Fluoride Activation of the Rho Family GTP-binding Protein Cdc42Hs*

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Aluminum tetrafluoride (AlF₄⁻) activation of heterotrimeric G-protein α-subunits is a well established aspect of the biochemistry of these proteins; however, until recently it has been thought that AlF₄⁻ does not mediate effects on the Ras superfamily of low molecular weight GTP-binding proteins. Recent work demonstrating aluminum fluoride-induced complex formation between Ras and its GTPase-activating proteins (RasGAP and NFP1) has provided important insights into the mechanism of GAP-stimulated GTP hydrolysis. We have characterized the AlF₄⁻-induced complex formation between the GDP-bound form of the Rho subfamily G-protein Cdc42Hs and a limit functional domain of the Cdc42-GAP using a variety of biochemical techniques. Our results indicate that the apparent affinity of GAP for the AlF₄⁻-mediated complex is similar to the affinity observed for the activated (GTP-bound) form of Cdc42 and that beryllium (Be) can replace aluminum in mediating fluoride-induced complex formation. Additionally, the AlF₄⁻-induced interaction is weakened significantly by the catalytically compromised GAP(R305A) mutant, indicating that this arginine is critical in transition state stabilization. Unlike Ras, we find that AlF₄⁻ and BeF₃ mediate complex formation between Cdc42Hs-GDP and downstream target/effecter molecules, indicating that there are important differences in the mechanism of effector binding between the Ras and Rho subfamily G-proteins.

The interaction between members of the Ras superfamily of GTPases and GTPase-activating proteins (GAPs) is a critical step in the signaling pathways of these proteins. GTP-binding proteins exhibit intrinsic hydrolytic activity, allowing them to cycle between an active GTP-bound state and an inactive GDP-bound state (1). The binding of a GAP significantly increases this hydrolytic rate, down-regulating the signal carried by the GTPase (2–4).

The α-subunits of heterotrimeric G-proteins are comprised of two domains: a Ras-like GTP binding domain and a large helical domain (5–7). Heterotrimeric G-proteins exhibit a much higher intrinsic hydrolytic rate than GTPases in the Ras superfamily (1, 8, 9). A large body of evidence suggests that the helical domain of the α-subunits serves as an internal GAP by contributing a critical arginine residue to the GTP binding site (Arg-174 in α-transducin or Arg-178 in Gi₃α). Covalent modification of this arginine by cholera toxin or mutations at this site lead to a constitutively active α-subunit that is incapable of hydrolyzing GTP (10–12). The x-ray crystallographic structures of α-subunits bound to GTP·S show that this arginine is coordinated with the γ-phosphate (α-transducin) or positioned nearby (Gi₃α), indicating that this may be an essential catalytic residue involved in GTP hydrolysis (6, 7). The analogy between the helical domain of α-subunits and GAPs for the small G-proteins is supported by mutational analyses of the GAPs identifying catalytically important arginine residues similar to the catalytic arginine of the heterotrimers (13, 14).

An interesting aspect of the biochemistry of heterotrimeric G-protein α-subunits is the ability of AlF₄⁻ to stimulate their activation (15). Upon binding of AlF₄⁻ to the GDP-bound α-subunit, the G-protein adopts an activated conformation capable of signaling to downstream effectors. Crystallographic and biochemical studies indicate that AlF₄⁻ binds in the position of the γ-phosphate to induce this active conformation (7, 16, 17). Crystal structures of AlF₄⁻-bound α-transducin and Gi₃α show important structural differences from the corresponding GTP·S-bound forms. The unique coordination of the aluminum suggests that the AlF₄⁻ structure represents a transition state analog of the GTP-hydrolytic pathway. GTP hydrolysis proceeds through a penta-covalent intermediate in which the γ-phosphate acts as the general base, de-protonating the attacking water (18). The planar structure of AlF₄⁻ approximates the transition state structure of the γ-phosphate (7, 17). Importantly, the catalytic arginine has a critical role in coordinating and stabilizing this transition state structure.

Recently, Mittal et al. (14) made an important connection between the high and low molecular weight GAP-binding proteins by demonstrating AlF₄⁻-mediated effects on Ras (14). Two GAPs, NFP1 and RasGAP, form stable complexes with the GDP-bound form of Ras in the presence of aluminum fluoride, indicating that it also mimics the transition state of GTP hydrolysis in low molecular weight GAP-binding proteins. This observation provides an important opening for investigation of the GTPase mechanism in Ras and other low molecular weight GTPases.

In the study presented below, we demonstrate that AlF₄⁻ induces complex formation between the Rho family GTPase Cdc42Hs and the Cdc42-GAP, and we characterize this interaction using spectroscopic methods. Unlike Ras, we observe that AlF₄⁻ is able to induce GDP-bound Cdc42 to form stable complexes with effectors as well as GAPs, suggesting important differences in effector binding between the Ras and Rho

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§ The abbreviations used are: GAP(s), GTPase-activating protein(s); GTP·S, guanosine 5′-3′-O-(thio)triphosphate; MOPS, 4-morpholinepropanesulfonic acid; PBD, PKA binding domain; PAGE, polyacrylamide gel electrophoresis; mant, N-methylanthraniloyl; GppNHP, guanosine 5′-O-(3′-O-methyl)triphosphate; GDP·P·NP, guanosine 5′-O-(3′-O-methyl)triphosphate; GTP·P·NP, GTPase inhibitory protein.

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subfamilies. As shown for heterotrimeric G-proteins, BeF\(_2\) behaves in a manner similar to AlF\(_3\) and is also capable of mediating complex formation; however, unlike AlF\(_3\), the BeF\(_2\) clearly acts as a true GTP analog and is even more effective than AlF\(_3\) at mediating complex formation between Cdc42 and its target/effectors proteins.

**EXPERIMENTAL PROCEDURES**

**Proteins**

Cdc42Hs and Cdc42Hs(G61L)—Cdc42Hs was prepared as described previously with some modifications (19). The DNA encoding Cdc42Hs (or the Cdc42Hs(G61L) GTPase-defective mutant) was inserted into the pET15b expression vector immediately downstream of the hexa-histidine tag and transformed into *Escherichia coli* BL21 cells. The bacteria were grown in 4-liter fermentors, and protein expression was induced by the addition of 100 μM isopropyl-1-thio-β-D-galactopyranoside when the cultures reached an OD\(_{600}\) of 4.0.

The bacterial pellet was resuspended in 100 ml of lysose buffer (20 mM Tris-HCl, pH 8.0, 5 mM imidazole, 500 mM NaCl, 1 mM NaN\(_{3}\), 200 μM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.1 μg/ml GDF, and 0.5 mg/ml lysozyme) and homogenized. 10 mg of DNase I and 5 ml of 1 M MgCl\(_2\) were added to degrade the chromosomal DNA. The lysate was subjected to ultracentrifugation (30 min, 10,000 g) and affinity purified over a 25-ml imidodiacetic acid column (Sigma). The samples were then analyzed as described above.

To detect AlF\(_3\) and AlCl\(_3\), 0.1 mg/ml of α-fodrin was added to the sample, and the fluorescence anisotropy data were fit using a single-exponential equation for AlF\(_3\) and a single-exponential equation for AlCl\(_3\) that accounts for the 1 mM NaF added to the column buffer. The fluorescence measurements were taken.

**Fluorescence Spectroscopy**

Anisotropy measurements of complex formation were taken on an SLM 8000 spectrofluorometer operating in the T-format. Excitation of the mant-moiety was accomplished with a xenon-arc lamp monochromated to 350 nm with an 8-nm bandwidth. Excitation in the vertical orientation was used to obtain a correction value, and anisotropy measurements were taken with horizontally polarized excitation light. Vertically polarized emission was measured on channel A over a 16-nm band selected at 445 nm, using a monochromator, and horizontal emission was measured on channel B selected at 440 nm with a glass filter. An integration time of 10 s was used for all measurements, and data points represent the average of at least three measurements on the same sample.

All titrations were carried out in 1 ml of HMN buffer (20 mM HEPES, pH 8.0, 5 mM MgCl\(_2\), 100 mM NaCl) in a quartz cuvette. To observe AlF\(_3\)-induced complex formation, the HMN buffer was premixed with 60 μM AlCl\(_3\) and 25 mM NaF for 1 h. The fluorescence cuvette was then titrated with either the GAP or PBD. Additions were made directly to the fluorescence cuvette from a concentrated stock (100–200 μM) and allowed to equilibrate for 2 min before anisotropy measurements were taken.

**Data Analysis**

The fluorescence anisotropy data were fit using a simple equilibrium model for the bimolecular interaction between Cdc42Hs and GAP.

**Gel Filtration Analysis of Complex Formation**

A Superdex 75 16/60 gel filtration column coupled to a Pharmacia fast performance liquid chromatography system was used to analyze complex formation between Cdc42Hs and the GAP. The column was run in buffer B (20 mM MOPS, 5 mM MgCl\(_2\), 1 mM NaN\(_{3}\), pH 7.5) at a flow rate of 0.5 ml/min. Purified samples of Cdc42 and GAP (0.5 ml at 5 mg/ml) were applied to the column, and elution profiles were monitored by UV absorption.

Complexes between GAP and the GTPase-defective Cdc42Hs(G61L) mutant were prepared by combining 2.5 mg of each protein. The sample was then loaded onto a Superdex 75 16/60 gel filtration column and 20 ml of 20 mM Tris-HCl, 5 mM MgCl\(_2\), and 25 mM NaCl was applied to the center of the column. The gel filtration conditions were essentially as described previously (21, 22) and stored at 4 °C. The fraction containing the complex mixture was pooled and again subjected to NH\(_4\)SO\(_4\) precipitation.

**Preparation of mant-Nucleotides and Cdc42Hs-mant-Nucleotide Complexes**

N-Methylanthranilyl (mant)-labeled nucleotides (mant-dGDP and mant-dGTP) were synthesized as described previously (24). 5′-Deoxy-nucleotides were used to obtain a single isomer of the mant-labeled nucleotide (the mant-moiety is covalently attached to the 3′-OH of the ribose ring). Cdc42Hs (or Cdc42Hs(G61L)) was preloaded with mant-nucleotide by incubation with a 20-fold excess of purified mant-nucleotide and 25 mM EDTA in HMA at 4 °C for 1 h. The exchange reaction was quenched with 50 mM MgCl\(_2\). The reaction mixture was passed over a 10-ml Sephadex G-25 column equilibrated against HMA to remove unbound nucleotide. The protein was then concentrated to a final volume of 0.5 ml at 20 °C. Spectroscopic analysis confirmed that more than 90% of the protein was loaded with mant-nucleotide after this procedure.
It has been well established that the GAP normally interacts with the GTP-bound form of Cdc42Hs. However, stable complexes between Cdc42Hs-GTP and GAP are not formed because of the rapid hydrolysis of GTP to GDP and the subsequent loss of affinity between the two proteins. Stable complexes between Cdc42Hs and the GAP can be obtained using nonhydrolyzable GTP analogs or GTPase-deficient mutants of the protein. For comparison with the AlF₄⁻-induced complex, Fig. 1D shows a gel filtration experiment where stable complexes were formed between GAP and the GTPase-deficient Cdc42Hs(Q61L) mutant. As expected, the appearance of a 47-kDa peak is observed, consistent with the formation of a Cdc42Hs-GAP complex.

SDS-PAGE analysis of the column fractions confirmed that the high molecular mass peak induced by the addition of AlF₄⁻ was indeed a complex of Cdc42Hs-GAP and GDP. Fig. 2 shows SDS-PAGE analysis of the column fractions from the gel filtration experiment shown in Fig. 1E. The high molecular mass peak (fractions 60–62 in Fig. 2) clearly contained both Cdc42Hs and GAP, indicating that these proteins were eluted from the gel filtration column as a stable complex of approximately 47 kDa. The two proteins appeared to be present in equivalent amounts which is in agreement with the 1:1 stoichiometry expected for this interaction.

19F NMR Spectroscopy of the AlF₄⁻-induced GAP Complex—19F NMR spectroscopy was performed to probe fluoride binding directly. This method was used successfully to study the interaction of AlF₄⁻ with α-transducin (16). Free AlF₄⁻ shows a single peak at −10 ppm (Fig. 3A). No change in the spectrum was observed for the 19F NMR spectrum of Cdc42Hs-GDP in the presence of AlF₄⁻ (Fig. 3B), suggesting that free Cdc42Hs did not bind to AlF₄⁻. The 19F NMR spectrum obtained for AlF₄⁻-induced Cdc42Hs-GDP-GAP complex was caused by the binding of fluoride in the position normally occupied by the γ-phosphate.

Characterization of AlF₄⁻-induced Complex Formation Using Fluorescence Spectroscopy—Fluorescence anisotropy measurements can be used to monitor changes in the rotational life time mass of a fluorescently labeled protein. This technique provides a sensitive read-out for protein-protein interactions and was used to characterize the AlF₄⁻-induced Cdc42Hs-GAP interaction in more detail. Addition of GAP to preparations of Cdc42Hs-mant-dGDP in the presence of AlF₄⁻ caused dramatic changes in the anisotropy which were indicative of complex formation. Fig. 4A shows a typical titration of 1 μM Cdc42 Hs-mant-dGDP with increasing amounts of GAP in the presence of 60 μM AlCl₃ and 25 mM NaF. The binding isotherm was well fit by a simple bimolecular equilibrium model for Cdc42Hs-GAP complex formation (see “Experimental Procedures”) and yielded an apparent $K_d$ of 239 nm ± 79 (n = 10). Increasing the concentration of AlF₄⁻ did not lead to a significant change in the $K_d$ value, suggesting that AlF₄⁻ is saturating under these conditions. Additionally, both aluminum and fluoride were required such that the addition of either compound alone was not sufficient to promote the interaction between Cdc42Hs-mant-dGDP and GAP (data not shown). A significantly weaker affinity was observed between Cdc42Hs-GDP and GAP in the absence of AlF₄⁻. Assuming an identical anisotropy value at saturation, a lower limit $K_d$ of −50 μM can be assigned to this interaction.

**Results**

**Demonstration of Stable Complex Formation between Cdc42Hs and the GAP in the Presence of AlF₄⁻ by Gel Filtration Analysis**—Fig. 1 compares elution profiles from the gel filtration analysis of AlF₄⁻-induced complex formation between GDP-bound Cdc42Hs and GAP. Panels A and B show the elution profiles of native Cdc42Hs and GAP, respectively. The elution volume of these proteins was consistent with their monomeric molecular masses of 22 and 25 kDa. In the absence of GAP, a mixture of Cdc42Hs-GDP and GAP eluted as two peaks (Fig. 1C) corresponding to their native molecular masses. When the same analysis was performed in the presence of AlF₄⁻ (Fig. 1E), a new peak corresponding to an approximate molecular mass of 47 kDa appeared, which matched the expected size of a complex between Cdc42Hs and GAP. This result indicated that AlF₄⁻ induced stable complex formation between the GDP-bound form of Cdc42Hs and the GAP and is in agreement with similar results recently reported by Ahmadian et al. (25).

![Diagram](image_url)
In the case of the heterotrimeric G-proteins, beryllium was found to be the only cation capable of replacing aluminum in the fluoride-induced activation response (15). Fig. 4B shows the titration of Cdc42Hs mant-dGDP with increasing amounts of GAP in the presence of BeF$_3^2$. The best fit to this binding isotherm yielded an apparent $K_d$ value of 388 nM$^6_47$ ($n = 2$), which was similar to that determined for the AlF$_4^2$-induced Cdc42Hs GAP interaction.

To compare carefully the $K_d$ values for Cdc42Hs-GAP complex formation induced by AlF$_4^2$ and BeF$_3^2$ versus the values for GAP interactions with activated (GTPase-defective) Cdc42Hs, experiments were performed with the Cdc42(Q61L) mutant. Fig. 4C shows the titration of 1 mM Cdc42Hs(Q61L)-mant-dGTP with increasing amounts of GAP. The titration profile was fit using the same bimolecular model as above and yielded an apparent $K_d$ of 279 nM$^{134}$. This value is not significantly different from that determined for the AlF$_4^2$-induced Cdc42Hs-GAP interaction.

The Affinity of the AlF$_4^2$-induced Complex Formation Is Reduced Significantly by a Catalytically Deficient GAP Mutant—To pursue the hypothesis that AlF$_4^2$ represents a transition state analog of the GTP-hydrolytic pathway, fluorescence anisotropy experiments were performed with a GAP that was mutated at arginine 305 (according to the sequence in Refs. 13 and 28), a residue that appears to be essential for catalysis. The R305A mutant of GAP, while binding to Cdc42Hs with wild type affinity, shows a significantly reduced ability to catalyze the GTPase reaction (i.e. 25-fold lower GAP activity). Similar results have also been shown for the analogous mutations in a number of other GAPs (13).

Fig. 5 shows a titration of 1 mM Cdc42Hs mant-dGDP in the presence of AlF$_4^2$ with increasing amounts of the GAP(R305A) mutant. The best fit to this titration profile yielded an apparent $K_d$ of 2.15 mM, which represents a significantly weaker affinity than that measured for the interaction between GAP and the GTP-bound state of Cdc42Hs (see above). A similar loss of affinity for the BeF$_3^2$-induced complex was also observed (data not shown). The titration of GTP-bound Cdc42Hs with the GAP(R305A) mutant gives an apparent $K_d$ value of 245 nM, i.e. an affinity nearly identical to that observed for the wild type GAP.

Characterization of AlF$_4^2$- and BeF$_3^2$-induced Interactions between the GDP-bound Form of Cdc42Hs and Effectors—The interaction between Ras and its effector Raf was shown to be unaffected by AlF$_4^2$ (14). To investigate this possibility in the Rho subfamily, the effects of AlF$_4^2$ on the binding of PBD were studied by fluorescence spectroscopy. PBD is the binding domain of mPAK-3 (21) and contains the CRIB (Cdc42Hs-Rac-
interactive binding) motif, originally shown to be a limit domain found on a number of proteins that bind Cdc42Hs or Rac (27). Fig. 6A shows the binding isotherm for Cdc42Hs-mant-dGDP titrated with increasing amounts of GAP. Titrations were carried out either in the absence (○) or in the presence (●) of AlF$_4^-$). The solid lines represent best fits of the data to the bimolecular binding models described under “Materials and Methods.” The lower curve represents additions of equivalent volumes of GAP storage buffer (■).

Panel B, Cdc42Hs-Mant-dGDP (1 μM) was titrated with the indicated amounts of GAP in the presence of 60 μM AlCl$_3$ and 25 mM NaF (●) or 60 μM BeCl$_2$ and 25 mM NaF (○). Panel C, Cdc42Hs(Q61L)-mant-dGTP (1 μM) was titrated with the indicated amounts of Cdc42-GAP (○). For comparison, the titration of Cdc42Hs-mant-dGDP (1 μM) in the presence of 60 μM AlCl$_3$ and 25 mM NaF with the indicated amounts of GAP is also pictured (●).

A

B

C

FIG. 4. Fluorescence anisotropy titration of AlF$_4^-$ and BeF$_3^-$-induced Cdc42Hs-GAP complex formation using Cdc42Hs-mant-dGDP. Panel A, Cdc42Hs-mant-dGDP (1 μM) was titrated with the indicated amounts of GAP. Titrations were carried out either in the absence (○) or in the presence (●) of AlF$_4^-$. The solid lines represent best fits of the data to the bimolecular binding models described under “Materials and Methods.” The lower curve represents additions of equivalent volumes of GAP storage buffer (■).

Panel B, Cdc42Hs-Mant-dGDP (1 μM) was titrated with the indicated amounts of GAP in the presence of 60 μM AlCl$_3$ and 25 mM NaF (●) or 60 μM BeCl$_2$ and 25 mM NaF (○). Panel C, Cdc42Hs(Q61L)-mant-dGTP (1 μM) was titrated with the indicated amounts of Cdc42-GAP (○). For comparison, the titration of Cdc42Hs-mant-dGDP (1 μM) in the presence of 60 μM AlCl$_3$ and 25 mM NaF with the indicated amounts of GAP is also pictured (●).
Fluoride Activation of Cdc42Hs

We have demonstrated that AlF$_4^-$ induces the formation of a stable complex between Cdc42Hs-GDP and GAP using a variety of biochemical and spectroscopic techniques. The $K_d$ value measured for the AlF$_4^-$-induced complex formation was similar to that determined for the binding of activated (GTP-bound) Cdc42Hs to GAP. We have also found that beryllium was able to replace aluminum in mediating Cdc42Hs-GDP-GAP complex formation in the presence of fluoride, consistent with results that have been obtained for heterotrimeric G-proteins. Our results indicate that the aluminum fluoride-mediated complex formation reported between Ras and RasGAP is likely to be a general phenomenon observed between all low molecular mass GTP-binding proteins and their GAP molecules.

The $^{19}$F NMR data are crucial to understanding the mechanism of the AlF$_4^-$-induced complex formation. There are two potential mechanisms by which AlF$_4^-$ could promote the interactions between the GDP-bound form of Cdc42Hs and its effectors. The first mechanism is analogous to that seen with the heterotrimeric GTP-binding proteins, where the AlF$_4^-$ binds directly to the GDP-bound Cdc42Hs, inducing an activated conformation capable of interacting with the GAP. Mittal et al. (14), however, proposed an alternative mechanism for the aluminum fluoride-induced complex formation between Ras and its GAPs. The first step in this mechanism is the formation of a weakly interacting complex between the Ras and RasGAP. The transient formation of this low affinity complex creates a pocket for the aluminum fluoride which then binds to stabilize the complex.

The results of the $^{19}$F NMR spectroscopy with Cdc42Hs clearly implicate the second of these two mechanisms. The addition of the GDP-bound form of Cdc42Hs to a solution containing AlF$_4^-$ did not give rise to additional fluoride peaks in the NMR spectrum, indicating that AlF$_4^-$ did not bind to Cdc42Hs uncomplexed. The addition of GAP produced an additional up-field peak not observed with Cdc42Hs alone. Thus, the GAP must contribute important residues to the active site, forming a binding pocket capable of stabilizing the transition state and thereby allowing AlF$_4^-$ to bind. The chemical shift observed for the binding of AlF$_4^-$ to the Cdc42Hs-GDP-GAP complex was similar to that observed for α-transducin, suggesting that the environment of the AlF$_4^-$ binding pocket is similar in these two proteins. AlF$_4^-$ did not bind to the Cdc42Hs-GppNHp-GAP complex indicating that AlF$_4^-$ occupies the position that normally contains the γ-phosphate, as expected from studies on heterotrimeric G-proteins.

GAPs for the Rho family GTPases make up a diverse family with highly divergent amino acid sequences. A few highly conserved residues have been isolated which are critical to the catalytic activity of these proteins; in particular, mutation of the universally conserved arginine (Arg-305 in Cdc42-GAP) catalytic residues have been isolated which are critical to the active site (14). The recent x-ray crystallographic structures for the Ras-GDP-AlF$_4^-$RasGAP complex formation reported between Ras and RasGAP provides critical catalytic residues to the active site (14). The recent x-ray crystallographic structures for the Ras-GDP-AlF$_4^-$RasGAP complex formation reported between Ras and RasGAP provides critical catalytic residues to the active site (14).
complex, as well as complexes between RhoGAP and Rho family GTPases in both the active (GppNHp-bound) and transition state (GDP-AlF$_4^-$-bound) conformations, show that GAPs contribute an arginine residue to the nucleotide binding site which specifically stabilizes the transition state for GTP hydrolysis (29–31). The results presented here as well as the observations of these crystallographic studies are summarized in Fig. 7A, showing the catalytically important Arg-305 mediating the AlF$_4^-$-induced complex formation between Cdc42 and GAP.

As stated in the Introduction, a great deal of biochemical and structural data has implicated AlF$_4^-$ as a transition state analog for the GTPase reaction in heterotrimeric G-proteins. In the crystal structure of a subunits complexed to AlF$_4^-$, the critical arginine residue, which is located within the large helical domain, interacts with the coordinating fluorides. Considering the data presented here in light of the work performed on heterotrimeric G-proteins and Ras, it seems likely that the GTP-hydrolytic mechanism is conserved across the families of both large and small G-proteins and that Arg-305 in Cdc42-GAP plays the critical role of stabilizing the transition state in the GTP-hydrolytic pathway, analogous to arginine 174 in α-transducin, arginine 178 in G$_i$, and arginine 789 in the RasGAP.

We obtained interesting and rather unexpected results when examining the effects of AlF$_4^-$ on the interactions between Cdc42Hs and its downstream target/effector molecules. Specifically, we found that AlF$_4^-$ enhanced the affinity of the GDP-bound form of Cdc42Hs for its target/effector proteins. This was unexpected in light of the finding that AlF$_4^-$ does not influence Ras-target interactions and suggests that the Ras and Rho subfamily G-proteins have distinctly different modes of effector binding. The $^{19}$F NMR data clearly demonstrate the inability of AlF$_4^-$ to bind to free Cdc42Hs, and thus the AlF$_4^-$-induced complex formation between Cdc42Hs and PBD must proceed in a manner similar to that proposed for the GAP where PBD must first form a transient complex with Cdc42Hs and thereby create a binding site for AlF$_4^-$ as it appears to be the case for the GAP, the creation of the binding pocket for AlF$_4^-$ apparently relies on the introduction of a specific residue by the effector molecule. The crystal structure of the Ras-related G-protein Rap in complex with the Ras-binding domain of Raf demonstrates that targets for Ras do not introduce such a residue into the GTP-hydrolytic state (32). Unlike Raf, the PKs and other Rho family target/effectors must introduce a residue into the active site, enabling AlF$_4^-$ to bind and mediate complex formation.

Another unique property of Rho family targets is their role as GTPase inhibitory proteins (GIPs) (33). Such GIP activity is not observed for effectors of Ras (34). One interesting possibility is that the GIP activity of Rho family target/effectors is mediated by a residue introduced into the γ-phosphate binding pocket of the G-protein which stabilizes the GTP-bound state in a manner analogous to the stabilization of the transition state for GTP hydrolysis by the GAP, but with an opposite effect. This interpretation is supported further by the complex formation observed between Cdc42-GDP and PBD in the presence of AlF$_4^-$ and is summarized in Fig. 7B. Comparison of the crystal structures of myosin-ADP bound with either AlF$_4^-$ or BeF$_3^-$ has shown that although the AlF$_4^-$ complex represents a distinct transition state conformation, BeF$_3^-$ is a true ATP analog, inducing a conformation indistinguishable from the ATP-bound protein (35). The affinity of PBD for Cdc42Hs-GDP in the presence of BeF$_3^-$ is identical to that observed for the activated form of Cdc42Hs, whereas the affinity in the presence of AlF$_4^-$ is significantly weaker than that measured for the ATP-bound state. This is consistent with the hypothesis that the fluoride-mediated interaction of Cdc42Hs with effectors relies on the GIP activity of these proteins which should stabilize a GTP analog (BeF$_3^-$) more effectively than a transition state analog (AlF$_4^-$). This implies a direct role for target/effector molecules in modulating the GTPase activity of Rho family G-proteins.

In conclusion, it is clear that aluminum fluoride represents a transition state analog in the GTP-hydrolytic pathway of Cdc42Hs and is capable of inducing complex formation with the Cdc42-GAP. Moreover, we propose a role for BeF$_3^-$ as a true GTP analog, capable of inducing high affinity complex formation between Cdc42Hs and target/effector molecules. Such fluoride-mediated complex formation is singular to the Rho subfamily of GTPases and is a direct result of the unique biochemical structure of the G-protein-target interaction in this important family of signaling molecules.

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Note Added in Proof—It has been reported that the transition-state complex for the GTP hydrolytic reaction for Ras contains AlF$_4^-$ (29).

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