0013-7227/01/$03.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 86(10):4622–4626

Editorial: The Stimulatory G Protein α-Subunit Gene: Mutations and Imprinting Lead to Complex Phenotypes

G_S is one of about 20 heterotrimeric guanine nucleotide-binding proteins (G proteins) that transmit signals from cell-surface receptors to effector enzymes or ion channels to produce intracellular "second messengers." Each G protein is composed of three subunits: α, β, and γ. The α-subunit binds guanine nucleotide and is important for receptor coupling and effector activation. In the inactive state the α-subunit has GDP bound in its guanine nucleotide-binding site and is associated with a βγ dimer. On activation by a ligand-bound receptor, GDP is released and replaced by GTP. On binding GTP, the α-subunit switches to an active conformation and dissociates from βγ. The "turn-off" mechanism is an intrinsic GTPase activity of the α-subunit that hydrolyzes bound GTP to GDP, allowing reassociation of the α-subunit with a βγ dimer. The G_s α-subunit ($G_s\alpha$) is ubiquitously expressed and couples receptors for many peptide and glycoprotein hormones, biogenic amines, and other neurotransmitters and circulating factors to the enzyme adenylyl cyclase, and is, therefore, required for hormone-stimulated intracellular cAMP generation (1). Its single copy gene *GNAS1* is located at chromosome 20q13.

Surprisingly, $G_s\alpha$ is the only G protein α-subunit involved in hormone signaling, to date, that has been shown to have genetic defects associated with human disease. Somatic missense mutations affecting two residues (Arg^{201} and Gln^{227}) that are catalytically important for the GTPase turn-off reaction lead to constitutively active forms of $G_s\alpha$ protein, and are present in ~40% of GH-secreting pituitary adenomas (2). In these tumors, the increased intracellular cAMP resulting from activating $G_s\alpha$ mutations stimulates both proliferation and GH secretion.

Similar mutations affecting Arg^{201} are also present in the McCune-Albright syndrome (MAS), which in its most severe presentation is associated with hyperpigmented skin (café-au-lait) lesions, fibrous dysplasia of bone, and both increased growth and hormone oversecretion from various endocrine organs (including gonads, adrenal cortex, thyroid, and pituitary somatotrophs) (3, 4). The diverse manifestations of MAS result from increased intracellular cAMP in melanocytes, osteoblastic precursor cells, and endocrine tissues, respectively. In MAS the somatic mutation presumably occurs during early embryonic development, leading to a widespread distribution of cells bearing the activating $G_s\alpha$ mutation. The disease is never inherited, indicating that activating $G_s\alpha$ mutations are embryonically lethal. Similar mutations are also present in bone lesions of patients who have fibrous dysplasia in the absence of other manifestations of MAS and in skeletal muscle myxomas. Recent studies suggest that increased cAMP leads to fibrous dysplasia by altering the differentiation program of bone marrow stromal cells (5). Cholera toxin, an exotoxin of *Vibrio cholerae*, catalyzes a reaction resulting in covalent modification of $G_s\alpha$ Arg^{201}. This posttranslational modification leads to an activated form of $G_s\alpha$ protein and increased intracellular cAMP in intestinal epithelial cells, which underlies the severe secretory diarrhea that is characteristic of intestinal cholera.

Heterozygous inactivating $G_s\alpha$ mutations lead to Albright hereditary osteodystrophy (AHO), a disorder characterized by short stature, brachydactyly, sc ossifications, centripetal obesity, and, in some cases, mental deficits (1). Consistent with the presence of heterozygous mutations, $G_s\alpha$ expression and/or function is decreased by ~50% in erythrocytes and other tissues obtained from AHO patients. Similar mutations have also been found in patients with more aggressive ossifications that invade deeper tissues (progressive osseous heteroplasia) (6).

In this issue of the *JCEM*, Ahrens *et al.* (7) report on the genetic analysis of *GNAS1* in 29 unrelated AHO patients with decreased erythrocyte $G_s\alpha$ bioactivity and show that 21 of these patients have a heterozygous mutation within 1 of the 13 $G_s\alpha$ coding exons that presumably affects $G_s\alpha$ expression or function. These findings are consistent with the results of prior studies showing that the majority of AHO patients have a $G_s\alpha$ mutation (8). Among the 15 mutations identified in this study, 11 have not been previously reported, providing further evidence for the heterogeneity of inactivating $G_s\alpha$ mutations. This and prior studies demonstrate that such mutations can occur in any of the $G_s\alpha$ encoding exons (except perhaps exon 3, which can be spliced out and still encode a biologically active form of $G_s\alpha$).

On the other hand, the study by Ahrens *et al.* (7) also provides evidence that there may be some mutation "hot spots." A 4-bp deletion mutation in exon 7 previously reported in several AHO patients was present in five of their unrelated patients. They also identified four other missense mutations that are present in at least two unrelated patients and a mutation of a residue (Arg^{231} to cysteine) that was previously shown to be mutated to histidine in another patient. Arg^{231} is important for $G_s\alpha$ activation (7), but it is unknown if the other residues that are mutated in more than one patient are functionally important. Iiri *et al.* (9) showed that mutation of Ala^{366} in two unrelated males leads to AHO and precocious puberty, due to increased release of GDP in the basal state. At core body temperature the major effect of Ala^{366} substitution is thermolability of the protein (leading to AHO), but at the slightly lower temperature of the testes the major effect is increased G_s signaling due to greater GDP-GTP exchange (leading to gonadotropin-independent precocious puberty). Other $G_s\alpha$ residues that are mutated in single AHO patients have also been shown to be functionally

Abbreviations: AHO, Albright hereditary osteodystrophy; MAS, McCune-Albright syndrome; PHPIa and Ib, pseudohypoparathyroidism types Ia and -Ib; PPHP, pseudopseudohypoparathyroidism.

important (1). The failure of the study by Ahrens *et al.* (7) to identify mutations in eight patients may be due to the fact that some patients have $G_s\alpha$ mutations within regulatory regions such as the promoter. There are also technical limitations of the study (sensitivity of the mutation screening method or inability to amplify and analyze all of exon 1) that may also account for the failure to identify mutations in these patients. Overall this study demonstrates the usefulness of *GNAS1* genetic analysis for confirming the diagnosis of AHO.

Unlike MAS, AHO is inherited in an autosomal dominant manner. The genetics of this disorder are complicated by the fact that some AHO patients also present with renal resistance to PTH and milder resistance to TSH and the gonadotropins [a condition referred to as pseudohypoparathyroidism type Ia (PHPIa)] whereas other affected patients within the same kindred have AHO without evidence of hormone resistance [referred to as pseudopseudohypoparathyroidism (PPHP)]. Because the receptors for PTH, TSH, and the gonadotropins all activate G_s, one might predict that $G_s\alpha$ mutations might lead to target-tissue resistance to these hormones. However, simple haploinsufficiency of $G_s\alpha$ due to heterozygous inactivating mutations that are present in both PHPIa and PPHP patients cannot fully explain why some patients develop multihormone resistance (PHPIa) while others do not (PPHP). It cannot also explain why PHPIa patients seem to be resistant to these hormones, but do not show resistance to other hormones that also activate G_s in their target-tissues (*e.g.* ACTH, vasopressin effects in the renal collecting ducts).

The first clue to understanding this apparent paradox was provided by Davies and Hughes (10), who noted that all offspring who inherit AHO from their mother also develop multihormone resistance (PHPIa) whereas those who inherit AHO from their father do not develop multihormone resistance (PPHP). This inheritance pattern has been supported by subsequent studies and is consistent with the results now published by Ahrens *et al.* (7), in which 12 of 12 PHPIa patients from 11 unrelated kindreds inherited the $G_s\alpha$ mutation from their mother, regardless of whether the mother had PHPIa or PPHP.

One possible explanation for the effect of parental inheritance on phenotype of the offspring is that the $G_s\alpha$ gene is imprinted. Genomic imprinting is an epigenetic phenomenon affecting a small number of genes that results in partial or total loss of expression from one parental allele (11). Imprinted genes contain one or more regions where the two parental alleles are differentially methylated. Often, although not always, the differentially methylated region is the gene promoter, which is methylated on the transcriptionally silent allele. The imprint is presumably erased in primordial germ cells and is reestablished in either the male or female germ line during gametogenesis or before pronuclear fusion in the zygote (the only time during development when the two parental genomes are physically separated).

In AHO patients, if $G_s\alpha$ is primarily expressed from the maternal allele in specific hormone target tissues (*e.g.* renal proximal tubules, the primary renal target for PTH), then mutations inherited on the active maternal allele would markedly reduce $G_s\alpha$ expression and lead to PTH resistance (PHPIa) whereas mutations inherited on the inactive paternal allele would have little effect on $G_s\alpha$ expression or renal PTH sensitivity (PPHP). This model is consistent with the observation that the acute urinary cAMP response to administered PTH is markedly reduced in PHPIa patients but is totally unaffected in PPHP patients (12). $G_s\alpha$ imprinting would have be tissue specific, because $G_s\alpha$ has been shown to be biallelically expressed in human lymphocytes (13) and fetal tissues (14), and its expression is equally reduced by ~50% in several tissues from both PHPIa and PPHP patients (12), consistent with no parent-of-origin effect in these tissues.

This model of tissue-specific imprinting of $G_s\alpha$ has been confirmed in a $G_s\alpha$ knockout mouse model (15). Analogous to patients with AHO, maternal inheritance of the $G_s\alpha$ mutation resulted in biochemical hypoparathyroidism and renal PTH resistance whereas paternal transmission of the same mutation had no effect on renal PTH action. Consistent with maternal-specific expression of $G_s\alpha$, $G_s\alpha$ was poorly expressed in proximal tubules isolated from mice with the maternal $G_s\alpha$ defect but was expressed normally in proximal tubules isolated from mice with the paternal $G_s\alpha$ defect. Interestingly, $G_s\alpha$ appeared not to be imprinted in many other tissues, including other portions of the kidney (glomeruli, distal nephron, collecting ducts). Variable imprinting between the proximal and distal nephron might explain why PHPIa patients are resistant to the effects of PTH on the proximal portion of the nephron (phosphaturia, 1α-hydroxylation of vitamin D) but do not seem to be resistant to the effects of PTH on the distal nephron (calcium reabsorption) (16). Tissue-specific imprinting of $G_s\alpha$ might also explain why PHPIa patients do not show resistance to all hormones that activate G_s (*e.g.* vasopressin in the renal collecting ducts). In humans $G_s\alpha$ has been recently shown to be imprinted in the pituitary with expression only from the maternal allele (17).

The $G_s\alpha$ imprinting story is further complicated by the fact that the *GNAS1* gene produces multiple other gene products through the use of at least four alternative promoters and first exons, which themselves are imprinted (see Fig. 1; reviewed in Ref. 1). All four alternative first exons splice onto a common set of downstream exons (exons 2–13). The most downstream first exon (exon 1) generates transcripts encoding $G_s\alpha$. Its promoter is not methylated. Alternative exons located 47 and 35 kb upstream of $G_s\alpha$ exon 1 generate transcripts encoding the chromogranin-like protein NESP55 and XLαs (a $G_s\alpha$ isoform with a long amino-terminal extension), respectively. Both are primarily expressed in neuroendocrine tissues and are oppositely imprinted: NESP55 is only expressed from the maternal allele and its promoter is methylated on the paternal allele while XLαs is only expressed from the paternal allele and its promoter is methylated on the maternal allele. Little is known about their biological function. A fourth alternative first exon (exon 1A) generates transcripts that are ubiquitously expressed but do not appear to encode a functional protein. Like XLαs, this exon is methylated on the maternal allele and is transcriptionally active on the paternal allele. In mice maternal-specific methylation of the exon 1A region is established during oogenesis and is

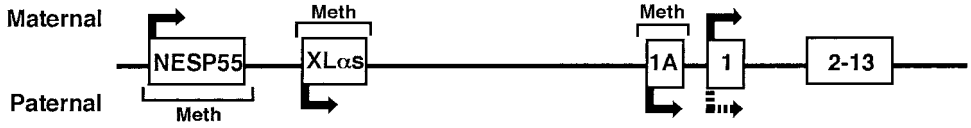

FIG. 1. Organization and imprinting of the *GNAS1* locus. *GNAS1* has four alternative first exons (labeled NESP55, XLαs, 1A, and 1) that splice onto a common set of downstream exons (labeled 2–13) to generate transcripts for the NESP55, XLαs, and $G_s\alpha$ proteins (exon 1). Exon 1A generates transcripts that are presumably untranslated. The methylation and transcriptional activation (*arrows*) of the maternal and paternal allele are shown above and below, respectively. The *hatched arrow* for exon 1 on the paternal allele indicates that $G_s\alpha$ is expressed from the paternal allele in most, but not all, tissues. A paternally expressed *GNAS1* antisense transcript that traverses the NESP55 exon is not shown in the figure.

maintained throughout development, suggesting that this region may be critical for establishment of *GNAS1* imprinting. Recently paternally expressed antisense transcripts that traverse the NESP55 upstream exon have also been identified.

Some patients present with renal PTH resistance in the absence of AHO, a condition referred to as pseudohypoparathyroidism type Ib (PHPIb). As in PHPIa, the urinary cAMP response to administered PTH is markedly reduced, implicating a PTH signaling defect proximal to cAMP generation. However, in contrast to what is observed in PHPIa, $G_s\alpha$ expression and function is normal in erythrocytes from PHPIb patients, ruling out typical inactivating mutations within the $G_s\alpha$ coding exons. However, in four families the PHPIb locus was mapped to 20q13, in the vicinity of *GNAS1* (18). Moreover, in this study PTH resistance was only observed when the trait was inherited maternally, reminiscent of the parental inheritance pattern for PTH resistance that is observed in patients who also have AHO. Recently several studies have shown that PHPIb is associated with a *GNAS1* imprinting defect whereby maternal imprinting of exon 1A is lost in 38 of 38 patients, leading to a paternal-specific imprinting pattern (unmethylated, transcriptionally active) within the exon 1A region on both alleles (19–21). Similar imprinting abnormalities affecting the NESP55 and XLαs regions are present in only a small subset of PHPIb patients, ruling out these regions or their transcripts as being important in the pathogenesis of PHPIb. Paternal uniparental disomy of the long arm of chromosome 20, which produces the same abnormal imprinting pattern, has been described in one PHPIb patient (22).

The defect commonly found in PHPIb patients is the abnormal imprinting of the exon 1A region, suggesting that this defect is important for the pathogenesis of PHPIb. While it is possible that PHPIb is the direct consequence of exon 1A-specific mRNA overexpression, this seems unlikely given the fact that these transcripts do not encode a known functional protein and the central role that $G_s\alpha$ plays in PTH signaling. More likely, the exon 1A region is an important element for the tissue-specific imprinting of $G_s\alpha$, and, therefore, abnormal imprinting of this region leads to loss of $G_s\alpha$ expression in specific tissues. One hypothetical model (shown in Fig. 2) would predict that the exon 1A region contains a silencer element which is a binding site for a tissue-specific repressor protein that is expressed in only some tissues, such as renal proximal tubule. In these tissues the repressor binds to the paternal allele and inhibits transcription from the $G_s\alpha$ promoter, but is unable to bind to the maternal allele because its binding site is methylated, therefore, allowing $G_s\alpha$ to be expressed from this allele. In most

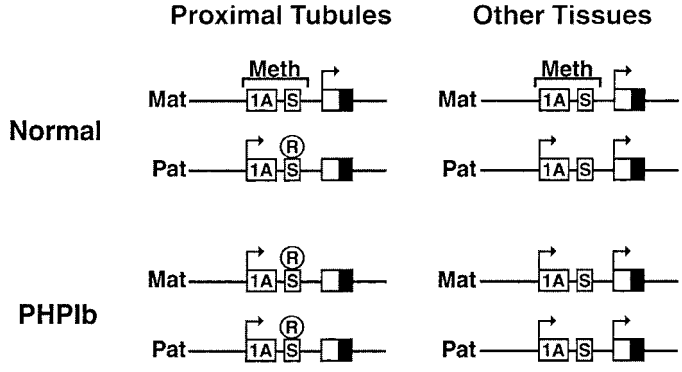

FIG. 2. One potential model for the tissue-specific imprinting of $G_s\alpha$ and the consequences of an imprinting defect in PHPIb. In the tissue-specific repressor model the exon 1A region has a *cis*-acting silencer element (S) that is able to bind a repressor (R), whose expression is limited to specific tissues such as renal proximal tubules. Normally the repressor binds to the paternal allele and inhibits $G_s\alpha$ expression ($G_s\alpha$ exon 1 shown as a *partially filled box*), but is unable to bind to the maternal allele because the silencer is methylated, allowing the $G_s\alpha$ to remain active. In most other tissues, the repressor is not expressed, and, therefore, $G_s\alpha$ is expressed from both alleles even though only the maternal allele is methylated. In PHPIb the exon 1A region is unmethylated on both the maternal and paternal alleles. In proximal tubules the repressor can bind to both alleles, leading to loss of $G_s\alpha$ expression and PTH resistance. In most other tissues abnormal methylation has no impact because the repressor is not present. Such a model could explain why PHPIb patients have normal levels of $G_s\alpha$ expression in other tissues, such as erythrocytes (and perhaps, why they lack the AHO phenotype).

other tissues the repressor is not expressed, and, therefore, $G_s\alpha$ is expressed biallelically, even though the exon 1A methylation pattern is the same. In PHPIb patients exon 1A is not methylated on the maternal allele, allowing the repressor to bind to both alleles in proximal tubules, leading to near total loss of $G_s\alpha$ expression and PTH resistance. In contrast, $G_s\alpha$ expression is unaffected in most other tissues where the repressor is not expressed. Other potential mechanisms for the tissue-specific imprinting of $G_s\alpha$ have been proposed elsewhere (1).

Regardless of the exact mechanism, a paternal-specific imprinting pattern in both alleles is predicted to lead to loss of $G_s\alpha$ expression in tissues where it is normally maternally expressed but has no effect on $G_s\alpha$ expression in tissues where it is normally biallelically expressed. This would explain why PHPIb patients have PTH resistance (due to loss of $G_s\alpha$ expression in proximal tubules) but lack the AHO phenotype (due to normal levels of $G_s\alpha$ in most other tissues, as demonstrated in erythrocytes). Several facts suggest that loss of NESP55 or XLαs expression do not contribute to the

AHO phenotype. First, in some PHPIb patients total loss of NESP55 associated with biallelic methylation of the NESP55 promoter does not produce an AHO phenotype (19). Also, mutation of either the maternal or paternal allele leads to AHO, while only paternal allele mutations would be expected to disrupt XLαs expression. The known roles of $G_s\alpha$ and cAMP in the regulation of osteoblast differentiation, lipid metabolism, and neurological function provide potential mechanisms by which $G_s\alpha$ haploinsufficiency could lead to the skeletal, metabolic, and neurological abnormalities associated with AHO (1). Interestingly, PHPIb does not generally present with short stature, even though the imprinting defect would be expected to produce low $G_s\alpha$ levels and GHRH resistance in the pituitary.

The models for the maternal inheritance of renal PTH resistance in AHO (PHPIa) and PHPIb described above are all predicated on the assumption that $G_s\alpha$ is expressed primarily or exclusively from the maternal allele in renal proximal tubules, the primary site of PTH action in the kidney. Although this has been shown to be true in mice (15) and $G_s\alpha$ has been shown to be expressed only from the maternal allele in human pituitaries (17), there is still no direct evidence that $G_s\alpha$ is imprinted in renal proximal tubules in humans. In this issue of *JCEM*, Zheng *et al.* (23) try to address this question by examining the allele-specific expression of $G_s\alpha$ and the other *GNAS1* gene products in renal cortex and other tissues derived from human fetuses that were heterozygous for an informative polymorphism within an exon common to all *GNAS1* sense transcripts. After isolating RNA from these tissues, they amplified NESP55, XLαs, exon 1A, and $G_s\alpha$-specific transcripts by RT-PCR and determined whether or not each were mono- or biallelically expressed. As expected, NESP55 was expressed from one parental allele (presumably maternal) whereas XLαs and exon 1A transcripts were expressed only from the opposite (presumably paternal) allele in all tissues examined. This is consistent with previous results and the known methylation patterns of their respective promoters (see Fig. 1). However, $G_s\alpha$ seemed to be biallelically expressed in all kidney cortex samples examined. Based on this observation, the authors conclude that $G_s\alpha$ is not imprinted in kidney cortex and, therefore, the PTH resistance in PHPIb (and presumably PHPIa) is not due to loss of $G_s\alpha$ expression in renal proximal tubules.

There are at least two other potential explanations besides lack of $G_s\alpha$ imprinting in renal proximal tubules for the failure of Zheng *et al.* (23) to detect $G_s\alpha$ imprinting in renal cortex. The first possibility is that imprinting was not detected because their cortex samples contained many elements in addition to proximal tubules, including glomeruli, condensing mesenchyme, developing nephron structures, and medullary tubular structures. $G_s\alpha$ has been shown to be biallelically expressed in glomeruli and more distal (including medullary) segments of the nephron (16), and is likely to also be biallelically expressed in condensing mesenchyme and developing nephron structures. Our studies in mice showed that $G_s\alpha$ mRNA expression is very low in proximal tubules relative to neighboring structures (15). If this is also true in humans, then only a small fraction of $G_s\alpha$ mRNA in their samples may have been derived from proximal tubules, even if proximal tubules were highly represented in their samples.

The second possible explanation for the failure to detect imprinting in the study by Zheng *et al* (23) relates to the fact that fetal renal cortex is not the same as fully mature (postnatal) renal cortex. Histologically, nephrons and glomeruli are not fully developed during the fetal period and continue to mature even during the postnatal period. This is consistent with the presence of condensing mesenchyme and developing nephron structures in their cortex samples. It is possible that $G_s\alpha$ is not imprinted in this immature fetal cortical tissue but becomes imprinted as the proximal tubules fully mature in early postnatal development. This is one possible explanation for why PHPIa patients do not show evidence of renal PTH resistance at birth, but subsequently develop PTH resistance over the first years of life (24–26). There are other examples of genes whose imprinting is developmentally regulated (27). In the repressor model described above, the repressor may only be expressed in proximal tubules once they are fully differentiated in the postnatal period. In my view, the large weight of clinical and experimental evidence in both humans and mice suggests that $G_s\alpha$ is imprinted in postnatal renal proximal tubules. The definitive answer as to whether this is true in humans awaits studies that examine allele-specific expression of $G_s\alpha$ in proximal tubules that are isolated from postnatal kidneys.

Recent evidence suggests that imprinting of $G_s\alpha$ may also have an effect on the clinical manifestations in patients with activating $G_s\alpha$ mutations. In MAS patients, one would expect that in tissues where $G_s\alpha$ is imprinted the expression of the constitutively activated form of $G_s\alpha$ would be much greater when the mutation is present on the active maternal allele. Therefore, the manifestations within an individual patient might be a function of both the distribution of cells bearing the mutation and the parental allele that has the mutation. Evidence for this is provided by Hayward *et al.* (17), who recently showed that in 21 of 22 GH-secreting pituitary tumors with an activating $G_s\alpha$ mutation, the mutation was on the maternal allele.

In summary, our present knowledge suggests that tissue-specific imprinting contributes to the multiple clinical manifestations that result from genetic defects involving $G_s\alpha$. Future studies in both mouse and humans will allow us to determine the physiological mechanisms by which $G_s\alpha$ deficiency leads to the AHO phenotype, the mechanisms underlying the complex imprinting of *GNAS1*, and how this mechanism goes awry in PHPIb. Clinical genetic studies in patients with diseases such as MAS, AHO, and PHPIb provide a good example of how important insights into gene regulation and protein function can be derived from careful examination of patients with relatively uncommon diseases.

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Acknowledgments

Received July 30, 2001. Accepted July 30, 2001.

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