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Formation of a Transition-State Analog of the Ras GTPase Reaction by Ras·GDP, Tetrafluoroaluminate, and GTPase-Activating Proteins

Rohit Mittal,* Mohammad Reza Ahmadian,* Roger S. Goody, Alfred Wittinghofer†

Unlike the α subunits of heterotrimeric guanosine triphosphate (GTP)–binding proteins, Ras-related GTP-binding proteins have hitherto been considered not to bind or become activated by tetrafluoroaluminate (AlF_4^-). However, the product of the proto-oncogene *ras* in its guanosine diphosphate (GDP)–bound form interacted with AlF_4^- in the presence of stoichiometric amounts of either of the guanosine triphosphatase (GTPase)–activating proteins (GAPs) $p120^{GAP}$ and neurofibromin. Neither oncogenic Ras nor a GAP mutant without catalytic activity produced such a complex. Together with the finding that the Ras-binding domain of the protein kinase c-Raf, whose binding site on Ras overlaps that of the GAPs, did not induce formation of such a complex, this result suggests that GAP and neurofibromin stabilize the transition state of the GTPase reaction of Ras.

The α subunit of heterotrimeric GTP-binding proteins (G proteins) in the GDP-bound form can be activated by the addition of the complex AlF_4^- ion. This ion is thought to mimic the terminal phosphate of GTP because the structure of the Gα·GDP·AlF_4^- complex resembles that of the GTP-bound form of the protein and because binding of AlF_4^- triggers the interaction of the α subunit with downstream effectors (*1*). Furthermore, x-ray structure analysis of $G\alpha_{i1}$ and the α subunit of transducin bound to GDP and AlF_4^- revealed that AlF_4^- activates Gα·GDP by binding with a geometry resembling that of the putative pentacovalent phosphorus intermediate, or perhaps transition state, of the GTPase mechanism (*2*), a possibility that had been discussed (*3*). In these structures, the Al ion is octahedrally coordinated with four F ligands, forming the equatorial plane, and two O ligands, supplying the apical ligands. Because the O ligands—one contributed by a water molecule and the other by the β phosphate of GDP—correspond to the attacking nucleophile and the leaving group of the GTPase reaction, respectively, and because conserved Gln and Arg residues stabilize the complex, these structures are thought to resemble the putative pentacovalent intermediate of the GTPase reaction.

The Ras-related proteins have slow GTPase reaction rates. For Ras, this rate is 0.028 min^{-1}, which is about one-hundredth of the reaction rate of an average Gα protein [2 to 5 min^{-1} (*4*)]. A number of GAPs that stimulate the GTPase reaction rate have been identified for Ras (*5*) and other related small G proteins (*6*). Four Ras-specific GAPs have been described. The $p120^{GAP}$ and neurofibromin proteins increase the rate of GTP hydrolysis by four to five orders of magnitude at saturation (*7–9*). Because it seemed reasonable to assume that all G proteins have a similar mechanism of GTP hydrolysis (on the basis of structural and other similarities), it was surprising to find that Ras-related proteins do not bind AlF_4^- (*10*), implying that the active site of Gα proteins in the ground state or transition state, or both, is different from that of Ras-related proteins, although differential effects of AlF_4^- on the ribosome-stimulated GTPase reaction of elongation factor G have been reported (*11*).

The addition of AlF_4^- did not change the emission [or excitation (*12*)] spectrum of 0.1 μM Ras bound to the fluorescent GDP-analog 2′(3′)-O-(*N*-methylanthraniloyl) GDP (mantGDP, referred to subsequently as mGDP). Such fluorescent analogs are sensitive to changes in the local structure of the protein (*13, 14*). Even with 5 μM Ras·mGDP and excess AlF_4^-, there was little or no change (<3%) of fluorescence (*12, 15*). It has been proposed that the role of GAP might be to stabilize or induce the formation of a transition state in the GTPase reaction (*16*), and that GAP supplies the helical domain or similar structure that exists in Gα proteins (*4*) and is necessary for GTP hydrolysis (*17*). To test this, we added an excess of the catalytic fragment of neurofibromin NF1-333 to the solution of Ras·mGDP containing AlF_4^- (Fig. 1A). The fluorescence spectrum of mGDP revealed that the wavelength of absorption maximum changed from 448 to 430 nm and the fluorescence increase at 448 nm was 20 to 25% (and 40 to 50% at 430 nm), indicating a conformational change around the active site, which we attribute to the formation of an analog of the GTP-bound ground state or transition state of the GTPase reaction. This change was not caused by the addition of neurofibromin alone (Fig 1B); no change in fluorescence was produced after the addition of NF1-333 to Ras·mGDP, but after addition of AlF_4^-, a similar large increase of fluorescence was seen. Furthermore, the change in fluorescence was inhibited by addition of excess Ras bound to unmodified GDP, supporting the notion that complex formation is not simply due to the fluorescent label on GDP (*12*).

The change in fluorescence properties did not occur in the presence of catalytic amounts of neurofibromin. The effective dissociation constant (K_d) between Ras·mGDP and NF1-333 under the conditions used was 60 nM (Fig. 1C). This value is close to the K_d for the complex between

R. Mittal, M. R. Ahmadian, A. Wittinghofer, Abteilung Strukturelle Biologie, Max-Planck-Institut für Molekulare Physiologie, Rheinlanddamm 201, 44139 Dortmund, Germany.
R. S. Goody, Abteilung Physikalische Biochemie, Max-Planck-Institut für Molekulare Physiologie, Rheinlanddamm 201, 44139 Dortmund, Germany.

*These authors contributed equally to this work.
†To whom correspondence should be addressed.

Ras·GTP and neurofibromin and the Michaelis constant K_m of the GTPase reaction catalyzed by NF1 (*7–9, 18, 19*). However, this effective K_d cannot be compared directly to constants obtained for the interaction of neurofibromin with Ras·GTP or Ras·guanylylimidodiphosphate (GppNHp), because it must be a complex function of equilibrium constants for the multistep formation of the complex. Because the K_d between $p120^{GAP}$ and the triphosphate state (ground state) of Ras and also the K_m of the $p120^{GAP}$-stimulated reaction are about 50 times higher than those with neurofibromin (*7–9, 18, 19*), the above experiment was repeated with 2 μM Ras·mGDP and 20 μM GAP-334; higher concentrations of AlF_4^- were also necessary for full response. These concentrations resulted in an increase in and a shift of the emission maximum similar to those seen with neurofibromin (Fig. 2A). The fitted effective K_d in this case was 1.34 μM (Fig. 2B). The results indicate that, as with the ground-state complex between Ras and GAPs, the effective affinity of neurofibromin is higher than that of $p120^{GAP}$ in the transition-state analog of the GTPase reaction.

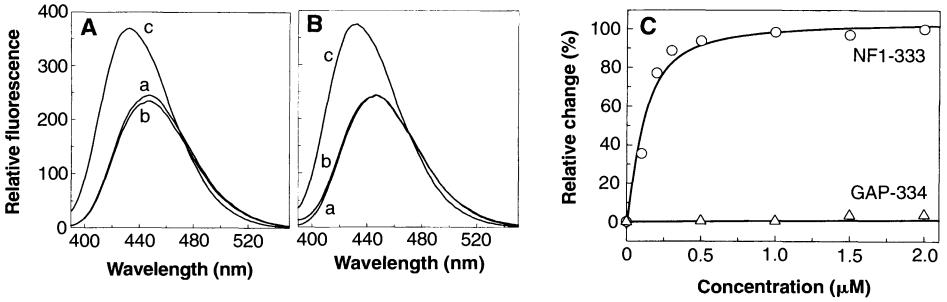

Fig. 1. Fluorescence emission spectra of 0.1 μM Ras·mGDP. (**A**) The spectra of Ras·mGDP alone (curve a), after addition of 30 μM $AlCl_3$ and 10 mM NaF (curve b), and after further addition of 1 μM NF1-333 (curve c) recorded on a spectrofluorometer; excitation wavelength, 366 nm. (**B**) Same as (A) with the order of additions changed: Ras·mGDP (curve a), NF1-333 (curve b), and AlF_4^- (curve c). Ras·GDP was isolated from *Escherichia coli*, and its fluorescent derivatives were prepared as described (*29*); NF1-333 (residues 1198 to 1530 from human neurofibromin) and GAP-334 [residues 714 to 1047 of human $p120^{GAP}$ (*8*)] were also isolated from *E. coli* (*30*). (**C**) Titration of the fluorescence response seen in (A), with identical concentrations of Ras·mGDP and AlF_4^- and various concentrations of either NF1-333 or the $p120^{GAP}$ fragment GAP-334. Relative fluorescence change represents the peak area from 415 to 450 nm.

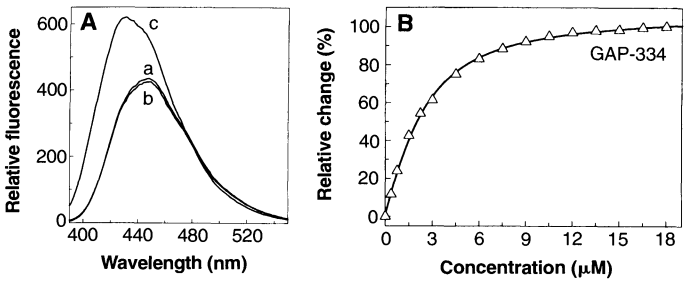

Fig. 2. The AlF_4^--mediated fluorescence increase in the presence of GAP-334. (**A**) Fluorescence emission spectrum of 2 μM Ras·mGDP alone (curve a), in the presence of 60 μM $AlCl_3$ and 25 mM NaF (curve b), and with the addition of 20 μM GAP-334 (curve c). The blue shift of the spectrum and the fluorescence increase were similar to those in Fig. 1A, and reversal of the order of addition gave the same effect as shown in Fig. 1B. (**B**) Fluorescence yield of Ras·mGDP in the presence of AlF_4^- (as above) titrated with various concentrations of GAP-334.

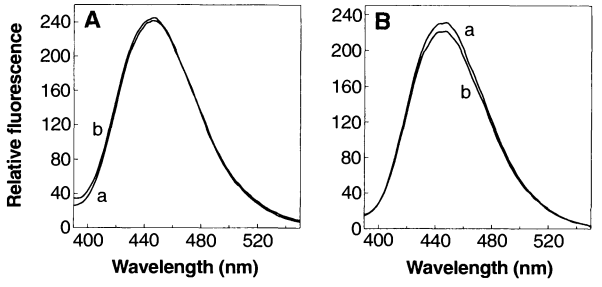

Fig. 3. Failure of mutant forms of NF1 or oncogenic mutant forms of Ras to display interaction with AlF_4^-. (**A**) Spectra of 0.1 μM Ras·mGDP in the presence of AlF_4^- (30 μM $AlCl_3$, 10 mM NaF) (curve a) and 2 μM NF1-333 with a mutation of Arg^{1391} (to Met) in the Phe-Leu-Arg-Phe region of the protein (curve b), recorded as in Fig. 1. (**B**) Spectra of oncogenic Ras(Q61L)mGDP in the presence of 2 μM Nf1-333 without (curve a) and with (curve b) AlF_4^- (30 μM $AlCl_3$, 10 mM NaF).

It has been suggested that GAPs supply residues to the GTPase reaction that stabilize the transition state or otherwise change the chemistry of the reaction (*4, 16*). Obvious candidates for such amino acid side chains are Arg residues. One such Arg has been postulated to stabilize the developing charge on the β,γ-bridging oxygen and one oxygen of the pentacovalent phosphorous in the transition state of the GTPase reaction in $G\alpha_{i1}$ and in α transducin (*2*). Arginine residues are also thought to stabilize the presumed transition state in phosphoryl transfer enzymes that use adenosine triphosphate, such as adenylate kinase (*20*). RasGAPs contain two totally conserved Arg residues and one Lys residue, and the mutation of either of these reduces the GAP activity of neurofibromin and GAP by one or two orders of magnitude (*9, 21*). We mutated the Arg residue to Met in the highly conserved Phe-Leu-Arg-Phe sequence of neurofibromin. Adding this mutant (R1391M) to Ras·mGDP did not produce the spectroscopic change induced by wild-type neurofibromin (Fig. 3A), even though the mutant NF1 bound to Ras complexed with the nonhydrolyzable GTP analog 5′-GppNHp (*12*), as is the case for the analogous mutation in $p120^{GAP}$ (*19*).

Oncogenic mutants of Ras have a reduced intrinsic rate of hydrolysis, and this rate cannot be stimulated by either $p120^{GAP}$ or neurofibromin (*5, 7, 18, 22*). Correspondingly, addition of NF1-333 to neither Ras(Q61L)·mGDP (Fig. 3B) nor Ras(G12V)·mGDP (*12*) in the presence of AlF_4^- induced a fluorescent change indicative of the formation of the transition-state analog.

Further information on the nature of the state induced in the presence of Ras·mGDP, AlF_4^-, and neurofibromin or GAP was obtained from experiments with the Ras-binding domain (RBD) of an effector of Ras action, the protein kinase c-Raf-1 (*23*). The RBD binds the activated form of Ras (and the Ras-related protein Rap) at the so-called effector binding region localized between amino acids 32 and 40 on Rap (*24*). This is also the region on Ras that is a likely area of contact with GAPs (*25*). It was thus of interest to determine whether the addition of RBD to Ras·mGDP in the presence of AlF_4^- could give rise to a complex similar to that observed with GAPs. However, in keeping with the interpretation that a transition state–like structure for the GTP hydrolysis reaction cannot be reached in the absence of GAP, addition of RBD did not cause the formation of such a complex (Fig. 4A). Furthermore, addition of RBD did not prevent the formation of or induce dissociation of the

complex between Ras·mGDP, AlF_4^-, and neurofibromin (Fig. 4A), even though it did efficiently dissociate the complex between Ras·mGppNHp and neurofibromin (Fig. 4B). These two results provide corroborative evidence that a GTP-like state of Ras cannot be induced starting from Ras·GDP and AlF_4^- in the absence of GAP.

The fluorescence properties of the complex between Ras·mGppNHp and neurofibromin are different from those obtained by mixing Ras·mGDP, AlF_4^-, and neurofibromin. Upon adding NF1-333 to Ras·mGppNHp, there was a smaller shift in absorption maximum (from ~448 to 442 nm) and a smaller increase in the fluorescence yield (12% at 448 nm). These results support the interpretation that the state induced by AlF_4^- is different from the GAP·Ras·GTP ground state and indicates structural differences in the two states that affect the surroundings of the fluorescent group, which is on the sugar moiety of GDP.

The structures of $G\alpha_{i1}$ and the α subunit of transducin complexed with GDP and AlF_4^- suggested that an Arg and a Gln residue participate in the stabilization of what is believed to be a transition-state analog. The role of this Gln residue appears to be conserved between Ras-related (except Rap) and Gα proteins, because this residue (Gln^{61} in Ras) is conserved in the primary sequence of the proteins. The mutation of this Gln in Gα proteins or Ras leads to transforming properties (*4*, *26*). A comparable conserved Arg (Arg^{174} in the α subunit of transducin) is, however, not observed in Ras-related proteins. The modification of this Arg by cholera toxin or its mutation to another amino acid activates the Gα proteins, because they no longer hydrolyze GTP efficiently (*27*). The presence of such an Arg in the active site of Gα proteins is likely to be responsible for the fact that Gα proteins (but not Ras-related proteins) in their GDP-bound form interact strongly with AlF_4^- and the fact that heterotrimeric G proteins usually have faster GTPase reaction rates (*17*). That the helical domain of Gα mediates the stabilization of the transition state is supported by the observation that the AlF_4^- effect on heterotrimeric G proteins needs the interaction between the Ras-like domain and the helical domain (*28*).

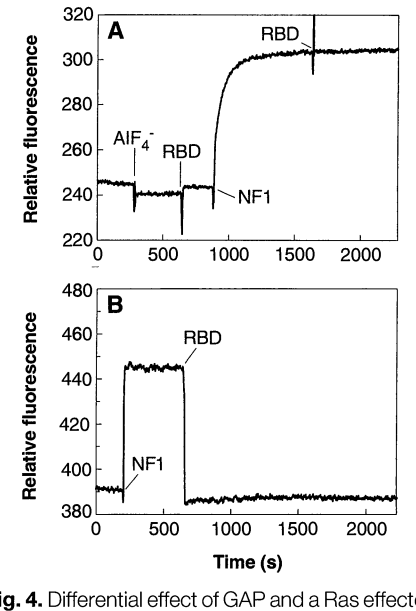

Fig. 4. Differential effect of GAP and a Ras effector on complex formation. (**A**) Effect of the RBD of the protein kinase c-Raf-1 on the formation of a Ras·mGDP·AlF_4^- complex. Time course of the fluorescence change (at 448 nm) of 0.1 μM Ras·mGDP after successive additions of AlF_4^- (30 μM $AlCl_3$ and 10 mM NaF), 2 μM RBD, 1 μM NF1-333, and a further 2 μM RBD. (**B**) Time course of fluorescence emission of 0.1 μM Ras·mGppNHp (the fluorescent form of the GTP analog) at 448 nm, after addition of 1 μM NF1-333 and the subsequent addition of 2 μM RBD.

The results presented here are directly relevant to the discussion of the mechanism of GAP activation of Ras GTPase activity (*16*). According to one hypothesis, the rate-limiting step (that is, the one that must be accelerated by GAPs) in the intrinsic GTPase mechanism is a conformational change preceding the actual hydrolysis step (*13*). Alternatively, the cleavage step itself may be rate-limiting (*14*, *16*), implying that GAP has a direct effect on the cleavage rate, possibly by supplying additional amino acid side chains. Our results indicate that active site residues important for efficient catalysis are supplied by GAP, rendering unlikely a mechanism in which the active site machinery is already present in Ras and only needs to be rearranged by the GAP.

The addition of Ras GAPs to Ras induces an active site that perhaps resembles that of Gα proteins. The GAPs do so by supplying one (or more) critical positive charges to the γ-phosphate binding site of Ras, allowing the binding of AlF_4^- in a conformational state probably analogous to the transition state of the GAP-mediated GTPase reaction of Ras. This transition state is presumably different (of lower energy) from that for the intrinsic GTPase reaction. We expect that other G proteins that do not normally form stable complexes with GDP and AlF_4^- may behave similarly in the presence of their corresponding GTPase-activating proteins.

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