-Raf-1; alanines predominate, but there is also a serine in Xenopus c-Raf-1 and a leucine in LIN-45 from Caenorhabditis elegans. The conservation of uncharged amino acids in position 85 suggests a functional significance for these residues, even though Ala85 has not been implicated in the RBD binding site (1, 2). Clearly, these neutral residues are not required for the maintenance of the RBD structure, because their replacement with lysine or arginine leads to elevated binding (Fig. 3C). Despite their positive contribution to binding, there is no instance of a basic residue in the RBD positions aligned with Ala85 of human c-Raf-1 (see Fig. 2). Similarly, there is no instance of a basic amino acid in position 64 of c-Raf (Fig. 2). Asparagine is highly conserved in all cases, with the exception of Lin-45 from C. elegans, where it is replaced by phenylalanine, a nonpolar aromatic residue.

Similarly, with the exception of the C. elegans Raf analogue, Lin-45, there are no positively charged residues aligned with position 88 of c-Raf. Lin-45 contains lysine in the aligned position. Of the three basic residues tested, lysine had the least impact in position 88, increasing RBD binding to Ras to only 170%, compared with 270% for histidine and 410% for arginine. Furthermore, the Ras binding site of Lin-45 may not be identical to RBD, because out of eight highly conserved interactive residues (Arg69, Asn84, Glu88, Arg89, Thr89, Val93, Lys94, and Arg95) only the three that contribute to high affinity binding (Glu88, Lys94, and Arg95 (1)) are preserved in Lin-45 (Fig. 3A). The Lin-45 lysine aligned with c-Raf position 88 might be expected to compensate for the possible absence of the other favorable interactive sites usually provided by positions equivalent to 59, 64, 67, 68, and 69 of c-Raf-1.

It is interesting to speculate that evolutionary selection may be responsible for the absence of basic residues from the RBD positions where such residues would result in elevated Ras binding. If such an evolutionary pressure did exist, it would imply that elevated binding to Ras is in some way suboptimal for biological function. Geyer et al. (66) and Ito et al. (67) showed that Ras exists in the form of multiple conformational isomers. Both reports postulated that different Ras isomers may function to discriminate among multiple Ras effectors for binding.

In the present experiments the concentrations of Ras and RBD are an order of magnitude higher than the reported $K_d$ for the interaction (15–160 nM (1, 29, 64, 68–72)). Therefore any difference in binding is not expected to reflect a difference in affinity. A detailed kinetic analysis of the v-Ha-Ras-RBD interaction is presented in a separate paper, where we show v-Ha-Ras exists as a population of isomeric conformers and that A85K-RBD binds a greater proportion of these conformers than wt-RBD, giving rise to the difference in Ras saturation levels.

The ability of Raf mutants to bind a wide range of conformations may impede appropriate interaction with other effectors (6, 7), resulting in evolutionary pressures against such mutants. The finding that the binding interface of Ras can accommodate several more favorable interactions than are found in wild type RBD is not entirely surprising. Two mutants of another Ras-binding protein, NF1, were found to bind 5–10-fold more Ras than wild type NF1 (73). Furthermore, random mutagenesis of yeast Ras2 uncovered 57 point mutants that displayed increased binding to Raf (74). Until now, no corresponding Raf mutants have been described.

The most immediate application for the A85K-RBD mutant is as an activation-specific probe for Ras-GTP. GST-RBD fusion protein has been used as a probe for endogenous activated Ras in insulin-stimulated A14 cells and glial-derived neurotrophic factor-stimulated SK-P2 cells (28). A85K-RBD increases the sensitivity of the assay 4–6-fold. De Rooij and Bos (28) speculated that such probes have potential applications in research and clinical diagnosis. Ras mediates signals from a myriad of cellular stimuli, and a sensitive simple method for measuring
its activation state would help clarify its role in tumor cells or signal transduction.

Around 30% of human cancers carry oncogenic mutations in Ras (32, 75, 76). However, the aberrant Ras activation in the incidence of cancer could be even more pervasive. Activating mutations in signaling proteins upstream of Ras may elevate the level of Ras-GTP even in the absence of oncogenic mutations in the Ras molecule itself. It may be possible to use an A58K-RBD fusion protein as an activation-specific probe to test tumor samples for the presence of activated Ras-GTP.

In vivo, RBD has been shown to suppress the transformed phenotype in v-Ha-Ras-transfected NIH-3T3 cells (17), suggesting that this molecule acts as a Ras antagonist. In the present report we have described six RBD mutants with elevated binding to recombinant and cellular Ras. The increased binding of RBD mutants to Ras-GTP could increase their potential as Ras antagonists, hence as anti-cancer drugs. Such molecules need to be effective not only in their role as Ras antagonists but also in their ability to penetrate cells and resist degradation or excretion. The additional interactions generated by the Arg64, Lys85, or Arg88 mutations would expand the range of possible molecules that fulfill these criteria and increase the chance of finding a useful therapeutic drug based on the RBD prototype.