Pseudohypoparathyroidism, type Ia (PHP-Ia) is a dominantly inherited endocrine disorder characterized by resistance to hormones that act by stimulating adenyl cyclase. It is caused by inheritance of an autosomal allele that inactivates the \( \alpha \) subunit \((\alpha_s)\) of \( G_s \), the stimulatory regulator of adenyl cyclase. In three members of a family, the PHP-Ia phenotype is associated with a mutation \((R231H)\) that substitutes histidine for arginine at position 231 in \( G_s \). We assessed signaling function of \( \alpha_s \)-WT versus \( \alpha_s \)-R231H transiently transfected in HEK293 cells. Hormone receptor-dependent stimulation of cAMP accumulation in cells expressing \( \alpha_s \)-R231H is reduced by \( \sim 75\% \) in comparison to cAMP accumulation in cells expressing \( \alpha_s \)-WT. A second mutation, \( \alpha_s \)-R201C, inhibits the GTPase turnoff reaction of \( \alpha_s \) thus producing receptor-independent stimulation of cAMP accumulation. The double mutant, \( \alpha_s \)-R231H/R201C, stimulates cAMP accumulation almost as well \((\sim 80\%)\) as does \( \alpha_s \)-R201C itself, indicating that the \( R231H \) mutation selectively impairs receptor-dependent signaling. In three-dimensional structures of \( G_s \) heterotrimers, Arg-231 is located in a region, switch 2, that is thought to interact with the \( \beta \gamma \) subunit rather than with the hormone receptor. Thus, the \( R231H \) phenotype suggests that switch 2 (perhaps in concert with \( \beta \gamma \)) mediates \( G_s \) protein activation by receptors at a site distant from the receptor-\( G_s \) protein contact surface.

Heterotrimeric \( G_s \) proteins relay information from receptors for extracellular stimuli to effector enzymes and ion channels that mediate cell responses. Receptors interact with the \( G_s \) protein \( \alpha/\beta \gamma \) complex, causing release of GDP bound in the guanine nucleotide binding pocket of the \( G_s \) protein \( \alpha \) subunit and its replacement by GTP. GTP-induced conformational changes cause \( G_s \) GDP to dissociate from the \( \beta \gamma \) dimer, allowing both subunits to interact with effectors. Effector stimulation is then terminated by the intrinsic GTPase activity of \( \alpha_s \), followed by reassociation of \( G_s \) GDP and \( \beta \gamma \). With respect to this cycle, recently published crystal structures have revealed details of GTP-induced conformational change in \( G_s \) \((1-3)\), a plausible structure for the catalytic intermediate in the GTPase reaction \((4, 5)\), and three-dimensional structures for the \( G_s \) \( \beta \gamma \) heterotrimer \((6, 7)\). Because we do not have the structure of a receptor-\( G_s \) \( \beta \gamma \) complex, we cannot yet describe in molecular detail the pivotal event in receptor-\( G_s \) protein signaling, receptor-triggered release of GDP from \( G_s \).

At present we can try to understand this event by interpreting effects of instructive mutations. A potential source of such mutations is an inherited disease of \( G_s \) protein signaling, pseudohypoparathyroidism, type Ia (PHP-Ia) \((8)\). PHP-Ia patients inherit a defect in one of the two autosomal alleles of the gene for \( \alpha_s \), the \( \alpha \) subunit of \( G_s \), the stimulatory regulator of adenyl cyclase. Although \( G_s \) mediates effects of many hormones, the \( \sim 50\% \) decrease in \( G_s \) activity produced by loss of an autosomal allele causes clinically evident impairments of responsiveness to two hormones, parathyroid hormone (PTH) \(^\text{1}\) and thyroid stimulating hormone (TSH). Resistance to PTH causes hypocalcemia and hyperphosphatemia, while resistance to TSH produces hypothyroidism.

Since the discovery of \( G_s \) deficiency in PHP-Ia \((9, 10)\), investigators have reported a large number of \( \alpha_s \) mutations in patients with this disorder \((8)\). Unfortunately, most of these \( \alpha_s \) mutations globally inactivated the protein \((e.g. \text{by premature chain termination})\) and therefore proved useless for understanding detailed mechanisms of \( G_s \) protein function. One PHP-Ia mutation appeared specifically to impair interaction of the mutant \( \alpha_s \) with receptors, in keeping with the location of the mutation \((R385H)\) at a site, near the \( G_s \) C terminus, that was already known to interact with receptors \((11)\). We characterized another \( \alpha_s \) mutation, A3665, found in patients with a rare syndrome that combines testotoxicosis and PHP-Ia; in this case, the PHP-Ia phenotype resulted from thermal instability of the mutant \( \alpha_s \) \((12)\). Here we report a third PHP-Ia mutation, \( R231H \). Because this mutation impairs responsiveness of \( \alpha_s \) to receptor stimulation, its location at a site that interacts with \( \beta \gamma \) rather than with receptor is of special interest.

**EXPERIMENTAL PROCEDURES**

Analysis of the \( \alpha_s \) Gene—Using genomic DNA from peripheral blood leukocytes, exons 2–13 and their flanking intron sequences in the human \( \alpha_s \) gene \((13)\) were amplified by the polymerase chain reaction (PCR), as described previously \((14)\). Amplified DNA fragments were sequenced directly, using a Promega fmol Sequencing Kit.

Plasmid Construction and Transfection—An \( \alpha_s \) cDNA containing an internal hemagglutinin epitope, previously described \((15)\), was used as a PCR template to introduce a histidine substitution for arginine at position 231 and (in other constructs) a cysteine substitution for arginine at position 201. The mutated region was ligated back into pDNAJ, and the sequence of the PCR-generated fragment was confirmed by dyeoxy sequencing. HEK293 cells were propagated and transiently transfected exactly as described \((16)\), using the DEAE-dextran method \((17)\).

CAMP Assay—One day after transfection, cells were reseeded into wells of a 24-well plate, and \(^3\text{H}\)labeled adenine \((2 \mu\text{Ci/ml})\) was added. One day later, cells were washed in 0.5 ml of assay medium containing 1 mM

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1 The abbreviation used are: PTH, parathyroid hormone; TSH, thyroid stimulating hormone; PCR, polymerase chain reaction; \( \alpha_2\)-AR, \( \alpha_2 \)-adrenergic receptor.
isobutylmethylxanthine, with or without the indicated concentrations of UK-14304. Reactions were terminated after 30 min by removing the medium and lysing the cells in 5% trichloroacetic acid containing ATP and cAMP (each at 1 mM). [3H]cAMP and [3H]ATP were separated on AG 50W-X4 Dowex and alumina columns as described, and data are presented as the ratio of [3H]cAMP to [3H]cAMP plus [3H]ATP, as described previously (16, 18).

Immunoblots—Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with monoclonal antibody 12CA5 as described (15). Immunoreactive proteins were specifically detected by incubation with horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham) and developed with enhanced chemiluminescence (Amersham).

RESULTS

Three of five family members showed clinical characteristics of PHP-Ia (Fig. 1, Table I), including the distinctive skeletal abnormalities of Albright's hereditary osteodystrophy, disturbed Ca\(^2+\) homeostasis (elevated PTH, low Ca\(^2+\), and elevated PO\(_4\)), and hypothyroidism (elevated TSH and decreased thyroxine). The mother (patient I-1) had mild Albright's hereditary osteodystrophy and normal or only slightly abnormal endocrine function. Only the three clinically affected individuals (I-1, II-1, and II-3) showed the R231H mutation (substitution of an adenine for a guanine residue) in exon 9 of one \(\alpha\_s\) gene (Fig. 2).

To assess the ability of wild type or mutant \(\alpha\_s\) to mediate receptor-stimulated cAMP synthesis, we co-transfected the \(\alpha\_s\) with a porcine \(\alpha\_2\)-adrenoreceptor (\(\alpha\_2\)-AR) and measured cAMP accumulation after stimulation with an \(\alpha\_2\)-AR agonist, UK-14304, administered at 10 \(\mu\)M (Fig. 3A) or in a concentration-response curve (Fig. 3B). In both cases, UK-14304 stimulated cAMP accumulation much more effectively in cells expressing exogenous \(\alpha\_s\)-WT than in cells transfected with vector alone; in cells expressing \(\alpha\_s\)-R231H, the \(\alpha\_2\)-AR agonist also stimulated cAMP accumulation, to an extent slightly greater than observed with transfection of vector alone, but much (~75%) less effectively than in cells expressing \(\alpha\_s\)-WT. From these results we infer that the R231H mutation markedly impairs ability of \(\alpha\_s\) to stimulate cAMP accumulation in response to receptor stimulation. As in the past (16), we measured cAMP accumulation in response to an agonist acting on the transiently transfected \(\alpha\_2\)-AR, which stimulates \(G_s\) quite weakly, rather than in response to a receptor that efficiently stimulates \(G_s\) because the latter kind of receptor, but not the \(\alpha\_2\)-AR, can efficiently stimulate the limited concentration of endogenous \(\alpha\_s\) use of the \(\alpha\_2\)-AR agonist allowed us to measure selectively the cAMP accumulation.

**FIG. 1. Pedigree of the affected family.** Filled symbols indicate patients carrying the \(\alpha\_s\)-R231H mutation. Squares = male, circles = female.

**TABLE I**

| Clinical characteristics of family members |
|-------------------------------------------|
| Patient | I-1 | I-2 | II-1 | II-2 | II-3 |
| Albright's hereditary osteodystrophy | + | - | + | - | + |
| Ca\(^2+\), PO\(_4\) | - | - | + | - | - |
| PTH | - | - | + | - | + |
| Thyroxine | - | - | + | - | + |
| TSH | - | - | + | - | + |
| Mutation | R231H | R231H | R231H | R231H | R231H |

**FIG. 2.** Sequence analysis of exon 9 fragment in DNA of subjects I-1 and II-1, exhibiting a heterozygous substitution of A for G in codon 231. N = normal; P = patients.

**FIG. 3.** Effects of the \(\alpha\_2\)-AR agonist, UK-14304, on cAMP accumulation in cells transfected with \(\alpha\_2\)-AR DNA (1 \(\mu\)g) plus 4 \(\mu\)g of pCDNA1 containing no construct (vector), \(\alpha\_s\)-WT, or \(\alpha\_s\)-R231H. A, effect of 10 \(\mu\)M UK-14304 (cross-hatched bars) versus no agonist. Each bar represents mean ± S.E. of 9 experiments. B, effect of indicated concentrations of UK-14304. C, immunoblots of recombinant \(\alpha\_s\)-WT or \(\alpha\_s\)-R231H, detected by virtue of the HA epitope. 5 × 10\(^6\) transfected cells were dissolved in 120 \(\mu\)l of sample buffer; in each case, lanes were loaded with 7.5, 15, or 30 \(\mu\)l of this cell extract.
accumulation that depended predominantly on transiently transfected \( \alpha \) (16). Note that the R231H defect was not associated with reduced expression of the mutant protein; indeed, immunoblots show that \( \alpha_s \)-R231H was expressed at least as well as \( \alpha_s \)-WT in these experiments (Fig. 3C).

The R231H defect observed in Fig. 3 could have resulted from intrinsic inability of the mutant \( \alpha_s \) to stimulate adenyl cyclase, rather than from a selective defect in ability to respond to receptor. Accordingly, we assessed \( \alpha_s \)-dependent, receptor-independent \( cAMP \) accumulation after transfecting cells with \( \alpha_s \) containing a second mutation, R201C; the R201C mutation reduces the GTPase activity of \( \alpha_s \), thereby preventing the GTP-bound protein from turning itself off and making receptor stimulation unnecessary (19). In these experiments (Fig. 4), expression of \( \alpha_s \)-R201C caused substantial \( cAMP \) accumulation almost as well as \( \alpha_s \)-R201C itself. We infer that the R231H mutation impairs \( \alpha_s \)-adenyl cyclase interaction only slightly, to an extent considerably less than its impairment of \( \alpha_s \)-receptor interaction.

**DISCUSSION**

Arg-231 in \( \alpha_s \), which corresponds to Arg-204 in the \( \alpha \) subunit of retinal transducin, is a highly conserved residue in the “switch 2” region, an \( \alpha \) helix (a2) that interacts directly with the \( \beta y \) subunit and that also plays an important role in GTP-dependent conformational changes in \( \alpha \). By interacting with a main-chain amide at the N terminus of a2, the \( \gamma \)-phosphate of GTP induces a change in orientation of a2 and a twist about its axis (1, 2); in this conformation the side chain corresponding to Arg-231 binds a negatively charged glutamate in the “switch 3” loop. Thus, Arg-231 might be important for mediating the GTP-induced changes that cause dissociation from \( \beta y \) and allow stimulation of effectors. Although the three-dimensional structures make it easy to imagine that a substitution of histidine at position 231 could impair effector stimulation, \( \alpha_s \)-R231H in fact stimulates adenyl cyclase almost as efficiently as does \( \alpha_s \)-WT (Fig. 4).

Instead, we must ask how the R231H mutation could impair receptor-induced replacement of GDP by GTP. In the \( \alpha \beta y \) heterotrimer (6), which is likely to serve as the receptor target, the arginine in this position (Arg-204 in \( \alpha_s \)) is oriented toward the \( G \) hydrophobic core rather toward the \( \beta y \) subunit, which interacts directly with the a2 residues on either side of the arginine. It is hard to see how a histidine at this position could interfere with the interaction of \( G \) protein and receptor, because no possible orientation of the receptor would allow it to contact Arg-204. Consequently, we are left with two possibilities. In the absence of \( \beta y \), receptors cannot release GDP from \( G \); thus, it is possible that histidine at this position could block receptor activation by somehow impairing the interaction of \( G \) with \( \beta y \). Alternatively, or in addition, the receptor may interact with the a2/\( \beta y \) loop at the C terminus of the a2 helix; thus, this helix could transmit conformational change from the receptor to the guanine nucleotide binding pocket, near the N terminus of a2, and histidine substituted for arginine at position 231 might block this conformational change.

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