The Ras-RasGAP Complex: Structural Basis for GTPase Activation and Its Loss in Oncogenic Ras Mutants

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The three-dimensional structure of the complex between human H-Ras bound to guanosine diphosphate and the guanosine triphosphatase (GTPase)–activating domain of the human GTPase-activating protein p120GAP (GAP-334) in the presence of aluminum fluoride was solved at a resolution of 2.5 angstroms. The structure shows the partly hydrophilic and partly hydrophobic nature of the communication between the two molecules, which explains the sensitivity of the interaction toward both salts and lipids. An arginine side chain (arginine-789) of GAP-334 is supplied into the active site of Ras to neutralize developing charges in the transition state. The switch II region of Ras is stabilized by GAP-334, thus allowing glutamine-61 of Ras, mutation of which activates the oncogenic potential, to participate in catalysis. The structural arrangement in the active site is consistent with a mostly associative mechanism of phosphoryl transfer and provides an explanation for the activation of Ras by glycine-12 and glutamine-61 mutations. Glycine-12 in the transition state mimic is within van der Waals distance of both arginine-789 of GAP-334 and glutamine-61 of Ras, and even its mutation to alanine would disturb the arrangements of residues in the transition state.

Fig. 1. Stereo view of a segment of the 2Fo–Fo electron density map (contoured at 1.2σ) covering the active site region in the complex, with Ras in blue, GAP-334 in red, and waters in light blue.
Fig. 2. The complex between GAP-334 and Ras. (A) Ribbon representation of the complex model drawn with Molscript (52) and Raster3D (53) according to the assignment of secondary structure elements obtained with the program DSSP (54). The extra and catalytic domains of GAP-334 are shown in green and red (respectively), regions of GAP contacting Ras in light brown, Ras in yellow, and GDP and AlF₃ as ball-and-stick models. Regions involved in the interface are labeled, Sw I and Sw II indicating the switch regions, C the COOH-terminal, and N the NH₂-terminal. (B) Schematic drawing with selected interactions. Polar interactions between individual residues of GAP-334 and Ras are shown as red lines for interactions of side chains, and as red arrows for contacts from side chain to main chain atoms, where the arrowhead marks the residue contributing the main chain group. Yellow lines indicate van der Waals or hydrophobic interactions. Some water molecules (marked W) from the interface region are included. Residues belonging to the interacting regions of Ras indicated in (A) are denoted with specified boxes, as indicated. Interaction between Lys⁸₈ and Thr⁷₉¹ is shown by a dashed arrow, because the electron density in this region is presently not of sufficient quality to unambiguously define the contact. Amino acid abbreviations are in (55).

Table 1. Summary of crystallographic analysis.

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<tr>
<td><strong>GAP-334(718–1042)</strong></td>
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<tr>
<td><strong>H-Ras(1–166)</strong></td>
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<td><strong>rmad bond angles (degrees)</strong></td>
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*R_sym = Σh|F₀| - |F_c|/Σ|F₀|, where |F₀| is the scaled intensity of the h symmetry-related observation of reflection h and |F_c| is the mean value.

‡Rcocryst = Σ|F₀| - |F_c|/Σ|F₀|, where |F₀| and |F_c| are the observed and calculated structure factor amplitudes for reflection h.

†Value of Rcryst for 10% randomly chosen reflections not included in the refinement.
The overall structures of GAP-334 and Ras are similar to those of the isolated molecules. The most significant differences are seen in the switch II region of Ras and the loop L6, of GAP-334. The values for the root-mean-square deviation (rmsd) for superimposition of corresponding Cα atoms are 0.9 Å for GAP-334 and 0.54 Å for Ras, using free GAP-334 (11) and Ras GppNHp (13) for the comparison. The orientation of the two proteins in the complex structure is similar to that of our earlier docking model (figure 4 in (11)). Activated Ras binds in the shallow groove of the central catalytic domain GAPc of GAP-334, with the tip of its triangular shape penetrating most deeply into the groove. A large amount of solvent-accessible surface area (3145 Å²) becomes buried in the interface. A large amount of solvent accessible surface area (3145 Å²) becomes similar to that of our earlier docking model (figure 4 in (11)).

Fig. 3. Sequence alignment of the catalytic domain of RasGAPs from the indicated organisms, together with secondary structure assignment according to the program DSSP (54). Rs, Homo sapiens; m, Rattus norvegicus; ca, Caenorhabditis elegans; dm, Drosophila melanogaster; sc, Saccharomyces cerevisiae; RA1 and IRA2 are yeast homologs of RasGAPs. Residues participating in catalysis are marked as closed circles, residues involved in Ras-GAP interaction as crosses, and residues that have been found mutated in neurofibromin from patients with type I neurofibromatosis and solid tumors as closed triangles. Amino acid abbreviations are in (PDB).

Fig. 2A) is sufficient for GTPase activation (23). Contact with the switch regions of Ras. Switch I overlaps with the Ras effector region (24). It interacts predominantly with the central helices α6 and α7, and with the variable loop. Hydrophobic interactions are formed by the invariant Leu502, and Leu910 on α6, of GAP and by Pro134, Ile136, Tyr32, and Tyr69 of Ras (Figs. 2 and 4A). These interactions are presumably the target of inhibitory lipids such as phosphatidic acid and arachidonic acid (25, 26), the latter of which inhibits the Ras-RasGAP interaction in a partly competitive mode with respect to RasGTP (27). The partly hydrophobic nature of the interface might also explain the finding that the oncogenic mutation Gln711→Leu in Ras, although unable to hydrolyze GTP efficiently, has a higher affinity than the wild type for either GAP-334 or neurofibromin (26, 28–30), and explain why apparently small perturbations such as the mutation Leu69→Ile strongly inhibit catalysis (29, 30).

Switch 1 contains five acidic residues, Asp52, Glu54, Asp55, Glu57, and Asp58, that create a negatively charged surface patch used for interaction with effectors (21) and with GAP-334 (Figs. 2 and 4, A and B). Lysine-949, located on the tip of a conspiciously exposed part of loop L6c, points into the highly negatively charged surface patch. Whereas the side chains of Asp30, Glu31, and Glu35 are not directly involved in the interaction, Asp33 and Asp38 are oriented toward Lys949, with only Asp33 making a direct contact and Asp38 and Thr35 making water-mediated polar contacts, which is mostly consistent with mutational studies (17, 26, 31).

From the invariant residues on helix α7c, only Asn342 directly contacts Ras. It forms a hydrogen bond with Asp333 of Ras, and its own orientation is stabilized by the carbonamide group of Gln343 (Figs. 2B and 4B). Lysine-959 forms an intramolecular salt bridge with Glu351 in loop L6c, whose carboxylate group also contacts the main chain amide group of Ser39 of Ras (Fig. 4B). Lysine-1423 in neurofibromin (Lys355 in p120GAP) is mutated to Glu or Gln in neurofibromas and solid tumors (32), and it is thought to be important for catalysis because GAP activity is reduced in such mutants, with conflicting results on its affinity for Ras (32, 33). On the basis of the intramolecular salt bridge between Lys355 and Glu350, it appears unlikely that Lys355 is directly involved in catalysis or binding. One would postulate instead that most of its mutations should destabilize the protein, consistent with experimental findings on the Lys355→Met mutation in neurofibromin (34).

Two consecutive residues in loop L6c, Lys345 and Gln346, form a bifurcated clamp that fixes helix α7c, and the effector loop of Ras, and these residues appear to be of crucial importance for complex formation with Ras (Fig. 4B). We are proposing a structure-based alignment (Fig. 3) where the KE-motif (K is Lys and E is Glu) is
Gln61 homolog stabilizes the transition state of the GDP or GTP hydrolysis while the other, Lys1436 of the KE-motif, contributes to the stabilization of switch II. In position 12 of Ras, normally glycine, an alanine side chain has been introduced (gray) into the model to show that even small substitutions would be within van der Waals distance to Gln61 (NH2, blue sphere) and Arg789 (CO, red sphere). Glycine-13 is more remote from GAP-334. (A) Residues 32 to 39 from the effector region of Ras, with side chains shown as ball-and-stick models (in yellow), contact GAP-334 (ribbon colored red and light brown as in Fig. 2A) in the vicinity of helices \( \alpha_6 \) and \( \alpha_7 \). Selected side chains of GAP-334 are shown as ball-and-stick models with Arg789 and Lys949 penetrating into the effector region from opposite sides, similar to the view in Fig. 1. Water molecules are light brown spheres. (B) Interaction of the KE-motif of GAP-334 with part of the effector region in Ras, with water molecules in orange. (C) Close approach of the finger loop of GAP-334 to loops L1 (P-loop) and L4 of Ras. In position 12 of Ras, normally glycine, an alanine side chain has been introduced (gray) into the model to show that even small substitutions would be within van der Waals distance to Gln61 (NH2, blue sphere) and Arg789 (CO, red sphere). Glycine-13 is more remote from GAP-334.

Switch II comprises loop L4 and helix \( \alpha_2 \) in Ras. In contrast to the structures of isolated Ras where L4 appears highly mobile (13, 20, 36), this region is well defined in the complex with GAP-334. Residues 61 to 63 are arranged in a short \( 3_10 \) helix preceding \( \alpha_2 \). Tyrosine-64 participates in the formation of the hydrophobic interface between Ras and GAP-334 and forms a polar contact with the main chain carbonyl group of Leu902, consistent with the observation that it can be mutated to Phe but not to Glu without affecting Ras-RasGAP interaction (17). Interactions between Glu62 and Arg749 or between Glu63 and Arg903 may contribute to the stabilization of switch II. Glutamine-61 is essential for GTP hydrolysis: Its mutation to any other amino acid (except Glu) blocks Ras-mediated GTP hydrolysis (18, 19, 26, 37, 38) and leads to tumor formation. In Gp proteins in the GDP-AlF4 conformation, the Gln61 homolog stabilizes the transition state of the GTPase reaction (10), and a similar role was suggested for Gln61 of Ras (39). In the complex with GAP-334, Gln61 points toward the phosphate chain of the nucleotide and is stabilized in its orientation by a hydrogen bond with the main chain carbonyl group of the invariant Arg789. The temperature factor distribution shows a local minimum for the L4 region (40), suggesting that binding of GAP to Ras results in stabilization of switch II.

Structural basis for oncogenicity of Gly12 mutants. Glycine-12 of the P-loop is critical for oncogenic activation of Ras because any mutation of this residue [except Gly12→Pro (G12P)] activates the oncogenic potential of Ras (41). Ras(G12P), although insensitive to GAP, does not have transforming abilities, presumably because its intrinsic rate of GTP hydrolysis is slightly increased (6, 22, 42). No consistent explanation has so far been put forward to explain the defect in both the intrinsic and GAP-accelerated GTP hydrolysis of Gly12 mutants, although some have more obvious rearrangements of the active site (28, 43). In the structure of the complex, Gly12 contacts the loop L1 region of GAP-334. The main chains approach each other, the closest encounter being a van der Waals contact between the CO atom of Gly12 and the main chain CO of Arg789 (Fig. 4C). This mode of interaction imposes constraints on the space that may be filled by amino acids in position 12 of Ras and provides an explanation for the block in GAP-accelerated GTP hydrolysis. Although the interaction with GAP has not been investigated for all Gly12 mutants, it appears from the structure that even replacement with alanine would be highly unfavorable, leading to steric clashes with the main chain of Arg789 and with the side chain NH2 of Gln61 (31). Glycine-12 mutants of Ras bind to GAP with an affinity similar to that of the wild type (38) without subsequent GTP hydrolysis. This suggests that the close neighborhood of Arg789 and Gly12 is not as crucial for the RasGTP-GAP ground state as for the transition state.

The active site and catalytic mechanism. GDP is bound in a mode similar to that observed in isolated RasGDP or RasGppNHp complexes, with Mg2+ present in the expected position (13, 20).
Thr785 (CO) (Fig. 5A), and communicates functionally stabilized by interaction with analysis, consistent with mutational analysis "finger loop"), which participates in catalysis of arginine finger (hence the designation water molecules, one of which contacts the B). This residue in loop L1 c is the postulated Phe788, Arg 789, and Ala 790. Arginine with the main chain carbonyl oxygens of side chain. As in uncomplexed GAP-789, Arg 789 forms a hydrogen bond lengths are very similar (Fig. 5, A and B). Because Al-F and P-O bond lengths are very similar (46), and because an AlF3 trigonal base mimics the trinuclear region around loop L4 containing Gln61 is very mobile with respect to the rest of the molecule (13, 20, 36). Glutamine-61 is invariant in Ras-like GTP-binding proteins except Rap. In the Ras-GAP-334 structure, Gln61 contacts one of the fluoride ions and the axial ligand derived from the nucleophilic water molecule, thereby contributing to the stability of the transition state. It gives an explanation why mutants of Gln61 have reduced GTPase activity and are oncogenic (18, 19, 26, 37, 38).

**Comparison with Gα proteins.** Compared with Ras and other small GTP-binding proteins, the α subunits of heterotrimeric G proteins contain an extra helical domain as an insertion into the G domain, the topological domain responsible for the biochemical properties of GTP-binding proteins. The helical domain positions an invariant arginine that appears to be critical for the GTPase reaction because its

gests an indirect role of Arg903 in catalysis, whereby it stabilizes the position of the crucial arginine.

In the structures of activated Gαi and Gαo, and of myosin, the putative transition state mimics were identified as GDP-AlF4- and adenosine diphosphate (ADP)-AlF4-, respectively (10, 44). This difference is not understood and may be clarified if higher resolution data become available. AlF3 has also been found in the transition-state mimics of nucleoside di- and monophosphate kinase structures (45), reflecting the ability of aluminum fluoride to form trigonal-bipyramidal complexes (46).

During phosphoryl transfer reactions, a partial negative charge develops. If this is on the γ-phosphate, the reaction is associative and goes through a pentavalent phosphorous intermediate. If the charge is on the leaving group, which in the hydrolysis of GTP is the β,γ-bridging oxygen atom, the mechanism is dissociative and shows a metaphosphatase-like transition state (7). Proteins such as GAP could stabilize the transition state by neutralizing these developing charges and thus catalyze the chemical reaction. The intrinsic GTPase reactivity of Ras proceeds by means of substrate-assisted catalysis in which the substrate GTP itself acts as the general (or specific) base (6), which would only be favorable in the case of an associative mechanism (7).

The structure suggests a polar interaction between the charged guanidinium group of Arg789 and a fluoride ligand of aluminum (Fig. 5, A and B). Because Al-F and P-O bond lengths are very similar (46), and because an AlF3 trigonal base mimics the trinuclear base of the transferred phosphoryl group, we assume that the observed geometry in the active site is a close mimic of that of the transition state (or intermediate) of the GTPase reaction. We can identify one axial ligand of the pentavalent aluminum as the oxygen of the GDP leaving group, the second being derived from the attacking nucleophilic water. This water molecule matches the site of Wat127 found in the GTP conformation of Ras (13) and is located within hydrogen-bonding distance to the carbonyl group of the Glu61 side and the Thr15 main chains. On the basis of the arguments put forward earlier (7) that residues that neutralize charges on the γ-phosphate would act anticatalytically in a dissociative mechanism, and because Arg789 has its strongest interaction with the fluoride ion and a weaker one with the leaving group, the arrangement of active site residues presently seen would support a mostly associative mechanism in the GTPase reaction.

The second major factor in the acceleration of the Ras-GTPase reaction by GAP appears to arise from the stabilization of the switch II region, as postulated before (6, 10). It has been shown both by the crystallographic analysis of Ras in the di- and triphosphate forms and by nuclear magnetic resonance spectroscopy that the region around loop L4 containing Gln61 is very mobile with respect to the rest of the molecule (13, 20, 36). Glutamine-61 is invariant in Ras-like GTP-binding proteins except Rap. In the Ras-GAP-334 structure, Gln61 contacts one of the fluoride ions and the axial ligand derived from the nucleophilic water molecule, thereby contributing to the stability of the transition state. It gives an explanation why mutants of Gln61 have reduced GTPase activity and are oncogenic (18, 19, 26, 37, 38).

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modification by cholela toxin reduces the GTPase activity of Goα (47) and because its mutation in Goα or Goγ renders the proteins oncogenic (48). In the Goα-GDP-AIF complex of both transducin and Goα, this arginine contacts the fluorides of AIF as well as the βγ-bridging oxygen and is thus involved in stabilization of the transition state (10). From the superimposition of the G domains of RasGAP-334 and of Goαγ, the transition state mimics for the two GTPases look very similar (Fig. 5C). The guanidinium groups of the respective arginines are in a similar position, although they point into the active site from different directions. In Goγ, the arginine, located in the linker connecting the helical and the G domain, is supplied in cis from the same molecule, whereas in the RasRasGAP system it is supplied in trans by RasGAP. The corresponding glutamine residues are also in close proximity, confirming a similar role for these conserved residues in the stabilization of the transition state and their involvement in oncogene formation (37, 48).

Recently GAPS for Goα proteins, called RGS (for regulator of G protein signaling), have been described that increase the GTPase reaction rate and that have no sequence homology to RasGAPS or other GAPS for Ras-like proteins (49). Although the maximal rate of GTP hydrolysis has not been reported, it is estimated to be on the same order of magnitude as the maximally stimulated GTPase on Ras. Some RGS proteins bind with higher affinity to the GDP-AIF complex of Goα proteins than to the guanine 5′-O-(3′-thiotriphosphat)e (GTPγS)-bound state, which indicates that they act by stabilizing the transition state (50). The recent structure determination of a complex of RGS4 with Goα-GDP-AIF− (51) shows that RGS is a purely helical protein that contacts the switch residues of Goαγ, similar to RasGAP contacting and stabilizing the switch regions of Ras.

Ras-mediated GTP hydrolysis accelerated by RasGAP is required for physiological control of a number of important signal transduction processes. Indeed, oncogenic mutants of Ras escape regulation by RasGAP. The model of the RasGDP-AIFγ; GAP-334 complex shows that GAP catalyzes the GTPase reaction by stabilizing the switch II region and by supplying a catalytic residue. In addition, it provides an explanation why mutations in both Gly18 and Gln61 activate the transforming potential of Ras: Both are in van der Waals distance from each other and from GAP in the transition state mimic. The structural view of the communication between Ras and GAP-334 may aid in the design of small molecules with the capacity to induce GTP hydrolysis on mutant oncogenic forms of Ras found in human tumors. Together with the biochemical data, it shows that for signal transduction to function properly, nature has designed Ras to be an inefficient GTPase, which can be induced to be an efficient switch-off enzyme in the presence of another signal transduction molecule.

REFERENCES AND NOTES

2. M. S. Boguski and F. McCormick, Nature 366, 643 (1993); A. Wittinghofer, K. Schefzik, M. R. Ahmadian, F. McCormick, Science 288, 240 (1990). See also the superimposition of the G domains of RasGAP-334 complex shows that GAP catalyzes the GTPase reaction by stabilizing the transition state (50). The recent structure determination of a complex of RGS4 with Goα-GDP-AIF− (51) shows that RGS is a purely helical protein that contacts the switch residues of Goαγ, similar to RasGAP contacting and stabilizing the switch regions of Ras.

2.5 Å is 65%; 2027 reflections of nonpositive intensity were mixed along with 2 mM AlCl3 and 20 mM NaF. Crystals were grown by the hanging drop method with 15 to 20% polyethylene glycol 3350 in 100 mM Crystals were grown by the hanging drop method with 15 to 20% polyethylene glycol 3350 in 100 mM Hepes (pH 8) with 20 mM ammonium sulfate and 20

12. GAP-334 and H-Ras (residues 1 through 166, missing the COOH-terminal 23 amino acids), hereafter referred to as Ras, in the GDP-bound conformation were expressed in Escherichia coli and prepared as described (36). For complex formation, equal amounts of both proteins (20 mg/ml in 20 mM Hepes, pH 8) were mixed along with 2 mM GTPγS and 20 mM NaF. Crystals were grown by the hanging drop method with 15 to 20% polyethylene glycol 3350 in 100 mM Hepes (pH 8) with 20 mM ammonium sulfate and 20 mM NaF as the precipitant, and improved by seeding prototroplasmic forms described by K. Schefzik et al. (Proteins Struct. Funct. Genet. 27, 315 (1997)) together with 100 mM guanidinium hydrochloride as additive. The crystals belong to the monoclinic space group P21, with unit cell dimensions a = 71.9 Å, b = 41.1 Å, c = 89 Å, α = 90°, β = 108.4°, and γ = 90°; they contain one RasGAP-334 complex in the asymmetric unit. Few crystals decide on resolution higher than 3 Å. A data set from a single crystal (cooled to 4°C) was collected by the rotation method with mirror-focused x-rays from a rotating anode (Enax G1; 38 K/150 mA and a Siemens/Nicolet area detector for data recording. Space group determination, data processing, and scaling were done with the programs XDS and XSSCALE (W. Kabsch, J. Appl. Crystallogr. 25, 795 (1992)). We used data to 2.7 Å resolution on a double—single crystal pair between 2.8 and 2.5 Å is 65%; 2027 reflections of nonpositive intensity were excluded from the refinement.

19. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; Leu, M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
20. We thank A. Becker, I. Schlichting, I. Vetter, M. Geyer, A. Scherer, and A. Lavie for helpful discussions, H. Wagner for maintenance of the x-ray facilities at the Max-Planck-Institut für medizinische Forschung Heidelberg, R. Scheffzek for secretarial assistance, and K. Holmes for continuous support. Supported by the Peter and Traudl Engellhorn Stiftung (Germany) and the National Neurofibromatosis Foundation (United States) (K.S.). The coordinates have been submitted to the Brookhaven data base, accession number 1WQ1.