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The Gsα Gene: Predominant Maternal Origin of Transcription in Human Thyroid Gland and Gonads

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Mutations in the guanine nucleotide binding α-subunit 1 gene (GNAS1) cause Albright's hereditary osteodistrophy, and the parent of transmission determines variable phenotypic expression of the disease. This has suggested that GNAS1 may be under tissue-specific imprinting control, although studies so far available have failed to clearly define the pattern of GNAS1 expression in humans. To establish if GNAS1 is imprinted in human endocrine tissues, we selected 14 thyroid, 10 granulosa cell, 13 pituitary (3 normal glands, 7 GH-secreting adenomas, and 3 nonfunctioning adenomas), 3 adrenal, and 11 lymphocyte samples shown to be heterozygous for a known polymorphism in exon 5. RNA from these tissues was analyzed by RT-PCR, and expression from both parental alleles was evaluated by enzymatic digestion and subsequent quantification of the resulting fragments. The parental origin of Gsα was assessed by evaluating neuroendocrine secretory protein 55 and extra large αs-like protein transcripts, which have been shown to be monoallelically and parent-specifically expressed from the maternal and paternal allele, respectively. By this approach, the great majority of thyroid (n = 12), ovarian (n = 7), and pituitary (n = 11) samples showed an almost exclusive or significantly predominant expression of the maternal allele over the paternal one, whereas in lymphocyte and adrenal samples both alleles were equally expressed. Our results provide evidence for a predominant maternal origin of GNAS1 transcripts in different human adult endocrine tissues, particularly thyroid, ovary, and pituitary, and strongly suggest that this mechanism may play a crucial role in the determination of the phenotypic expression of Albright's hereditary osteodistrophy. (*J Clin Endocrinol Metab* 87: 4736–4740, 2002)

GENOMIC IMPRINTING IS an epigenetic phenomenon affecting a small number of genes by which one allele (maternal or paternal) undergoes, either during the embryogenesis or in the postnatal period, a partial or total loss of expression (1). In humans, imprinting plays a role in the pathogenesis of various disorders, such as Angelman, Beckwith-Wiedeman, and Prader-Willi syndromes. More recently, Liu *et al.* (2) identified in patients affected with pseudohypoparathyroidism (PHP) type Ib (3) an imprinting defect of guanine nucleotide binding α-subunit 1 gene (GNAS1), which is likely to be involved in the pathogenesis of the disease.

GNAS1 maps on human chromosome 20q13 and on mouse distal chromosome 2. Both regions have been recently shown to be under complex imprinting control (4–8). In fact, by using alternative promoters and first exons splicing into a common site in exon 2, the GNAS1 locus gives rise not only to the Gsα gene, but to at least three other gene products, *i.e.* the extra large αs-like protein (XLαs) (9), the neuroendocrine secretory protein 55 (NESP55) (10) and a nontranslated transcript deriving from Exon 1A (11) (Fig. 1). A fourth antisense transcript is believed to play a role in regulating NESP55 expression (12). The XLαs, antisense, and 1A transcripts are expressed only from the paternal allele, whereas NESP55 is expressed specifically from the maternal allele.

Heterozygous loss of function mutations in GNAS1 inherited from the mother lead to PHP type Ia, in which Albright's hereditary osteodistrophy (AHO), a disorder characterized by a constellation of physical features, is associated to end organ resistance to the action of different hormones, primarily PTH, TSH, and gonadotropins. Interestingly, when the same mutations are inherited from the father, patients show the physical abnormalities of AHO, without any evidence of hormone resistance (pseudo-pseudohypoparathyroidism, PPHP) (13, 14).

Although the pattern of inheritance of PHP Ia is consistent with imprinting of GNAS1 paternal allele, different expression studies on RNA from various human fetal tissues have failed to demonstrate monoallelic expression of this gene (15, 16). Consistent with the view that genomic imprinting may be a phenomenon restricted to selective tissues and evolving in postnatal life, monoallelic expression of the Gsα gene has been recently shown in the human adult pituitary, although this gland is not classically included among the target organs resistant to hormone action in AHO (17–19). The aim of this study was to investigate Gsα allelic expression and parental origin in human adult endocrine tissues that are differentially involved in the determination of PHP Ia phenotype.

Materials and Methods

Tissues

The study included 25 normal perinodular thyroid tissues obtained from patients undergoing surgery for multinodular goiter or toxic adenoma; 22 granulosa cell specimens (from ovarian follicular fluid) from patients undergoing *in vitro* fertilization because of idiopathic infertility; 27 pituitary tissues, including 6 normal glands obtained at autopsy, 13 GH-secreting and 7 nonfunctioning pituitary adenomas surgically removed from patients with pituitary adenomas; 6 normal perinodular

Abbreviations: AHO, Albright's hereditary osteodistrophy; GNAS1, guanine nucleotide binding α-subunit 1 gene; NESP55, neuroendocrine secretory protein 55; PHP, pseudohypoparathyroidism; PPHP, pseudo-pseudohypoparathyroidism; XLαs, extra large αs-like protein.

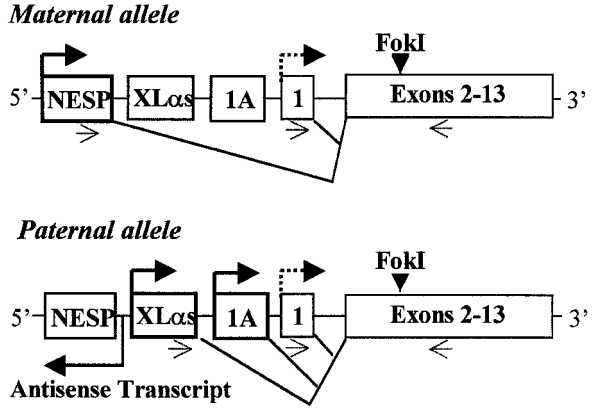

FIG. 1. Genomic organization of human GNAS locus. The figure shows four alternative first exons that splice into exon 2, generating four different transcripts: Gsα, NESP55, XLαs, and a noncoding gene product from exon 1A. Both the maternal and paternal alleles are shown: *arrows* designate transcription start sites. An additional antisense transcript has been recently demonstrated to be expressed only from the paternal allele. The *arrowhead* marks the polymorphic *Fok*I site in exon 5; *gray arrows* indicate RT-PCR primers.

adrenal glands obtained from patients undergoing surgery for benign cortical adenomas, and 20 blood samples from normal subjects.

Informed consent was obtained in all cases, and prior project approval was given by the local ethic committee.

PCR, RT-PCR, and digestions

DNA and RNA were extracted from all samples with standard methods. Exon 5 (GenBank accession no. M21741 J03647) was amplified from genomic DNA by PCR using the primers and under the conditions described previously (sense: 5′-ATGAAAGCAGTACTCCTAACTGA-3′; antisense: 5′-TGGATGCTCCTGCCCATGTG-3′) (20). The amplified products were then screened by digestion for a silent T to C polymorphism (Ile 131, nucleotides 297–299) that creates an *Fok*I site (1 h at 37 C, with 1 U of enzyme). Heterozygosity for this polymorphism was then confirmed by direct sequencing (ABI-PRISM 310, PE Applied Biosystems, Foster City, CA).

To examine allele-specific gene expression, 5 μg total RNA were reverse transcribed (Promega Corp., Madison WI) and then subjected to PCR (28 cycles at 94/58/72 C for 45/45/45 sec) using a common downstream primer (5′-CCTTGGCATGCTCATAGAATTC-3′) (5) located in exon 6 and 3 exon 1-specific upstream primers amplifying Gαs (GenBank accession no. M21139 J03647, nucleotides 789–807), XLαs (GenBank accession no. AJ 251760, nucleotides 16000–16021), and NESP55 (GenBank accession no. AJ 251760, nucleotides 1432–1451) genes, respectively (Gαs: 5′-CCATGGGCTGCCTCGGGAACA-3′; XLαs:5′-CGCAGTAAGCTCATCGACAAAG-3′; NESP55: 5′-AGCCCGAGGACAAAGATCCA-3′). RT-PCR products were digested with *Fok*I as for genomic DNA and visualized on 2% Nusieve-1% agarose gels. Bands from Gsα RT-PCR were finally evaluated by an imaging densitometer (GS-700, Bio-Rad Laboratories, Inc., Richmond, CA), and the maternal contribution was calculated as a percentage of the sum of the normalized values for the two alleles.

Somatotroph adenomas were also screened for somatic mutations at codon 201 or 227 of the Gαs gene (the *gsp* oncogene) as previously described (21).

Results

The evaluation of parental origin of transcription of the Gsα gene was carried out on samples found to be heterozygous for the polymorphism in exon 5, that is 14 thyroid, 10 granulosa cell, 13 pituitary (3 normal glands, 7 GH-secreting adenomas, 3 of which were *gsp*+, and 3 nonfunctioning adenomas), 3 adrenal, and 11 lymphocyte samples. The parental origin of Gsα transcripts was established by *Fok*I digestion and direct sequencing of NESP55 and XLαs transcripts. Both analyses showed the expected monoallelic expression from opposite alleles. In particular, when a NESP-derived transcript showed to carry the polymorphism, *i.e.* the correspondent band was digested by *Fok*1, the Xlαs-derived transcript from the same sample always displayed a wild-type sequence and was not digested by the same enzyme. In contrast, Gsα-derived transcripts were always biallelically expressed but, with the exception of two samples, all thyroid tissues showed a significant predominant expression of the maternal allele over the paternal one (Figs. 2A and 4), *i.e.* the most expressed allele was always the same represented in NESP55 transcripts (ratio maternal/total × 100 ± SD: 75.7 ± 13.4%). In particular, in five samples we observed a striking predominance of the maternally derived transcript (89.6 ± 4.4%), whereas in the other seven this phenomenon was seen at a lesser but still significant extent (72.7 ± 4.3%). Moreover, in two patients we could compare Gsα allelic expression between thyroid gland and lymphocytes from peripheral blood. As shown in Fig. 2B, the predominance of the maternal allele observed in the thyroid was absent in lymphocyte samples.

Seven of 10 granulosa cell samples showed a less striking but still significant predominance of the maternal allele (68.8 ± 6.7%) (Figs. 3 and 4).

A striking predominance of the maternal allele was observed in both normal and adenomatous pituitary tissues. In particular, all the normal pituitaries (n = 3) displayed an almost exclusive Gsα expression from the maternal allele (92.3 ± 3%), with a maximum 11% contribution from the paternal one. With the exception of two adenomas (one *gsp*-GH-secreting and one nonfunctioning tumor) in which the two alleles were equally expressed, a similar pattern of expression was demonstrated in the adenomatous samples (87.8 ± 7.8%), with no difference observed between nonfunctioning and GH-secreting adenomas (either *gsp*+ or *gsp*−) (Figs. 2C and 4).

In all adrenal and lymphocyte samples, both the paternal and the maternal alleles were expressed at the same extent (Fig. 4).

Discussion

Our study first demonstrates that, in specific endocrine tissues, GNAS1 transcription mainly derives from the maternal allele. In particular, a predominant, though not exclusive, maternal origin of Gsα was observed in adult human thyroid, ovarian cells and pituitary, whereas in the adrenal both the paternal and maternal alleles were equally expressed. The cell specificity of GNAS1 imprinting was strongly supported by the experiments showing a similar maternal and paternal contribution to GNAS1 expression in lymphocytes obtained from subjects in whom the removed thyroid only expressed the maternally derived GNAS1. In tissues mainly expressing the maternal allele, the predominance of the maternally derived transcript occurred at a variable extent in different samples, being almost exclusive in the majority of pituitary samples, largely predominant in most thyroid samples and less striking, but still significantly

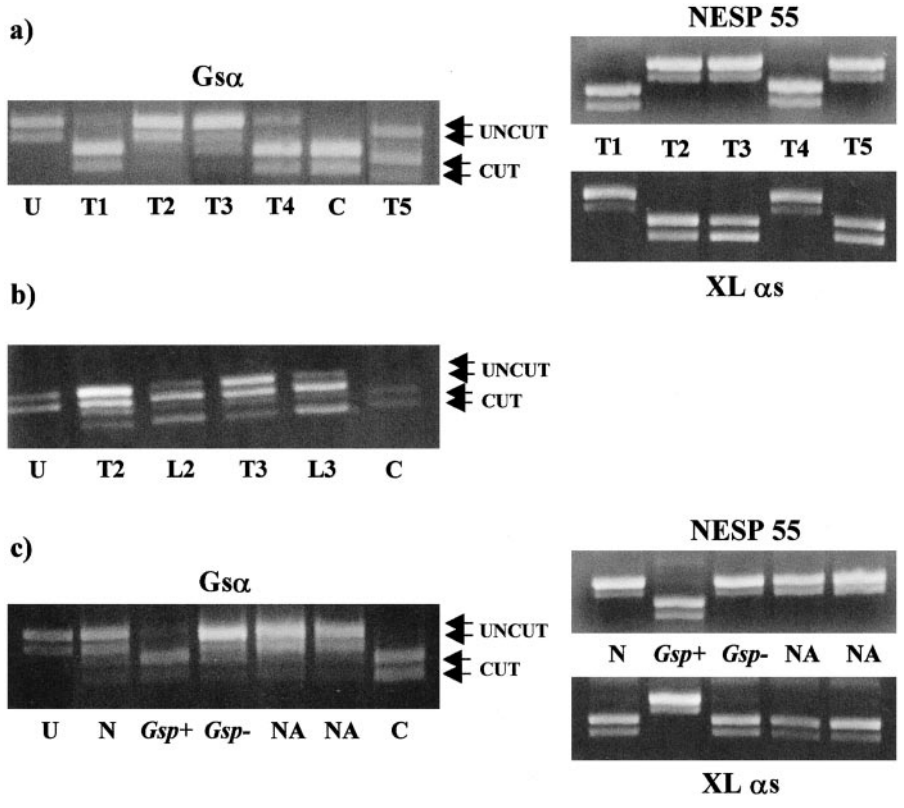

FIG. 2. GNAS1 expression patterns in different human tissue samples derived from RT-PCR of mRNA. In each figure, *arrows* indicate undigested and digested bands respectively. Double bands observed in all samples are due to alternative splicing of exon 3, characteristic of all GNAS1 transcripts. A, Expression of Gsα, NESP55, and XLαs genes after 1 h digestion with *Fok*I in four different thyroid (T) samples heterozygous for the polymorphism in exon 5. With the exception of sample T5, which shows a comparable intensity of bands from the two alleles, the others display a striking predominance of one allele over the other. In all cases, study of NESP55 and XLαs expression indicated the maternal allele to be the predominant one. Uncut (U) and cut (C) controls are also shown in the figure. B, Comparison of Gsα expression in thyroid (T) and lymphocytes (L) from two samples: the predominance of maternal origin of transcription observed in thyroid samples appear to be lost in lymphocyte ones, thus providing evidence for tissue-specific imprinting of this gene. C, Analysis of allelic expression of GNAS1 transcripts in human pituitary glands: N, normal pituitary; NA, nonfunctioning adenoma; *gsp*+ and *gsp*−, GH-secreting adenoma positive and negative, respectively, for mutations of the *gsp* oncogene.

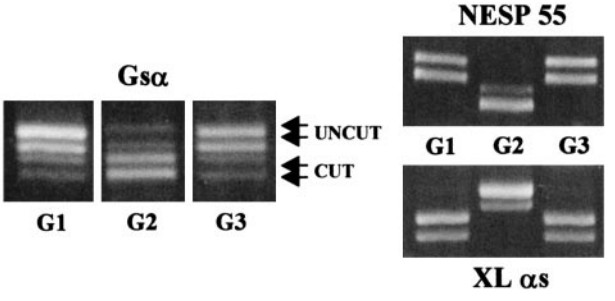

FIG. 3. Expression of GNAS1-derived transcripts (Gsα, NESP55, and XLαs) in granulosa cell extracts (G1, G2, and G3); as observed in thyroid and pituitary samples, the maternal allele resulted to be more expressed respect to the paternal one. However, densitometric evaluation of these bands demonstrated a higher contribution from the paternal allele compared with the one seen in the pituitary and the thyroid (see *Results* for more details).

higher than the paternally derived transcript, in granulosa cells. This variable pattern of GNAS1 imprinting is consistent with the incomplete transcriptional repression of other imprinted genes that have been previously observed in individual tissues (22–24), indicating that imprinting is not necessarily an all-or-nothing phenomenon. Mechanisms underlying these findings include either biallelic expression by a subset of cells within a tissue or an only partial silencing of one allele in all cells of such tissues. Moreover, the difference observed among different samples, particularly among granulosa cell preparations, might also be, at least partially, attributed to the different degree of contamination of tissue samples by the peripheral blood, which biallelically expressed GNAS1.

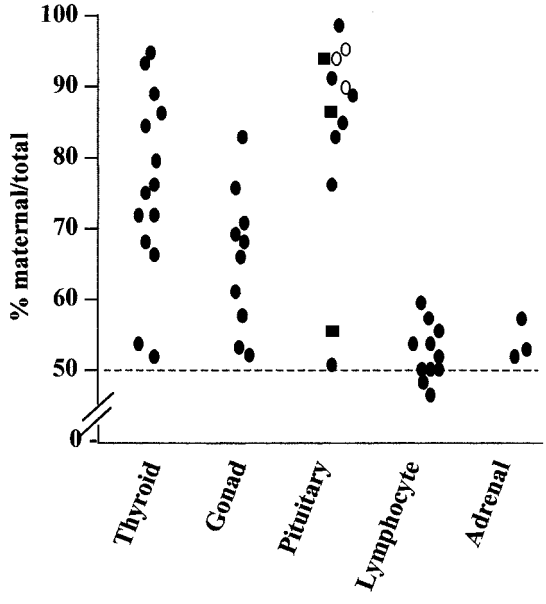

FIG. 4. The diagram shows the estimated percentage of maternally derived transcripts in 14 thyroid glands, 10 gonads (granulosa cells), 12 pituitary glands (*open circles*, normal ones; *filled circles*, somatotroph adenomas; *filled squares*, nonfunctioning adenomas), 11 lymphocyte samples, and 3 adrenal glands.

The data reported here strongly support the hypothesis that imprinting of the Gsα gene is the potential mechanism responsible for occurrence of variable resistance to hormone action in patients with GNAS1 mutations. First, the predominant, though not exclusive, maternal origin of Gsα is consistent with the pattern of inheritance of PHP Ia. Indeed,

mutations in GNAS1 inherited from the mother lead to PHP type Ia, in which AHO is associated with multiple hormone resistance, whereas the same mutations inherited from the father lead to PPHP in which AHO is not accompanied by any evidence of hormone resistance (13, 14). Although imprinting of GNAS1 has been proposed to explain the occurrence of PHP Ia and PPHP in patients with AHO (14), expression studies carried out up to now in various human fetal tissues have failed to demonstrate monoallelic expression of this gene (15, 16). The recent report of GNAS1 biallelic expression in human kidney cortex (16) is only apparently in contrast with our findings. In fact, Zheng *et al*. (16) carried out their study on fetal tissues, and it has been demonstrated that imprinting can be a process beginning and evolving in postnatal life (25). This hypothesis is also supported by the observation that PHP Ia patients do not show any sign of hormone resistance at birth, but they generally develop hypocalcemia, hypothyroidism, and hypogonadism over childhood or adolescence. Moreover, the kidney cortex was studied as a whole, whereas data from mice indicate imprinting of GNAS1 as a cell-specific phenomenon, limited to the proximal renal tubule, *i.e*. the main PTH target tissue.

Secondly, imprinting of GNAS1 paternal allele was present in selective tissues, such as the thyroid and the gonad, the two endocrine tissues that, besides the kidney, are mostly affected in PHP Ia patients. The observation of a different extent of maternal contribution to GNAS1 expression in thyroid and gonadal samples may explain why hypothyroidism is present in the totality of patients with PHP Ia, whereas hypogonadism is demonstrated in a lower percentage of cases, though it is possible that the slight differences observed in this still restricted number of samples may not have a clinical impact. In agreement with the notion that, with the exception of a single report (26), patients with PHP Ia show a normal responsiveness to ACTH, both the paternal and maternal allele was equally expressed in the adrenal gland, thus justifying the occurrence of resistance to some (PTH, TSH, and gonadotropins), but not all hormones that activate Gs-coupled pathways in patients with PHP Ia.

The findings of a striking predominance of the maternal allele in normal pituitary glands confirms the recent report by Hayward *et al.* (17). These data are in contrast with what observed in PHP Ia patients, who do not display, with the exception of anecdotal case reports (27–29), any sign of GH deficiency, as it would be expected by the coupling of the GHRH receptor to the cAMP-dependent pathway via Gs protein. However, the observation that GHRH is also effective in triggering intracellular calcium increase in somatotrophs could partially explain the preserved GH secretion in these patients (30). Alternatively, it is also possible to hypothesize a compensatory expression of the paternal allele in the presence of a defective GNAS1 transcription, though it remains unclear why such compensation does not occur in the kidney, the thyroid or the gonad.

Finally, in contrast with the report by Hayward *et al*. (17) of a partial loss of imprinting in somatotroph adenomas, our data from both nonfunctioning and GH-secreting adenomas (either carrying mutations of the *gsp* oncogene or not) are similar to those observed in normal glands. Based on the present study and given the relatively low number of cases reported in the literature, additional studies are required to confirm a role for GNAS1 imprinting in the pathogenesis of acromegaly.

In conclusion, our study demonstrates a predominant maternal origin of GNAS1 transcripts in different human adult endocrine tissues, particularly in the thyroid and the gonad. These data strongly support the hypothesis that imprinting of the GNAS1 gene is the potential mechanism responsible for occurrence of variable resistance to hormone action in patients with AHO.

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