Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 617–621, January 1988
Medical Sciences

Genetic deficiency of the α subunit of the guanine nucleotide-binding protein G_s as the molecular basis for Albright hereditary osteodystrophy

(G proteins/adenylyl cyclase/hormone resistance/pseudohypoparathyroidism)

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Communicated by M. Daniel Lane, September 30, 1987 (received for review July 16, 1987)

ABSTRACT **Patients who have pseudohypoparathyroidism type I associated with Albright hereditary osteodystrophy commonly have a genetic deficiency of the α subunit of the G protein that stimulates adenylyl cyclase (αG_s) (ATP pyrophosphate-lyase, EC 4.6.1.1). To discover the molecular mechanism that causes αG_s deficiency in these patients, we examined eight kindreds with one or more members affected with Albright hereditary osteodystrophy or pseudohypoparathyroidism and αG_s deficiency. In these families, αG_s deficiency and the Albright hereditary osteodystrophy phenotype were transmitted together in a dominant inheritance pattern. Using a cDNA hybridization probe for αG_s, restriction analysis with several endonucleases showed no abnormalities of restriction fragments or gene dosage. RNA blot and dot blot analysis of total RNA from cultured fibroblasts obtained from the patients revealed ≈50% reduced mRNA levels for αG_s in affected members of six of the pedigrees but normal levels in affected members of the two other pedigrees, compared to mRNA levels in fibroblasts from unaffected individuals. By contrast, mRNA levels encoding the α subunit of the G protein that inhibits adenylyl cyclase were not altered. Our findings suggest that several molecular mechanisms produce αG_s deficiency in patients with pseudohypoparathyroidism type Ia and that major gene rearrangements or deletions are not a common cause for αG_s deficiency in pseudohypoparathyroidism type I.**

The G proteins, a family of guanine nucleotide-binding proteins, mediate numerous transmembrane hormonal and sensory transduction processes in eukaryotes. These proteins are essential for coupling the extracellular signals generated by activated membrane receptors to intracellular effector enzymes and ion channels (1). The G proteins share a heterotrimeric protein structure with subunits designated α, β, and γ. The α subunits (αG), which are distinctive to each G protein, contain the GTP binding site, confer specificity for binding of the G protein to specific membrane-associated receptors, and have GTPase activity. At least two different G proteins modulate activity of the hormone-sensitive adenylyl cyclase (ATP pyrophosphate-lyase, EC 4.6.1.1): αG_s is responsible for stimulation of catalytic activity, and αG_i mediates inhibition of the enzyme (2). In mammalian cells, hormones stimulate cAMP synthesis via activation of αG_s (2).

Decreased activity of αG_s occurs in most patients who have Albright hereditary osteodystrophy (AHO) (3–5). AHO is a heritable disorder characterized by a distinctive body habitus that includes round facies, short stature, obesity, brachydactyly, and heterotopic subcutaneous ossification. In addition, most patients with AHO have pseudohypoparathyroidism type Ia (PHP-Ia), a metabolic disorder characterized by resistance to parathyroid hormone (6) and other hormones that act by stimulating adenylyl cyclase activity (7), mental retardation (8), and impaired olfaction (9). In patients with PHP-Ia, decreased αG_s activity has been demonstrated in all tissues tested (10–12), including plasma membranes from kidney (a target tissue for parathyroid hormone) (13) and cultured cells (14–16). The generalized biochemical defect of αG_s in these patients apparently results in reduced responsiveness of diverse tissues to hormones and neurotransmitters that act by stimulating adenylyl cyclase (7). However, in the same kindreds, some members affected by AHO lack hormone resistance despite a reduction in αG_s activity that is similar to that in their PHP-Ia relatives (3, 5). This variant of AHO has been termed pseudo-pseudohypoparathyroidism (pseudo-PHP) to emphasize the morphologic similarity (i.e., AHO) but metabolic dissimilarity (i.e., normal hormone responsiveness) with PHP-I (17). The dominant, apparently autosomal (18), inheritance pattern that is found in most pseudohypoparathyroid (PHP) pedigrees (3) suggests that the causative molecular lesion affects one of two genes that encode αG_s in the human genome.

The availability of cDNA probes encoding the α subunits of G_s and G_i (19) permitted investigation of the molecular basis of αG_s deficiency in patients with AHO. We report here that αG_s deficiency is not due to a detectable gene deletion or rearrangement of the αG_s structural gene. From the amount of mRNA for αG_s present in cultured fibroblasts, patients with AHO and PHP-Ia can be divided into two groups: (*i*) those with reduced levels of αG_s mRNA, and (*ii*) those with normal levels of this mRNA. Our findings suggest that deficiency of the αG_s protein results from several different gene mutations.

MATERIALS AND METHODS

Subjects. We studied 13 subjects with AHO and αG_s deficiency from eight families. The diagnosis of AHO was

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Abbreviations: AHO, Albright hereditary osteodystrophy; PHP, pseudohypoparathyroidism; PHP-I, PHP type I; PHP-Ia, PHP type Ia, which includes PHP with αG_s deficiency; pseudo-PHP, pseudo-pseudohypoparathyroidism; αG_s, α subunit of the G protein that stimulates adenylyl cyclase; αG_i, α subunit of the G protein that inhibits adenylyl cyclase.

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based on the presence of obesity, short stature, round facies, heterotopic ossification, and brachydactyly. Detailed endocrinologic studies on many of these patients have been previously reported (5, 7). Six unrelated and unaffected individuals were also studied. Informed consent was obtained from all participants.

αG_s Activity in Cell Membranes. Plasma membranes were prepared from erythrocytes (8) or cultured fibroblasts (13) and stored at −70°C before assay. αG_s activity in these membranes was evaluated using a complementation assay (13) in membranes from a cyc$^-$ murine S49 lymphoma cell line (no. 94.15.1), which lacks αG_s. Detergent extracts made from the membrane preparations were used to reconstitute the hormonal responsiveness of adenylyl cyclase to isoproterenol in membranes from cyc$^-$ S49 murine lymphoma cells.

Culture of Fibroblasts. Skin biopsies were obtained from each subject, and fibroblast cultures were established in Richter's improved modified Eagle's medium supplemented with fetal bovine serum (10%, vol/vol), insulin (100 pM), penicillin (100 units/ml), and streptomycin (100 μg/ml) as described by Levine and co-workers (13). Membranes were prepared from fibroblasts between the 5th and 15th subculture.

RNA Isolation and Analysis. Total cellular RNA was isolated from confluent fibroblast monolayer cultures (20) and measured using spectrophotometry. RNA yields from cells of unaffected subjects were comparable to those from cells of subjects with αG_s deficiency. For RNA blot analysis, 10 μg of RNA was denatured with formaldehyde, size-fractionated by electrophoresis in agarose gels containing 2.2 M formaldehyde (21), and transferred to GeneScreen*Plus* (DuPont). Filters were hybridized overnight at 42°C in hybridization solution containing 1 M NaCl, 50% formamide (vol/vol), 50 mM Tris·Cl (pH 7.5), 1.0% $NaDodSO_4$, 0.25 mg of denatured salmon sperm DNA per ml, 10% dextran sulfate, 0.5 mg of heparin sulfate per ml, and radiolabeled hybridization probe (10^6 dpm/ml). Blots were then washed twice for 10 min at 25°C in a solution containing the following: 0.3 M sodium chloride, 0.03 M sodium citrate (pH 7.5), and 1% $NaDodSO_4$; then blots were washed twice for 1 hr at 65°C in 30 mM sodium chloride, 3 mM sodium citrate, and 1% $NaDodSO_4$. Autoradiography was completed at −70°C on Kodak XAR-5 film using an intensifying screen.

Dot Blot Analysis. The relative amounts of αG_s mRNA in cultured fibroblasts from subjects with αG_s deficiency were determined by dot blot analysis. Equal quantities of total cellular RNA (6 μg) from fibroblasts of each subject were denatured with formaldehyde and serially diluted before application to nylon filters mounted on a 96-well manifold (Schleicher & Schuell). The filters were hybridized as described above with a radiolabeled αG_s cDNA probe. The probe was then removed prior to rehybridization with either radiolabeled αG_i or γ-actin cDNA probes. Hybridization intensity for each cDNA probe was measured by scanning the autoradiographs using two-dimensional transmission densitometry (22). The relative amount of αG_s mRNA present was expressed as a ratio of hybridization signal intensities: $\alpha G_s/\alpha G_i$ or $\alpha G_s/\gamma$-actin. Similar studies were completed with RNA isolated from the fibroblasts of individuals unaffected by AHO or PHP. Two or more independent measurements were made on all RNA samples.

Isolation and Analysis of Genomic DNA. DNA was extracted from peripheral blood leukocytes (22). Restriction endonucleases were purchased from Bethesda Research Laboratories or New England Biolabs and used according to the suppliers' directions. Each DNA sample (5 μg) was digested with endonuclease (3–10 units/μg of DNA) for 15 hr, size-fractionated by electrophoresis on an agarose gel, transferred to GeneScreen*Plus*, and probed with radiolabeled cDNAs (23).

DNA Hybridization Probes. Plasmid pGM13.1 contained a 1110-base-pair (bp) insert encoding the 3′ terminus (nucleotides 398–1492) of the murine αG_s in the *Eco*RI site of pUC13 (19); plasmid pGM1.3 contained a full-length insert of 1350 bp that encodes murine αG_i (19). The coding portion of the murine αG_s cDNA clone (19) has 95% base-sequence identity with that of the human cDNA encoding αG_s (24). A cDNA clone encoding human γ-actin was supplied by D. W. Cleveland (Johns Hopkins University) (25). Hybridization probes were prepared by radiolabeling plasmid DNA by

Table 1. Patient descriptions with cellular G_s and mRNA levels

Subject (kindred)*	Clinical diagnosis	αG_s activities,† %		RNA hybridization intensity ratios‡, %	
		RBC	Fibroblast	$\alpha G_s/\gamma$-actin	$\alpha G_i/\gamma$-actin
AHO group I					
1 (B-I-2)	Pseudo-PHP	55	52	12	75
2a (C-I-2)	Pseudo-PHP	48	50	55	154
2b (C-II-1)	PHP-Ia	37	7	54	109
3a (D-II-2)	PHP-Ia	44	20	10	69
3b (D-I-2)	Pseudo-PHP	46	ND	39	139
4a (E-II-3)	PHP-Ia	63	41	51	79
4b (E-I-2)	Pseudo-PHP	55	39	62	84
5 (I-2)	Pseudo-PHP	53	52	53	64
6a (H-II-1)	PHP-Ia	47	58	53	100
6b (H-II-3)	PHP-Ia	54	52	43	84
		50 ± 7	41 ± 17	43 ± 18	96 ± 30
AHO group II					
7 (A-II-2)	PHP-Ia	22	35	80	108
8a (F-I-2)	PHP-Ia	41	19	105	90
8b (F-II)	PHP-Ia	38	ND	110	118
		34 ± 10		98 ± 16	105 ± 14
Control subjects (n = 6)		ND	100 ± 21	100 ± 18	100 ± 18

ND, not done; RBC, red blood cells.
*Pedigrees and detailed endocrinologic studies of these patients have been reported (5, 7, 27).
†Expressed as percent of mean activity measured in cell membranes from unaffected individuals.
‡Expressed as percent of mean value obtained with RNA isolated from fibroblasts of unaffected subjects.

random primer extension (26) using [α-^{32}P]dCTP (10^9 dpm/μg of DNA).

Statistical Analysis. Measured differences were analyzed for statistical significance using a two-tailed Student's *t* test. Unless otherwise noted, data are expressed as $\bar{x} \pm$ SD.

RESULTS

Patient Characterization. The study population consisted of 13 patients with AHO from eight kindreds (Table 1). Categorization of patients to PHP-Ia or pseudo-PHP was based on the results of detailed endocrinologic studies; eight patients with PHP-Ia had characteristic clinical or biochemical resistance to one or more hormones, including subnormal urinary cAMP excretion in response to a standard i.v. infusion of parathyroid hormone (5, 7, 27). Five patients had pseudo-PHP based on morphologic criteria with a normal parathyroid hormone infusion study. Erythrocyte membranes from both groups of patients had ≈50% reduced αG_s activity compared to erythrocytes from unaffected, unrelated subjects. In fibroblast membranes from these patients, activity of αG_s was also similarly reduced in both groups (Fig. 1).

Restriction Blot Analysis of the αG_s Gene. To determine whether αG_s deficiency in PHP-Ia is due to a major deletion or rearrangement within or near the αG_s gene, DNA samples from affected and control individuals were digested with *Eco*RI, *Bam*HI, *Msp* I, *Pvu* II, or *Taq* I and subjected to Southern blot analysis using the cDNA probe encoding αG_s. For each restriction endonuclease used, DNA from the affected individuals examined produced αG_s restriction fragment patterns that were indistinguishable from the patterns produced by DNA samples from control subjects (Fig. 2, Table 2). The relative hybridization intensities of the αG_s restriction fragments were the same in DNA samples from AHO patients and controls. Absence of αG_s gene-dosage differences between DNA from patients with AHO and control individuals was confirmed (C.V.D. and M.A.L., unpublished observations) using cDNA probes that encode human αG_s (donated by A. D. Levinson, Genentech, South San Francisco, CA) and human prepro-parathyroid hormone (donated by H. M. Kronenberg, Harvard University, Cambridge, MA). Comparison of the hybridization intensity of the simple restriction patterns that were found using these two probes suggests that αG_s occurs as a single-copy gene in the human genome.

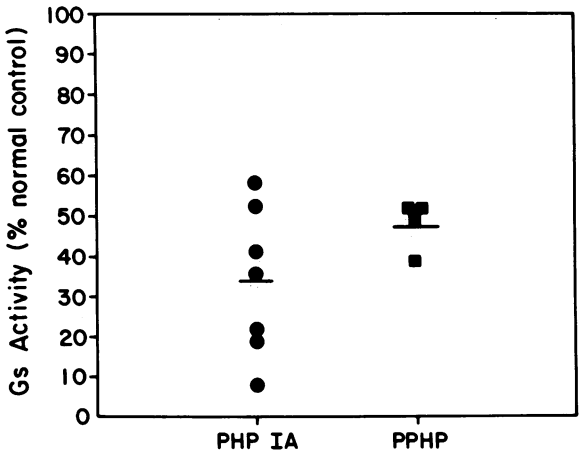

FIG. 1. Activity of αG_s in fibroblast membranes. Cell membranes were isolated from skin fibroblasts cultured from patients with AHO and PHP-I or pseudo-PHP (PPHP), and from unaffected controls. Results are expressed as % mean activity of control membranes.

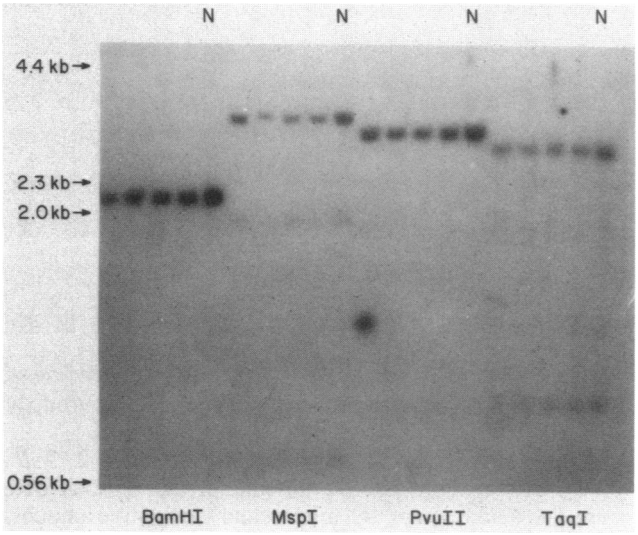

FIG. 2. Autoradiograph of Southern blot from DNA of patients with PHP-Ia probed with radiolabeled αG_s cDNA. N, DNA isolated from an unaffected individual and analyzed on the same blot. Digestion with *Eco*RI (data not shown) yielded a single 10.7-kilobase (kb) restriction fragment in all patients and normal subjects.

Analysis of αG_s mRNA. To examine the possibility that αG_s deficiency in PHP-Ia is due to defective expression of the structural gene for αG_s, cellular RNA isolated from fibroblast monolayers derived from all subjects was subjected to RNA blot analysis. The cDNA probe for αG_s hybridized only to a single mRNA band (1.9 kb) in RNA isolated from cells of either affected or normal subjects (Fig. 3*A*). By contrast, RNA from cyc$^-$ murine S49 lymphoma cells lacks the αG_s transcript (28). To confirm that comparable amounts of RNA from each sample were present on this blot, we rehybridized it with a cDNA probe encoding human γ-actin that demonstrated similar hybridization intensities among the samples (data not shown).

To measure the relative amount of αG_s mRNA present in fibroblasts from AHO individuals, we subjected RNA samples to dot blot analysis using cDNA probes encoding αG_s, αG_i, and γ-actin. Two-dimensional densitometry (22) of autoradiograms demonstrated a linear relationship between autoradiographic spot densities and the amount of RNA applied to the nylon membrane (data not shown). As all skin fibroblasts used in these studies were grown under the same culture conditions, the relative amounts of various mRNAs in these cells should be similar. From the relative amount of αG_s mRNA in their fibroblasts, the AHO kindreds could be separated into two distinct groups. Patients from five kindreds (AHO group I) had decreased amounts of αG_s mRNA relative to total RNA or mRNA encoding either αG_i or γ-actin; this group consisted of 10 subjects, five individuals with PHP-Ia and five with pseudo-PHP. Although there was some variation in the measured reduction in levels of αG_s mRNA, all affected members in these families had a reduction of mRNA levels. By contrast, the cells from patients in

Table 2. Restriction fragments of the αG_s gene in control subjects and in patients with AHO

Restriction endonuclease	Restriction fragment length, kb
*Eco*RI	10.7
*Bam*HI	2.1
Msp I	3.1, 1.9, 0.7
Pvu II	2.7
Taq I	2.7, 1.0

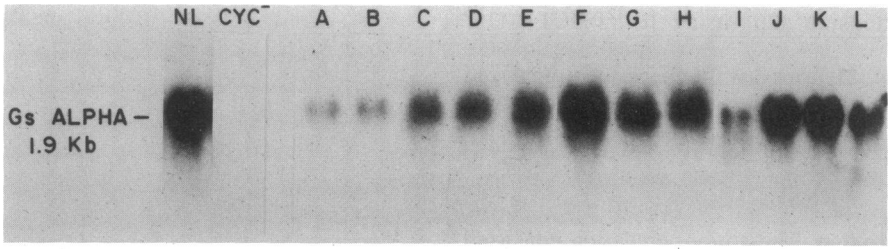

FIG. 3. Autoradiograph of RNA blot prepared using RNA isolated from cultured skin fibroblasts of patients with AHO that had been probed with radiolabeled αG_s cDNA. NL, normal subject; CYC⁻, RNA isolated from cyc⁻ S49 lymphoma cells; patients are described in Table 1: A, 1; B, 3a; C, 2a; D, 2b; E, 4a; F, 7; G, 4b; H, 5; I, 3b; J, 8a; K, 8b; L, 6b.

two pedigrees had normal amounts of mRNA encoding αG_s (AHO group II); all three affected members of this group had PHP-Ia (Table 1).

There was a highly significant correlation ($r = 0.79$, $P < 0.001$) between the relative amount of αG_s mRNA determined as a function of αG_i mRNA and the relative amount of αG_s mRNA determined as a function of γ-actin mRNA (Fig. 4). However, the relative amounts of αG_s mRNA did not correlate with either values of αG_s activity or clinical phenotype. Normal amounts of αG_s mRNA were present in all family members examined who lacked AHO (data not shown). In contrast to the results for αG_s mRNA, we found normal levels of αG_i mRNA in fibroblasts from all AHO patients.

DISCUSSION

All patients included in this study had several clinical characteristics of the AHO phenotype. In these eight pedigrees, reduced αG_s activity occurred only in individuals with AHO. Furthermore, αG_s was reduced similarly in membranes isolated from either erythrocytes or cultured fibroblasts. The finding of reduced αG_s activity in fibroblast membranes from patients with pseudo-PHP extends our previous observations of αG_s deficiency in fibroblasts from

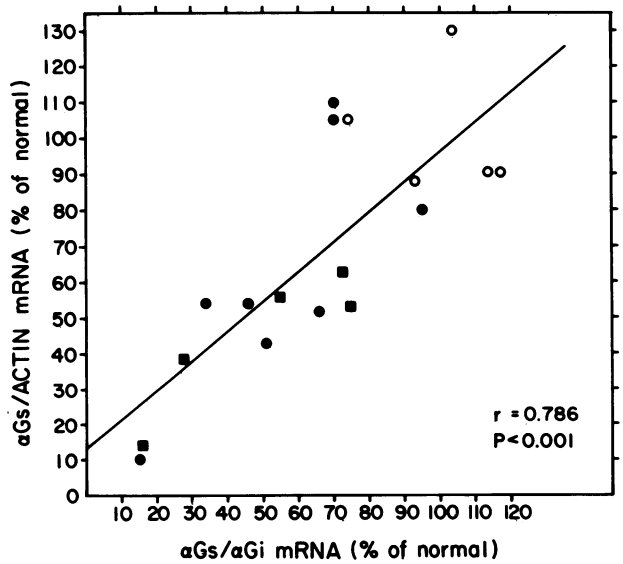

FIG. 4. Correlation of RNA hybridization intensity ratios for αG_s. Autoradiographic densities were measured for dot blots prepared from total RNA that had been isolated from cultured fibroblasts probed with radiolabeled cDNAs encoding either αG_s, αG_i, or γ-actin. A ratio of hybridization intensities was calculated for $\alpha G_s/\alpha G_i$ and $\alpha G_s/\gamma$-actin; this ratio was then standardized by dividing with the mean ratio found for the same probes in RNA samples from unaffected controls. ●, PHP-Ia individuals; ■, pseudo-PHP individuals; and ○, unaffected control individuals.

patients with PHP-Ia (14, 16). Although there was complete concordance between the occurrence of AHO and αG_s deficiency, characteristic endocrinopathies were not demonstrable in all of these individuals. This clinical heterogeneity may result from as yet incompletely metabolic modifiers or other influences (29).

The inheritance patterns of αG_s deficiency among these pedigrees is most consistent with an autosomal dominant pattern of genetic transmission. As an individual has only two αG_s genes, each subject affected by AHO has presumably inherited one αG_s allele that cannot produce functional αG_s protein. Our finding that restriction fragment patterns are unchanged on restriction blots of genomic DNA from patients with AHO suggests that αG_s deficiency did not result from large deletions or rearrangements within or near the αG_s gene. However, small deletions inside the structural gene, or deletions within the promoter region, cannot be excluded. Finally, lack of any gene-dosage effect on Southern blots makes it unlikely that a complete gene deletion caused αG_s deficiency in any of these pedigrees.

Distinct species of human G_s mRNA have been described that correspond to the 52,000 and 45,000 M_r forms of αG_s (24). Although the molecular basis for the existence of these multiple mRNA forms is not known, recent evidence suggests that they result from alternative RNA splicing of a primary transcript encoded by a single gene (24).

Of the eight pedigrees with AHO that were examined, reduced cellular levels of αG_s mRNA were found in the cultured fibroblasts from affected members in six kindreds. In these six pedigrees, αG_s deficiency appears to result from a defect in transcription or processing of mRNA encoding αG_s. These patients appear to be heterozygous for a pretranslational defect analogous to that in cyc⁻ S49 cells, where total deficiency of αG_s activity is associated with an absence of mRNA encoding αG_s (28). Levels of both the 52,000 and 45,000 M_r forms of αG_s are reduced to a similar degree in fibroblast membranes from patients with AHO (16), thus suggesting that both major forms of αG_s mRNA may be reduced. Elucidating the specific steps in transcription or RNA processing that account for this defect in patients with αG_s deficiency may require comparison of nucleotide sequences from genomic clones for αG_s isolated from these patients and from normal subjects and possibly direct studies of αG_s gene transcription.

The molecular basis for αG_s deficiency in the three patients who had normal levels of αG_s mRNA is more difficult to explain. Significantly, fibroblast cultures for assaying αG_s activity and for preparations of RNA were grown and harvested cotemporally. Thus, it is unlikely that spontaneous mutation, sampling error, clonal selection, or altered growth conditions account for our findings in these two kindreds. At least two mechanisms may explain the αG_s deficiency in these patients: (*i*) decreased translational efficiency of the defective αG_s mRNA, leading to diminished total synthesis of αG_s protein, and (*ii*) point mutations in the coding region that specify frame shifts, premature stop

codons, or amino acid substitutions that yield structurally abnormal or unstable αG_s proteins that are nonfunctional. The previous description of various mutant S49 lymphoma cells, including one with G_s activity that is not only diminished but also inhibits wild-type extracts in complementing adenylyl cyclase of cyc^- membranes (30), suggests that a variety of biochemical defects can reduce activity of cellular G_s. Similarly, a range of biochemical defects have been described in the low-density lipoprotein receptor of patients with familial hypercholesterolemia (31).

Finally, fibroblasts from patients with AHO had unchanged levels of mRNA encoding αG_i as assessed by comparison to levels of mRNA encoding γ-actin (Table 1). These results are consistent with our earlier demonstration of normal levels of αG_i protein in erythrocyte membranes from patients with PHP-Ia who had low αG_s activity (32). This finding further emphasizes the specificity of αG_s deficiency in these patients and makes improbable the explanation that differences in αG_i level account for distinction between PHP-Ia and pseudo-PHP. However, cDNAs encoding at least three very similar αG_i proteins have been identified in some mammalian tissues (33). These proteins may each have specific functional effects on adenylyl cyclase; alterations in their ratios, with a constant total concentration, could modify regulation of adenylyl cyclase by αG_s.

In conclusion, we have evaluated the molecular basis for αG_s deficiency in AHO. Our data indicate that at least two distinct mutation mechanisms produce the biochemical phenotype of αG_s deficiency. The challenge now will be to define the precise molecular defects that account for these abnormalities.

This work was supported in part by grants from the March of Dimes (M.A.L., H.R.B., and C.V.D.), and by National Institutes of Health Grants DK-36085, GM-27800, GM-28310, and DK-34281.

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