A New Function of p120-GTP-activating Protein

PREVENTION OF THE GUANINE NUCLEOTIDE EXCHANGE FACTOR-STIMULATED NUCLEOTIDE EXCHANGE ON THE ACTIVE FORM OF Ha-Ras p21

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This work studies the coordination of the action of GTPase-activating protein (GAP) and guanine nucleotide exchange factor (GEF) on activated human c-Ha-Ras p21. Purified human p120-GAP was obtained with a new efficient procedure. To distinguish the GTPase-activating effect of p120-GAP from other effects dependent on the interaction with activated Ha-Ras, the nonhydrolyzable GTP analogue guanosine 5'-O-(thiotriphosphate) (GTPγS) was used. The results showed that the GTPγS/GTPγS exchange enhanced by the C-terminal catalytic domain of the yeast GEF Sdc25p (S-Sdc25p) is prevented by p120-GAP. This effect is strictly specific for the activated form of Ha-Ras, the target of GAP; no effect on Ha-Ras-GDP was detectable. The GAP catalytic domain also inhibited C-Sdc25p but to a lower extent. The interfering effect by p120-GAP was also evident in a homologous mammalian system, using full-length mouse RasGEF, its C-terminal half-molecule, or C-terminal catalytic domain. As a consequence of this inhibition, presence of p120-GAP enhanced the regeneration of Ha-Ras-GTP by GEF at a GDP/GTPγS ratio mimicking the in vivo GDP/GTP ratio. Our work describes a novel function of p120-GAP and suggests a mechanism by which GAP protects Ha-Ras against unproductive exchanges. This constrain is likely involved in the regulation of the physiological GDP/GTP cycle of Ras and in the action of p120-GAP as downstream effector of Ras. Helix α3 is proposed as a Ras element playing a key role in the interference between GAP and GEF on Ras.

Ras proteins are molecular switches of a pathway regulating cell growth and differentiation by cycling between two conformations: the active GTP-bound state and the inactive GDP-bound state (1). As for most GTPases, the GDP/GTP cycle of Ha-Ras is controlled by two kinds of regulators, the GTPase activating protein (GAP)1 and the GDP/GTP exchange factor (GEF). Thus, the level of Ha-Ras-GTP depends on the ratio of the activities of these two regulators and consequently, conditions influencing their activity affect the function of Ha-Ras. The mechanisms involved in the regulation of these two ligands have only been in part clarified. For instance, why only in particular conditions is the decrease in GAP activity associated with the increase in the concentration of p21-GTP (2, 3)? Why an overexpression of Ras be associated with a very low percentage of activated GTP-bound state (<0.3%) (4)? Upon activation of receptor or nonreceptor tyrosine kinase, p120-GAP is phosphorylated in vivo, but it is as yet unclear whether this modification is involved in the regulation of its negative effect on Ha-Ras-GTP (5). Concerning GEF, the activation of Ha-Ras by tyrosine kinase-linked receptors has been reported to depend on the transient translocation of the ubiquitous Ras-GEF SOS to the cell membrane (5), a process whose regulation still presents several unclear aspects. Recent work in vivo has suggested that Ca2+, calmodulin (6), and phosphorylation (7) control the activity of the neuronal CDC25-like Ras-GEF, but how these effects are coordinated, remains an open question. The biochemical characterization of CDC25Mm has shown that the activity of its C-terminal catalytic domain is negatively regulated by the N-terminal moiety and that the Ca2+-dependent proteins calmodulin and calpain may be involved in this mechanism (8). Another important aspect, the effect of the simultaneous action of p120-GAP and GEF on the activated Ha-Ras state p21-GTP, has yet to be investigated. Indeed, GAP and GEF have both as common target GTP-bound Ha-Ras, with the specific difference that p120-GAP has a much higher affinity for Ha-Ras-GTP than for Ha-Ras-GDP (9), whereas GEF acts on Ras-GTP and Ras-GDP with nearly the same efficiency (10–13). In this context, it is also worth mentioning that the GTP-bound elongation factor (EF) Tu, a model GTPase in bacterial protein biosynthesis sharing structural and functional homology with Ras, is protected against the action of its GEF EF-Ts by amino acid RNA and the ribosome that is since long known to exert a GAP-like activity on the EF-Tu GTPase (14, 15). The importance of a coordination between the various Ras ligands is moreover suggested by the increasing number of ligands found to interact with Ha-Ras besides GAPs and GEFs (for references, see Wittighofer and Hermann (16)).

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1 The abbreviations used are: GAP, GTPase-activating protein; p120-GAP, human RasGAP; GEF, guanine nucleotide exchange factor; CDC25Mm, mouse RasGEF (p140-GEF); CDC25Mm31, C-terminal half-molecule of CDC25Mm of 631 amino acid residues; CDC25Mm285, catalytic domain of CDC25Mm of 285 amino acid residues; GTPγS, 5'-O-(thiotriphosphate); EF, elongation factor; GST, glutathione S-transferase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis.
Starting from these considerations, we have studied the influence of GAP on the action of GEF on activated Ha-Ras. For this purpose, we have tried to distinguish possible effects of GAP from its ability to enhance the Ha-Ras-dependent GTPase by substituting GTP with its nonhydrolyzable analogue GTPγS. As a system, we have chosen human p120-GAP, prepared by a novel method, and the catalytic domain of Saccharomyces cerevisiae Sdc25p (C-Sdc25p), a model GEF acting equally well on Ha-Ras p21, and Ras2p (10, 12, 17, 18) or the mammalian RasGEF CDC25Mm (p140-GRF). The results suggest that p120-GAP exerts an additional function that prevents GEF from interacting with p21-GTP. The evidence that this effect is involved in the modulation of the signals of the Ras pathway is discussed.

MATERIALS AND METHODS

Expression and Purification of Full-length p120-GAP—The Escherichia coli strain SCS-1 was transformed with a pGEX vector containing the NaeI-EcoRI fragment from pUC101, a vector kindly provided by Dr. F. McCormick. It encoded a p120-GAP lacking the first 5 amino acids, thus comprising residues 6–1047. The bacteria were grown at 37 °C in 800 ml of LB medium containing 50 μg/ml ampicillin. Expression of the GST-fused protein was induced overnight at 24 °C by adding low amounts of IPTG (0.01 mM) at a cell density of 0.4 A600 unit. The cell culture, chilled to 4 °C at 2.0 A600 was centrifugated at 4,000 × g for 10 min, and the pellet was resuspended in 70 ml of buffer A (150 mM NaCl, 16 mM Na3HPO4, 4 mM NaH2PO4, pH 7.3) containing 2 mM phenylmethylsulfonyl fluoride. After sonication and addition of 1% triton, the suspension was centrifuged at 17,000 × g for 30 min and the supernatant was mixed batchwise with 4 ml of glutathione-Sepharose 4B (Pharmacia), gently shaken for 1 h at 4 °C. The resin was washed twice with buffer A and the protein eluted with glutathione (3 mg/ml) was concentrated to 1.5 ml with Aquacide II (Calbiochem) and then to 400 μl on a Centricon-100 (Amicon) at 3,000 × g, diluting several times with 50 mM Tris-HCl, pH 7.5, 50 mM NaCl and 28 mM 2-mercaptoethanol. The protein solution, mixed with an identical volume of glycerol, was stable for at least several months when stored at −20 °C.

The GST fusion was cleaved by incubation with 0.1 unit of thrombin (Sigma), 1 μg of protein in 25 mM Tris-HCl, pH 8.0, 25 mM NaCl, 25 mM CaCl2, 7 mM mercaptoethanol, and 10% glycerol at 30 °C for 30 min. The thrombin was removed by washing on Centricon-100 as above.

Dissociation Rate Constants—Dissociation of the p21 complex was determined at 30 °C by the nitrocellulose binding assay. The effect is involved in the modulation of the signals of the RasGEF from interacting with p21. The dissociation rate of p21 stimulation by GAP is defined as the amount of GAP inducing the hydrolysis of 1 nmol of Ha-Ras p21-GTP/min at 25 °C (20).

Other Methods and Materials—The catalytic domain of p120-GAP (C-terminal 350 amino acid residues) was purified as GST fusion protein with the same method as p120-GAP. C-Sdc25p (C-terminal 550 amino acid residues) was produced and purified to near homogeneity as described previously (12). Purified Ha-Ras p21 (21), full-length CDC25Mm (1262 amino acid residues), its C-terminal half-molecule of 631 residues (C-CDC25Mm631) (8) and the catalytic domain of 285 residues (C-CDC25Mm285) (13) were obtained as reported. SDS-PAGE was carried out using 7.5% acrylamide gel (22). Immunoblot analysis was performed using specific antibodies anti-GST produced in rabbit and peroxidase-conjugated antibodies revealed by the diaminobezamidine method. Protein concentration was determined by the Bio-Rad protein assay, using BSA as standard, and in the case of p21 also checked by GDP binding.

RESULTS

Overproduction and Purification of p120-GAP—The pGEX system allowed an easy reproducible production and purification of the recombinant human p120-GAP in good yield and, noteworthy, in the absence of proteolytic phenomena (Fig. 1A). As shown by Western blot analysis with specific antibodies anti-GST (Fig. 1B), virtually 90% of the produced GST-p120-GAP remained in the supernatant fraction, after centrifugation for 30 min at 17,000 × g. The temperature of induction and the concentration of IPTG were important parameters to obtain high overproduction and solubility. In fact, induction at 24 °C and 0.01 mM IPTG yielded higher production and solubility of GST-p120-GAP than at 37 °C and 0.1 mM IPTG (not shown). No partial translation products of p120-GAP were detectable. After the affinity chromatography and Centricon-100 treatment, the GST-p120-GAP was at least 90% pure and the yield was ~1 mg/L for cell culture of 2.0 A600 density (Fig. 1A, lane 5). The contamination essentially consisted of a faster migrating component unreactive to GST antibodies. Purified GST-p120-GAP was stable for several months, when kept at −20 °C in storage buffer.

GST-p120-GAP was cleaved by thrombin (Fig. 1A, lane 4). However, since fused GST-p120-GAP was essentially as active as the unfused product, most experiments were carried out with the fused protein. The specific activity of purified GST-p120-GAP (1510 units/mg) and thrombin-cleaved p120-GAP (1100 units/mg) were comparable to those reported for full-length p120-GAP isolated from the baculovirus/sf9 insect cells.

FIG. 1. SDS-PAGE and immunoblot analysis of p120-GAP prior to and after purification. A, SDS-PAGE of a 7.5% acrylamide gel stained with Coomassie Brilliant Blue. Lane 2, total cell extract after induction of p120-GAP; lane 3, cell extract supernatant after induction of p120-GAP; lane 4, thrombin-cleaved GST-p120-GAP; lane 5, purified GST-p120-GAP. B, immunochromatographic Western blot analysis. Lane 2, total cell extract before induction of p120-GAP; lane 3, total cell extract after induction; lane 4, supernatant of extract after induction; lane 5, pellet of cell extract after induction. Lane 1 in both A and B, 2 μg of markers with the indicated relative molecular mass (kDa).
system (1100 units/mg) (20, 23).

Stimulation by C-Sdc25p of the Ha-Ras GTP\textsubscript{G}S/GTP\textsubscript{G}S

Exchange Is Specifically Inhibited by p120-GAP and by Its Catalytic Domain C-GAP—To determine the effect of GAP on the activity of the exchange factor on both active and inactive states of Ras, the complex formed by p21 with the nonhydrolyzable GTP analogue [\textsuperscript{\textgamma}S]\textsuperscript{35}GTP\textsubscript{G}S or with [\textsuperscript{3H}]GDP was used. It is known that p120-GAP and C-GAP have an affinity for GTP-bound p21 much higher (\(\sim 100\) times) than for GDP-bound p21 (9, 24).

Fig. 2 illustrates the action of C-Sdc25p on Ha-Ras p21-\([\textsuperscript{\textgamma}S]\textsuperscript{35}GTP\textsubscript{G}S) in the presence and in the absence of p120-GAP or C-GAP. At a concentration of 20 nM C-Sdc25p, the apparent dissociation rate constant (\(k^{-1}\)) of the Ha-Ras-\([\textsuperscript{\textgamma}S]\textsuperscript{35}GTP\textsubscript{G}S complex was decreased by the presence of 130 nM p120-GAP from 3.5 \times 10^{-2}\text{min}^{-1} to 1.9 \times 10^{-2}\text{min}^{-1}. Since the apparent dissociation rate constant of p21-\([\textsuperscript{\textgamma}S]\textsuperscript{35}GTP\textsubscript{G}S) is a measure of the GEF activity, this corresponds to a 46% inhibition of the GEF activity in the GTP\textsubscript{G}S/GTP\textsubscript{G}S exchange. Most important, this effect was specific for the active form of p21, since no GAP inhibition was detectable if p21-\([\textsuperscript{\textgamma}S]\textsuperscript{35}GTP\textsubscript{G}S was replaced by p21-[\textsuperscript{3H}]GDP (Fig. 2B). The inhibition by p120-GAP of the action of C-Sdc25p increased with increasing the concentration of p120-GAP (Figs. 3, A and B). A similar inhibitory effect by p120-GAP was also observed on the stimulation by C-Sdc25p of the GTP\textsubscript{G}S/GDP exchange reaction of Ha-Ras (not illustrated). We could estimate that the IC\textsubscript{50} of p120-GAP on the C-Sdc25p activity was around 140 nM (Fig. 3B). When we replaced p120-GAP with its isolated catalytic domain C-GAP (350 amino acid), the inhibition of the C-Sdc25p was about 2.5-fold lower (Fig. 3, C and D), the IC\textsubscript{50} being approximately 350 nM. This lower effect is in good agreement with the 2-fold lower times affinity observed for a similar catalytic domain of p120-GAP, GAP334, as reported previously (20).

To our surprise, in the course of these measurements we observed a slight but reproducible stimulation of the intrinsic GTP\textsubscript{G}S/GTP\textsubscript{G}S exchange of Ha-Ras by p120-GAP, an effect that increased with increasing the concentration of p120-GAP and was not observed if C-GAP replaced p120-GAP (cf. Fig. 3, A and C). Worth mentioning, no p120-GAP-dependent hydrolysis of 

![Fig. 2. Specific inhibition by p120-GAP or C-GAP of the C-Sdc25p-stimulated nucleotide dissociation activity of Ha-Ras. 70 nM p21-[\textgamma]S\textsuperscript{35}GTP\textsubscript{G}S (A) or p21-[\textsuperscript{3H}]GDP (B) were incubated for 10 min at 30 °C without (square) and with 130 nM p120-GAP (●) or C-GAP (○). The dissociation reaction was started by adding a 500-fold excess of cold nucleotide and 20 nM C-Sdc25p. At the given times 10-\mu\text{L} samples were withdrawn and the amount of the p21-bound [\textsuperscript{3H}]GDP or [\textgamma]S\textsuperscript{35}GTP\textsubscript{G}S determined by the nitrocellulose filtration procedure. In the equation \(\ln(C_{t}/C_{0}) = -k^{-1} t\), \(C_{0}\) represents the initial concentration of the p21-nucleotide complex and \(C_{t}\) represents the concentration at time \(t\).](image)

![Fig. 3. Effect of p120-GAP or C-GAP concentration on the C-Sdc25p activity. In the panels A and C, the dissociation rate constants of p21-[\textgamma]S\textsuperscript{35}GTP\textsubscript{G}S complexes (70 nM), in the presence of C-Sdc25p (20 nM) and the given concentrations of p120-GAP (△) or C-GAP (●), were calculated from 30 min kinetic experiments. In the absence (square, ○) of C-Sdc25p, the dissociation rate constants were calculated from 60-min kinetic experiments. In panels B and D, the percentages of inhibition of C-Sdc25p were determined taking the C-Sdc25p-dependent p21 exchange activity in the absence of GAP proteins as 100% activity.](image)
GAP Hinders GEF on Activated Ha-Ras

In the set of experiments described of Fig. 4, we examined the dependence of the GEF activity on the molar ratio C-Sdc25p:p120-GAP, using increasing concentrations of C-Sdc25p and a fixed amount of p120-GAP (136 nM), corresponding to the concentration giving approximately a 50% inhibition in the experiments of Fig. 3. An increase in C-Sdc25p from 10 to 100 nM considerably reduced the inhibition by p120-GAP, as shown by the decrease in the difference between the $k_{on}$ values in the absence and in the presence of p120-GAP (Fig. 4, I–IV). In fact, with the lowest concentration of C-Sdc25p (10 nM) the inhibition by p120-GAP on the dissociation rate constant of p21-[$\gamma^{35}$S]GTP was nearly 70%, whereas in the presence of 100 nM C-Sdc25p the inhibition by p120-GAP was practically absent.

p120-GAP also Inhibits the GEF Activity of Mammalian CDC25Mm—The interference by GAP on the GEF activity was also tested in a homologous mammalian system by using three forms of the mammalian RasGEF mouse CDC25M: the full-length molecule, the C-terminal half-molecule, and the short catalytic domain CDC25Mn285. Noteworthy, the full-length CDC25Mm has a GEF activity 5 times lower than CDC25Ms and 25 times less than CDC25Mn285 (8). Also in this system, p120-GAP exerted an inhibitory activity, in which specific differences depending on the GEF form could be observed. As shown in Fig. 5, in which the concentration of the diverse CDC25Mm forms was chosen to give similar dissociation rates, the inhibition of the stimulation of the Ha-Ras GTP S/GTP S exchange in the case of full-length CDC25Mm and C-terminal half-molecule was about 50%. The effect was lower (inhibition, 18%) when the short catalytic domain CDC25Mn285 was used.

Presence of p120-GAP Enhances the Regeneration of the Ha-Ras Active Form by GEF—To evaluate the implication of the inhibitory effect of p120-GAP on GEF in the regeneration of the active form of Ha-Ras and, consequently, to examine the possibility of correlating these observations in vitro with the physiological conditions in the cell, we have investigated the influence of p120-GAP under conditions in which the molar ratio of the free nucleotides GDP and GTP (as GTP S) corresponded to the range of values estimated to occur in the cell. As shown in Fig. 6, at a GDP to GTP S ratio of 1 to 10, the addition of 130 nM p120-GAP enhanced the CDC25Mm-dependent stimulation of the GDP/GTP S exchange on Ha-Ras. This result supports the possibility that the inhibition by GAP of the GEF activity via sequestration of the active form of Ha-Ras favors the interaction between the exchange factor and Ha-Ras-GDP, its physiological substrate.

DISCUSSION

The increasing number of interactions attributed to Ha-Ras, a central crossing point for the regulation of the signal transmission in the cell, suggests the existence of mechanisms coordinating the complex pattern of interactions of ligands on Ha-Ras (for references, see Wittinghofer and Hermann (16)). In an attempt to shed some light on possible mechanisms, we have chosen a system in vitro analyzing whether GAP can directly affect the activation of Ha-Ras by GEF, since GAP and GEF are both known to affect the GTP-bound Ha-Ras. For this study, it was important to have available the whole molecule of p120-GAP. We therefore developed a simple procedure to isolate highly purified p120-GAP, by defining precise, reproducible conditions for its efficient production in E. coli. Despite the lack of the first 5 residues, the ability of our p120-GAP to stimulate the p21 GTPase was virtually the same as reported for full-length p120-GAP produced and purified from baculovirus/sf9 insect cells (20, 23). No proteolytic phenomena were observed during its preparation and the stability of the purified form was verified over months. This method facilitates the utiliza-

![Figure 4](image-url)

**Fig. 4.** Inhibition of the GEF activity by p120-GAP as a function of the concentration of C-Sdc25p. The four small panels (I–IV) illustrate the different dissociation rates of p21-[$\gamma^{35}$S]GTP S complexes in the presence (○) and in the absence (▲) of 136 nM p120-GAP using 10 (I), 20 (II), 40 (III), 100 (IV) nM of C-Sdc25p, respectively. The percentage of the inhibition of C-Sdc25p was determined as described in the legend to Fig. 3.
Inhibition by p120-GAP of the GTP/GTP exchange rate on Ha-Ras p21 stimulated by full-length CDC25Mm, CDC25\textsuperscript{Mm621}, or CDC25\textsuperscript{Mm285}. The reaction mixtures containing p21-[\gamma-\textsuperscript{35}S]GTP/GTP exchange (70 nM) were incubated for 10 min at 30 °C with or without 130 nM p120-GAP. The dissociation reaction was started by the addition of an excess of cold nucleotide and 200 nM full-length CDC25\textsuperscript{Mm}, 35 nM CDC25\textsuperscript{Mm621}, or 10 nM CDC25\textsuperscript{Mm285}. The concentrations of the different CDC25\textsuperscript{Mm} forms were chosen to give the same k\textsuperscript{-1} in the absence of p120-GAP.

In the GDP/GTP cycle of Ras proteins, the reversibility of all the partial steps of the nucleotide exchange on p21 is correlated from being fully understood and it is possible that the protective association of p120-GAP with activated p21 is correlated with the function of GAP as transmitter of downstream signals and specific effects on the ability of this GAP to enhance the GTPase activity of Ras. However, inhibitory effects on the ability of this GAP to enhance the GTPase activity of Ha-Ras have been reported as response to extracellular stimuli. For example, stimulation of the T cell receptors in T lymphocytes (2) or treatment with erythropoietin of human erythroleukemia cells (3) results in a large increase in Ras activation in vivo. p120-GAP is phosphorylated in vitro upon activation of receptors or nonreceptors tyrosine kinases. Phosphorylated p120-GAP associated with the p190 phosphoprotein was found to have a decreased GTPase activity by GAP in cell lysate. Phosphorylated p120-GAP associated with the p190 phosphoprotein was found to have a decreased GTPase activating effect (27). Mitogenic lipids such as phosphatidic acid and arachidonic acid can inhibit p120-GAP in vitro (28). All these data are consistent with the possibility that under specific in vivo conditions the GAP/GTP exchange activating effect of p120-GAP may be inhibited. Evidence also exists that p120-GAP may act as downstream effector (for refs, cf. Pronk and Bos (5)). The dual role of p120-GAP as negative regulator and effector of Ras is far from being fully understood and it is possible that the protective association of p120-GAP with activated p21 is correlated with the function of GAP as inhibitor of downstream signals of Ha-Ras.

As model GAP we have used for most experiments the highly purified catalytic domain of \textit{S. cerevisiae} Sdc25p that is the best biochemically characterized exchange factor so far.
form of Ha-Ras in a homologous mammalian system using the mouse GEF CDC25Mn. The GEF activity of full-length CDC25Mn and of the C-terminal half-molecule, CDC25Mn631, were inhibited by p120-GAP more efficiently than C-Sdc25p, in agreement with their lower affinity for Ha-Ras. The observation that the inhibitory effect on the isolated catalytic domain CDC25Mn285 is weak suggests that noncatalytic domains of GEF are also involved in the interference with GAP.

The observed reproducible slight stimulation by p120-GAP of the intrinsic GTPPs exchange of Ha-Ras was not inhibited by C-GAP. Since the noncatalytic regions of p120-GAP are needed for a fully productive Ras-GAP interaction (20), it is possible that these regions may induce some destabilization of the Ras-nucleotide interaction, very likely via intramolecular arrangement of the Ras nucleotide binding pocket.

We have demonstrated the inhibition by GAP of the GEF activity in vitro. The reciprocal effect, the influence of GAP on the GEF activity is also likely to exist, as suggested by preliminary experiments indicating an inhibition by C-Sdc25p of the p120-GAP-dependent stimulation of the Ha-Ras p21-GT. Since the investigation of this aspect presents several technical difficulties, because the GTP hydrolysis produces the inactive form p21-GDP (Fig. 7) that is also a substrate of the competitor GEF, other experimental approaches may be necessary to define in an unequivocal manner the reciprocal nature of the constraints induced by GAP on GAP.

Since it is known that besides GEF and GAP, the active form of Ha-Ras can bind other ligands, such as c-Raf, Rin, protein kinase C, and PI(3)K (for references, see Wittinghofer and Hermann (16)), it is likely that not only GAP but also these effectors contribute to protect p21-Ras for binding to Ha-Ras-GTPase. The reciprocal effect, the influence of GEF on GAP, the loop L2 plus the N-terminal strand \( \alpha_2 \) and the loop L6/N-terminal helix \( \alpha_2 \) are structural elements implicated in the binding site of GAP (32–35). Loop L1 has been shown to play a key role in the molecular mechanism of the GTPase activation (20, 24, 25, 32, 36–38). As this loop contains a residue (Ala31) in Ha-Ras and Gly10 in Ras2p important for the specificity toward RasGAP, it can be considered as potential binding site (35). Concerning the interaction between Ras and GEF, a direct role has been reported for helix \( \alpha_2 \) (12, 39–45) and the region spanning residues 101–105, that corresponds to the C-terminal helix \( \alpha_3/N \)-terminal loop L7 (46, 47). Therefore, the present indication of the Ras interaction sites with GAP and GEF indicates that helix \( \alpha_3 \) represents a secondary element concerning both regulators, GAP interacting with its N-termi

\[ \text{FIG. 7. Three-dimensional model of Ha-Ras p21, derived from the triphosphate conformation (49), illustrating the structures most probably involved in direct interactions with GAP (red) and GEF (yellow) regulators.} \]

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