A Novel Mutation in the Switch 3 Region of Gsα in a Patient with Albright Hereditary Osteodystrophy Impairs GDP Binding and Receptor Activation*

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Albright hereditary osteodystrophy (AHO), a disorder characterized by skeletal abnormalities and obesity, is associated with heterozygous inactivating mutations in the gene for Gsα. A novel Gsα mutation encoding the substitution of tryptophan for a nonconserved arginine within the switch 3 region (Gsα R258W) was identified in an AHO patient. Although reverse transcription-polymerase chain reaction studies demonstrated that mRNA expression from wild type and mutant alleles was similar, Gsα expression in erythrocyte membranes from the affected patient was reduced by 50%. A Gsα R258W cDNA, as well as one with arginine replaced by alanine (Gsα R258A), was generated, and the biochemical properties of in vitro transcription/translation products were examined. When reconstituted with cyc- membranes, both mutant proteins were able to stimulate adenylyl cyclase normally in the presence of guanosine-5’-O-(3-thiotriphosphate) (GTPγS) but had decreased ability in the presence of isoproterenol or AlF4 (a mixture of 10 μM AlCl₃ and 10 mM NaF). The ability of each mutant to bind and be activated by GTPγS or AlF₄⁻ was assessed by trypsin protection assays. Both mutants were protected normally by GTPγS but showed reduced protection in the presence of AlF₄⁻. The addition of excess GDP (2 mM) was able to rescue the ability of AlF₄⁻ to protect the mutants, suggesting that they might have reduced affinity for GDP. A Gsα R258A mutant purified from Escherichia coli had decreased affinity for GDP and an apparent rate of GDP release that was 10-fold greater than that of wild type Gsα. Sucrose density gradient analysis demonstrated that both Gsα R258W and Gsα R258A were thermolabile at higher temperatures and that denaturation of both mutants was prevented by the presence of 0.1 mM GTPγS or 2 mM GDP. The crystal structure of Gsα demonstrates that Arg²⁵⁸ interacts with a conserved residue in the helical domain (Gln¹⁷⁹). Arg²⁵⁸ substitutions would be predicted to open the cleft between the GTPase and helical domains, allowing for increased GDP release in the inactive state, resulting in enhanced thermolability and reduced AlF₄⁻-induced adenylyl cyclase stimulation and trypsin protection, since activation by AlF₄⁻ requires bound GDP.

Heterotrimeric guanine nucleotide-binding proteins (G pro-

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1 The abbreviations used are: G protein, guanine nucleotide binding protein; Gsα, stimulatory G protein; GαS, Gα subunit; GαS-R258W, GαS mutant with Arg²⁵⁸⁻ to tryptophan substitution; GαS-R258A, GαS mutant with Arg²⁵⁸⁻ to alanine substitution; AHO, Albright hereditary osteodystrophy; PPHP, pseudopseudohyoparathyroidism; DTT, dithiothreitol; AlF₄⁻, mixture of 10 μM AlCl₃ and 10 mM NaF; GTPγS, guanosine-5’-O-(3-thiotriphosphate); PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR.
mutations of the gene encoding G_sα (14, 15). Within AHO kindreds, patients may have the somatic features of AHO alone (termed pseudopseudohypoparathyroidism [PPHP]) or AHO in association with resistance to multiple hormones that activate G_s-coupled signaling pathways (termed pseudopseudohypoparathyroidism type Ia). While most mutations associated with AHO are frameshift deletions or splice junction mutations, some encode missense mutations that have specific effects on the functional properties of the G_sα protein. Examples include mutations that affect receptor coupling (16), guanine nucleotide binding (17, 18), and activation (19).

In the present report, we describe a novel G_sα missense mutation from an AHO patient in which an arginine residue in switch 3 (Arg^{258} in rats) is substituted with tryptophan. Arg^{258} is a nonconserved residue adjacent to a highly conserved glutamic acid residue (Glu^{259}) that is important for contact between switch 2 and 3 in the active state (7, 12). We present evidence that substitution of Arg^{258} leads to defective G_sα binding, resulting in increased thermolability and decreased activation by AlF_4-. This mutation also leads to decreased receptor activation. In the crystal structure of G_sα in the active state, Arg^{258} associates with a residue (Glu^{177}) located between the αD and αE helices of the helical domain, forming a “lid” over the guanine nucleotide binding pocket. Mutation of Arg^{258} is predicted to disrupt these interactions as well as an interaction between Asp^{177} in the helical domain and Lys^{293} in the GTPase domain previously shown to be important for receptor- and AlF_4- induced activation (20). Analysis of this mutation suggests that switch 3 residues, in addition to being involved in the activation mechanism, are also involved in maintaining the basal state (i.e., sustaining G_sα in the guanine nucleotide binding state).

EXPERIMENTAL PROCEDURES

Patient—The patient was a 24-year old white male with a diagnosis of AHO and PPHP. His birth weight was 6 pounds, 11 ounces. By age 10 months, developmental delay, brachycephaly, and decreased muscle tone were noted. Throughout childhood he was small for his age and had a stocky appearance. By 6 years, learning disabilities as well as impulsive and aggressive behavior were noted, and over the past 2 years the patient has demonstrated increased compulsive behavior. During evaluation at NIH in 1995, the patient was noted to have short stature (62 inches), moderate obesity, and a rounded face with mildly depressed nasal bridge. The patient had brachydactyly involving the distal phalanx of the first digit and the fourth metacarpals bilaterally. Calcium metabolism, evaluation revealed no evidence for resistance to parathyroid hormone or thyroid hormone.

Preparation of Erythrocyte Membranes and Determination of G_s Activity and G_sα Expression—Erythrocyte membranes were prepared from the patient, his parents, and three normal subjects as described previously (18). Genomic DNA was isolated from whole blood and screened for mutations within the gene encoding G_sα (GNAS1) using PCR and temperature gradient gel electrophoresis (15, 23). PCR fragments were purified using Centricon 100 filters (Amicon) and directly sequenced using the Sequenase kit (U.S. Biochemical Corp.). RNA was isolated from 100-μl aliquots of whole blood, and RT-PCR was performed as described previously (18). For amplification of the GNAS1 exon 10 coding region, the primers were as follows: upstream, 5′-ATGTTTGGAGCTGGGGTCAGGC-3′; downstream, 5′-CACAGAGATGGTGCCGACACCAT-3′ (24). The mutation was confirmed by digestion of PCR and RT-PCR products with the restriction enzyme MspI.

Construction of G_sα Plasmids and In Vitro Transcription/Translation—To generate G_sα R258W, RT-PCR fragments amplified from the patient’s RNA were digested with HincII and Sse8387I and ligated into the transcription vector pBluescript II SK (Stratagene, La Jolla, CA) that contained wild type human G_sα cDNA (splice variant G_sα-1, Ref. 24) from the same HincII/Sse8387I restriction fragments were then removed. A control plasmid was created using the identical approach with material obtained from a normal control. G_sα R258A (CGG to GCG) was generated by PCR using a mutagenic primer. The primers were as follows (1 μM each): upstream, 5′-GACAAATGCACATCTTCCA- CATTTTGACGTTGGGCGACGGGATGAAAGCG-3′; downstream (mutagenic), 5′-GGGCTCCTGCAAGGCGGGTTGGCTGTCGCTGACCCTCGGGATGATGTTGG-3′. The DNA template was linearized vector containing the wild type G_sα cDNA (0.2–1 μg/ml). The PCR mixtures were denatured at 94 °C for 1 min, annealed at 65 °C for 1 min, and extended at 72 °C for 30 s for 20 cycles. PCR products were digested with HincII and Sse8387I and ligated into the pBluescript II SK containing G_sα as described above. Mutations were verified by DNA sequencing, and synthesis of full-length G_sα was confirmed by immune precipitation of in vitro translated products with RM antibody. In vitro transcription/translation was performed on G_sα plasmids as described previously (18) using the TNT coupled transcription/translation system from Promega, with the exception that in most experiments no RNase inhibitor was added. We have found that deletion of this component reduces nonspecific initiation from downstream Met codons with no detectable loss of full-length G_sα.

Adenyl Cyclase Assays—Wild type and mutant G_sα in vitro transcription/translation products (10 μl of translation medium) were constituted into 25 μg of purified S49 cec- plasma membranes and tested for stimulation of adenyl cyclase in the presence of various concentrations of GMP (1 nM) and GTP (10 μM) and GDP were added to attain final concentrations of 200 μM GMP and GTP. The zero time point, trypsin was added prior to incubation buffer with 100 μM EDTA, 100 μM DTT, 100 μM NaCl, 0.1% Lubrol-PX, and additions as described previously (15). The background activity was determined from mock transcription/translation reactions and were subtracted for final presentation of the data. Data were normalized to the relative amount of G_sα synthesis as determined by quantitation of [35S]methionine-labeled in vitro transcription/translation products.

Trypsin Protection Assays—Limited trypsin digestion of in vitro translated G_sα was performed as described previously (16). Briefly, 1 μl of in vitro translated [35S]methionine-labeled G_sα was incubated in incubation buffer (20 mM HEPES, pH 8.0, 10 mM MgCl_2, 1 mM EDTA, 1 mM DTT) with or without 100 μM GTPγS or 10 mM NaF/10 μM AlCl_3 at various temperatures for 1 h and then digested with 200 μg/ml tosylphenylalanyl chloromethyl ketone-treated trypsin for 5 min at 20 °C. In some experiments, GMP was also included in the preincubation buffer. Aliquots 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 PhosphoImager analysis. The percentage of protection is the signal in the 38-kDa protected band divided by the signal in the undigested full-length G_sα band times 100. For experiments examining the time course of GDP displacement, [35S]methionine-labeled in vitrotranslated were incubated with 2 mM GDP for 1 h at 30 °C; chilled on ice and diluted 20-fold into incubation buffer with 100 μM GTPγS, and then transferred to a 30°C water bath. At the indicated times, aliquots were removed, and trypsin and GDP were added to attain final concentrations of 200 μg/ml and 1 mM, respectively. For the zero time point, trypsin was added prior to transfer to 30 °C. After the addition of trypsin, the samples were immediately placed in an ice water bath and incubated for 1 h. GTPγS binds to a negligible degree within 1 h at 0 °C (data not shown).

Sucrose Density Gradient Centrifugation—[35S]methionine-labeled G_sα was synthesized, and rate zonal centrifugation was performed on linear 5–20% sucrose gradients (200 μl) as described previously (18, 27). Gradients were prepared in 20 mM HEPES, pH 8.0, 1 mM MgCl_2, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 0.1% Lubrol-PX, and additions as described previously (15). The fractions were obtained and analyzed by SDS-polyacrylamide gel electrophoresis, and the relative amount of G_sα in each fraction was quantified as described previously (18). Gβγ was isolated from bovine brain (28).

Expression and Purification of G_sα from E. coli—Plasmid pQE60 containing the long form of bovine G_sα cDNA with a hexahistidine extension at the carboxyl terminus was a generous gift of A. G. Gilman.
and R. K. Sunahara. The Arg258 residue was mutated by site-directed mutagenesis using the Quickchange kit (Stratagene). After mutagenesis, each cDNA was sequenced to confirm the presence of the desired mutation and to rule out PCR artifacts. After transformation into *E. coli* strain JM109, cultures were grown, Gα expression was induced, and cleared lysates were prepared as described previously (29). His-tagged Gα proteins were purified on 2.5-ml Ni²⁺-nitrilotriacetic acid resin columns (Qiagen) equilibrated with TβP buffer (50 mM Tris-HCl, pH 8.0, 20 mM β-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride). Cleared lysates were loaded onto each column, and then each column was washed with 25 ml of TβP (TβP buffer supplemented with 50 mM GDP) containing 500 mM NaCl, followed by 40 ml of TβPG containing 50 mM NaCl and 10 mM imidazole. Gα was eluted with TβPG containing 50 mM NaCl, 150 mM imidazole, and 10% glycerol and then exchanged into 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM DTT, 50 μM GDP, and 10% glycerol and stored at −80 °C at greater than 1.5 mg/ml.

Guanine Nucleotide Binding Assays—Assays measuring the rate of binding of GTP-S were performed as described previously (30). Briefly, 25 mM purified Gα was incubated with 1 μM [35S]GTP-S (30,000 cpm/pmol) in 25 mM HEPES, pH 8.0, 1 mM EDTA, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT, and 0.01% Lubrol-PX in a final volume of 2 ml. At various times, 50-μl aliquots were removed and diluted with 2 ml of ice-cold stop solution (25 mM Tris-HCl, 100 mM NaCl, 25 mM MgCl₂, and 10% glycerol) and maintained on ice until all samples were collected. Samples were then filtered under vacuum through nitrocellulose filters (Millipore Corp.), washed twice with 10 ml of stop solution without GTP, and dissolved in 10 ml of scintillation mixture. *k*_*off* values for GTP-S binding were calculated using GraphPad Prism software.

**RESULTS**

**Identification of Gα Biochemical and Genetic Defect in an AHO Patient**—The patient had somatic features of AHO but no evidence of hormone resistance. Gα bioactivity in the patient’s erythrocyte membranes measured by the cyc-2 assay in the presence of isoproterenol (10 μM) and GTP (10 μM) was 52% of that present in normal subjects. Gα expression in these same membranes was 50% of normal based upon quantitative immunoblotting. The patient’s mother and father, who do not have clinical evidence of AHO and who do not have a GNAS1 mutation (see below) showed no decrease in Gα bioactivity or Gα expression (data not shown). The GNAS1 gene was screened for mutations by temperature gradient gel electrophoresis analysis of PCR-amplified genomic DNA fragments encompassing GNAS1 exons 2–13 and their intron-exon splice junctions (15, 24). Temperature gradient gel electrophoresis analysis of a genomic fragment encompassing GNAS1 exons 10 and 11, which was amplified from the patient’s genomic DNA, revealed abnormally migrating bands that were not present in either parent’s sample or in numerous other normal control or patient samples (data not shown). By direct sequencing of the genomic DNA fragment (Fig. 1), the patient was shown to have a heterozygous single base substitution (C to T) within the coding region of exon 10 that encodes the substitution of tryptophan (TGG) for arginine (CGG) at codon 258 (Arg258). Arg258 is within residues within switch 2 (most likely Arg228 and Arg231, based upon sequence homology with transducin) are sensitive to tryptophan digestion, leading to specific defects in activation by activated receptor or AlF₄⁻. 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 Gα and induce the active conformation (31). 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 small molecular weight fragments. When Gα attains the active conformation, these residues are inaccessible to trypsin digestion (7); therefore, trypsinization of activated Gα generates a partially protected 38-kDa product. Wild type Gα was well protected by AlF₄⁻ or GTPγS at temperatures up to 37 °C (Fig. 3, Table II).
**TABLE I**

| Gα mutation | GTP (100 μM) | Isoproterenol (10 μM) + GTP (100 μM) | GTP-αS (100 μM) | AlF₄⁻ |
|-------------|--------------|-------------------------------------|----------------|-------|
| Wild type   | 20 ± 6       | 231 ± 6                             | 167 ± 7        | 350 ± 41 |
| R258W       | 24 ± 5       | 73 ± 9 (32 ± 4)                     | 155 ± 7 (93 ± 6) | 177 ± 12 (49 ± 7) |
| R258A       | 8 ± 1        | 56 ± 7 (24 ± 3)                     | 221 ± 4 (132 ± 6) | 223 ± 19 (62 ± 9) |

* 10 mM NaF, 10 μM AlCl₃, and 100 μM GDP.

At 37 °C, GTP-αS was able to protect Gα-R258A and Gα-R258W to levels of 85 and 68% of wild type Gα, respectively (Fig. 3, Table II). Consistent with the results of the cyc- reconstitution assays, AlF₄⁻ was less effective than GTP-αS in protecting either mutant from trypsin digestion at higher temperatures, with Gα-R258W being more severely affected than Gα-R258A (Fig. 3, Table II). AlF₄⁻ protected Gα-R258A normally at 25 and 30 °C but only about 40% as well as wild type Gα at 37 °C. For Gα-R258W, trypsin protection by AlF₄⁻ was 70, 59, and 6% of wild type Gα at 25, 30, and 37 °C, respectively. These data demonstrate that both mutants are capable of attaining the activated conformation with GTP-αS or AlF₄⁻ (although for AlF₄⁻ more efficiently at lower temperatures). The somewhat decreased trypsin protection of Gα-R258W in the presence of GTP-αS at 37 °C or AlF₄⁻ at lower temperatures may be due to steric effects resulting from the bulky tryptophan side chain.

Substitution of Gα Arg²⁵⁸ Leads to Decreased Affinity for GDP—GTP-αS was a more effective activator of both mutants than AlF₄⁻ at 37 °C. It is possible that both mutants have decreased affinity for AlF₄⁻ or activate poorly when GDP and AlF₄⁻ are bound. Another possibility is that at higher temperatures the mutants bind GDP more poorly, which would result in decreased activation by AlF₄⁻ since GDP binding is a prerequisite for AlF₄⁻ binding and activation. To address these possibilities, the effect of increasing the concentration of GDP on AlF₄⁻ induced trypsin protection of Gα-R258W and R258A was determined (Fig. 3, Table II). In the presence of increased GDP concentrations, the ability of each mutant to be protected by AlF₄⁻ was partially or fully restored to normal. The effect was dose-dependent with maximum protection attained at a GDP concentration of 2 mM (data not shown). These data suggest that decreased protection of the mutants by AlF₄⁻ is not due to a specific defect in AlF₄⁻ binding but rather to decreased ability of the mutants to maintain the GDP-bound state at higher temperatures. GTP was equally effective in enhancing protection in the presence of AlF₄⁻ (data not shown). In the absence of AlF₄⁻, no protection was observed with either GDP or GTP. The latter observation suggests that the mutants have intact GTPase function.

We next indirectly determined the relative rate of GDP release in the inactive state from both mutants and wild type

3 We estimate that the carryover guanine nucleotide from the in vitro transcription/translation reaction into the trypsin protection assays is 50–100 μM, based on the concentrations required to support coupled transcription and translation reported by Craig et al. (40). This concentration is well above the the Kₘ of Gα for guanine nucleotides (~1 μM) (41). The distribution between GDP and GTP is unknown, but presumably more GDP would be present due to the nucleotide-regenerating system present in the translation reaction. However, we have determined that GTP is equally effective in the trypsin protection assay when AlF₄⁻ is used (data not shown). The carryover into the samples loaded onto sucrose gradients (Fig. 6) is estimated to be 240–320 μM.

Gα, [³⁵S]methionine-labeled in vitro translation products were preincubated with 2 mM GDP and then diluted 20-fold into a solution with 100 μM GTP-αS, and the level of trypsin protection was determined at various time points (Fig. 4). Since the rate of GTP-αS binding and activation is limited by the rate of GDP release, the rate of increase of trypsin protection is a function of the rate of GDP release (17, 32, 33). Wild type Gα reached maximum protection by 8–10 min, whereas for both Gα-R258A and R258W equal maximal protection was achieved by 2 min (the first time point examined). These data suggest that the rate of GDP release from the mutants in the inactive state is at least 4–5 times greater than the rate of GDP release from wild type Gα.

To confirm these observations and to more accurately determine the relative rates of GDP release, we expressed and purified bovine wild type Gα and Gα-R258A from E. coli and...
Table II

| Temperature | Treatment | Gs<sub>a</sub> Mutation in Albright Osteodystrophy |
|-------------|-----------|-------------------------------------------------|
| 25 °C       | AlF<sub>4</sub> | % protection % of wild type % of wild type |
|             | R258W     | 60 ± 4 70 ± 6 107 ± 10 |
|             | R258A     | 66 ± 4 75 ± 7 104 ± 8 |
| 30 °C       | AlF<sub>4</sub> | 67 ± 5 59 ± 7 87 ± 10 |
|             | R258W     | 66 ± 4 64 ± 3 85 ± 3 |
|             | R258A     | 48 ± 2 52 ± 2 40 ± 3 |
| 37 °C       | AlF<sub>4</sub> | 57 ± 5 48 ± 8 78 ± 4 |
|             | R258W     | 68 ± 4 68 ± 4 85 ± 3 |
|             | R258A     | 48 ± 2 52 ± 2 40 ± 3 |

These data were obtained from four experiments of the type presented in Fig. 3. The amount of the 38-kDa trypsin-stable Gs<sub>a</sub> fragment was determined by PhosphorImager analysis and for wild type Gs<sub>a</sub> is expressed as a percentage of undigested Gs<sub>a</sub> (mean ± S.E.). No protection was observed when AlF<sub>4</sub> and GTP-Gs were excluded. Maximum trypsin protection has a theoretical limit of 71%, based on the removal of Gs<sub>a</sub> on digestion was terminated, and the percentage of trypsin protection was determined as described under “Experimental Procedures.” The data for Gs<sub>a</sub> are expressed as percentage of wild type under each condition (mean ± S.E.).

a The percentage protection of Gs<sub>a</sub> R258W was significantly less than that of wild type Gs<sub>a</sub> under all conditions (Student’s t-test).

b The percentage protection of Gs<sub>a</sub> R258A was significantly less than that of wild type Gs<sub>a</sub> at 37 °C with all agents and at 30 °C in the presence of AlF<sub>4</sub> and GDP (Student’s t-test).

c,d The percentage protection of Gs<sub>a</sub> R258A was significantly less than that of wild type Gs<sub>a</sub> at 37 °C with all agents and at 30 °C in the presence of AlF<sub>4</sub> and GDP (Student’s t-test).

![Fig. 5. Time course of GTP-Gs binding to purified Gs<sub>a</sub>. Bovine wild type Gs<sub>a</sub> and Gs<sub>a</sub> R258A with carboxyl-terminal hexahistidine extension were expressed and purified from E. coli, and the rate of GTP-Gs binding for each was determined. Wild type Gs<sub>a</sub> (●) and Gs<sub>a</sub> R258A (●) were incubated in 1 μM [35S]GTP-Gs (1-30,000 cpm/μmol) at 20 °C in 25 mM HEPEs, pH 8.0, 1 mM EDTA, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT, and 0.001% Lubrol-PX. At the indicated times, the reaction was terminated, and bound GTP-Gs was determined as described under “Experimental Procedures.” Data were fit (r<sup>2</sup> > 0.995) to the equation B = B<sub>max</sub>(1 - e<sup>-kt</sup>), where B represents GTP-Gs bound at time t, B<sub>max</sub> represents maximal GTP-Gs bound, and t represents the apparent on rate. Each point is the mean ± S.D. of triplicate determinations. The B<sub>max</sub> values were 3.0 pmol for wild type Gs<sub>a</sub> and 1.3 pmol for Gs<sub>a</sub> R258A. k<sub>app</sub> values averaged from three independent experiments were 0.04 ± 0.00 min<sup>-1</sup> for wild type Gs<sub>a</sub> and 0.36 ± 0.02 for Gs<sub>a</sub> R258A.](image-url)
GDP or 100 μM GTPγS (Fig. 6A). When in vitro translations of G_sα R258A and R258W were held on ice, the gradient profile was virtually the same as that of wild type G_sα and consistent with the overall proper conformation (sedimentation coefficient ~3.7 S; Ref. 18). When preincubated on ice with purified bovine brain βγ, the sedimentation coefficient of both mutants and wild type G_sα increased from 3.7 to 5.0 S (Fig. 6B), demonstrating that at low temperatures each mutant maintained the ability to interact with βγ (18). Similar results were obtained with G_sα R258W after incubation with βγ at 30 °C (data not shown).

When preincubated for 1 h at 30 °C, some of the G_sα R258W protein was present in a ~6.3 S or higher peak, presumably due to denaturation and aggregation, while the profiles for G_sα R258A and wild type G_sα were the same as after the 0 °C preincubation. After preincubation for 1 h at 37 °C, both mutants displayed a more severe pattern of aggregation, with most of each located in the latter half of the gradient with sedimentation values greater than 7.2 S. This pattern is most likely the direct result of denaturation, although we have not conclusively proven that this material in fact represents denatured G_sα. Wild type G_sα, although showing some loss of the native 3.7 S peak, was significantly more stable than either mutant at 37 °C. When 2 mM GDP or 100 μM GTPγS was included in the preincubation, G_sα R258A (as well as wild type G_sα) was fully protected from the denaturing effects of mild heat treatment (37 °C). Denaturation of G_sα R258W at 37 °C was mostly prevented by 100 μM GTPγS and to a somewhat lesser extent by 2 mM GDP. AlF_4 provided no stabilization of G_sα R258W above that observed with 2 mM GDP, whereas AlF_4 alone was sufficient to stabilize wild type completely against denaturation at 37 °C (data not shown). These results suggest that both G_sα R258W and R258A are more thermolabile than wild type G_sα and that this is due to decreased affinity for guanine nucleotides, since the addition of guanine nucleotides can partially or fully reverse denaturation of these mutants at higher temperatures. For G_sα R258W, decreased protection by AlF_4 and increased thermolability were observed at both 30 and 37 °C, while for G_sα R258A these abnormalities were only observed at 37 °C. For both mutants, these defects were reversed by the addition of excess GDP. It therefore appears that both mutants have a similar underlying biochemical abnormality (decreased GDP binding) but that the abnormality is more severe in G_sα R258W than in G_sα R258A.

DISCUSSION

We identified a heterozygous missense mutation (G_sα R258W) in a patient with AHO and PPHP. This mutation, as well as a mutation that replaces Arg^258 with alanine (G_sα R258A), encodes a G_sα protein with impaired function. Both mutants stimulated adenyl cyclase and attained the active conformation normally in the presence of GTPγS. However, their ability to stimulate adenyl cyclase in the presence of AlF_4 or activated receptor (isoproterenol plus GTP) was significantly attenuated. Consistent with this result, GTPγS was better able than AlF_4 to protect both mutants from trypsin digestion. Excess GDP was able to partially or fully restore AlF_4 protection, suggesting that decreased activation by AlF_4 was due to decreased binding of GDP in the inactive state. Both mutants were shown to have a markedly increased rate of guanine nucleotide turnover, reflecting an increased rate of GDP release. Defective guanine nucleotide binding in these mutants probably results from disruption of interactions between the helical and GTPase domains (see below). Although the overall conformation (and ability to bind βγ) of both mutants was normal at lower temperatures, both denatured more rapidly at physiological temperatures. The mutants were pro-

![Fig. 6. Sucrose density gradient centrifugation of G_sα in vitro translation products. A. [35S]methionine-labeled in vitro translations of both G_sα Arg^258 mutants and wild type G_sα were preincubated for 1 h at 0 °C, 30 °C, or 37 °C in the presence of an added 2 mM GDP. The samples were layered over a 200-μl sucrose density gradient and centrifuged for 1 h at 4 °C and 436,000 × g. B. [35S]methionine-labeled in vitro translations of both G_sα Arg^258 mutants and wild type G_sα were preincubated for 1 h at 37 °C in the presence of an added 2 mM GDP. The samples were layered over a 200-μl sucrose density gradient and centrifuged for 1 h at 4 °C and 436,000 × g. Fractions (6 μl each) were collected, and odd numbered fractions were analyzed by SDS-polyacrylamide gel electrophoresis and PhosphorImager analysis (18). The data are expressed as the percentage of total G_sα present in each fraction. Fraction 1 represents the top of the gradient. For samples in which either GDP or GTPγS was included in the preincubation, it was also present in the gradient itself. The positions of the peak concentrations of standard proteins in the gradient are shown at the top and are as follows: soybean trypsin inhibitor, 2.3 S; carbonic anhydrase, 2.7 S; ovalbumin, 3.5 S; bovine serum albumin, 4.4 S, and phosphorylase b, 8.4 S. B. [35S]methionine-labeled in vitro translations of both G_sα Arg^258 mutants and wild type G_sα were preincubated for 1 h at 0 °C in the presence or absence of purified bovine brain βγ at 20 μg/ml and subjected to sucrose density gradient centrifugation. In the presence of βγ, both mutants and wild type G_sα shifted to a peak of ~5 S. For clarity, each mutant is shown only in the presence of βγ.
suggesting that substitution of Arg 258 is disrupting specific sections of the conserved guanine nucleotide binding motif NKXD (Fig. 7). Codina and Birnbaumer (20) showed that mutating either residue Arg 258 results in normal activation by GTPγS but decreased activation by AlF4− or activated receptor, similar to what we observed with Arg258 substitutions. Since both Asp173 and Gln170 lie within the interhelical loop between αD and αE, which is in close contact with the GTPase domain, it is possible that mutations of Arg258 may also disturb the salt bridge between Asp173 and Lys293, which would lead to defective activation. Repositioning of the conserved lysine in the NKXD motif could directly result in decreased guanine nucleotide binding by altering the conformation of the guanine nucleotide binding pocket.

The exact mechanism by which substitution of Arg258 leads to defective receptor-mediated activation is not well defined. Several regions in transducin that are critical for receptor binding and activation were identified by scanning mutagenesis, although the switch 3 region was not mutagenized in that study (37). Decreased receptor-mediated activation could be the direct result of decreased binding to βγ or receptor. The Arg258 mutants were capable of binding to βγ. Li and Cerione (13) demonstrated that deletion of the whole switch 3 region of transducin had no effect on interactions with βγ or its receptor (rhodopsin). Moreover, crystal structures of transducin and Gα do not demonstrate direct interactions between switch 3 and βγ (9, 11). Disturbance of the salt bridge between the helical and GTPase domains results in severely decreased receptor activation (see above; Ref. 20). Studies on GαGαGβGγ chimeras suggest that interactions between switch 3 residues and the helical domain are critical not only to maintain the basal state but also for receptor-mediated activation (38).

Decreased receptor activation could be the direct result of a GTP binding defect, as demonstrated by mutation of a conserved switch 2 arginine (Gα R231H) in an AHO patient (19, 39). Similar to the Arg258 mutations, this mutation leads to normal GTPγS-mediated but decreased AlF4− and receptor-mediated activation. In the GTP-bound state, Arg231 interacts with several residues in the α3 helix and switch 3 regions, including the conserved glutamic acid residues Glu259 and Glu268. Disrupting these interactions presumably leads to a GTP binding defect, resulting in decreased receptor-mediated activation (19). It is of interest that mutation of Glu259 results in a similar phenotype.6 In contrast to the Arg258 mutants, Gα R231H did not appear to have a GDP binding defect. GTP alone did not protect either Arg258 mutant or wild type Gα from trypsin protection, but it was able to restore trypsin protection of Gα R258W in the presence of AlF4− with a similar dose response as GDP (data not shown), suggesting that GTP as well as GDP binding may be altered by substitution of Arg258. While GTPγS binding might also be predicted to be decreased, it has been proposed that defective GTP binding may result in a conditional activation defect that is only obvious in states in which guanine nucleotide binding is destabilized (such as interaction with activated receptor; Ref. 19). It was postulated that decreased activation of Gα R231H by AlF4− is due to an inability of the mutant to stably maintain the GDP-AlF4− complex in the guanine nucleotide binding pocket, and it was demonstrated that the complex could be stabilized by high concentrations of Mg2+ (19). The Arg258 mutants showed decreased activation by AlF4− even in the presence of 10 mM Mg2+. Moreover the defect was corrected with high concentrations of GDP, suggesting that for Gα R258W and R258A, decreased activation by AlF4− is due to decreased GDP binding.

In summary, genetic analysis of the gene encoding Gα in an

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4 Asp173 and Lys293 are referred to as Asp158 and Lys278, respectively, in Ref. 20, since in that paper the numbering was based upon the Gα-3 isoform (24).

5 D. Warner and L. S. Weinstein, unpublished observations.
AHO patient identified a residue in the switch 3 region that is critical for normal guanine nucleotide binding and receptor activation. This residue interacts with a residue in the helical domain and underscores the importance of interdomain interactions in both guanine nucleotide binding and receptor-mediated activation. This study demonstrates that identification and analysis of G\textsubscript{s} mutations in AHO patients can further our understanding of G protein function.

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