p120 Ras GTPase-activating Protein Interacts with Ras-GTP through Specific Conserved Residues*

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(Received for publication, November 28, 1995, and in revised form, April 9, 1996)

Ras is a ubiquitous GTPase that plays a critical role in cell growth, division, and differentiation as a signal transducer (1, 2). As a GTPase, the GTP bound form of Ras is active and able to transduce signals from surface transmembrane receptors to the Raf protein kinase, resulting in the activation of mitogen-to transduce signals from surface transmembrane receptors to growth, division, and differentiation as a signal transducer (1, 2). As a GTPase, the GTP bound form of Ras is active and able to transduce signals from surface transmembrane receptors to the Raf protein kinase, resulting in the activation of mitogen- to transduce signals from surface transmembrane receptors to growth, division, and differentiation as a signal transducer (1, 2). As a GTPase, the GTP bound form of Ras is active and able to transduce signals from surface transmembrane receptors to the Raf protein kinase, resulting in the activation of mitogen-

1 The abbreviations used are: RasGAP, Ras GTPase-activating protein; GAP, GTPase-activating protein; GST, glutathione S-transferase; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

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The Journal of Biological Chemistry Vol. 271, No. 26, Issue of June 28, pp. 15322–15329, 1996

Printed in U.S.A.

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eration of previously characterized Ras mutations suggests that discrete sites of interaction exist on RasGAP that interact separately with the Ras switch I and II regions, affecting both protein binding and GTPase activation.

EXPERIMENTAL PROCEDURES

Materials—All chemical reagents were purchased from Sigma or Aldrich. [γ-32P]GTP (4500 Ci/mmm) was purchased from ICN Pharmaceuti- cals, Inc. Glutathione-Sepharose 4B beads were from Pharmacia Biotech Inc. Prepared 4–20% Tris-Glycine polyacrylamide gels were from NOVEX. Centricon-10 concentrators were from Amicon, Inc. Brad- ford reagent was from Bio-Rad. All other chemicals and reagents were obtained from Sigma Chemical Co.

Expression and Purification of GST-Fusion Proteins in Esche- richia coli RR1 lac—The 1-kilobase pair BamHI-EcoRI fragments en- coding wild type and mutant bovine RasGAP [702–1044] (known as GAP-C) were cloned into the corresponding sites of the pGEX-2T expression vector. The mutations were confirmed by DNA sequencing. Using these constructs, the wild type and mutant GAP-C protein expression in the pGEXlac—E. coli strain was confirmed as glutathione S-transferase (GST) fusion proteins. The transformed cells were grown in 1 liter of Luria Broth with 125 μg/ml ampicillin (LBA) at 37°C to an OD of 0.4–0.8 and induced with 0.5 mM isopropyl-1-thio-galactoside to an OD of 3. The induced cells were grown at 30°C for an additional 3 h. Cells were pelleted and frozen at −80°C. The cells were then lysed by sonication in 260 μl of lysis buffer (as above) followed by the addition of 0.1% Triton X-100, 10 μg/ml DNAase I, and 10 μg/ml MglCl. The cell lysates were then centrifuged at 14,000 rpm for 10 min. The supernatants were collected and kept on ice for GAP assays and Western blotting. GAP assays were performed as above except that 10 μl of cell extract were added to 40 μl of prewarmed Ras-GTP.

Kinetic Assays for the GST-Mutants with Altered Activity— Kinetic assays were used to determine the catalytic activity (Kcat) and binding affinity (Km) for Ras-GTP of wild type GST-GAP-C, and the K83L, Q93L and V897L mutant proteins. In these assays, Ras-GTP was used as the substrate and GST-GAP-C as the enzyme. Different amounts of nonradioactive Ras-GTP (from 0.012 to 12.34 μM) were prewarmed with [γ-32P]GTP charged GST (about 0.8 nM) in order to lower the specific activity of radioactive Ras-GTP and render a higher concentra- tion of substrate over enzyme. Ras-GTP substrate was prepared in 20 μl NaHEPES buffer, pH 7.5, 1 mM MgCl2, prewarmed at 30°C for 2 min followed by addition of GST-C-Proteins. The reactions were then allowed to proceed at 30°C for 30 s, 1 min, 2 min, and 5 min and quenched with 5% cold charcoal in 50 mM phosphate buffer, pH 8.0. The reaction mixtures were centri- fuged briefly to pellet the charcoal, the supernatants were collected, and the hydroydized [32P]phosphate was detected by Cherenkov counting.

Chemical Coupling of Glutathione to BSA—To a 10 mg/ml solution of BSA (Sigma) in degassed 50 mM sodium phosphate, pH 8.0, was added 12.5 mg/ml of m-maleimidobenzyl-hydrazine-N-hydroxy-sulfosuccinimidyl carbonate (Pierce). The pH was readjusted to 8.0 with 1N NaOH, and the solution was allowed to stand at room temperature for 1 h. The reaction solution was then prewarmed at 30°C for 5 min followed by the addition of 1% Triton X-100, 100 μg/ml DNase I, and the pH was readjusted to 6.5 by the addition of 20 μl of NaOH. The reaction was stopped by the addition of 100 μl of 1-stepTM ABTS sub- strate (Pierce) was added to each well and allowed to react at room temperature for 30 min. The reaction was stopped with 100 μl of 10% H2O2. The absorbance at 405 nm was measured.

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amount of solid phase GST-GAP-C protein used in the assay was confirmed by removing the bound protein from parallel wells with boiling SDS-PAGE sample buffer and quantitation by Coomassie Blue-stained SDS-PAGE gels.

Circular Dichroism Spectropolarimetry—Circular dichroism analyses were performed to study the secondary structure of wild type and mutant GAP catalytic domains. GAP-C proteins purified after thrombin cleavage were concentrated and exchanged into 25 mM phosphate buffer, pH 7.4, 1 mM MgCl₂ using Centricon-10 concentrators (Amicon). The protein concentrations were determined by Bradford assays and SDS-polyacrylamide gels stained with Coomassie Blue. A Jasco J-720 spectropolarimeter (Japan Spectroscopic Co.) was used to measure the 190–250-nm spectra of 0.1 mg/ml GAP-C protein samples in 0.2-cm path length cuvettes at room temperature. The scanning speed was 20 nm/min. Each spectrum was an average of three scans. The resolution of the scans was 0.5 nm, and the sensitivity was 200 mdeg.

Data Processing—For GAP kinetic assays, the time course at each substrate concentration was used to calculate the initial velocity of the reaction. The initial velocities (V) and their corresponding substrate concentrations were used to estimate the Michaelis-Menten constant (Kₘ) and the maximal velocity of the reaction (Vₘ) by fitting the data to the Michaelis-Menten equation using the Enzfitter program (Elsevier Biosoft). The catalytic constant (Kcat) was calculated by dividing Vₘ with the GST-GAP-C protein concentration used in the reaction. For the circular dichroism studies, the percentages of secondary structure in the GAP-C proteins were estimated using the SSE338 program provided with the J 700 system software (Japan Spectroscopic Co.) based on the reference spectra of Yang et al. (27). The data were plotted as mean residue ellipticity verses wavelength. Mean residue ellipticity is the ellipticity generated by one molar amino acid residues/dm pathlength.

RESULTS

Expression Properties of Mutant GST-GAP-C Proteins—Using oligonucleotide-directed mutagenesis, 11 of the most highly conserved residues in the Ras catalytic domain were changed to semi-conserved amino acids (Fig. 1). The conserved residue substitutions used in this study are: F757A, E774K, R786Q, L811T, E826K, K831Q, W882M, F898A, R925E, and K958Q. Two of the substitutions in the highly conserved block 1 region (E774K and R786Q), one was in block 2 (W882M), one was in block 3A (F898A), and two were in block 3B (R925E and K958Q).

One indication of structural instability introduced by the loss or substitution of residues important for folding is a large decrease in soluble polypeptide upon expression in E. coli. The level of soluble expression of each mutant RasGAP protein was measured by growing small volumes of transformed cell cultures under identical conditions, followed by purification of GST-GAP-C fusion protein on glutathione-Sepharose 4B beads. The captured proteins were stripped from the beads by boiling in SDS-PAGE sample buffer and visualized by SDS-PAGE and Coomassie Blue staining. The results of one such experiment is shown in Fig. 2. Compared with wild type GST-GAP-C, four mutants (R786Q, K831Q, Q935H, and K958Q) showed the same level of expression and were considered structurally stable. Three mutants (L811T, R925E, and F757A) were expressed about 5-fold less than wild type GST-GAP-C, suggestive of a minor effect on protein stability. Four mutants (E774K, E826K, W882M, and F898A) were expressed at very low levels suggesting gross destabilization of the RasGAP catalytic fragment.

RasGAP Activities of GST-GAP-C and Nonfusion GAP-C Mutant Proteins—Using purified proteins, the GAP activity profile of the seven most stable GST-GAP-C mutants was compared with wild type GST-GAP-C. The results were similar to those obtained with the GST fusion proteins. The expression levels of the E774K, E826K, W882M, and F898A mutants were too low to obtain sufficiently purified protein for assay. Instead, crude extracts of cells expressing these mutants were assayed for GAP activity and compared with wild type GST-GAP-C and GST alone. No GAP activity was detected for any of the mutants (data not shown). However, because of low levels of expression, no definite conclusion concerning relative GAP activities can be made for these unstable mutants.

Kₘ and Kcat Determinations for Reduced Activity, Stable GAP-C Mutants—Either an inability to bind Ras-GTP or a defective catalytic mechanism could explain the lack of Ras GTPase activating function for the impaired mutant GAP proteins. To distinguish between these two possibilities, kinetic assays were performed with the stable K831Q and Q935H mutants using Ras-GTP as the substrate. From these assays the Kₘ for Ras-GTP and Kcat for GTPase stimulation were calculated (Table I). In the case of K831Q, Ras affinity was

| Position | 575 | 774 | 786 | 811 | 826 | 831 | 882 | 897 | 998 | 925 | 975 | 989 |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Mutation |
| bGAP    | 575 | 774 | 786 | 811 | 826 | 831 | 882 | 897 | 998 | 925 | 975 | 989 |
| NF1     | 575 | 774 | 786 | 811 | 826 | 831 | 882 | 897 | 998 | 925 | 975 | 989 |
| rGAP1    | 575 | 774 | 786 | 811 | 826 | 831 | 882 | 897 | 998 | 925 | 975 | 989 |
| hP4BP    | 575 | 774 | 786 | 811 | 826 | 831 | 882 | 897 | 998 | 925 | 975 | 989 |
| dGAP1    | 575 | 774 | 786 | 811 | 826 | 831 | 882 | 897 | 998 | 925 | 975 | 989 |
| IRA1    | 575 | 774 | 786 | 811 | 826 | 831 | 882 | 897 | 998 | 925 | 975 | 989 |
| IRA2    | 575 | 774 | 786 | 811 | 826 | 831 | 882 | 897 | 998 | 925 | 975 | 989 |
| sar1    | 575 | 774 | 786 | 811 | 826 | 831 | 882 | 897 | 998 | 925 | 975 | 989 |
| Block | 1 | 2 | 3A | 3B |

Fig. 1. Sequence alignment of the catalytic domains of Ras GTPase-activating proteins. The abbreviations are: bGAP, bovine GAP; NF1, human neurofibromatosis type 1 (21); rGAP1, rat brain GAP (22); hP4BP, human P14.5P binding protein (23); dGAP1, Drosophila Gap1; IRA1 and IRA2, Saccharomyces cerevisiae GAP homologs; and sar1, Schizosaccharomyces pombe GAP homolog (18). The residues mutated in this study are shown along with the substitutions used. The four conserved blocks indentified by Gutmann et al. (17) are shown at the bottom of the figure.

Fig. 2. Comparison of the expression levels of wild type and mutant GAP-C proteins. GST-GAP-C fusion proteins were precipitated from equivalent extracts of induced E. coli with glutathione-Sepharose 4B. The beads were washed and boiled in SDS-PAGE sample buffer and analyzed on a 10% SDS-PAGE gel stained with Coomassie Blue. Lane 1, wild type GST-GAP-C; lane 2, Q935H; lane 3, E826K; lane 4, R786Q; lane 5, F757A; lane 6, R925E; lane 7, K958Q; lane 8, E774K; lane 9, V897Y; lane 10, L811T; lane 11, F898A; lane 12, K831Q; lane 13, W882M.

![Graph showing expression levels of wild type and mutant GAP-C proteins](http://www.jbc.org/)

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The abbreviations are: bGAP, bovine GAP; GAP homologs; and sar1, Schizosaccharomyces pombe GAP homolog (18). The residues mutated in this study are shown along with the substitutions used. The four conserved blocks indentified by Gutmann et al. (17) are shown at the bottom of the figure.
reduced with the calculated $K_r$ three times greater than that of wild type GST-GAP-C. The Q935H mutant was observed to be catalytically impaired with a 39-fold reduction in $K_{cat}$, although the $K_m$ of this mutant for Ras-GTP was actually reduced.

**Detection of Binding Defects between Mutant GST-GAP-C Proteins and Ras by ELISA—** Kinetic assays were not possible for the R786Q and R925E mutants due to a complete lack of detectable GTPase stimulating activity. As an alternative method for measuring Ras-GTP association, an ELISA assay was developed. In essence, ELISA plates were coated with GST-GAP-C proteins and allowed to associate with the GAP-insensitive, high affinity Ras[L61]-GTP protein. The assay was GAP-specific and was able to clearly distinguish between known binding and nonbinding forms of the Ras protein (Fig. 4). As predicted by the kinetic assays, the K831Q mutant bound Ras[L61]-GTP more poorly and the Q935H mutant bound Ras[L61]-GTP better than wild type GST-GAP-C. The R786Q and R925E mutants were severely impaired for Ras association, consistent with their inability to stimulate the Ras GTPase.

**Mutant Protein Secondary Structure Estimations by Circular Dichroism Spectropolarimetry—** Of the fully stable GAP proteins analyzed, the R786Q and K831Q mutants appeared to be defective for Ras binding, whereas the Q935H mutant was catalytically impaired. CD spectropolarimetry was used to measure the total secondary structure motifs in the nonfusion form of these GAP mutants as a gauge of subtle alterations of structure (28). When plotted as mean residue ellipticity versus scanned wavelength, the spectra of these three mutants were indistinguishable from the wild type spectrum. Boiled wild type GAP-C and GAP-C[R786Q] had significantly different spectra, indicating little secondary structure (Fig. 5). Analysis of the spectral data provided estimations of the total secondary structure (Table II). All four GAP-C proteins had a high percentage of $\alpha$-helices and random coils and a low percentage of $\beta$-strands and $\beta$-turns. These data are supported by the Garnier secondary structure prediction algorithm (Table II) (29).

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**Table I**  
**Km and $K_{cat}$ values of GST-GAP-C fusion proteins**  
The initial velocity at each Ras-GTP concentration was calculated from the time course as described under “Experimental Procedures.” $K_m$ and $V_m$ values were estimated by fitting the initial velocities and their corresponding substrate concentrations from GAP kinetic assays with Enzfitter program (Elsevier Biosoft). $K_{cat}$ was calculated by dividing $V_m$ with the enzyme concentration in the reaction. The GST-GAP-C enzyme concentrations used in the reactions were: wild type, 78.8 nM; K831Q, 342 nM; Q935H, 453.8 nM; and V897Y, 123.8 nM. ND, not determined.

| GST-GAP-C | $K_m$ | $K_{cat}$ |
|-----------|-------|-----------|
| Wild type | 115.1 | 4.64      |
| K831Q    | 309.6 | 1.97      |
| Q935H    | 32.9  | 0.12      |
| V897Y    |       | ND        |

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**Fig. 3.** GAP activities of wild type and mutant GST-GAP-C proteins. GAP activity assays were performed using serial dilutions of purified GST-GAP-C fusion proteins (A) and nonfusion GST-C proteins (B). Each reaction included less than 0.1 nM [$\gamma$-32P]GTP charged Ras protein. GAP activities are expressed as the percentage of saturated wild type activity. A, GST fusion proteins: □, wild type; ○, K958Q; ◊, L81T; △, F757A; crossed diamond, K831Q; ●, Q935H; ▽, R786Q; ▪, R925E; B, nonfusion GST-C proteins: □, wild type; crossed diamond, K831Q; ●, Q935H; ▽, R786Q. Each data point is an average of five independent assays for the wild type protein and three independent assays for each mutant.

**Fig. 4.** Detection of complex formation between GST-GAP-C mutants and Ras using ELISA. The ability of defective RasGAP mutants to bind Ras was determined by ELISA. The detailed method described under “Experimental Procedures.” Buffer blanks were subtracted from the results, and relative binding values were normalized to wild type GST-GAP-C and Ras[L61]-GTP. Finally, the numeric values were normalized to the measured concentration of each GST-GAP-C protein bound on the surface of the wells. Ras protein used in the assays: •, Ras[L61]-GTP; □, Ras[34A38]-GTP.

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**Fig. 5.** Detection of complex formation between GST-GAP-C mutants and Ras using ELISA. The ability of defective RasGAP mutants to bind Ras was determined by ELISA. The detailed method described under “Experimental Procedures.” Buffer blanks were subtracted from the results, and relative binding values were normalized to wild type GST-GAP-C and Ras[L61]-GTP. Finally, the numeric values were normalized to the measured concentration of each GST-GAP-C protein bound on the surface of the wells. Ras protein used in the assays: •, Ras[L61]-GTP; □, Ras[34A38]-GTP.
No significant differences in total secondary structural elements were observed between wild type GAP-C and the three mutants, suggesting that the functional differences observed between these mutants and wild type GAP-C were not due to gross alterations in protein structure.

RasGAP Residues 891–903 Share Conserved Sequence and Function with Yeast Adenylyl Cyclase Residues 1528–1540—Previously, one of us identified a mutation in the yeast RAS-responsive adenylyl cyclase gene, CYR1, which suppressed inactive RAS effector mutants (24). The mutation, SSR2–1, substituted a tyrosine for aspartate at CYR1p residue 1574, which maps in one of two separate sites of RAS interaction on the adenylyl cyclase protein (30, 31). This substitution increased enzyme responsiveness to RAS, presumably by increasing affinity for RAS proteins. Examination of the sequence flanking CYR1p residue 1574 revealed an intriguing similarity with the highly conserved block 3A region of RasGAP (see Fig. 6A) with valine 897 analogous to CYR1p aspartate 1547. To determine if these regions of CYR1p and RasGAP also shared a conserved function, valine 897 of RasGAP was substituted with a tyrosine, analogous to the SSR2–1 mutation. The resulting mutant GST-GAP-C protein was expressed at moderate levels (see Fig. 2), purified, and analyzed kinetically. Fig. 6B shows that the V897Y mutant had significantly increased Ras GTPase stimulating activity when compared with wild type GST-GAP-C. Kinetic analysis revealed that the $K_m$ value of the V897Y mutant for Ras-GTP was 69 m$\mu$M compared with 115 m$\mu$M for wild type GST-GAP-C (Table I).

**DISCUSSION**

Regulation of Ras activity is an important physiological role of the Ras GTPase-activating proteins. Extensive mutagenesis of the Ras proteins has identified regions of the protein critical for binding and GTPase activation by RasGAP. Similar studies of RasGAP have been less revealing and have failed to identify a discrete site of Ras binding analogous to the Ras effector region. Peptides based on the sequence of GAP block 3A inhibited Ras GTPase activation (32), although mutagenesis of the conserved residues in block 3A impaired catalytic function but not Ras binding (17, 18). Mutation of the conserved lysine 1423 of neurofibromin (lysine 932 in RasGAP) and valine 853 of...
RasGAP were found to impair Ras binding (21). However, substitution of either of these residues also had a severe destabilizing effect upon the protein, which might suggest that they are not directly involved in Ras binding but rather alter the Ras binding site through gross structural changes (28).

The eight members of the RasGAP family share significant homology with the p120 RasGAP catalytic domain, retaining ten amino acid positions that are completely conserved and seven that are moderately conserved (17, 18, 22, 23). In a previous study we have reported that residues involved in the association of the Raf-1 and Ras proteins were highly conserved during evolution and that many had charged side chains (34). These observations suggest that the Ras binding residues of the RasGAP family of proteins may also be conserved and involve charged residues. A biochemical approach was used in order to test this hypothesis. Eleven of the conserved and semi-conserved residues within the RasGAP catalytic domain were mutated semi-conservatively and tested for effects on protein structure and Ras interaction.

Mutation of amino acid residues involved in protein folding and tertiary structure frequently results in poor solubility or rapid degradation when expressed recombinantly in E. coli (18, 33). Of the 11 mutations introduced into RasGAP, four had severe consequences for the apparent stability of the protein upon expression in E. coli. This result suggests that glutamate 774, glutamate 826, tryptophan 882, and phenylalanine 898 are important for the folding or tertiary structure of RasGAP and probably are not involved directly in Ras binding or GTPase activation. Mutation of leucine 811, arginine 925, and phenylalanine 757 moderately reduced soluble protein expression, indicating a possible role in protein structure. The substitutions at positions arginine 786, lysine 831, glutamine 935, and lysine 958 did not affect protein stability, suggesting that these residues may be located on the surface of the protein. Based on these results, analysis was focused on phenylalanine 757, arginine 786, leucine 811, lysine 831, arginine 925, glutamine 935, and lysine 958 to determine their roles in Ras binding and GTPase stimulation.

We observed that the F757A, L811T, and K958Q mutants had GAP activity profiles similar to wild type RasGAP, K831Q, and Q935H which had greatly reduced GAP activities and that the R786Q and R925E mutants had no detectable GAP activity. Kinetic assays revealed that the lower GAP activity of K831Q was primarily a result of its lower affinity for Ras-GTP. In contrast, the Q935H mutant had increased affinity for Ras-GTP. Reduction in the GAP activity of Q935H was the result of a catalytic activity that was nearly 39-fold lower than wild type GST-GAP-C. By ELISA we demonstrated that the complete loss in GAP activity observed for the R786Q and R925E mutants was primarily due to an inability to bind to Ras-GTP. Additionally, arginine 786, lysine 831, and lysine 958 were found not to be required for protein structure based upon circular dichroism spectropolarimetry, suggesting that each of these residues has a direct role in Ras-GTP binding and catalysis.

The kinetic assays performed in this study found the \( K_m \) of wild type GST-GAP-C for wild type Ras-GTP to be 115 \( \mu \)M. This value is larger than the reported 19 \( \mu \)M for GAP-C for two possible reasons (4). First, the GST fusion has been shown to partially reduce binding affinity of GAP-C for Ras-GTP (18). In fact, as shown in Fig. 3 (A and B), nonfusion GAP-C proteins have higher GAP activity than the corresponding GST fusion proteins. Second, the GAP-C used in previous kinetic studies was expressed in insect cells, whereas we used GAP proteins expressed in bacteria. Although not formally reported, RasGAP expressed in insect, bacterial, or mammalian cells all differ severalfold in their relative binding affinities and catalytic rates.\(^2\) The \( K_{cat} \) of wild type GST-GAP-C determined from this study is 4.64 s\(^{-1}\), which is similar to the reported 4.2 s\(^{-1}\) (4).

Homology mapping of the members of the RasGAP family of proteins has identified four highly conserved regions in the RasGAP catalytic domain designated blocks 1, 2, 3A, and 3B. Within the 27-amino acid block 1, we have identified two conserved residues important for both protein structure (glutamate 774) and Ras binding (arginine 786). The residue equivalent to glutamate 774 in the GAP-related domain of neurofibromin (glutamate 1264) was also mutated by Gutmann et al. in their study (17). The substitution of this glutamate by tyrosine suppressed the heat shock sensitivity of a yeast ira strain, suggesting retention of GAP function in vivo, although no activity was detected in crude lysates. Substitution of RasGAP tyrosine 798 in block 1 with histidine also resulted in a highly unstable protein with poor Ras binding in vitro (18). Residue Arg786 was shown in this study to play a direct role in binding Ras-GTP. This residue might also play a role in catalysis, because the R786Q mutant had no detectable GAP activity even though it did associate weakly with Ras. Based on these data, block 1 is probably most important for the structural integrity of the GAP catalytic domain with arginine 786 playing an individual role in the direct binding of Ras-GTP.

The block 2 homology is the longest conserved region in the GAP catalytic domain containing about 37 amino acids. A previous study showed this region may not be essential for binding RasGTP or catalysis, because a functionally active splicing variant of neurofibromin contains a 21-amino acid insertion in block 2 (34). We observed that the W882M mutation in this block resulted in a very unstable protein. Previously we mutated seven residues in block 2, including two highly conserved positions. These mutations resulted in mostly extremely unstable, active proteins but not in stable, binding-defective mutants (18). Therefore, we conclude that block 2 is a structurally important region with no direct role in Ras binding or catalysis.

Block 3A is the most highly conserved homology block, containing six invariant residues out of 23. For this reason block 3A has been assumed to have an important role in GAP function and has been extensively studied. Neurofibromin mutants, with substitutions corresponding to the conserved GAP residues phenylalanine 898, proline 904, alanine 905, and proline 909 had normal GAP activity in vivo or in vitro (17, 21). Mutation of RasGAP residues leucine 902, arginine 903, and isoleucine 906 resulted in proteins that still bound Ras yet had impaired GTPase stimulating activity (18, 35). In this study, we changed the final two unstudied conserved residues in block 3A. We found that the F898A mutant was extremely unstable, preventing any conclusion about a functional role for this residue other than structural. The other mutant analyzed was V897Y, which resulted in a 2-fold increase in affinity for Ras and a corresponding increase in GAP activity. This particular substitution was chosen because of a curious sequence similarity between block 3A in RasGAP and a Ras-binding site in the yeast adenyl cyclase. Our data suggest that these sequences are conserved functionally as well. Mutation of adenyl cyclase aspartate 1547 to tyrosine, which positionally corresponds to GAP valine 897, resulted in an enzyme that could be stimulated by Ras effector mutants (24). The phenotype of the yeast mutant was suggestive of an increased affinity for Ras proteins, as we observed with the RasGAP V897Y mutant. Additionally, a synthetic peptide corresponding to a sequence within block 3A (residue 891-906) inhibited GAP activity of wild type RasGAP in an in vitro assay, indicating the region

\(^2\) M. Marshall, unpublished observations.
891–906 has the ability to bind to Ras-GTP (32). Based on the available data, we conclude that block 3A is not a high affinity binding site for Ras but rather a secondary site. This is possibly the only example of a conserved Ras binding motif found in functionally distinct Ras effector proteins.

Mutation of multiple conserved residues in RasGAP block 3B suggests that this region is a site of Ras interaction as proposed by Poulet et al. (17, 21). The R925E mutant demonstrated a complete loss of binding to Ras-GTP, whereas the Q935H mutant had increased affinity for Ras-GTP and a dramatic decrease in catalytic activity. Numerous studies of neurofibromin have indicated that lysine 1423 in block 3B (lysine 932 in GAP) also has an essential role in GAP function (19–21). Poulet et al. showed that lysine was the only functional amino acid at that position (21). Block 3B is the only region of the GAP/neurofibromin catalytic domain to harbor multiple residues found to be important for both high affinity Ras binding and GTPase enhancement.

The only conserved residue in the RasGAP catalytic domain found to be important for Ras binding and located outside of the homology blocks was lysine 831. This residue is located between block 1 and block 2. Substitution of lysine 831 with glutamine resulted in a very stable protein with greatly reduced affinity for Ras-GTP.

In this study, the amino acid residues found to have the greatest contribution to Ras binding were arginine 786, lysine 831, arginine 925, and glutamine 935. Circular dichroism spectroscopy showed that the R786Q, K831Q, and Q935H mutations had wild type secondary structure content, indicating that changes in affinity for Ras were not due to gross structural changes. It is likely that these residues are located on the surface of the protein and directly interact with residues on the Ras protein. Interestingly, three out of these four residues are basic, drawing a parallel with the Ras binding domain of Raf-1, which utilizes multiple salt bridges between conserved basic residues and the Ras effector region (33, 36). This hypothesis, that Ras-GAP association is mediated by ionic interactions, is supported by the fact that GAP binding is very sensitive to salt concentration (37). The CD spectra secondary structure estimation of the GAP catalytic domain indicated a much higher percentage of $\alpha$-helices than the Ras binding domain of Raf-1, which contains mostly $\beta$-sheets (36). This might explain the lack of sequence similarity between the Ras binding domains of RasGAP and Raf-1 as well as their different affinities for Ras-GTP (4, 38).

The known GAP binding regions on Ras include switch I, switch II, and an undefined region around aspartate 92 (2, 5, 39). Many of the mutations in the switch I region reduce affinity for RasGAP or neurofibromin and are insensitive to GAP activity (11). In contrast, mutations in the switch II region often lead to impairment of GTPase activity and even an increase in binding affinity for GAP (4). Substitution of glutamate 99 in yeast Ras2 (aspartate 92 in H-Ras) with lysine greatly increased affinity for RasGAP (5).

Integration of the available information about the respective regions of association of the Ras and RasGAP proteins suggests a model for binding and GTPase activation. We propose that the conserved basic RasGAP residues, Arg789, Lys831, and possibly Arg925 interact directly with conserved acidic residues in the Ras effector region and possibly aspartate 92 to promote association with the GTP-bound form of Ras. The interactions between these respective protein regions could be largely electrostatic in nature. According to this model, block 3A, and to some extent 3B, constitute a secondary site of interaction binding to the Ras switch II region. Interactions between block 3A/B and switch II would then lead to activation of the Ras GTPase, possibly by stabilizing the transition state of GAP hydrolysis reaction or assisting the formation of an optimal microenvironment for the catalytic site (40, 41). An interaction between block 3A and Ras switch II is supported by the similarity of phenotypes caused by mutations in each region. Specifically, most mutations in either region result in a loss of GTPase stimulation with no decrease in the affinity of binding. Mutations in RasGAP block 3B and Ras switch II often significantly increase the affinity of RasGAP/Ras association. The interaction between block 3A and the Ras switch II region is also supported by the observation that a block 3A dioligopeptide can bind to Ras-GTP but not Rap1A-GTP (32). Rap1A and Ras share the same switch I region, but their switch II regions are different. The ability of this peptide to distinguish Ras from Rap1A implies that block 3A is involved in a direct interaction with the Ras switch II region.

Acknowledgments—We thank Dr. Eric Long and Paula Eason for assistance with the CD measurements and Dr. Ellen Chuang and Lisa Hetcht for making the GAP mutants.

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J. Biol. Chem. 1996, 271:15322-15329.
doi: 10.1074/jbc.271.26.15322

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