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Potent constitutive cyclic AMP-generating activity of XLas implicates this imprinted *GNAS* product in the pathogenesis of McCune-Albright Syndrome and fibrous dysplasia of bone

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Abstract

Patients with McCune-Albright syndrome (MAS), characterized primarily by hyperpigmented skin lesions, precocious puberty, and fibrous dysplasia of bone, carry postzygotic heterozygous mutations of *GNAS* causing constitutive cAMP signaling. *GNAS* encodes the α -subunit of the stimulatory G protein (G_{α}), as well as a large variant (XLas) derived from the paternal allele. The mutations causing MAS affect both *GNAS* products, but whether XLas, like G_{α} , can be involved in the pathogenesis remains unknown. Here, we investigated biopsy samples from four previously reported and eight new patients with MAS. Activating mutations of *GNAS* (Arg201 with respect to the amino acid sequence of G_{α}) were present in all the previously reported and five of the new cases. The mutation was detected within the paternally expressed XLas transcript in five and the maternally expressed NESP55 transcript in four cases. Tissues carrying paternal mutations appeared to have higher XLas mRNA levels than maternal mutations. The human XLas mutant analogous to G_{α} -R201H (XLas-R543H) showed markedly higher basal cAMP accumulation than wild-type XLas in transfected cells. Wild-type XLas demonstrated higher basal and isoproterenol-induced cAMP signaling than G_{α} and co-purified with $G\beta 1\gamma 2$ in transduced cells. XLas mRNA was measurable in mouse calvarial cells, with its level being significantly higher in undifferentiated cells than those expressing preosteoblastic markers osterix and alkaline phosphatase. XLas mRNA was also expressed in murine bone marrow stromal cells and

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preosteoblastic MC3T3-E1 cells. Our findings are consistent with the possibility that constitutive XLas activity adds to the molecular pathogenesis of MAS and fibrous dysplasia of bone.

Keywords

GNAS; McCune-Albright syndrome; fibrous dysplasia of bone; *gsp* oncogene; stimulatory G protein; XLas

1. INTRODUCTION

The stimulatory guanine nucleotide binding protein mediates receptor-activated adenylyl cyclase stimulation and, thereby, intracellular cAMP accumulation [1-3]. The α -subunit of the stimulatory G protein ($G\alpha$) is encoded by exons 1-13 of the human *GNAS* gene [4]. *GNAS* is a complex locus giving rise to additional products including XLas, XXLas, NESP55 (neurosecretory protein 55), and the A/B transcripts (also known as 1A or 1') [5-12]. In addition, a transcript that is antisense to NESP55 (AS) is derived from *GNAS* [13-15]. XLas, XXLas, A/B, and AS are expressed paternally, while NESP55 is expressed maternally in all investigated tissues. In contrast, $G\alpha$ expression is biallelic in most tissues, including the bone [16]; however, a small subset of cells and tissues, such as renal proximal tubules, pituitary, testis, and thyroid, express $G\alpha$ predominantly from the maternal allele [17-21]. $G\alpha$ and its variant XLas are identical in the C-terminal portion encoded by exons 2-13 of *GNAS* but differ in their N-terminal domains encoded by different first exons [9,10,22]. Towards its C-terminal end, the N-terminal portion of XLas encoded by its first exon (the XL domain) becomes increasingly homologous to the corresponding region of $G\alpha$. Consistent with the high degree of homology between $G\alpha$ and XLas, both rat and human XLas can couple to typical $G\alpha$ -coupled receptors and can stimulate intracellular cAMP generation in transfected cells [23-25].

McCune-Albright syndrome (MAS) is a heterogeneous disease, in which affected individuals can present with various abnormalities, including fibrous dysplasia of bone, hyperpigmented (café-au-lait) skin lesions, sexual precocity and, sometimes, other endocrine dysfunctions (thyroid nodules, acromegaly, Cushing syndrome) [26,27]. Fibrous dysplasia of bone (FD) is a disorder with characteristic bone lesions containing a distinctive pathologic fibrous tissue [28,29]. FD can manifest as an isolated entity restricted to a single bone or it can affect multiple bones, typically as part of MAS. Both patients with MAS and those with isolated FD carry postzygotic missense mutations of *GNAS* that involve Arg201 [30-34] or, much less frequently, Gln227 of $G\alpha$ [35]. The identified amino acid changes at these residues inhibit the intrinsic GTP hydrolase activity, thereby resulting in constitutive $G\alpha$ signaling [36]. Arg201 and Gln227 are encoded by nucleotide sequences derived from exon 8 or exon 9, respectively [4], and are thus shared by $G\alpha$ and XLas. The mutations in patients with MAS/FD are heterozygous and can occur on the maternal or the paternal allele [37]. Therefore, these mutations are predicted to affect either $G\alpha$ alone or both $G\alpha$ and XLas. However, the possibility that the mutations on the paternal allele enhance signaling due to constitutive XLas activity, in addition to constitutive $G\alpha$ activity, and that this enhanced signaling contributes to the pathogenesis of MAS/FD have remained unexplored.

In this study, we addressed whether MAS mutations that occur on the paternal *GNAS* allele affect the XLas transcript and investigated the expression of this transcript in biopsy samples from MAS patients. Furthermore, we compared the activities of $G\alpha$ and XLas in cultured cells with respect to cAMP generation. We also investigated the expression of XLas transcript in mouse multipotent cells and bone cell precursors.

2. MATERIAL AND METHODS

2.1 Subjects

Except for two (patients 9 and 11), all samples were obtained from patients with at least two of the clinical triad of MAS (café-au-lait spots, precocious puberty, and fibrous dysplasia) and, in some cases, additional endocrine abnormalities (cortisol excess due to adrenal hyperplasia, hyperthyroidism, or GH excess) (Table 1). Patient 9 presented with hyperthyroidism, absence of anti-TSH receptor antibodies, and an adenoma on scintigraphy, which was removed surgically. Patient 11 had café-au-lait spots and neonatal Cushing's syndrome only; because he was only 6 months old (Table 1), fibrous dysplasia and precocious puberty could not be considered. Patients 3, 5, and 8 underwent ovarian surgery for the removal of a large cyst that could otherwise cause ovarian torsion; the surgery was not done for diagnosis. For all patients, the samples were obtained from biopsies during surgery, snap frozen in liquid nitrogen, and kept at -80°C until experiments were performed. Patients 1, 2, 3 and 6 correspond to patients 1, 3, 5 and 10, respectively in reference [37]. All patients, or their parents in the case of children, gave their informed consent to the genetic analysis presented herein, and the study was approved by the local institutional review board.

2.2 Plasmids and cell culture experiments

Complementary DNA encoding wild-type(wt) *Gsa* was previously subcloned into pcDNA3.1 (Invitrogen), as described [38]. To engineer cDNA encoding wt human XLas, the XLas portion was PCR-amplified from human genomic DNA with *Xba*I/*Avr*II linkers, and the portion encoded by exon 2-13 was PCR-amplified from wt *Gsa* cDNA with *Avr*II/*Hind*III linkers. Note that the *Avr*II site was introduced as a silent mutation to enable ligation of these two PCR products, which were subcloned into pcDNA3.1. The substitution of the Arg to His at position 201 in *Gsa* cDNA and the analogous substitution in XLas cDNA (Arg to His at position 543) were carried out by using the QuickChange site-directed mutagenesis kit (Stratagene). Both plasmid constructs were confirmed by complete sequencing of cDNA inserts. The adenoviral constructs have been described previously [25].

Human osteoblastic sarcoma (HOS) cells were kindly provided by Dr. Pierre Lebon (Hospital Saint Vincent de Paul). Mouse embryonic cells null for *Gsa* and XLas due to homologous disruption of *Gnas* exon 2 (*Gnas*^{E2-/E2-}) have been described [24]. *Gnas*^{E2-/E2-} cells and HOS cells cultured in 12-well plates in DMEM (Life Technologies, Paisley, Scotland) with 10% fetal bovine serum were cotransfected with (i) 500 ng per well plasmid DNA encoding wt-*Gsa*, R201H-*Gsa*, wt-XLas, R201H-XLas or the empty vector, and ii) 50 ng β -Gal-pCRE-Luc plasmid (Promega), which is a luciferase reporter gene used to assess the efficacy of transfection. Transfections were performed by using Effectene (QIAGEN, Courtaboeuf, France) at a DNA/Effectene ratio of 1/5. To compare *Gsa* and XLas mediated cAMP accumulation, *Gnas*^{E2-/E2-} cells were transduced with adenoviral vectors in order to obtain more than 90% of cells expressing the transduced vectors [25]. MC3T3-E1 cells were purchased from American Type Tissue Collection.

2.3 Total RNA extraction and RT-PCR amplification

Total RNA was extracted from tissues using Trizol® Reagent (Life Technologies, Paisley, Scotland) followed by a treatment in the RNeasy Mini Kit (QIAGEN) according to the supplied protocol and eluted in sterile water. One μg total RNA was reverse transcribed using the kit Superscript III First Strand Synthesis System (Invitrogen) according to the supplied protocol and then subjected to PCR. Each PCR reaction mixture contained 2 μL of cDNA, 1 X PCR buffer, 10 mM dNTP mixture, 50 mM MgCl_2 , 10 μM of each primer, and 1 U of Taq DNA Polymerase (Invitrogen) in a final reaction volume of 50 μL . PCR samples

were amplified for 1 cycle at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min. PCR products were purified and sent to be sequenced. The sequences of the primers used were as follows: *Gsa* forward primer 5'-CGTGAGGCCAACAAAAAGAT-3' and reverse 5'-ATGGCAGTCACATCGTTGAA-3' (674 bp); *NESP* forward primer 5'-CTTCCAAAAAGGGACCCATC-3' and reverse 5'-ATGGCAGTCACATCGTTGAA-3' (702 bp); *XLas* forward primer 5'-CGCAGTAAGCTCATCGACAA-3' and reverse 5'-ATGGCAGTCACATCGTTGAA-3' (668 bp).

2.4 Quantitative real time PCR

Real-time PCR experiments were done with the LightCycler LC480 device (Roche, Meylan, France) using the LightCycler® 480 SYBR Green I Master. The reaction mixture for the real-time PCR contained 1 µl of cDNA solution in a total volume of 10 µl containing 0.3 µM of each PCR primer, and master mix in accordance with the manufacturer's advices. The annealing temperature for PCR was 53°C (to amplify *XLas* and *GAPDH*) or 54°C (to amplify *Gsa*). Specificity of amplified products was verified by performing a melting curve analysis at the end of amplification (by stepwise increase of the temperature from 55 to 95 °C).

The primers and the product sizes were: for detecting *Gsa* F (5' AAGGACAAGCAGGTCTACCG 3') and R (5' TCTGCTTCACAATGGTGCTT 3') 121 bp, for detecting *XLas* F (5' GGACGAAAAGATGGGCTACA 3') and R (5' TCTGCTTCACAATGGTGCTT 3') 115 bp. The primers for the "house-keeping" gene, *GAPDH*, chosen as an endogenous internal control, were: F (5' GAGAAGGCTGGGGCTCAT 3') and R (5' TGCTGATGATCTTGAGGCTG 3'); product size was 130 bp. For all quantitative RT-PCR assays involving *GAPDH* amplification, the following controls were always undertaken: (1) human *GAPDH* cDNA was used as a positive control for the effectiveness of RNA extraction and RT-PCR reactions; (2) to control for false positive PCR amplification of contaminating genomic DNA, some samples did not include reverse transcriptase; (3) water was added instead of template to test for contamination with extraneous DNA. The *XLas* and *Gsa* transcripts in mouse bone marrow stromal and preosteoblastic cells were quantified by using the β -actin transcript as internal control. Each product was amplified with an efficiency of ~2. Primers used for the amplification of these transcripts were previously described [38].

2.5 cAMP measurements

After transfected cells were washed once with phosphate buffered saline (PBS), IBMX buffer (DMEM medium supplemented with 35 mM Hepes pH7.4, 2 mM IBMX, 0.1% BSA) was added into the well, and cells were incubated at 37°C for 15 min. Upon removal of the buffer, cells were subjected to one round of freeze-thaw lysis, before adding 0.5 ml 50 mM HCl into each well. Samples were kept at -20°C until the quantification of cAMP, which was accomplished by using the DELFIA cAMP kit (Perkin Elmer) according to the manufacturer protocol. In adenovirally transduced *Gnas*^{E2-/E2-} cells, basal cAMP accumulation and isoproterenol stimulation was carried out the same way, but cAMP concentration in each well was determined by a radioimmunoassay, as previously described [24]. The concentration of isoproterenol was 10⁻⁵M, which has been shown to be saturating with respect to cAMP accumulation [24,39].

2.6 Protein extraction and Western Blot analysis

Total cellular extracts were prepared from transfected cells, which were pelleted, washed twice in PBS, and lysed in a buffer containing 25 mM Hepes, 150 mM NaCl, 5 mM EDTA, 10% Glycerol, 0.02% sodium azide, 10 mM β -glycerophosphate, 10 mM sodium fluoride,

and a protease inhibitor cocktail (Sigma, St. Louis, MO). Following separation by 9% SDS-PAGE, proteins were blotted onto HyBond C membranes (Amersham), and reacted to specific antisera in a Tris-HCl buffered saline solution containing 0.1% Tween-20. An antibody against the common C-terminus of Gsa and XLas (sc-383) were used to identify both of these proteins. An antibody against β -actin (sc-8432) was used as a gel loading control. Bound antibodies were detected by horseradish peroxidase-conjugated secondary antibodies, followed by chemiluminescence (ECL, Amersham). Primary antibodies were purchased from Santa Cruz Biotechnology, and the antibody against rabbit IgG (#4010-05) from Southern Biotech.

2.7 Purification of G β subunits with XLas or Gsa

COS7 cells were triply transduced with adenovirus encoding XLas or Gsa together with adenovirus encoding G γ 2 and his6-tagged G β 1. Forty-eight hours after transduction, cells were harvested and membrane extracts were prepared by gentle lysis with hypotonic saline solution, followed by homogenization with a Dounce homogenizer. Following further extraction with 1% Na-cholate, proteins were purified through the use of immobilized nickel affinity chromatography, as previously described [40]. Eluted proteins were separated by 9% SDS-PAGE and Western blot was performed by using either the aforementioned anti-Gsa C-terminal antibody (for Gsa), an anti-human XLas antiserum directed against an epitope within the XL domain of human XLas (for XLas) [25], or an anti-pan G β antibody (Santa Cruz Biotechnology, sc-378).

2.8 Osteogenic differentiation of mouse calvarial osteoblastic cells

Experiments using the mouse were done in compliance with the International Animal Care and Use Convention and approved by Massachusetts General Hospital (MGH) subcommittee on research animal care. Primary calvarial osteoblastic cells were isolated from wild-type mice at postnatal day 6 by serial collagenase digestion [41]. Adherent cells were plated at 12,500 cells/cm² and osteogenic differentiation induced with 10 mM β -glycerol phosphate and 50 μ g/ml ascorbic acid. At specified time points some wells were harvested for total RNA isolation, while others were stained with 1% alizarin red to assess mineralization. Alizarin red deposition was then quantified by extraction with cetylpyridinium chloride [42]. Mouse bone marrow stromal cells were isolated from four-week old mice upon flushing the marrow space by Hank's buffered saline solution (Invitrogen). Adherent stromal cells were cultured in α -MEM medium (Invitrogen) containing 10% fetal bovine serum for two days before RNA isolation.

2.9 Isolation of Osteoblastic Cells by fluorescence-activated cell sorting (FACS)

Osteoblastic cells were harvested from neonatal calvariae of *Osx-GFP/+* mice (kindly provided by Stephen Rodda and Andrew McMahon, Harvard Medical School) [43] by serial collagenase digestion. Fractions 3–6 were pooled and resuspended in phosphate-buffered saline (PBS) with 2% fetal bovine serum. Cells were incubated with a biotinylated antibody directed against alkaline phosphatase (ALP) (kindly provided by Dr. Mark Horowitz, Yale University) followed by APC-conjugated streptavidin (eBioscience). Cells were isolated based on expression of *Osx-GFP* and ALP using a Dako MoFlo high-speed sorter.

2.10 Statistical Analysis

Experiments measuring cAMP accumulation in transfected *Gnas*^{E2⁻/E2⁻} and HOS cells were analyzed by taking the mean and standard error (S.E.M) of independent experiments. Statistical significance of observed differences was determined by using Student's *t*-test for unpaired samples. A *P* value of less than 0.05 was considered significant.

3. RESULTS

To investigate whether disease-causing *GNAS* mutations that lead to constitutive cAMP signaling affect not only *Gsa* but also *XLas*, we examined twelve patients, eleven of whom presented with clinical features of MAS and one with toxic thyroid adenoma. An activating *GNAS* mutation, along with its allelic origin, had been revealed in four of the cases [37]. To find out whether the eight previously unreported patients carried missense *Gsa* mutations at either Arg201 or Gln227, the two residues at which activating mutations have been previously identified, we isolated total RNA from biopsy samples and amplified, by conventional RT-PCR, three different *GNAS* transcripts with documented biallelic (*Gsa*), paternal (*XLas*), or maternal (*NESP55*) expression. Direct nucleotide sequence analysis of the RT-PCR products indicated that five of the 8 cases carried mutations (patients 4, 9, 10, 11 and 12) at position 201 (according to *Gsa*) (Table 1). The remaining 3 cases demonstrated the wild-type sequence both at this residue and at position 227 (patients 5, 7 and 8). The mutation was detected in the *XLas* transcript in patient 9, 10, 11 and 12, indicating a paternal origin. In contrast, the *NESP55* transcript carried the mutation in patient 4, indicating a maternal origin (Table 1). The allelic origin of the mutation in biopsy samples from the previously reported cases was also confirmed, and thus, the number of patients with paternal or maternal mutations was five and four, respectively (Table 1).

To quantify the relative levels of *XLas* transcript in the biopsy samples, we performed real-time RT-PCR, using *GAPDH* as a reference control. Although this analysis could not be performed for patients 11 and 12 due to insufficient amount of total RNA from the biopsy material, analysis of the remaining samples revealed that the level of *XLas* transcript was higher in cases with paternal mutations than maternal mutations (Fig. 1). For example, whereas *XLas* transcript could not be amplified in the bone biopsy samples from patients 2 and 4, who had the mutations on the maternal allele, it could easily be amplified in the bone biopsy sample from patient 10, who had the mutation on the paternal allele. In contrast, no similar correlation was evident between the level of *Gsa* expression and the allelic origin of the mutation (Fig. 1).

A rat *XLas* mutant corresponding to the *Gsa*-Q227L mutant has been shown to display constitutive activity in transfected cells [23]. To determine whether mutations affecting Arg201, which are exceedingly more frequent in patients with MAS/FD, also result in constitutive activity when placed in the human *XLas* backbone, we examined the basal signaling activity through *XLas*-R543H, which is analogous to *Gsa*-R201H. Agonist-independent basal cAMP accumulation in *Gnas*^{E2-/E2-} cells (mouse embryonic fibroblasts *null* for endogenous *Gsa* and *XLas*) transiently expressing *XLas*-R543H was significantly higher than cells transiently expressing wild-type *XLas* (3.5 ± 1 fold), and this finding was similar to that observed for *Gsa*-R201H compared to wild-type *Gsa* (1.7 ± 0.2 fold) (Fig. 2A). Likewise, HOS cells (a cell line derived from human osteosarcoma) transiently transfected with cDNA encoding either wild-type or mutant forms of *XLas* and *Gsa* demonstrated that *XLas*-R543H, like *Gsa*-R201H, is constitutively active. Basal cAMP accumulation in HOS cells transiently expressing *XLas*-R543H or *Gsa*-R201H was 19 ± 3.9 fold or 8.6 ± 4.9 fold ($p < 0.05$) higher than in those expressing native forms of these proteins, respectively (Fig. 2B). Western blot analyses showed that in *Gnas*^{E2-/E2-} cells both constitutively active *XLas* and *Gsa* mutants were expressed slightly lower than wild-type *XLas* and *Gsa* (Fig. 2C), confirming that the higher cAMP accumulation through the mutants was not due to their higher expression levels than wild-type proteins. The expression level of the constitutively active *XLas* mutant attained in transfected HOS cells appeared to be slightly higher than other transiently expressed proteins (Fig. 2C). However, this expression difference was not sufficient to account for the enhanced basal signaling through the *XLas* mutant, because

when normalized for expression levels, XLas-R543H showed ≈ 11 -fold higher basal cAMP than wild-type XLas.

A previous study by our group has shown markedly higher basal cAMP accumulation in OK cells transiently expressing XLas compared to Gs α [25], suggesting that wild-type XLas and wild-type Gs α may differ from each other in their abilities to stimulate cAMP formation. To address this question, we transduced *Gnas*^{E2-/E2-} cells with increasing multiplicity of infection of adenovirus encoding Gs α or XLas and, subsequently, determined basal cAMP accumulation. In XLas-expressing cells, basal cAMP accumulation was approximately 2-fold higher than in Gs α -expressing cells despite equivalent MOI used for the transductions (Fig. 3A). When these cells were stimulated by a saturating concentration of isoproterenol – acting on the endogenously expressed β -adrenergic receptor – cAMP accumulation was also higher in XLas expressing cells than Gs α expressing cells (Fig. 3A). The observed differences between XLas and Gs α mediated cAMP accumulation did not simply reflect the relative expression levels of these proteins, as Western blot (Fig. 3B) and real-time RT-PCR (Fig. 3C) analyses demonstrated that XLas was not expressed at higher levels than Gs α in the transduced *Gnas*^{E2-/E2-} cells.

G $\beta\gamma$ subunits are both involved in controlling the inactive state of G α subunits and ensuring effective G α activation by receptors (see references [44,45] for review). We thus asked whether XLas, like Gs α , is able to form a stable heterotrimeric complex with G $\beta\gamma$. To address this question, we performed immobilized metal affinity chromatography using membrane proteins of adenovirally transduced COS7 cells transiently expressing either Gs α or XLas in the presence or absence of his6-tagged G $\beta 1$ and G $\gamma 2$. On analysis of the purified samples by Western blots, Gs α and XLas immunoreactivity were detected in samples from cells expressing his6-tagged G $\beta 1$ and G $\gamma 2$ in combination with either Gs α or XLas, but not from those expressing either Gs α or XLas alone (Fig. 4). These results thus indicated that XLas can interact with G $\beta 1\gamma 2$.

To explore the expression of XLas in multipotent and preosteoblastic cells, we isolated mouse calvarial cells. RT-PCR experiments could detect XLas as well as Gs α expression in these cells both before and after osteogenic differentiation, although no significant alterations were detected in the levels of these transcripts during differentiation (Fig. 5A). However, real-time quantitative RT-PCR analysis of mouse calvarial cells upon fluorescent activated cell sorting revealed that XLas mRNA is more abundant in cells that lack the expression of both osterix and alkaline phosphatase compared to those that express these preosteoblastic markers (Fig. 5B). This trend was different from Gs α levels, which appeared to be similar in both cell populations (Fig. 5B). In all groups of sorted calvarial cells, however, XLas expression appeared to be markedly lower than Gs α expression, being $0.26 \pm 0.04\%$ of Gs α expression in cells lacking both osterix and alkaline phosphatase expression and $0.05 \pm 0.02\%$ of Gs α expression in cells expressing both of these markers. In addition to mouse calvarial cells, we were able to amplify the XLas transcript by using RT-PCR and total RNA from undifferentiated murine bone marrow stromal cells and the preosteoblastic MC3T3-E1 cells (Fig. 5C). Our attempts to detect endogenous XLas protein by Western blots using the aforementioned antibody against the common C-terminus of Gs α and XLas did not yield specific immunoreactive bands (data not shown).

4. DISCUSSION

In this study, we determined that constitutively activating *GNAS* mutations found in patients with MAS can occur on the paternal allele, thus affecting the XLas transcript. Our measurement of XLas transcript levels in biopsy samples revealed higher XLas expression in those tissues with paternal *GNAS* mutations than maternal mutations. Moreover, we

showed that MAS mutations can lead to constitutive cAMP accumulation not only through *Gsa* but also through *XLas* in transfected cells, that *XLas* is more potent than *Gsa* in mediating basal and isoproterenol-stimulated cAMP accumulation, and that *XLas* can interact with $G\beta 1\gamma 2$. Finally, we demonstrated that *XLas* mRNA is expressed in mouse calvarial osteoblasts, bone marrow stromal cells, and a pre-osteoblastic cell line.

We identified constitutively active *GNAS* mutations in nine of our 12 patients. No mutation at either Arg201 or Gln227 was detected in the remaining three cases. It is likely that the abundance of the mutant cells relative to wild-type cells is markedly low in the biopsy samples from these three patients, as conventional PCR approaches can sometimes fail to amplify the mutant allele in cases with low degree of mosaicism [46,47]. Analyzing the *Gsa* transcript, we also detected no activating mutations in patients 3 and 9, but the paternally expressed *XLas* transcript appeared to bear such a mutation (R201H) in each case. This finding is consistent with the tissue-specific paternal silencing of *Gsa* in the ovary (patient 3) and the thyroid (patient 9) [19,21].

A previous study [37] investigated the origin of mutations in different tissues from patients with MAS, identifying an almost equal allelic distribution (4 maternal and 5 paternal). In the five new patients with MAS that we describe here, the activating mutation is on the maternal allele in one patient, and on the paternal allele in four patients. Since these paternal mutations affect both *Gsa* and *XLas*, and since both of these *GNAS* products show imprinting (albeit tissue-specific with respect to *Gsa*), it is possible that certain features of MAS may develop depending on the allelic origin of the mutation. Indeed, cases with acromegaly have been associated with maternal mutations [18,37], which likely reflects the paternal silencing of *Gsa* expression in the pituitary [18]. Data from our study are consistent with those findings. None of the patients with paternal mutations developed clinical features of acromegaly, whereas patient 4, in addition to the previously reported patient 6 [37], who both carried maternal mutations, showed clinical features consistent with excess growth hormone. Although previous investigations have shown that *XLas* expression is restricted to the paternal *GNAS* allele, it is currently unknown whether any MAS features develop exclusively or predominantly in patients with paternal mutations. In our study group, hyperthyroidism was present only in patients 3 and 9, both of whom carried a paternal mutation. However, a maternal constitutively activating *GNAS* mutation has been previously documented in a case with autonomous thyroid adenoma [37], thus arguing against a direct correlation between paternal mutations and the involvement of thyroid in the MAS phenotype. Careful investigations of additional patients may reveal additional correlations between specific MAS features and the allelic origin of *GNAS* mutations, and may identify a role for constitutive *XLas* activity in the pathogenesis of this disorder. In fact, findings in patients 3 and 9 are rather interesting. *Gsa* is partially silenced from the paternal allele in the thyroid [19-21], and therefore, one might expect that the effect of a paternal mutation is more significant on *XLas* than *Gsa* in that tissue. It is thus tempting to speculate that constitutive *XLas* activity had an important role in the development of hyperthyroidism in these two patients.

A single study has shown that *XLas* is biallelic, although with some variation, in both normal and mutation-carrying bone marrow stromal cells of patients with fibrous dysplasia [48], raising the possibility that constitutively active *XLas* mutant is expressed regardless of the origin of the mutations causing MAS and fibrous dysplasia. Further investigations are needed to confirm this intriguing finding and address this possibility. Note, however, that our amplification of *Gsa*, *XLas*, and *NESP55* transcripts from MAS biopsy samples yielded data that are consistent with monoallelic expression of *XLas* in bone (see Table 1). The discrepancy in these results may reflect technical differences. In the study showing biallelic *XLas* expression [48], investigations were performed upon cloning the cells, and this may

have resulted in a loss of XLas imprinting. Disruption of allele-specific methylation as a result of cloning has been previously described [49].

Our findings are consistent with previous reports with respect to tissue-specific Gsa imprinting [19-21]. Only the maternal Gsa transcript was amplified from the biopsies of ovary and thyroid, whereas both wild-type and mutant Gsa transcripts were detectable in adrenals and bone. In fact, we also detected a heterozygous single nucleotide polymorphism (rs7121) in the Gsa transcript amplified from the healthy bone tissue of patient 10 (data not shown), further indicating biallelic Gsa expression in this tissue.

We could amplify the XLas transcript more readily from biopsy samples with paternal mutations than maternal mutations. Because the sequence of the RT-PCR products from paternally mutated samples did not indicate amplification of wild-type transcripts, it appears unlikely that the amplicons reflect both the wild-type and the mutant XLas transcript. As such, there may be a tendency of mutant XLas transcripts to be more abundant compared to wild-type XLas transcripts. However, it is possible that the difference between transcript levels from paternal and maternal mutations reflect the small sample size in our study. Analyses of additional biopsy samples will help address these possibilities, and if the difference between mutant and wild-type XLas transcript levels is confirmed, further investigations will be necessary in order to elucidate the underlying molecular mechanisms.

Our findings regarding the activity of wild-type human XLas are in keeping with the findings of a recent study, which showed, through the use of transient and stable transfections, that rat XLas has higher basal and isoproterenol-stimulated activity than Gsa [50]. The increased activity of XLas could possibly reflect a deficiency in the interaction between XLas and Gβγ subunits, but our findings argue against a prominent defect between XLas-Gβγ coupling. Supporting our findings are the results of a previous study [23], which provided indirect evidence suggesting that rat XLas forms a heterotrimeric complex with Gβ1γ2. On the other hand, although overt differences between XLas and Gsa regarding Gβγ interaction can thus be ruled out (at least regarding the interaction with Gβ1γ2), it is theoretically possible that subtle differences in the efficiency of interaction contribute to the differences between the potencies of XLas and Gsa in stimulating cAMP generation. Additionally, XLas may be unable to interact with certain other Gβγ combinations. This, however, is less likely to provide an explanation as to why XLas is more potent than Gsa, given that a variety of cell lines (mouse embryonic fibroblasts, HOS cells, HEK293 cells [50], and OK cells [25]), which likely differ from one another with respect to the repertoire and expression levels of the different Gβγ subunits, appear to be permissive for the relatively enhanced activity of XLas. Alternatively, Gsa and XLas may be subject to different regulatory constraints upon activation. For example, Gsa loses its membrane avidity upon activation [51-53], and this mechanism may not apply to XLas, thus allowing the latter to activate adenylyl cyclase in a sustained manner. Consistent with this possibility, a recent study have shown that, whereas a constitutively active Gsa mutant is localized predominantly to the cytoplasm, the cognate XLas mutant is localized to the plasma membrane [12]. Finally, it is also possible that the intrinsic ability of XLas to stimulate adenylyl cyclase is higher than that of Gsa. It will be important to dissect these possibilities and elucidate the differences between the cellular activities of XLas and Gsa as stimulatory G protein α-subunits.

The XLas transcript is expressed in cells that can differentiate into osteoblasts, albeit at much lower levels than the Gsa transcript. Although the expression of neither of these transcripts seemed to alter dramatically during osteogenic differentiation in vitro, the level of XLas, but not Gsa, was reduced in calvarial cells committed to the osteoblast lineage compared to others. This finding may simply indicate a correlation between the expression

level of XLas and the degree of cellular differentiation in mesenchymal cells. In agreement with this explanation, XLas expression in white adipose tissue has been shown to decline shortly after birth in mice [54]. Alternatively, our finding may suggest that differentiation into the osteoblastic lineage requires XLas levels to be suppressed, a hypothesis that is consistent with the potential role of constitutive XLas signaling in the pathogenesis of FD. Future investigations are necessary to elucidate the role of XLas in osteogenic cell fate and early osteoblastic differentiation.

In summary, we have shown that some patients with MAS/FD have paternal activating mutations within *GNAS* affecting both *Gsa* and XLas. Our results also indicate that XLas, which is expressed in multipotent stromal cells and preosteoblasts, has higher activity than *Gsa* with respect to adenylyl cyclase stimulation. These findings are consistent with the hypothesis that XLas-mediated constitutive signaling may contribute to the pathogenesis of MAS and FD.

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Abbreviations

MAS	McCune-Albright syndrome
FD	fibrous dysplasia of bone
Gsa	stimulatory G protein α -subunit
XLas	extra-large stimulatory G protein α -subunit
NESP55	55 kDa neuroendocrine secretory protein

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Figure 1.

RT-PCR analysis of *Gαs* and *XLαs* transcripts by using total RNA derived from biopsy samples of patients with MAS and a maternal or paternal mutation of *GNAS*. Total RNA was extracted from fresh frozen samples, and SYBR Green was used to detect the amplicons during real-time PCR, following reverse transcription. While the reverse primer was located in the junction of exons 9 and 10 for both transcripts, the forward primers were located in either exon 1 (for *Gαs*) or exon XL (for *XLαs*). GAPDH mRNA was used as a reference control to which the results were normalized. Data represent mean ± SEM of duplicate measurements from one representative of two independent experiments with similar results.

Figure 2.

Basal cAMP accumulation through XLαs-R543H is higher than wild-type XLαs. cAMP accumulation in *Gnas^{E2-/E2-}* (A) or HOS (B) cells transfected with either wild-type or constitutively active forms of Gsα and XLαs. Measurements were done following incubation at 37°C for 15 min a buffer containing 2 mM IBMX. Data, which are normalized to those obtained from respective wild-type proteins, are mean ± SEM of at least three independent experiments. ^a, $p < 0.05$ compared to wild-type according to Student's *t*-test. Basal cAMP in untransfected *GNAS* null cells = 0.28 ± 0.01 pmol/well; basal cAMP in empty vector transfected *GNAS* null cells = 0.28 ± 0.07 pmol/well; basal cAMP in untransfected HOS cells = 0.56 ± 0.18 pmol/well; basal cAMP in empty vector transfected HOS cells = 0.47 ± 0.05 pmol/well. Protein expression levels (C) of Gsα and XLαs in transfected *Gnas^{E2-/E2-}* (left) or HOS (right) determined by Western blot analysis using an antibody directed against the common C-terminal region of Gsα and XLαs. Actin immunoreactivity was determined as a gel loading control. Note that the transiently expressed Gsα protein is equivalent in molecular mass to the long Gsα variant, and that both the latter and short Gsα variant, which lacks the residues encoded by exon 3, were detectable in HOS cells. Transfection efficiency was controlled through co-transfection of a luciferase reporter gene (beta-gal-pCRE-luc), as indicated in Materials and Methods.

Figure 3.

Basal and isoproterenol-stimulated activity of XLαs is higher than Gsα. (A) Basal and isoproterenol (10^{-5} M)-induced cAMP accumulation in $Gnas^{E2-/E2-}$ cells adenovirally transduced with cDNA encoding either Gsα and XLαs. Incubations were performed for 15 min in the presence of 2 mM IBMX; MOI, multiplicity of infection. (B) Western blots showing the expression levels of XLαs and Gsα in the transduced cells. Immunoreactive bands indicating XLαs and Gsα were visualized on the same blots by using an antibody directed against the common C-terminal region of Gsα and XLαs. Actin immunoreactivity was used as a gel-loading control. (C) Real-time RT-PCR experiments showing the expression of XLαs and Gsα mRNA normalized to β-actin. Forward primers were located in exon 1 for Gsα and exon XL for XLαs, and the reverse primer, which was used for both transcripts, was located in exon 2. Data are mean $\pm$ SEM of duplicate measurements and representative of three independent experiments with similar results.

Figure 4.

Co-purification of $XL\alpha s$ and $G\beta 1\gamma 2$ expressed in COS7 cells. Membrane proteins of COS7 cells transduced with adenovirus encoding $Gs\alpha$ or $XL\alpha s$ with or without individual adenoviruses encoding $his6-G\beta 1$ and $G\gamma 2$ were subjected to immobilized metal (nickel) affinity chromatography (IMAC), and eluted proteins were analyzed by Western blot (IB) using an antibody against the common C-terminus of $Gs\alpha$ and $XL\alpha s$ (for $Gs\alpha$) or a polyclonal antiserum directed against the exon XL-encoded portion of $XL\alpha s$. $Gs\alpha$ or $XL\alpha s$ immunoreactivity was detected only in the eluate from cells co-transduced with $G\beta 1\gamma 2$. Anti-pan $G\beta$ antibody demonstrated the purified $G\beta$ subunit. Western blot analysis of proteins from extracted membranes is shown at the bottom. Data are representative of three independent experiments with similar results.

Figure 5.

XLαs mRNA is expressed in cells of the osteoblastic lineage. (A) Expression levels of Gsα and XLαs transcripts in mouse calvarial osteoblasts differentiated under osteogenic conditions in vitro. The levels were determined by real-time RT-PCR using total RNA isolated from the cells differentiated for indicated days in culture. Alizarin staining of the differentiated calvarial osteoblasts is shown below, including the quantification of the mineral content based on alizarin concentration. (B) Gsα and XLαs mRNA expression in cells from freshly isolated mouse calvarial cells sorted based on expression of green fluorescent protein driven by the osterix promoter (OsxGFP) and alkaline phosphatase (ALP). Total RNA was isolated directly from sorted cells before performing real-time RT-PCR. (C) Conventional RT-PCR amplification of XLαs and Gsα in a preosteoblastic cell line (MC3T3-E1) and mouse bone marrow stromal cells (BMSC). Total RNA was extracted from cells that were grown in normal growth medium. Data were normalized to the β-actin transcript, which was used as a reference control. A common reverse primer located in exon 2 and specific forward primers located in exon XL (XLαs) or exon 1 (Gsα) were used for both real-time and conventional PCR. Data in panels A and B are mean ± SEM of three independent experiments. Data in C are representative of two independent experiments with similar results.

Table 1

Clinical manifestations and *GNAS* mutation analysis in patients affected with MAS

Pt.	Age (yr)	Sex	FD	CAL	PP	GH	HT	CS	Biopsy	Gsa-201 ^c	XLas-543 ^c	NESP55 ^d	Origin
1 ^a	17	M	Yes	Yes	No	No	No	No	Bone lesion	CGT(R)/CAT(H)	na	CAT	Mat
2 ^a	16	M	Yes	Yes	No	No	No	No	Bone lesion	CGT(R)/TGT(C)	CGT(R)	TGT	Mat
3 ^a	28	F	Yes	Yes	Yes	No	Yes	No	Ovary	CGT(R)	CAT(H)	na.	Pat
4	18	F	Yes	Yes	Yes	Yes	No	No	Bone lesion	CGT(R)/CAT(H)	CGT(R)	CAT	Mat
5	5	F	Yes	Yes	Yes	No	No	No	Ovary	CGT(R)	CGT(R)	CGT	Wt
6 ^a	13	M	Yes	Yes	No	Yes	No	Yes	Adrenals	CGT(R)/TGT(C)	CGT(R)	TGT	Mat
7	12	M	Yes	Yes	Yes	No	No	No	Bone lesion	CGT(R)	CGT(R)	CGT	Wt
8	6	F	Yes	Yes	Yes	No	No	No	Ovary	CGT(R)	CGT(R)	CGT	Wt
9 ^b	11	F	No	No	No	No	Yes	No	Thyroid	CGT(R)	CAT(H)	na	Pat
10	12	F	Yes	Yes	Yes	No	No	No	Healthy Bone	CGT(R)	CGT(R)	CGT	Wt
10									Bone lesion	CGT(R)/CAT(H)	CAT(H)	CGT	Pat
11	0.5	M	No	Yes	No	No	No	Yes	Adrenals	CGT(R)/TGT(C)	TGT(C)	CGT	Pat
12	13	M	Yes	Yes	No	No	No	No	Bone lesion	CGT(R)/TGT(C)	TGT(C)	CGT	Pat

The age and the endocrine manifestations refer to the last evaluation performed. Pt., patient; FD, fibrous dysplasia; CAL, café-au-lait spots; PP, precocious puberty; GH, growth hormone secreting adenoma; HT, hyperthyroidism; CS, Cushing's syndrome; Mat, maternal; Pat, paternal; Wt, Wild type; na, not amplified.

^a previously reported by Mantovani et al.

^b patient with toxic thyroid adenoma.

^c letters in parentheses are amino acids encoded by the nucleotide triplets.

^d nucleotide sequence corresponds to that encoding Gsa-201 and XLas-543, but it is located in the 3' untranslated region of NESP55.