Mutagenesis of the Conserved Residue Glu\textsuperscript{259} of G\textsubscript{a} Demonstrates the Importance of Interactions between Switches 2 and 3 for Activation*

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We previously reported that substitution of Arg\textsuperscript{258} within the switch 3 region of G\textsubscript{a} impaired activation and increased basal GDP release due to loss of an interaction between the helical and GTPase domains (Warner, D. R., Weng, G., Yu, S., Matalon, R., and Weinstein, L. S. (1998) J Biol. Chem. 273, 23976–23983). The adjacent residue (Glu\textsuperscript{259}) is strictly conserved in G protein \textalpha-subunits and is predicted to be important in activation. To determine the importance of Glu\textsuperscript{259}, this residue was mutated to Ala (G\textsubscript{a}-E259A), Gln (G\textsubscript{a}-E259Q), Asp (G\textsubscript{a}-E259D), or Val (G\textsubscript{a}-E259V), and the properties of in vitro translation products were examined. The G\textsubscript{a}-E259V was studied because this mutation was identified in a patient with Albright hereditary osteodystrophy. S49 cys reconstitution assays demonstrated that G\textsubscript{a}-E259D stimulated adenyl cyclase normally in the presence of GTP\textgamma-S but was less efficient with isotoprenol or AlF\textgamma. The other mutants had more severely impaired effector activation, particularly in response to AlF\textgamma. To determine the importance of Glu\textsuperscript{259}, this residue was mutated to Ala (G\textsubscript{a}-E259A), Gln (G\textsubscript{a}-E259Q), Asp (G\textsubscript{a}-E259D), or Val (G\textsubscript{a}-E259V), and the properties of in vitro translation products were examined. The G\textsubscript{a}-E259D was studied because this mutation was identified in a patient with Albright hereditary osteodystrophy. S49 cys reconstitution assays demonstrated that G\textsubscript{a}-E259D stimulated adenyl cyclase normally in the presence of GTP\textgamma-S but was less efficient with isotoprenol or AlF\textgamma. The other mutants had more severely impaired effector activation, particularly in response to AlF\textgamma. In trypsin protection assays, GTP\textgamma-S was a more effective activator than AlF\textgamma for all mutants, with G\textsubscript{a}-E259D being the least severely impaired. For G\textsubscript{a}-E259D, the AlF\textgamma-induced activation defect was more pronounced at low Mg\textsuperscript{2+} concentrations. G\textsubscript{a}-E259D and G\textsubscript{a}-E259A purified from Escherichia coli had normal rates of GDP release (as assessed by the rate GTP\textgamma-S binding). However, for both mutants, the ability of AlF\textgamma to decrease the rate of GTP\textgamma/S binding was impaired, suggesting that they bound AlF\textgamma more poorly. GTP\textgamma-S bound to purified G\textsubscript{a}-E259D irreversibly in the presence of 1 mM free Mg\textsuperscript{2+}, but dissociated readily at micromolar concentrations. Sucrose density gradient analysis of in vitro translated demonstrated that all mutants except G\textsubscript{a}-E259V bind to AlF\textgamma at 0 °C and were stable at higher temperatures. In the active conformation Glu\textsuperscript{259} interacts with conserved residues in the switch 2 region that are important in maintaining both the active state and AlF\textgamma in the guanine nucleotide binding pocket. Although both G\textsubscript{a} Arg\textsuperscript{258} and Glu\textsuperscript{259} are critical for activation, the mechanisms by which these residues affect G\textsubscript{a} protein activation are distinct.

Heterotrimeric guanine nucleotide-binding proteins (G proteins) couple heptahelical receptors to intracellular effectors and are composed of three subunits (\alpha, \beta, and \gamma) (reviewed in Refs. 1–3). The \alpha-subunits, which are distinct for each G protein, bind guanine nucleotide and modulate the activity of specific downstream effectors. For G\textsubscript{a}, these include the stimulation of adenylyl cyclase and modulation of ion channels (4, 5). In the inactive state, GDP-bound \alpha-subunit is associated with a \beta\gamma-dimer. Upon receptor activation, the \alpha-subunit undergoes a conformational change resulting in the exchange of GTP for GDP and dissociation from \beta\gamma. While GTP is bound, the \alpha-subunit interacts with and regulates specific effectors. An intrinsic GTPase activity within the \alpha-subunit hydrolyzes bound GTP to GDP, returning the G protein to the inactive state. Analogs of GTP, such as GTP\textgamma-S and GDP-AlF\textgamma, lock the G protein in the active state.

X-ray crystal structures reveal that G protein \alpha-subunits have two domains, a ras-like GTPase domain, which includes the regions for guanine nucleotide binding and effector interaction, and a helical domain, which may prevent release of GDP in the inactive state (6–12). Comparison of the crystal structures of inactive (GDP-bound) and activated (GTP\textgamma-S-or AlF\textgamma-bound) \alpha-subunits demonstrates three regions (named switches 1, 2, and 3), the conformation of which changes upon switching from the inactive to active state. The movement of switches 1 and 2 is directly related to the presence of the \gamma-phosphate group, whereas switch 3 has no direct contact with bound guanine nucleotide. Upon activation, switches 2 and 3 move toward each other, and the two regions form multiple interactions that presumably stabilize the active state (7, 10). Switch 3 residues also make contacts with the helical domain that are important for high affinity guanine nucleotide binding (10, 15). At least for transducin, this region may also be important in effector activation (13).

We have previously shown that substitutions of the switch 3 residue Arg\textsuperscript{258} impairs activation by receptor or AlF\textgamma (15). The latter effect was the direct result of decreased GDP binding due to loss of contacts between the Arg\textsuperscript{258} side chain and residues within the helical domain. The adjacent residue (Glu\textsuperscript{259}) is invariant in all known G protein \alpha-subunits and is predicted to be important in activation, because it makes interactions with switch 2 residues in the active state (7, 12). Moreover, this residue is mutated to a valine in a patient with Albright hereditary osteodystrophy (16). In the present report, we provide evidence that substitution of Glu\textsuperscript{259} also leads to impaired activation, particularly by receptor or AlF\textgamma. How-
ever, impaired activation of these mutants by AlF₄⁻ is not the result of decreased GDP binding (as is the case for the Arg²⁵⁸ mutants) but rather is the result of a decreased ability to bind the AlF₄⁻ moiety. The crystal structure of GTPγS-bound Gα reveals interactions between the acidic side chain of Glu²⁵⁹ and basic residues within switch 2 that are important in maintaining the active state and in binding of AlF₄⁻ (12). Although adjacent switch 3 residues in Gα (Arg²⁵⁸ and Glu²⁵⁹) are both critical for activation, the mechanisms by which mutations of these residues result in defective activation are distinct.

**EXPERIMENTAL PROCEDURES**

**Construction of Gα Plasmids and in Vitro Transcription/Translation**—To generate Gα Glu²⁵⁹ mutants, polymerase chain reaction was performed as described previously (15) using linearized vector containing wild type Gα cDNA as template. The upstream primer was 5'-GACAAGATCAGTCCAGTGTGTCAGTGGGCGACCCCGATGAC-GAAG-3', and the downstream mutagenic primers were as follows: 5'-GAGCTCCTTGACGGGTGTGTTGCGTCACCCCGATGAC-GAAG-3' for E259V, 5'-GAGCTCCTTGAGGCTCTGCTGCTGACCCCGATGAC-GAAG-3' for E259A, 5'-GAGCTCCTTGAGGCTCTGCTGCTGACCCCGATGAC-GAAG-3' for E259Q, and 5'-GAGCTCCTTGACGGGTGTGTTGCGTCACCCCGATGAC-GAAG-3' for E259D. Each polymerase chain reaction product was digested with HincII and Sse8387I and ligated into the transcription vector pBluescript II SK (Stratagene, La Jolla, CA) in which HindIII-EcoRI restriction fragment had been removed. Migrations were verified by DNA sequencing, and synthesis of full-length Gα from each construct was confirmed by immune precipitation of in vitro translated products with RM antibody, directed against the carboxyl-terminal decapeptide of Gα (18). In vitro transcription/translation was performed on Gα plasmids as described previously (15, 19) using the TNT-coupled transcription/translation system from Promega, with the exception that in most experiments, no RNase inhibitor was added.

**Adenylyl Cyclase Assays**—Wild type and mutant Gα in vitro transcription/translation products (10 μl of translation medium) were reconstituted into 25 μg of purified S49 cyc plasma membranes and tested for stimulation of adenylyl cyclase in the presence of various agents as indicated in Table I (15, 19, 20). Reactions were incubated for 15 min at 30 °C, and the amount of [³²P]cAMP produced was measured as described previously (21).

**Trypsin Protection Assays**—Limited trypsin digestion of in vitro translated Gα was performed as described previously (15, 19). Briefly, 1 μl of in vitro translated [³²S]methionine-labeled Gα was incubated in incubation buffer (20 mM HEPES, pH 8.0, 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol) with or without 100 μM GTPγS or 10 mM NaF/10 μM AlCl₃ at various temperatures for 1 h and then digested with 200 μg/ml tosyl-L-phenylalanyl chloromethyl ketone-trypsin for 5 min at 20 °C. In some experiments, GDP was also included in the preincubation, and in other experiments the MgCl₂ concentration was varied. Reactions were terminated by boiling in Laemmli buffer. Digestion products were separated on 10% SDS-polyacrylamide gels, and the amount of 38-kDa protected fragment was measured by phosphorimaging.

**Sucrose Density Gradient Centrifugation**—[³²S]Methionine-labeled Gα was synthesized, and rate zonal centrifugation was performed on linear 5–20% sucrose gradients (200 μl) as described previously (19, 22). Gradients were prepared in 20 mM HEPES, pH 8.0, 1 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 0.1% Lubrol-PX. Six-μl fractions were obtained and analyzed by SDS-polyacrylamide gel electrophoresis, and the relative amount of Gα in each fraction was quantified as described previously (19). To assess the ability of Gα to bind to Gβγ, in vitro translation products were preincubated for 1 h at 0 °C in the presence or absence of Gβγ (20 μg/ml) prior to centrifugation. In order to optimize separation between free α-subunit and heterotrimer,
mutated cDNA was sequenced to confirm the presence of the desired site-directed mutagenesis using the Quickchange kit (Stratagene). Each nine residues by trypsin. For Gs, the percentage of undigested Gs was determined either in the presence or absence of AlF4. This experiment was representative of three experiments. The amount of the 38-kDa trypsin-stable Gs, which the carboxyl group is replaced by a neutral amide group.

Table II

**Effect of temperature and GDP on AlF4-induced trypsin protection**

These data were obtained from experiments of the type presented in Fig. 1. The amount of the 38-kDa trypsin-stable Gs fragment was determined by phosphorimaging, and for Gs-WT, it is expressed as a percent of wild type at each condition (mean ± S.E.). The percentage of protection of Gs when AlF4 was loaded with [35S]GTPγS at 30 °C for 45 min in the presence of various free Mg2+ concentrations. After addition of 100 μM cold GTPγS, bound [35S]GTPγS was determined at various time points as described above. kcat for GTPγS dissociation was determined by fitting the data to the function y = a – bx using the software GraphPad Prism, version 2.01. Free Mg2+ concentrations were calculated as described (26).

| Temperature (°C) | Treatment | WT | E259D | n  |
|-----------------|-----------|-----|-------|----|
| 25              | AlF4      | 56 ± 8 | 85 ± 15 | 4  |
| 30              | AlF4 + 2 mM GDP | 59 ± 6 | 60 ± 6   | 4  |
| 100 μM GTPγS | AlF4       | 59 ± 5 | 49 ± 7b | 8  |
| 37              | AlF4      | 59 ± 3 | 60 ± 6  | 4  |
| 100 μM GTPγS  | AlF4      | 59 ± 3 | 60 ± 6  | 4  |

* The percentage of protection of Gs-E259D was significantly less than that of Gs-WT at all conditions except at 30 °C in the presence of AlF4 (Student’s t test).

**RESULTS**

**Substitution of Gs Glu259 Leads to Decreased Activation**

Gs Glu259 substitution mutants were cloned into the transcription vector pBlueScript, and the in vitro transcription/translation products were compared with those of Gs-WT in various biochemical assays. We substituted Glu259 with valine (Gα-E259V) because a mutation encoding this substitution has been identified in a patient with Albright hereditary osteodystrophy (16), a human disorder associated with heterozygous loss-of-function mutations of Gα (27, 28). Because the presence of an amino acid with a bulky and branched side chain (valine) may introduce nonspecific steric effects, we also generated and analyzed additional mutants in which Glu259 was replaced by alanine (Gα-E259A), glutamine (Gα-E259Q), or aspartate (Gα-E259D). In Gα-E259A, the acidic side chain was removed, whereas in Gα-E259Q it is converted to a residue in which the carboxyl group is replaced by a neutral amide group. In Gα-E259D, the charge of the residue at position Glu259 is...
maintained, but the length of the side chain is shortened by one methylene group.

After reconstitution of translation products into purified S49 cyc membranes (which lack endogenous Gsα), Gsα-E259V had markedly decreased ability to stimulate adenylyl cyclase in the presence of GTPγS, AlF4−, or activated receptor (isoproterenol·GTP) (Table I). For Gsα-E259A and -E259Q, the ability to stimulate adenylyl cyclase was moderately reduced in the presence of GTPγS (~40% of Gsα-WT) and more markedly reduced in the presence of AlF4− or activated receptor. Stimulation of adenylyl cyclase by Gsα-E259D was normal in the presence of GTPγS but moderately reduced in the presence of AlF4− or activated receptor. Although the severity of the defect varied depending on which specific residue replaced Glu259, for each Gsα-Glu259 mutant, GTPγS was the most effective activator and AlF4− the least effective activator.

We next examined the ability of AlF4− or GTPγS to protect each mutant from trypsin digestion, which measures the ability of each agent to bind to Gsα and induce the active conformation (29). In the inactive, GDP-bound state, two arginine residues within switch 2 (most likely Arg228 and Arg231, based upon sequence homology with transducin) are sensitive to trypsin digestion, leading to the generation of low molecular weight fragments. When Gsα attains the active conformation, these residues are inaccessible to trypsin digestion (7) and therefore trypsinization of activated Gsα generates a partially protected 38-kDa product. Gsα-WT was well protected by AlF4− or GTPγS at temperatures up to 37 °C (Fig. 1, Table II). At 30 °C, Gsα-E259V, -E259A, and -E259Q showed little protection by GTPγS and no protection by AlF4− (Fig. 1). In contrast, both GTPγS and AlF4− were able to protect Gsα-E259D, with GTPγS being a more efficient activator than AlF4− (Fig. 1, Table II). Consistent with the results of the cyc reconstitution assays, AlF4− was less effective than GTPγS in protecting all Gsα-E259 mutants from...
GTP experiment was representative of three experiments. Maximum Gs rate of GTP activate the mutant Gs GDP Release in the Basal State—
from and 7% of Gs the presence of AlF4 amount of bound [35S]GTP significantly reverse the AlF4 result of impaired GDP binding (15). The inability of GDP to Arg258 mutants, impaired activation by AlF4 complex bound to Go is more stable than GDP alone (8). Because Gs-WT, -E259D, and -E259A have similar rates of GTPS binding in the absence of AlF4, the time course of GTPS binding in the presence of AlF4 should reflect the ability of each form of Gα to interact with AlF4. Similar to previously reported observations (8), the rate and extent of GTPS binding to Gs-WT was markedly reduced in the presence of AlF4 (Fig. 2). In contrast, AlF4 only partially reduced the rate and extent of GTPS binding to Gs-E259D and had a minimal effect on the GTPS binding curve for Gs-E259A (Fig. 2). These results are consistent with the results of adenyl cyclase and trypsin protection assays, which demonstrate that AlF4-induced activation is severely impaired in Gs-E259A but only partially impaired in Gs-E259D and suggest that the decreased ability of AlF4 to activate Gs-E259D mutants is primarily due to decreased ability of the mutants to maintain AlF4 in the guanine nucleotide binding pocket.

Effect of Mg2+ Concentration on Activation by AlF4 and GTPS Binding—Substitution of Gα Arg258, a residue in switch 2 that interacts with switch 3 residues in the active state, leads to a defect in activation by AlF4 that is more pronounced at low Mg2+ concentrations (33). We therefore examined the effect of varying Mg2+ concentration on the ability of AlF4 to protect Gs-E259D from trypsin digestion. In the trypsin protection experiments shown in Fig. 1 and Table II, the MgCl2 concentration was 10 mM (−9 mM free Mg2+). Lowering the MgCl2 concentration to 2 mM (−1 mM free Mg2+) had no effect on the ability of AlF4 to protect Gs-WT at 30 °C (Fig. 3). In contrast, lowering the MgCl2 concentration below 8 mM (−7 mM free Mg2+) further impaired the ability of AlF4 to protect Gs-E259D in a concentration-dependent manner. Increasing the MgCl2 concentration up to 100 mM did not reverse the defect at 37 °C (data not shown). These results are similar to those observed for the Gα-R231 mutant (33) and demonstrate that, like this mutant, the GDP-AlF4-bound form of Gs-E259D has a lower apparent affinity for Mg2+ than Gα-WT.

Next, we examined the effect of lowering the Mg2+ concentration on the dissociation of GTPS from Gα-E259D to determine whether or not the Mg2+ dependence was specific for the GDP-AlF4-bound form. The apparent Kd of GTPS-Gα-WT for Mg2+ is very low (5–10 mM), and binding of GTPS is essentially irreversible in the presence of micromolar concentrations of Mg2+ (34). Consistent with previously published results (34), no dissociation of GTPS from Gα-WT was observed at free Mg2+ concentrations of 30 μM or higher (Fig. 4 and data not

![Fig. 4. Effect of free Mg2+ concentration on dissociation of GTPS from purified Gα-GPT. Bovine Gα-WT and -E259D, each with a carboxyl-terminal hexahistidine extension, were expressed and purified from E. coli, and the time course of GTPS dissociation was determined for each at various free Mg2+ concentrations. Gα-WT (closed symbols) and Gα-E259D (open symbols) were preloaded with [35S]GTP-S (5,000–10,000 cpm/pmol) at 30 °C for 45 min in the presence of 1 mM (□), 30 μM (■ and □), or no (▲ and ▼) free Mg2+ (5 mM EDTA was added for the no Mg2+ condition). After addition of 100 μM cold GTP-S, the amount of bound [35S]GTP-S was determined at various time points. Each data point is the mean ± range of duplicate determinations. This experiment was representative of three experiments. Maximum [35S]GTP-S in the presence of 5 mM EDTA was 0.5 pmol for Gα-WT and 0.3 pmol for Gα-E259D, and it was 2.5 pmol for both in the presence of Mg2+.](image-url)
shown), although GTPγS dissociated rapidly ($k_{off} = 2.5$ min$^{-1}$) in the absence of Mg$^{2+}$ (5 mM EDTA). For Gs$\alpha$-E259D, GTPγS binding was essentially irreversible in the presence of 1 mM free Mg$^{2+}$, but in contrast to Gs$\alpha$-WT, GTPγS clearly dissociated from Gs$\alpha$-E259D in the presence of 30 $\mu$M free Mg$^{2+}$ (Fig. 4, $k_{off} = 0.05$ min$^{-1}$). Dissociation of GTPγS from Gs$\alpha$-E259D ($k_{off} = 3.7$ min$^{-1}$) was similar to that of Gs$\alpha$-WT in the absence of Mg$^{2+}$ (5 mM EDTA). Therefore, like GDP-ALF$\gamma$-Gs$\alpha$-E259D, GTPγS-Gs$\alpha$-E259D appears to have decreased affinity for Mg$^{2+}$, although the defects are apparent in the millimolar range for the former and micromolar range for the latter.

In contrast to Gs$\alpha$-E259D, there is a slow rate of dissociation of GTPγS from Gs$\alpha$-R231H in the presence of high Mg$^{2+}$ concentrations (33). Another Gs$\alpha$ mutant (Gs$\alpha$-G226A) also displays an abnormally high apparent $K_f$ for Mg$^{2+}$ to prevent GTPγS dissociation (34). Similar to Gs$\alpha$-E259D, GTPγS dissociates from Gs$\alpha$-G226A in the presence of micromolar concentrations of Mg$^{2+}$. There is also considerable dissociation of GTPγS from Gs$\alpha$-G226A even in the presence of maximal (millimolar) concentrations of Mg$^{2+}$, because this mutant cannot
attain the active conformation that stabilizes the Mg$^{2+}$-GTPγS complex. The ability of Gsα-E259D to irreversibly bind GTPγS in the presence of 1 mM Mg$^{2+}$ suggests that this mutant can attain the active conformation necessary to stabilize Mg$^{2+}$-GTPγS, consistent with the results obtained in the adenylyl cyclase and trypsin protection assays (Table I and Fig. 1).

Gsα-E259Q, E259A, and E259D, but not Gsα-E259V, Maintain Normal Overall Conformation and Gβγ Interaction—We examined the ability of each Gsα-E259 mutant to interact with Gβγ by subjecting in vitro translated sucrase density gradient centrifugation in the presence or absence of purified bovine brain Gβγ. We previously showed that Gsα has a sedimentation coefficient of ~3.7 S (15, 19). When in vitro translated each Gsα-E259 mutant was held on ice, the gradient profiles of all mutants were virtually the same as Gsα-WT and consistent with the overall proper conformation (sedimentation coefficient, ~3.7 S) (Fig. 5A). When preincubated on ice with purified bovine brain Gβγ, Gsα-WT, -E259Q, -E259A, and -E259D formed heterotrimers, as demonstrated by significant shifting of the peak toward the bottom of the gradient (Fig. 5B). In contrast, Gβγ had no effect on the sedimentation profile of Gsα-E259V, indicating that this mutant does not interact with Gβγ. After preincubation at 30 °C, gradient profiles demonstrate that all mutants except Gsα-E259V maintain the normal 3.7 S conformation, whereas for Gsα-E259V, the majority of the protein is a higher S value material and is presumably denatured (19). Therefore, the valine substitution probably alters the overall conformation and stability of the protein due to nonspecific steric effects of its bulky hydrophobic side chain. In contrast, the activation defect in Gsα-E259A, E259Q, and E259D is not secondary to defects in thermostability or Gβγ binding.

**DISCUSSION**

We previously reported that substitution of the Gsα switch 3 residue Arg258 leads to impaired activation in the presence of AlIF$_4^−$, or activated receptor (isoproterenol + GTP) but normal activation in the presence of GTPγS (15). The impaired activation by AlIF$_4^−$ was reversible in the presence of excess GDP, and further characterization demonstrated a defect in GDP binding, presumably due to loss of direct contact between Arg258 and a residue(s) in the helical domain that would open the cleft through which guanine nucleotide must exit. In this study, we examined the effect of substituting the adjacent switch 3 residue (Glu259) on Gsα function for the following reasons: 1) this residue is strictly conserved among G protein α-subunits and therefore might have an important role in the biochemical function of these proteins; 2) upon activation, the Glu259 side chain interacts with several residues in the switch 2 region (7, 12) and therefore substitutions of this residue might be predicted to directly impair G protein activation; 3) this Gsα residue is mutated to a valine in a patient with Albright hereditary osteodystrophy (16), a human disorder associated with heterozygous inactivating mutations within the Gsα gene (27, 28).

Substitution of Gsα Glu259 to valine had a marked effect on the conformation and stability of the protein. This mutant was unable to interact with Gβγ, even though Glu259 is not within the Gβγ interaction site (11). This mutant was also more thermolabile. Presumably, the presence of a bulky and branched side chain provided by valine introduces nonspecific steric effects that severely affect the conformation and stability of the protein. We would predict that the primary biochemical defect in the patient harboring this mutation is lack of expression of Gsα-E259V in the membrane at physiological temperatures, similar to what is observed in other patients with mutants encoding unstable forms of Gsα protein (15, 19, 32).

In order to determine whether residue Glu259 is critical in maintaining either the basal or activated state, we generated mutants with more subtle alterations of Glu259 side chain. The most subtle mutation was Gsα-E259D, in which the charge of the residue is maintained but the length of the side chain is shortened by one methylene group. We also made two mutants in which the side chain was either removed (Gsα-E259A) or converted from an acidic to neutral amino acid (Gsα-E259Q). In all three of these mutants, the overall conformation and stability, as well as the ability to interact with Gβγ, was maintained, as determined by sucrose density gradient experiments. Based upon adenylyl cyclase and trypsin protection assays, activation of Gsα-E259D by GTPγS was normal, demonstrating that this mutant has not lost its intrinsic ability to attain the active conformation and activate adenylyl cyclase. However, this mutant had decreased ability of to be activated by AlIF$_4^−$ or recep...
tor. Gα-ε259Q and -ε259A showed a more severe phenotype, with decreased activation in the presence of all agents. In all three mutants, GTP•S was the most efficient activator whereas AlF₄⁻ was the least efficient. Mutation of the analogous residue in transducin (Glu232) to leucine had no effect on the ability of the G protein to interact with βγ or its receptor (rhodopsin), but it did appear to decrease the ability of GTP•S to mediate trypsin protection and effecter activation (13).

One possible mechanism for impaired activation by AlF₄⁻ is decreased ability to maintain the GDP-bound state, because binding of GDP is a prerequisite for AlF₄⁻ binding and activation. This is the primary mechanism by which substitutions of Gα-ε Arg258 lead to impaired activation by AlF₄⁻ (15). However, the ability of Gα-ε259A mutants to maintain the GDP-bound state was similar to that of Gα-ε-WT, as demonstrated by both Gα-ε-E259A and -E259D having a rate of GDP release that was similar to Gα-ε-WT, as well as an inability for excess GDP to significantly reverse the AlF₄⁻-induced activation defect. Consistent with normal guanine nucleotide binding, both Gα-ε259A and -E259D were thermostable. Binding of AlF₄⁻ to the GDP-bound α-subunit results in formation of a stable and activated GDP-AlF₄⁻-protein complex that mimics the transition state of the GTPase reaction and will slow the rate of GTP•S binding, probably by inhibiting GDP release (8). The ability of AlF₄⁻ to inhibit the rate and extent of GTP•S binding to both Gα-ε259A and -E259D was significantly reduced, suggesting that in these mutants the activation defect in response to AlF₄⁻ is due at least in part to impaired AlF₄⁻ binding. The fact that the activation defect is greater for AlF₄⁻ than GTP•S suggests that mutation of Glu259 has a more dramatic effect on stabilizing the transition (AlF₄⁻-bound) state than the activated (GTP•S-bound) state.

It is of interest that the biochemical phenotype of our Gα-ε-Glu259 mutants is quite similar to that previously described for Gα-ε-E259Q and -E259A having a rate of GDP release that was similar to Gα-ε-WT, as demonstrated by both Gα-ε-E259Q and -E259A showed a more severe phenotype, for Mg²⁺ (34). Lambright, D. G., Sondek, J., Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1994) Nature 369, 621–628. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) Science 265, 1405–1412. Ball, M. A., Coleman, D. E., Lee, E., Iñiguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) Cell 83, 1047–1058. Moxon, M. B., Lee, E., Coleman, D. E., Berghuis, A. M., Gilman, A. G., and Sprang, S. R. (1995) Science 270, 954–960. Lambright, D. G., Sondek, J., Bohm, A., Sikita, N. P., Hamm, H. E., and Sigler, P. B. (1996) Nature 378, 279–299. Sunahara, H. K., Tesmer, J. J., Gilman, A. G., and Sprang, S. R. (1997) Science 278, 1943–1947. Li, Q., and Cerione, R. A. (1997) J. Biol. Chem. 272, 21673–21676. Iiri, T., Farfel, Z., and Bourne, H. R. (1998) Nature 394, 35–38. Warner, D. R., Weng, G., Yu, S., Matalon, R., and Weinstein, L. S. (1998) J. Biol. Chem. 273, 23976–23983. Ahmed, S. F., Dixon, P. H., Bonthron, D. T., Stirling, H. F., Barr, D. G. B., Kelnner, C. J. H., and Thakker, R. V. (1998) Clin. Endocrinol. 49, 525–531. Kokasa, T., Itoh, H., Tsukamoto, T., and Kazuyo, Y. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2061–2065. Simonds, W. F., Goldsmith, P. K., Woodard, C. J., Unson, C. S., and Spiegel, A. M. (1989) PERS. Lett. 249, 189–194. Warner, D. R., Geiman, P. C., Collins, R. M., and Weinstein, L. S. (1997) Mol. Endocrinol. 11, 1718–1727. Szebenwieser, P. C., Northrup, R. K., Smigel, M. D., and Gilman, A. G. (1991) J. Biol. Chem. 266, 11517–11526. Salomon, Y., Londos, C., and Rodbell, M. (1974) J. Biol. Chem. 249, 1823–1829. Miller, R. T., Masters, S. B., Sullivan, K. A., Beiderman, B., and Bourne, H. R. (1988) Nature 334, 712–715. Ferguson, K. M., Higashiguma, T., Smigel, M. D., and Gilman, A. G. (1986) J. Biol. Chem. 261, 7383–7399. Graziano, M. P., and Gilman, A. G. (1989) J. Biol. Chem. 264, 15475–15482. Iiri, T., Hermark, P., Nakamoto, J. M., Van Dop, C., and Bourne, H. R. (1994) Nature 371, 1412–1419. Weinstein, L. S., Geiman, P. C., Friedman, E., Kadokawa, T., Collins, R. M., Gerhert, E. F., and Smigel, A. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8287–8290. Miller, R. T., Masters, S. B., Sullivan, K. A., Beiderman, B., and Bourne, H. R. (1988) Nature 334, 712–715. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 940–950.