Abstract

Albright hereditary osteodystrophy (AHO) is characterized by multiple somatic defects secondary to mutations in the GNAS1 gene. AHO patients with mutations on maternally inherited alleles are resistant to multiple hormones (e.g., PTH, TSH), a variant termed pseudohypoparathyroidism (PHP) type 1a, due to presumed tissue-specific paternal imprinting of the α chain of Gα as demonstrated in murine renal proximal tubule and fat cells. Studies in human tissues thus far revealed imprinting only in pituitary. Because mild hypothyroidism due to TSH resistance occurs in most PHP type 1a patients, we investigated whether Gα is imprinted in thyroid. Examination of eight normal thyroids demonstrated significantly greater expression from the maternal GNAS1 allele, with paternal Gα transcripts accounting for only 25.9–40.4%. Expression of NESP55, XLαs, and 1A was uniallelic. We conclude that Gα is incompletely imprinted in the thyroid, which provides an explanation for mild TSH resistance in PHP type 1a. © 2002 Elsevier Science (USA). All rights reserved.

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Albright hereditary osteodystrophy (AHO) is a genetic disorder characterized by somatic defects that include short stature, obesity, delayed puberty, subcutaneous ossifications, and brachydactyly [1]. It is caused by heterozygous inactivating mutations in the GNAS1 gene that lead to reduced expression or function of the α chain of Gα, the heterotrimeric G protein that couples heptahelical receptors to stimulation of adenylyl cyclase (reviewed in [2,3]). AHO patients with GNAS1 mutations on maternally inherited alleles [4] also manifest resistance to multiple hormones such as PTH, TSH, gonadotropins, and glucagon [2], a condition termed pseudohypoparathyroidism type 1a (PHP type 1a). By contrast, AHO patients with GNAS1 mutations on paternally inherited alleles have only the phenotypic features of AHO without hormonal resistance, a condition termed pseudopseudohypoparathyroidism (pseudoPHP) [5]. This inheritance pattern, derived from analysis of published pedigrees, implicated genomic imprinting of the GNAS1 gene as a possible regulatory mechanism [4,6].

The GNAS1 locus located at chromosome 20q13.2 and the syntenic mouse Gnas locus at distal chromosome 2 exhibit a very complex pattern of genomic imprinting. The GNAS1 gene consists of 13 exons that encode Gαs. Upstream of exon 1 of GNAS1 are three alternative first exons [7–9] that each splice onto exons 2–13 to create novel transcripts: (1) XLαs that is expressed only from the paternal allele [7,8] and encodes a signaling protein that stimulates adenylyl cyclase but lacks a known receptor [10,11]; (2) NESP55 that is expressed only from the maternal allele and encodes a secretory protein [7,8,12,13]; and (3) the exon 1A (associated first exon) transcript that is derived from the paternal allele and does not encode a known protein [9,14,15]. Each of these alternative first exons is...
imprinted, and the promoter regions contain differentially methylated regions (DMR) that are methylated on the non-expressed allele [7,8,14,16].

Paternal imprinting of GNASI provides an explanation for the non-Mendelian inheritance pattern of PHP type 1a and pseudoPHP. However, experimental evidence for imprinting of Gαs in human tissues is lacking, as patients with PHP type 1a and pseudoPHP have a similar 50% reduction in Gαs expression in all tissues examined [2,17]. In virtually all human tissues examined thus far, Gαs expression has been found to be biallelic [7,8,18,19]. This biallelic expression is consistent with the apparent absence of DMR within/near the promoter region of Gαs [16,20]. However, recent evidence has shown that the maternal Gαs allele is preferentially expressed in the human pituitary, suggesting “relaxed” paternal imprinting in this tissue [21].

Murine models of AHO were developed through disruption of a single Gnas gene in murine embryonic stem cells [22,23], and have revealed an apparent tissue-specific pattern of Gαs imprinting. Analysis of heterozygous Gnas+/- knockout mice has demonstrated that Gαs expression is derived primarily from the maternal allele in imprinted cells, such as the renal proximal tubule and fat cells, but is derived from both alleles in other tissues examined [22,24,25]. Thus, mice that inherit a defective maternal Gnas allele have markedly reduced levels of Gαs protein in paternally imprinted tissues. In contrast, Gαs levels are 50% of normal in non-imprinted tissues of Gnas+/- mice whereas they have inherited a defective maternal or paternal Gnas allele, as both alleles are active in these tissues. Because mild TSH resistance occurs in most patients with PHP type 1a [26], we sought to determine whether Gαs expression is paternally imprinted in the thyroid. Here, we demonstrate that Gαs expression derives predominantly from the maternal GNASI allele in the human thyroid. These results are consistent with incomplete paternal imprinting of Gαs, and provide an explanation for mild TSH resistance in patients with PHP type 1a.

Materials and methods

Thyroid tissue was obtained from 16 subjects who underwent subtotal thyroidectomy for excision of a thyroid nodule. Histological examination confirmed that thyroid sections used in this study consisted of normal tissue and contained at least 90% follicular epithelial cells. The protocol was approved by the Institutional Review Board of Washington Hospital Center and The Johns Hopkins Hospital. Total RNA was isolated using the Trizol reagent according to the manufacturer’s instructions (Gibco/BRL). First strand cDNA was synthesized from 1 μg of total RNA using Superscript II (Gibco-BRL) reverse transcriptase and random hexamers according to the manufacturer’s recommendations. We amplified the transcript-specific PCR products that contained the GNASI exon 5 FokI polymorphism [27] using a common reverse primer corresponding to nucleotide sequences in exon 6 and specific forward primers corresponding to nucleotide sequences in the first exons for NESP55, XLαs exon 1A, and Gαs. These primers and the PCR conditions are described in Table 1. To optimize the likelihood of distinguishing differences between levels of maternally derived and paternally derived transcripts, PCR was performed using a number of cycles that yielded products within the linear range of amplification (data not shown). After PCR, 15 μl samples were electrophoresed through 6% acrylamide gels and after staining with ethidium bromide, appropriate-sized DNA bands were isolated and purified. DNA fragments were sequenced directly with the USB Radiolabeled Terminator Cycle Sequencing Kit (USB, Cleveland, OH). Quantification of band intensity was performed with the PhosphorImager system (Bio-Rad). To control for the variability in sample loading between the lanes, eight bands surrounding the polymorphic band were quantified (for both the T and C lanes). The mean value for each was calculated and the background density subtracted such that these lanes could be normalized to one another and adjusted for this loading variability. For the maternal and paternal alleles (determined from the NESP55 and XLαs sequences, respectively) the densities were adjusted for this normalization. The relative expression of the maternal and paternal alleles was then calculated as a percentage of the normalized values for each of the two alleles. Results are presented as the means ± SEM, and measurements were compared with the unpaired Student’s t test. p values smaller than 0.05 were considered significant. The relative expression of the paternal allele was calculated as the proportion of the paternal allele out of the sum of the normalized values for both alleles.

Results

We isolated RNA from normal regions of thyroids that had been removed at surgery from individuals with thyroid nodules. We then performed RT-PCR using an upstream primer specific for each of the four alternative first exons of GNASI that correspond to transcripts for NESP55, XLαs, 1A, and Gαs. A common downstream primer was within exon 6 of GNASI to genotype alleles using a highly variable single nucleotide polymorphism in codon 131 (T/C) of exon 5 (Fig. 1). Eight of 16 subjects were heterozygous for this polymorphism and were therefore informative. In each case expression was uniallelic for NESP55 (maternal), XLαs (paternal), and 1A (paternal) (data not shown), as is consistent with other studies [7,8,14].

RT-PCR analysis of Gαs mRNA revealed expression of both maternal and paternal alleles in these samples. However, further analysis of Gαs transcripts revealed that the intensity of the radiolabeled bands corresponding to the T and C nucleotides was not equivalent, indicating that expression of the maternal and paternal alleles was not equal (Fig. 2). In all eight cases there was greater expression of the maternal Gαs allele, which coincided with uniallelic expression of NESP55 transcripts. Expression of the maternal allele compared to the paternal allele was 0.713 ± 0.025 versus 0.309 ± 0.017 (p < 0.0001; Fig. 3). The range of expression of the paternal allele was 25.9–40.4% (Fig. 4). The preferential expression of the maternal allele was consistent in all eight samples studied, and was similar whether the maternal genotype was T (two cases) or C (six cases).
Expression of $G_{as}$ is biallelic in most tissues [7,8,18], but recent studies show that preferential expression of the maternal allele occurs in the human pituitary [21].

Table 1

<table>
<thead>
<tr>
<th>Transcript and primers</th>
<th>PCR conditions</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NESP55</td>
<td>$94°C \times 3 \text{ min}; 94°C \times 30 \text{ s}; 55°C \times 30 \text{ s}$</td>
<td>400</td>
</tr>
<tr>
<td>$5'\text{-GTCATAATGGAGGACGCGCTTCT}C176C2\text{-3'}$</td>
<td>$72°C \times 1 \text{ min}; \times 39 \text{ cycles}; 72°C \times 5 \text{ min}$</td>
<td></td>
</tr>
<tr>
<td>XL$_{a}$</td>
<td>$94°C \times 3 \text{ min}; 94°C \times 30 \text{ s}; 55°C \times 30 \text{ s}$</td>
<td>818</td>
</tr>
<tr>
<td>$5'\text{-GGATGCCTCCTGCTTGTGAGCATCGGCGG-3'}$</td>
<td>$72°C \times 1 \text{ min}; \times 39 \text{ cycles}; 72°C \times 5 \text{ min}$</td>
<td></td>
</tr>
<tr>
<td>Exon 1A</td>
<td>$95°C \times 3 \text{ min}; 94°C \times 30 \text{ s}; 60°C \times 3 \text{ min}$</td>
<td>510</td>
</tr>
<tr>
<td>$5'\text{-GGACACTCAGTCGCCGCGAC-3'}$</td>
<td>$\times 35 \text{ cycles}; 68°C \times 5 \text{ min}$</td>
<td></td>
</tr>
<tr>
<td>G$_{as}$</td>
<td>$95°C \times 3 \text{ min}; 94°C \times 30 \text{ s}; 66°C \times 2 \text{ min}$</td>
<td>482</td>
</tr>
<tr>
<td>$5'\text{-ATGGGCTGGTCCCGGGAACAGT-3'}$</td>
<td>$\times 31 \text{ cycles}; 68°C \times 5 \text{ min}$</td>
<td></td>
</tr>
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</table>

**Fig. 1.** The GNAS1 gene complex. The GNAS1 gene complex consists of 13 exons that encode the signaling protein $G_{as}$. Upstream of exon 1 are three alternative exons, labeled exon 1A, XL$_{a}$, and NESP55. These three alternative first exons are spliced to exons 2–13 to produce unique transcripts. NESP55 is transcribed exclusively from the maternal allele; XL$_{a}$ and exon 1A are transcribed exclusively from the paternal allele. RT-PCR using an upstream primer specific for each of the four alternative first exons of GNAS1 and a common downstream primer within exon 6 of GNAS1 enabled us to genotype the alleles using a highly variable single nucleotide polymorphism in codon 131 (T/C) of exon 5.

**Fig. 2.** Sequence analysis of the product of RT-PCR of $G_{as}$ exon 5 derived from thyroid RNA from 16 subjects. Eight of the 16 subjects were heterozygous for the polymorphism in codon 131 (T/C). The intensity of the radiolabeled bands corresponding to the T and C nucleotides for each of these eight subjects was not equivalent, thereby indicating unequal expression of the two alleles.

**Discussion**

Expression of $G_{as}$ is biallelic in most tissues [7,8,18], but recent studies show that preferential expression of the maternal allele occurs in the human pituitary [21].

**Fig. 3.** Relative expression of $G_{as}$ alleles in normal human thyroid. Quantification of band intensity of the polymorphic alleles was performed with the Phosphorimager system (Bio-Rad) and corrected for variability of sample loading between lanes. In all eight subjects heterozygous for the exon 5 polymorphism there was significantly greater expression of the maternal allele compared to the paternal allele; the mean ± SEM was calculated for the maternal and paternal alleles (0.713 ± 0.025 versus 0.309 ± 0.017).

**Fig. 4.** Relative expression of paternal $G_{as}$ alleles in normal human thyroid. The relative expression of the paternal allele was calculated as the proportion of the paternal allele out of the sum of the normalized values for both alleles. The range of expression of the paternal allele was 25.9–40.4%.
and in murine renal proximal tubule cells and adipocytes [22]. The tissue-specific paternal imprinting found in murine renal proximal tubule cells leads to PTH resistance in Gnas+/- mice that have inherited the defective Gnas allele maternally [22], and a similar mechanism has been proposed to explain PTH resistance in humans with PHP type 1a. Because most patients with PHP type 1a also have mild TSH resistance, we investigated the possibility that Gαs is paternally imprinted in the human thyroid. We report here that there is incomplete (i.e., “relaxed”) paternal imprinting of GNAS in the human thyroid, with preferential expression of the paternal allele compared to the paternal allele. The proportion of paternal Gαs transcripts ranged from 25.9% to 40.4% in the thyroid samples we analyzed.

The incomplete paternal imprinting of Gαs in the thyroid is consistent with previous observations of Gαs expression in the human pituitary as well as in murine renal proximal tubule cells [22]. Although the majority of expression of Gαs in these tissues derives from the maternal allele, there is some expression from the paternal allele. In the four adult pituitary samples reported by Hayward et al. [21] the proportion of paternal Gαs transcripts was as high as 14%. The expression of Gαs mRNA in renal proximal tubule cells of Gnas knockout mice that had inherited a defective Gnas allele maternally was 38 ± 4% normal and represents transcription derived from the normal (but presumably imprinted) paternal allele [22]. By contrast, Gαs expression was 89 ± 9% normal in mice that inherited a defective Gnas allele paternally [22]. Therefore, although there appears to be tissue-specific repression of transcription from the paternal Gαs allele, this effect is incomplete.

The partial imprinting of the paternal Gαs allele in the human thyroid could explain the development of mild TSH resistance seen in almost all patients with PHP type 1a [26]. In PHP type 1a, TSH levels are commonly elevated at birth and often precede the development of elevated levels of PTH (and PTH resistance) by several years [28–30]. Typically TSH levels are only mildly elevated, and serum levels of thyroid hormone are normal or only slightly reduced. PHP type 1a patients do not have circulating anti-thyroid antibodies, and do not develop a goiter despite elevated serum levels of TSH [26], a finding consistent with impaired responsiveness to TSH due to decreased expression of Gαs protein. A previous study of a single patient with PHP type 1a with mild primary hypothyroidism demonstrated reduced, but not absent, levels of Gαs protein in plasma membranes prepared from thyroid tissue [31]. Basal as well as sodium fluoride-stimulated adenyl cyclase activities in these membranes were normal, indicating that the number of Gαs molecules in these membranes, although reduced by comparison to normal, was still sufficient when directly activated to stimulate adenyl cyclase maximally. By contrast, 100 nM TSH stimulated adenyl cyclase activity 2.7-fold in membranes from the patient, whereas a 10-fold increase in activity was detected in membranes from normal subjects. These results are consistent with the current modes of stoichiometry and compartmentalization in G protein-coupled receptor signaling [32]. In the case of the β-adrenergic receptor-coupled signal cascade, the ratio of β-adrenergic receptor-Gs-adenyl cyclase is approximately 1:100-3, and receptor activation of Gs appears to be the critical factor for amplification of signaling [33–35]. Although this model would predict that either receptor or adenyl cyclase, but not Gs, would set the limit on the maximal efficiency of the system, this analysis fails to consider the effect of potential compartmentalization of signaling molecules in the plasma membrane. Thus, our current results, taken in the context of previous observations that demonstrated reduced TSH-stimulated adenyl cyclase in thyroid membranes from a patient with PHP type 1a [31], argue that in PHP type 1a an insufficient number of Gαs molecules are physically associated with TSH receptors to assure normal TSH signaling.

In the mouse, paternal imprinting of Gαs occurs in the proximal renal tubules, which accounts for the development of PTH resistance in Gnas+/- mice inheriting the mutated maternal allele and normal PTH responsiveness in Gnas+/- mice inheriting the mutated paternal allele [22]. Although these results provide an explanation for the development of PTH resistance in human subjects with PHP type 1a, studies of GNAS expression in human fetal tissues, including kidney cortex, have failed to demonstrate paternal imprinting of Gαs [19]. Rather than discard the notion that imprinting, with reduced expression of Gαs, accounts for hormone resistance in PHP type 1a, we believe our present results may offer an alternative explanation. Our results show biallelic expression of GNAS in the thyroid, but with preferential maternal expression rather than complete repression of paternal allelic transcription. Similar results have been demonstrated in GH-secreting tumors and normal pituitaries [21] and the renal cortex of Gnas+/- mice [22]. Thus, it is likely that the relatively heterogeneous population of cells examined, including not only proximal renal tubular areas but also condensing mesenchyme and developing nephrons, as well as the number of amplification cycles and non-quantitative technique used to analyze PCR products, may have accounted for the failure to detect preferential expression of the maternal allele in previous studies of fetal human kidney cortex [19]. In addition, fetal human kidney cortex may be quite different from the postnatal kidney cortex.

The absence of typical features of an imprinted transcription unit, as well as the incomplete repression of paternal allele transcription, suggest that Gαs transcription is regulated by a secondary mechanism such as
the insulator model that has been proposed to explain reciprocal expression of the H19 and IGF2 genes [36]. Further evidence in support of this model comes from recent studies of patients with PHP type 1b that associate loss of methylation of the exon 1A DMR with reduced expression of \( G_\alpha \) in the renal cortex [14,15,20].

In conclusion, our results demonstrate relaxed paternal imprinting of \( G_\alpha \) in human thyroid. Although the precise molecular mechanism for the temporal and spatial paternal imprinting of \( G_\alpha \) is not completely defined, cell-specific paternal imprinting provides an explanation for the unusual pattern of inheritance and distribution of hormone resistance in PHP type 1a.

Acknowledgments

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References


