A Conserved Region of c-Ha-Ras Is Required for Efficient GTPase Stimulation by GTPase Activating Protein but Not Neurofibromin*

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The effector binding domain and the switch II region of c-Ha-Ras are necessary for p120GAP-stimulated GTP hydrolysis. We report a third region of c-Ha-Ras located within the α3 helix (amino acids 101-103) which is also required for efficient p120GAP, but not neurofibromin-mediated hydrolysis. This highly conserved region of the Ras protein was investigated using an insertion-deletion mutant (Ras-100LIR104) originally characterized by Willumsen et al. (Willumsen, B. M., Adari, H., Zhang, K., Papageorge, A. G., Stone, J. C., McCormick, F., and Lowy, D. R. (1989) in The Guanine Nucleotide Binding Proteins: Common Structural and Functional Properties (Bosch, L., Kraal, B., and Parmeggiani, A., eds) pp. 165-178, Plenum Press, New York). The 100LIR104 substitution did not alter the intrinsic hydrolytic rate of the protein. The p120GAP-stimulated hydrolysis of Ras-100LIR104, however, was decreased by 2-3-fold compared to wild type Ras. This decrease in p120GAP-stimulated hydrolysis was not due to its inability to physically associate with Ras-100LIR104-GTP (as determined by competitive binding assays). Surprisingly, neurofibromin-stimulated GTP hydrolysis was unaltered by the mutation. Finally, no differences were observed in the ability of either the p120GAP catalytic domain or the neurofibromin GRD to accelerate Ras-100LIR104 GTPase activity, indicating that the amino-terminal noncatalytic GAP region is critical for p120GAP-stimulated GTP hydrolysis. This is the first report of a Ras mutation which differentiates between p120GAP and neurofibromin activity.

Ras proteins, which function in signal transduction pathways critical for cell growth and differentiation, are guanine nucleotide binding proteins with a Mr of approximately 21,000. The membrane-associated Ras protein cycles between an inactive GDP-bound and active GTP-bound state. The activity of Ras is negatively regulated by the hydrolysis of bound GTP, and positively regulated by specific guanine nucleotide exchange factors which facilitate the replacement of bound GDP by GTP (Lowy and Willumsen, 1993). Growth factor stimulation of quiescent cells increases the proportion of Ras-GTP, resulting in the initiation of a cascade of intracellular protein kinases (Satoh et al., 1990; Zhang et al., 1992).

GTPase activating proteins negatively regulate Ras activity by accelerating the hydrolysis of bound GTP. Two such proteins have been extensively characterized, p120GAP and neurofibromin (Lowy and Willumsen, 1993). The region of p120GAP which is responsible for stimulating GTP hydrolysis is located in the carboxyl-terminal third of the protein and is termed the p120GAP catalytic domain. The second GTPase activating protein for Ras, neurofibromin, is the product of the neurofibromatosis type-1 gene and has a Mr of 250,000. Neurofibromin contains a 350-amino acid stretch located in the central portion of the protein which contains extensive sequence homology to the GAP1 catalytic domain and is therefore termed the GAP related domain (GRD; Xu et al., 1990; Martin et al., 1990). Both the GAP catalytic domain and the neurofibromin GRD are highly active in accelerating the GTP hydrolysis by Ras.

Mutations in multiple regions of the Ras protein effect its interactions with p120GAP and neurofibromin. Mutations in the Ras effector binding domain (amino acids 32-40) result in the inability of p120GAP to enhance the intrinsic GTPase activity (Adari et al., 1988; Cales et al., 1988). Although not all mutations in this region abolish GAP activity, the effector region is thought to be essential for p120GAP binding. In addition, analysis of Ras/Rap1A chimeras shows that deletion of residues 61-65 also renders Ras insensitive to p120GAP stimulation (Zhang et al., 1991; Maruta et al., 1991). Many Ras mutations that are resistant to GAP stimulation have also been found to be resistant to stimulation by neurofibromin-GRD. Thus the effector binding domain (amino acids 32-40) and switch II region (amino acids 61-65) of Ras are thought to be the sites required for interaction with GTPase activating proteins. Recently a third GTPase activating protein has been identified. Its full characterization has not been reported (Maekawa et al., 1994).

In this paper we identify an additional region within the Ras protein which is important for p120GAP-stimulated hydrolysis. This region of the Ras protein, consisting of amino acids 101-103, is highly conserved among a number of small GTP binding proteins (Santos and Nebreda, 1989). The functional significance of this highly conserved region of the Ras protein was investigated using an insertion-deletion mutant originally characterized by Willumsen et al. (1989). This mutation, in which the amino acids KRV were substituted for LIR at position 101-103 was designated Ras-100LIR104. The intrinsic hydrolytic activity of Ras-100LIR104 was unaltered compared to wild type Ras. The p120GAP-stimulated hydrolysis by Ras-100LIR104, however, was reduced 2-3-fold, while neurofibromin hydrolytic activity remained unaffected. No differences were observed in the ability of either the p120GAP catalytic domain or the neurofibromin GRD to accelerate Ras-100LIR104 GTPase activity, indicating that regions outside of the catalytic GAP domain are involved in protein-protein in-

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Ras Sequence Required for GAP Activity

**EXPERIMENTAL PROCEDURES**

Reagents—The following reagents were used: [γ-32P]GTP (3000 Ci/ mmol) and [γ-32P]GTP (30 Ci/mmol) from DuPont NEN; GMP-PNP from Boehringer Mannheim; and p120GAP antibody from Upstate Biotechnology, Inc. The polyclonal antibody against the COOH-terminal region of neurofibromin is described elsewhere (Gudubic et al., 1992). All other reagents were purchased from standard vendors.

**Purification of Ras and Ras-100LIR104—p21c-Ha-Ras and p21c-Ha-Ras100LIR104 (previously classified as mutant number 1742) were expressed in *Escherichia coli* and purified from bacterial pellets by SDS-polyacrylamide electrophoresis, stained with Coomassie Blue, and the Ras or Ras-100LIR104 containing fractions were pooled. The fractions of peak protein remain bound to the antibody.**

**Statistics—These analyses were extensively repeated and summarized statistically. Statistical analyses were, therefore, performed with the ANOVA model (by Lisa Rybicki, Department of Biostatistics and Epidemiology, Cleveland Clinic Foundation) which is similar to the t test, but allows comparisons of all the data obtained. This approach, therefore, uses all available data to obtain an estimate of the variability within the data. In the text and figure legends "p" values indicate the likelihood that the ratio of GAPase activation for Ras compared to each of the different from 3.0 standard deviations of a given experiment are considered (p < 0.05 is significant). The ratios reported represent the ratio of GAPase stimulation with Ras-100LIR104 compared to GAPase stimulation with wild type Ras, reported as a range of ratios having a confidence level of 95%.”

**Competitive Binding Assay—Reagent Preparation—** Ras was first allowed to associate with [γ-32P]GTP (30 Ci/mmol), 2 mM EDTA, 1 mM GTP, and 10 mM MgCl2. The reaction was then diluted by 10-fold, mixed thoroughly, and designated volumes of the slurry were aliquoted (using a large mouthed pipette tip) for use in the GAPase filter binding assay described below. The 10-fold dilution insured that equal amounts of beads were reproducibly obtained.

**GAPase Filter Binding Assay—** A 70-μl reaction mixture composed of 80 μl of active Ras or Ras-100LIR104, as measured by the GAPase binding assay, was incubated with 1 mM EDTA, 1 mM DTT, 5 μg of bovine serum albumin, 20 mM Tris-HCl (pH 7.5), and 3 μl of [γ-32P]GTP (30 Ci/mmol) for 10 min at 30°C. This 70-μl reaction mixture was then added to a 700-μl mixture containing 20 mM Tris-HCl (pH 7.5), 10 μg of bovine serum albumin, 1 mM EDTA, 1 mM GTP, and 10 mM MgCl2. A 50-μl volume of the combined mixture was then added to tubes containing neurofibromin or p120GAP activity for a total volume of 100 μl and a final Ras concentration of 60 ngtube (28 pm). The reaction mixtures were incubated for various lengths of time depending on the assay conditions. Following incubation, 80-μl aliquots were filtered and washed with 5 ml of 25 mM Tris-HCl, 5 mM MgCl2, and 50 mM NaCl. The filters were then counted using liquid scintillation spectrometry. The amount of [γ-32P]GTP:128 pmol of active Ras or Ras-100LIR104 was determined using a filter binding assay described below. Care was taken to ensure that the specific binding activity for GAP by each Ras protein preparation was equivalent before the preparation was used for the work described herein.

**Immunoprecipitation of neurofibromin—** All purification steps were performed at 4°C. Rabbit brain cytosol was prepared in Buffer A (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT) as described previously (Golubic et al., 1992). Twenty ml of rabbit brain cytosol at 18–25 mg/ml containing 100 mM NaCl and 400 μl of neurofibromin antibody were incubated for 3 h at 4°C. Swollen Protein A-Sepharose beads (600 μl) were added to the lysate/antibody mixture and rotated on a mixing wheel at 4°C for 30 min. The Protein A-Sepharose beads were allowed to settle by gravity and were washed twice with buffer A (containing 0.2% Nonidet P-40 and 100 mM NaCl), twice with buffer A, and finally with 100 mM Tris (pH 8.0). A 1 ml column was constructed and washed with 5 column volumes of 100 mM Tris-HCl (pH 8.0) and 10 column volumes of 10 mM Tris (pH 8.0). Neurofibromin was eluted with 100 mM glycine (pH 3.00). Approximately, 200-μl aliquots were collected in tubes containing 50 μl of 1 mM Tris (pH 8.0) to neutralize the pH. Fractions were concentrated in a Centricon-10 filter and loaded on a 200-ml G-50 Sephacryl S-200 column equilibrated with 20 mM HEPES (pH 7.5) and 1 mM MgCl2. Reaction volumes were added, followed by a 75-min incubation at 4°C. The suspension was then centrifuged at 10,000 rpm for 30 min and the supernatant was concentrated in a Centriprep-10 filter and loaded on a 200-ml G-50 Sephacryl S-200 column equilibrated with 20 mM HEPES (pH 7.5) and 5 mM MgCl2. The column was run at 4°C with 1-ml fractions collected. Column fractions of 20 μl were counted directly using liquid scintillation spectrometry and another 20 μl was analyzed by a filter binding assay in order to distinguish Ras bound [γ-32P]GTP from Ras fractions containing free [γ-32P]GTP. Fraction 4 yielded 80% bound Ras-GTP (86 μmol) which was aliquoted and frozen at −70°C. For binding Ras or Ras-100LIR104 with GAP-PNP between 3 and 6 mg of Ras or Ras-100LIR104, 2.5 mM GMP-PNP, 1 mM EDTA, and 5 mM EDTA were incubated at 30°C for 20 min. Following incubation, 10 mM MgCl2 was added and the reaction volumes were diluted (approximately 5-fold) with 20 mM HEPES (pH 7.5) and 1 mM MgCl2. Reaction volumes were analyzed in a 9-ml G25 column which were then separated on a 4-ml G75 column and the sample was spun for 2 min in a microcentrifuge. The Protein A-Sepharose and the slurry was placed on a 1-ml sucrose cushion (1 × sucrose) and the sample was spun for 2 min in a microcentrifuge. The Protein A-Sepharose was then washed 3 times with 1 × TBS + Nonidet P-40 and 3 times with TBS + Nonidet P-40 followed by a 10-fold, mixed thoroughly, and designated volumes of the slurry were aliquoted (using a large mouthed pipette tip) for use in the GAPase filter binding assay described below. The 10-fold dilution insured that equal amounts of beads were reproducibly obtained.

**Protein A-Sepharose was washed 5 times with TBS + Nonidet P-40 (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Nonidet P-40). The prepared Protein A-Sepharose was then added to the lysate/antibody mixture and incubated for 30 min at 4°C. Samples were then spun in a microcentrifuge for 5 s to pellet the Protein A-Sepharose and remove the lysate. The Protein A-Sepharose was then washed 4 times with TBS + Nonidet P-40. Two hundred μl of TBS + Nonidet P-40 were then added to the Protein A-Sepharose and the slurry was placed on a 1-ml sucrose cushion (1 × sucrose) and the sample was spun for 2 min in a microcentrifuge. The Protein A-Sepharose was then washed 3 times with 1 × TBS + Nonidet P-40 and 3 times with TBS + Nonidet P-40 followed by a 10-fold, mixed thoroughly, and designated volumes of the slurry were aliquoted (using a large mouthed pipette tip) for use in the GAPase filter binding assay described below. The 10-fold dilution insured that equal amounts of beads were reproducibly obtained.

**Preparation of neurofibromin and p120GAP—** Neurofibromin (the amount used yielded 50% RasGAP hydrolysis of the [γ-32P]GTP:128 pmol of active Ras or Ras-100LIR104 was determined using a filter binding assay described below. The 10-fold dilution insured that equal amounts of beads were reproducibly obtained.
RESULTS

The Intrinsic Hydrolytic Activity of Ras and Ras-100LIR104—To investigate whether the 100LIR104 substitution would alter the intrinsic hydrolytic activity of the protein, wild type Ras and Ras-100LIR104 were compared using a GTPase filter binding assay. It is important to note that when equal concentrations of the two proteins were used, wild type Ras and the Ras-100LIR104 mutant were able to bind equal amounts of $[\gamma-32P]GTP$ (data not shown). Therefore, both Ras and Ras-100LIR104 expressed the same GTP binding activity. To measure intrinsic hydrolytic activity, identical concentrations (28 nM) of wild type Ras or Ras-100LIR104 were individually allowed to associate with $[\gamma-32P]GTP$, and then incubated at 30°C for the various times. Hydrolysis of bound $[\gamma-32P]GTP$ would result in the loss of protein-associated phosphate label. Following incubation, the amount of labeled phosphate remaining associated with Ras protein was quantitated by passing the samples over nitrocellulose filters (which retained the Ras protein and associated labeled phosphate) followed by liquid scintillation spectrometry. Both wild type Ras-GTP and Ras-100LIR104-GTP possessed the same intrinsic rate of GTP hydrolysis (Fig. 1).

Stimulation of Ras and Ras-100LIR104 GTPase Activity—In order to separately characterize the interaction of the mutant protein with the two GTPase activating proteins, neurofibromin and p120GAP had to be physically separated from each other. This was initially accomplished by immunoprecipitation. Antibodies specific for p120GAP or neurofibromin were incubated with 300 μl of rabbit brain lysate (which contains both GTPase activating proteins) followed by the addition of Protein A-Sepharose to bind the immunocomplexes. The activity of the immunoprecipitated proteins was assayed by incubating increasing amounts of either p120GAP or neurofibromin bound to Protein A-Sepharose with Ras-$[\gamma-32P]GTP$ or Ras-100LIR104-$[\gamma-32P]GTP$ for 45 min at 30°C. The stimulatory effects of immunoprecipitated p120GAP or neurofibromin on the intrinsic hydrolytic rates of these two proteins were assessed using the filter binding assay previously described. No differences in GTPase stimulation were observed when increasing amounts of neurofibromin-bound Protein A-Sepharose was incubated with Ras-$[\gamma-32P]GTP$ compared to Ras-100LIR104-$[\gamma-32P]GTP$ (Fig. 2a). The ability of neurofibromin to stimulate GTPase activity was therefore not altered by the Ras mutation. However, when p120GAP-bound Protein A-Sepharose was incubated with Ras-$[\gamma-32P]GTP$, the stimulation of GTP hydrolysis was greater than when p120GAP was incubated with Ras-100LIR104-$[\gamma-32P]GTP$ at all concentrations tested (Fig. 2b). The GTPase activity of p120GAP with the mutant was reduced approximately 2-3-fold compared to wild type Ras.

These results were repeated multiple times and the data collectively analyzed using the methods described under "Experimental Procedures." The probability that neurofibromin stimulated the GTPase activity of wild type Ras and mutant Ras to different extents was not significant (p = 0.5); while the probability that p120GAP stimulated wild type Ras and mutant Ras differently was highly significant (p = 0.0012).

In order to confirm the above results, neurofibromin and p120GAP proteins were separated from each other utilizing an entirely different approach. Neurofibromin was affinity purified by passage of rabbit brain lysate over a Protein A-Sepharose column bound to neurofibromin-specific antibodies. The neurofibromin-antibody complex was then released from the Protein A-Sepharose. The neurofibromin thus purified ap-
accurately reflect those observed in the previous determina-
tion, and indicate that only p120GAP has reduced activity with the
mutant Ras protein.

A final experiment was performed to confirm that p120GAP
(and not neurofibromin) has reduced ability to stimulate the
GTPase activity of Ras-100LIR104. The detergent n-dodecyl-
maltoside has been shown to specifically inactivate neurofi-
brin in brain lysates (Bollag and McCormick, 1991). When a rat
brain lysate was treated with maltoside to neutralize the ne-
urofibromin activity, a 2–3-fold decrease in its ability to stimu-
late GTPase was evident with the Ras-100LIR104 mutant com-
pared to wild type Ras, similar to results obtained above. In
untreated brain lysates on the other hand, where a preponder-
ance of the GTPase activating activity is due to neurofibromin,
the decrease in GTPase stimulation of Ras-100LIR104 was not
observed (data not shown; see “Discussion” for details).

Because of the difficulty in obtaining purified neurofibromin
or p120GAP, together with the loss of activity of these two
reagents experienced during purification, we elected to rapidly
separate the two activities from each other using three entirely
separate approaches. With each of these approaches almost
identical results were obtained indicating that the 101–103
region is required for efficient p120GAP stimulated hydrolytic
activity of Ras; but not for neurofibromin-stimulated hydroly-
sis. While the means of separating p120GAP from neurofibro-
min differed in each experimental approach, the similarity in
results obtained with each, together with the high degree of
reproducibility of the data, strongly argue in favor of the above
conclusion.

Activities of the p120GAP Catalytic Domain and the Neurofi-
brin GRD—Attempts were next made to determine which
part of the p120GAP molecule was responsible for the decreased
interaction seen with the mutant Ras protein. While p120GAP
and neurofibromin are largely unrelated to each other, the
catalytic domain of p120GAP and the GRD of neurofibromin
display similar activities and share approximately 26% se-
quenome homology (Martin et al., 1990). These peptide regions,
each of which possess the ability to efficiently induce GTPase
activation of Ras proteins, have been separately expressed
(Hettich and Marshall, 1994; Xu et al., 1990). The p120GAP
catalytic domain and the neurofibromin GRD were purified as
glutathione S-transferase fusion proteins produced from bac-
terial vectors and purified by affinity chromatography. These
two proteins were then incubated with Ras or Ras-100LIR104
in the previously described GTPase assay. No difference in the
neurofibromin GRD activity was observed when Ras and Ras-
100LIR104 were compared (Fig. 4a), similar to observations
with full-length neurofibromin. Furthermore, the catalytic car-
bboxy-terminal portion of p120GAP was equally effective in its
ability to accelerate wild type and Ras-100LIR104 GTPase
activity (Fig. 4b). As above, these conclusions are based upon
statistical analyses of all the data obtained from several sepa-
rate experiments (see figure legend for statistical summary).
The mutant Ras protein therefore retains the ability to be
stimulated enzymatically by the catalytic core of p120GAP. The
reduced stimulation observed with full-length p120GAP must
therefore result from an altered interaction between the amino
terminus of p120GAP and the altered structure of the mutant
Ras. Obviously this interaction is important in controlling
p120GAP activity.

Determination of Binding Affinities—The question then re-
 mains, does the inability of full-length p120GAP to efficiently
stimulate the GTPase activity of Ras-100LIR104 result from
the inability of p120GAP to bind to the mutant protein, or to its
inability to enzymatically accelerate its GTPase activity? Com-
petitive binding assays were preformed to distinguish between
these two possibilities. This assay is designed to determine how

![Figure 3. Hydrolysis by separately purified GTPase activating proteins.](http://www.jbc.org/)

A

![Graph A](http://www.jbc.org/)

B

![Graph B](http://www.jbc.org/)
much Ras protein bound to an unlabeled GTP analogue is required to competitively inhibit GTPase stimulation of a trace amount of Ras bound to labeled GTP. These assays were, therefore, performed with a low concentration (6 nM) of labeled, wild type Ras\[\gamma^{32P}]GTP incubated with baculovirus p120GAP or affinity purified neurofibromin. The extent of hydrolysis was then determined with a charcoal binding assay in the presence of increasing concentrations of competitive Ras protein bound to an unlabeled, nonhydrolyzable GTP analogue (GMP-PNP).

When concentrations of the unlabeled, competitive protein reach the binding constant, hydrolysis of the labeled GTP bound to wild type Ras would be reduced 50% (IC$_{50}$). The binding affinity of neurofibromin for Ras and Ras-100LIR104 were identical (IC$_{50}$ = 0.030 µM, Fig. 5a). Similarly, the binding of p120GAP to Ras and Ras-100LIR104 was also equal (IC$_{50}$ = 12 µM, Fig. 5b). The values obtained here for both neurofibromin and p120GAP are similar to those previously reported (Bollag and McCormick, 1991).

These experiments rule out the possibility that the decrease in p120GAP activity in the presence of Ras-100LIR104 results from a decreased binding affinity. The decrease in GTPase stimulation of Ras-100LIR104, therefore, is due to the inability of full-length p120GAP to efficiently stimulate the hydrolysis of the mutant protein once it has bound. Clearly, amino acids in the region of 101–103 of Ras are important for the interaction between p120GAP and Ras proteins, an interaction which apparently is important for full activity of p120GAP. It is interesting, however, that this region is apparently not involved in the interaction between Ras and neurofibromin. It is possible that the amino-terminal regions of p120GAP (which are necessary to achieve efficient GTPase activity, and which are totally distinct from any sequence in neurofibromin) physically interact with the carboxyl terminus of the a3 region of Ras or another region of Ras altered by the 101–103 mutation.

Lastly, in order to address the biological significance of the Ras-100LIR104 mutation, wild type Ras or the Ras-100LIR104 mutant were microinjected into NIH3T3 cells. The cells were subsequently labeled with [\textsuperscript{3}H]thymidine. The results indicated that there was no difference in the ability of the cell microinjected with Ras or Ras-100LIR104 to enter S phase (data not shown). In the future, experiments designed to transfected different cell types with Ras-100LIR104 may provide further insight regarding the biological role of this mutant.

DISCUSSION

The amino acids KRV located in the a3 helix of Ras at position 101–103 are highly conserved in Ras and several other small GTP binding proteins (Santos and Nebreda, 1989). This conserved region of the Ras protein was investigated using an insertion-deletion mutant Ras-100LIR104. The 100LIR104 substitution does not alter the intrinsic GTP binding or hydroly-
ysis of the protein but does diminish p120GAP-stimulated hydrolysis by 2-3 fold. Neurofibromin hydrolytic activity, however, was not altered in Ras-100LIR104 when compared to wild type Ras. This result was obtained regardless of the means by which p120GAP and neurofibromin were separated from each other. A biochemical purification of these two GTPase activating proteins was not attempted due to the extensive manipulations required and the consequent loss and potential alteration of their activities. Three methods were therefore utilized to rapidly separate the two proteins and assess their abilities to stimulate the GTPase activity of mutant and wild type proteins. First, the two proteins were separately immunoprecipitated and assayed directly. Second, the neurofibromin was affinity purified on an antibody column and the neurofibromin-immunoglobulin complex released and utilized in comparison to a lysate of baculovirus expressed p120GAP. Finally, crude brain lysate was assayed after the neurofibromin activity had been specifically inhibited by a detergent.

In the first instance the two proteins produced in mammalian cells were isolated as immunoprecipitates. In the second approach the p120GAP was produced in an insect cell where no endogenous GTPase activity has been observed (data not shown). In this case the protein would be free of other mammalian proteins. In the third procedure the p120GAP was assayed in the presence of other soluble mammalian proteins, but with inactivated neurofibromin. The fact that essentially identical results were obtained in repeated analyses of each type clearly indicate that there is a distinction in the interaction of these two GTPase activating proteins with the mutant Ras. On the other hand, the catalytic domain of p120GAP and the GRD of neurofibromin were found to stimulate the rate of GTP hydrolysis of Ras and Ras-100LIR104 efficiently, and to the same extent. It is therefore concluded that an interaction between the amino-terminal, noncatalytic region of p120GAP and the 101-103 position of Ras must be essential for efficient GTPase stimulation, while no such interactions are required for the activity of neurofibromin. Alternatively, alterations in the structure of distant regions of Ras resulting from the mutation might also play a role.

In an attempt to address the biological significance of the Ras-100LIR104 mutation, this protein was microinjected into NIH3T3 cells. We found, however, no difference in the ability of the cells microinjected with Ras or Ras-100LIR104 to enter S phase (data not shown). These results are, however, not unexpected when considering our data obtained using rat brain lysate. When rat brain lysate was used as a source of GAP (p120GAP and neurofibromin) activity, no difference was observed between Ras and the Ras-100LIR104 mutant. It was only the separation of p120GAP from neurofibromin which resulted in the identification of a decrease in p120GAP activity in the presence of Ras-100LIR104. It is therefore conceivable that microinjection of the Ras-100LIR104 mutant into cells containing both p120GAP and neurofibromin, would not produce different effects. Additional experiments in which the Ras-100LIR104 mutant is transfected into different cell types, however, may further our understanding regarding the biological significance of this mutation.

It has been previously reported that the Ras-100LIR104 mutation exhibits normal sensitivity to p120GAP. However, the source of GAP in these studies was an MCF-7 cell extract which would be expected to contain both p120GAP and neurofibromin (Adari et al., 1988). Those experiments were performed at a Ras concentration of 0.8 μM. Since the binding constant of Ras for neurofibromin and p120GAP is 30 nM and 12 μM, respectively, a Ras concentration of 0.8 μM would result predominantly in neurofibromin-mediated Ras-GTP hydrolysis. Thus, it is under-standable that no decrease in GTP hydrolysis was detected when Ras-100LIR104 was incubated with MCF-7 cell lysate. For the same reason we also did not observe differences in GTP hydrolysis between Ras and Ras-100LIR104 when rabbit brain lysate (which contains both neurofibromin and p120GAP) was used as a source of GAP (data not shown). Only when steps were taken to separate the p120GAP and neurofibromin activity were the differences seen.

The a3 helix of Ras has been implicated in other studies as a site for GAP interaction (Wood et al., 1994). The yeast Ras2-E99K mutation which exhibited intrinsic hydrolytic rates similar to wild type Ras, showed reduced sensitivity to three separate GTPase proteins: IRA2 GAP, E, colli expressed mammalian GAP, and neurofibromin. Furthermore, Wood et al. (1994) also showed by a competitive binding assay that the affinity of Ras2-E99K for neurofibromin was dramatically reduced. These results differ from ours, where neither the neurofibromin activity nor binding affinity for Ras-100LIR104 was diminished. The yeast Ras2-E99K mutation would be located at the beginning of the a3 helix of the protein, while the 100LIR104 mutation is located at the terminus of this helix.

While the individual characteristics of the two types of mutants differ, both result in clear alterations in interactions with GTPase activating proteins supporting the conclusion that this region of Ras, in addition to the effector binding domain and residues 61–65 of the switch II region, is an important site for interaction with GTPase activating proteins. It is also interesting to note that the effector domain, the switch II region, and a region of the a3 helix, all reside on the same surface of the Ras protein. These three regions are in close proximity to one another and could potentially provide a binding site for GAP proteins (Wood et al., 1994). Finally, the Ras-D38E mutant is not activated by p120GAP but is able to bind to p120GAP with an affinity similar to wild type. Interestingly, the D38E mutation causes a chain shift in the 101–103 region of Ras. Thus the D38E mutation may have low GTPase activity in part due to the chain shift in the 101–103 region of Ras (Krenge et al., 1990). Alternatively, both this mutation and the Ras-100LIR104 mutation might induce conformational changes at other sites of the protein which are important in modulating GTPase activation.

Other studies have also examined Ras-100LIR104 in the presence of GAP purified from placenta. Placental GAP, designated p100GAP, is generated from an alternative splicing product and encodes a protein product with a predicted molecular weight of 100,400 (Trahey et al., 1988). p100GAP lacks the hydrophobic amino terminus of the p120GAP species, but retains catalytic activity. No difference in hydrolysis was observed when Ras and Ras-100LIR104 were assayed for p100GAP activity (Downward et al., 1990). This observation is consistent with our data where decreased hydrolysis was observed with Ras-100LIR104 only in the presence of full-length p120GAP. Therefore, the hydrophobic, extreme amino terminus of p120GAP appears to be responsible for the differences in GTPase activation observed between Ras-100LIR104 and wild type Ras. While it is not clear exactly how Ras protein is positioned with respect to the plasma membrane, it is likely that the protein is oriented with the lipid-modified carboxyl terminus oriented toward the membrane. If so, it is further likely that the terminal region of the a3 helix of Ras containing amino acids 101–103 is located near the plasma membrane (de Vos et al., 1988; Pai et al., 1989). If, as postulated above, the amino terminus of p120GAP interacts with the terminus of the a3 region of Ras, this hydrophobic region of p120GAP would be expected to be positioned near, or perhaps even at, the plasma membrane. It is additionally clear from these studies that this
region of p120GAP plays a critical role in modulating GTPase activation.

Recently, a novel mammalian GTPase activating protein for Ras, Gap1m, has been identified (Maekawa et al., 1994). Because Gap1m is expressed in brain, it is possible (in those experiments in which rabbit brain lysate was used as a source of p120GAP) that Gap1m function is also impaired in the presence of the Ras-100LIR104 mutant. Further investigation, however, is needed in order to determine the interaction of Gap1m with 100LIR104 Ras.

It is also possible that associated protein(s) are in a complex with p120GAP. This possibility, however, does not diminish the importance of the observation that the conserved 101–103 amino acids in the a3 helix of Ras are specifically involved in p120GAP-mediated hydrolysis but not neurofibromin-mediated hydrolysis. The likelihood that associated proteins are bound to p120GAP is lessened by the fact that p120GAP is overexpressed in insect cells, therefore, the ratio of an associated protein to overexpressed p120GAP would differ significantly to that observed in rat brain lysate in which p120GAP is not overexpressed. Thus, if an associated protein were involved, one would expect different results from p120GAP overexpressed in baculovirus lysates compared to endogenous p120GAP in rat brain lysates. Since identical results were obtained in both cases, it is unlikely that an associated protein contributes significantly to the decrease in GTPase activity.

Another interesting possibility is that the decrease in p120GAP activity is not due to the deletion of the conserved KRV amino acids but the substitution of amino acids LIR. Future experiments, in which single substitutions or a deletion of the one or all three of the conserved amino acids may help us better answer this question or perhaps even lead to the identification of additional mutants of this type.

Lastly, the Ras-100LIR104 mutant may serve as an important tool to better understand the function of the GAP proteins. For example, such a mutant may differentiate between different GAPs or between neurofibromin and p120GAP. The construction of cell lines transfected with the Ras-100LIR104 mutation could thus provide a useful in vivo approach to address these types of questions.

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