GNAS Defects Identified by Stimulatory G Protein α-Subunit Signalling Studies in Platelets

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Context: GNAS is an imprinted region that gives rise to several transcripts, antisense transcripts, and noncoding RNAs, including transcription of RNA encoding the α-subunit of the stimulatory G protein (Gsα). The complexity of the GNAS cluster results in ubiquitous genomic imprints, tissue-specific Gsα expression, and multiple genotype-phenotype relationships. Phenotypes resulting from genetic and epigenetic abnormalities of the GNAS region include Albright's hereditary osteodystrophy, pseudohypoparathyroidism types Ia (PHPIa) and Ib (PHPIb), and pseudopseudohypoparathyroidism (PPHP).

Objective: The aim was to study the complex GNAS pathology by a functional test as an alternative to the generally used but labor-intensive erythrocyte complementation assay.

Design and Patients: We report the first platelet-based diagnostic test for Gsα hypofunction, supported by clinical, biochemical, and molecular data for six patients with PHPIa or PPHP and nine patients with PHPIb. The platelet test is based on the inhibition of platelet aggregation by cAMP, produced after Gsα stimulation.

Results: Platelets are easily accessible, and platelet aggregation responses were found to reflect Gsα signaling defects in patients, in concordance with the patient's phenotype and genotype. Gsα hypofunction in PHPIa and PPHP patients with GNAS mutations was clearly detected by this method. Mildly decreased or normal Gsα function was detected in patients with PHPIb with either an overall or exon 1A-only epigenetic defect, respectively. Platelet Gsα expression was reduced in both PHPIb patient groups, whereas XLαs was up-regulated only in PHPIb patients with the broad epigenetic defect.

Conclusion: The platelet-based test is a novel tool for establishing the diagnosis of Gsα defects, which may otherwise be quite challenging. **(*J Clin Endocrinol Metab* 93: 4851–4859, 2008)**

The *GNAS* imprinted cluster on chromosome 20q13 is characterized by many complex genetic and epigenetic regulatory aspects (1, 2). It encodes multiple gene products through the use of alternative promoters and first exons that splice onto a common set of downstream exons (Fig. 1). The most downstream "classical" first exon (exon 1) generates ubiquitous transcripts for Gsα, the G protein α-subunit that couples receptors to adenylyl cyclase and is therefore required for receptor-mediated cAMP generation. Two upstream exons generate overlapping transcripts encoding the chromogranin-like protein NESP55 and the extra large Gsα isoform XLαs, respectively (3). NESP55 and XLαs are primarily expressed in neuroendocrine tissues, but little is known about their biological function. Finally, the exon 1A-containing transcripts are presumably not translated into a func-

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Abbreviations: AHO, Albright's hereditary osteodystrophy; gDNA, genomic DNA; PHPIa, pseudohypoparathyroidism type Ia; PPHP, pseudopseudohypoparathyroidism; PRP, platelet-rich plasma.

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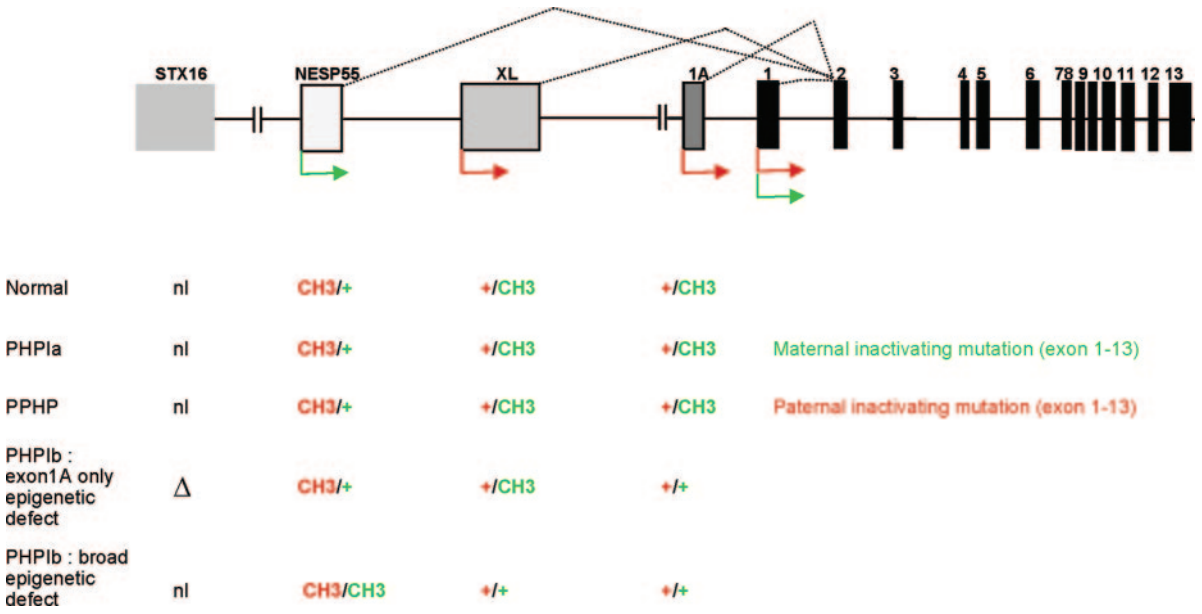

FIG. 1. Schematic representation of the human *STX16* loci and *GNAS* gene cluster on chromosome 20q13. Exons are depicted as *boxes*, and the *arrows* indicate the direction and allelic origin (*red* for paternal, *green* for maternal) of the different *GNAS* transcripts. Differential *GNAS* splicing is indicated with *dotted lines*. Paternal and maternal imprint settings are indicated as CH3 for the methylated allele and + for the transcriptional active allele. The different *GNAS*-associated pathologies with their responsible genetic abnormalities are indicated. nl, Normal; Δ, chromosomal deletion.

tional protein (4). All these additional gene products are oppositely imprinted: NESP55 is only expressed from the maternal allele and its promoter is methylated only on the paternal allele, whereas XLαs and exon 1A are expressed from the paternal allele, and their promoters are methylated only on the maternal allele (5, 6). Although the classical Gsα promoter is unmethylated on both alleles and mostly biallelically expressed in most human adult and fetal tissues (5–8), recent studies have reported prevalent maternal expression of the Gsα gene in some human postnatal tissues such as the pituitary (9), thyroid, and gonads (10). Studies in the mouse also revealed a predominant maternal Gsα expression in the renal proximal tubules (11–13), although this has not yet been demonstrated in humans.

Heterozygous inactivating Gsα mutations lead to Albright's hereditary osteodystrophy (AHO), an autosomal dominant disorder characterized by short stature, sc ossifications, brachydactyly, and, in many cases, mental retardation and/or developmental delay (14–17). In addition to AHO, maternal transmission of Gsα mutations can lead to end-organ resistance to several hormones that activate Gsα-coupled pathways, particularly resistance to PTH in the renal proximal tubule (12, 13). The presence of AHO, obesity, and multihormonal resistance is termed pseudohypoparathyroidism type Ia (PHPIa) (16–18). In contrast, paternal transmission of Gsα mutations leads to the AHO phenotype alone, a condition referred to as pseudopseudohypoparathyroidism (PPHP) (16–19). This different phenotype is explained by the differential expression of Gsα in the proximal renal tubule from the maternal allele only. Similar to what is observed in humans, maternal, but not paternal, transmission of the Gsα knockout allele in mice resulted in renal PTH resistance and reduced PTH-stimulated cAMP production in renal proximal tubules (13). Mice with targeted disruption of Gsα exon 2 in both alleles die in early gestation, demonstrating that Gsα expression is necessary for development (13, 20).

Patients with PHPIb present with renal PTH resistance, usually in the absence of the AHO phenotype (14). As in PHPIa, urinary cAMP responses to PTH are markedly reduced in patients with PHPIb, suggesting that Gsα expression is lost in renal proximal tubules. It was recently shown that PHPIb is caused by a *GNAS* imprinting defect that leads to a paternal-specific imprinting pattern (unmethylated, transcriptionally active) on both alleles in the exon 1A promoter region (21, 22). The familial form of the disease has been shown to be associated with an exon 1A-only methylation defect and a heterozygous 3-kb deletion mutation within the closely linked *STX16* gene (21–23). Similar methylation changes in the NESP55 and XLαs promoter regions are only present in patients with a sporadic form of PHPIb (23). The exon 1A region is believed to be critical for the tissue-specific imprinting of Gsα in the renal proximal tubules.

AHO is characterized by considerable genetic heterogeneity (24–26). In a recent study, Gsα activity was determined in isolated erythrocyte membranes from 106 patients with AHO, including 93 subjects with endocrine abnormalities (PHPIa) (27). Significantly reduced Gsα activity was found in 78 PHPIa and 13 PPHP patients, but an inactivating *GNAS* mutation was found only in 64 and 23% of these patients, respectively. In contrast, *GNAS* mutations were also found in 26% of patients with AHO and endocrinopathies who had normal Gsα erythrocyte function. This clinical entity is often referred to as PHP type Ic, but actually might rather reflect limitations of the assay used for the detection of Gsα activity, and probably represents a variant of PHPIa. The low mutation detection rate in patients with abnormal erythrocyte Gsα activity and the fact that *GNAS* mutations exist in patients with normal Gsα activity could be due to either a clinical or biological misdiagnosis of Gsα loss of activity. In some cases, a firm clinical diagnosis of PHPIa and especially PPHP may be hard to make because the AHO features can be nonspecific. Also, patients with PHPIb have a normal erythro-

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cyte Gsα activity (26). The generally used quantitative determination of erythrocyte Gsα functional activity is based on an *ex vivo* complementation assay in which adenylyl cyclase of S49 cyc$^-$ murine lymphoma cells, which genetically lack Gsα, is reconstituted with human erythrocyte membranes (27, 28). This method relies on Gsα activation via ADP ribosylation or GTPγS stimulation and is generally used but not readily applicable for screening purposes. In addition, a normal Gsα activity in the presence of an inactivating GNAS mutation can still be determined because direct Gsα-coupled receptor activation is not being measured by this method. There is a need for a robust and less cumbersome assay to study Gsα activity after Gsα-coupled receptor activation in children and adults, using routinely available cells such as platelets, which may subsequently also contribute to the identification of genetic defects.

We propose to use a platelet-based Gsα activity assay that we previously described in patients with a platelet Gsα hyperfunction due to an insertion in XLαs and in a PHPIb patient with a mild platelet Gsα hypofunction (29, 30). This test is based on the inhibition of platelet aggregation by cAMP produced by Gsα agonists and proved to be straightforward and reliable, requiring only 15 ml of blood. Platelet Gsα activity was studied in six patients with a clinical AHO phenotype and in nine patients with PHPIb having a broad or exon 1A-only epigenetic defect. We found that the platelet Gsα function test reflects the patients' clinical and molecular defects. In addition to leukocyte genomic DNA (gDNA), we also used platelet reversed-transcribed RNA to screen the *GNAS* coding region for mutations and platelet protein extracts to unravel underlying Gsα signaling defects further.

Subjects and Methods

Blood sampling

Healthy donors and AHO and PHPIb patients, not taking aspirin or other medication interfering with platelet function for at least 10 d, donated 15 ml of blood anticoagulated with 3.8% (wt/vol) trisodium citrate, as well as 10 ml of EDTA anticoagulated blood. Platelet-rich plasma (PRP) from these samples was prepared by centrifugation (15 min at 150 × *g*). Citrated PRP was adjusted by autologous plasma to 250×10^9 platelets per liter for platelet aggregations; 2 ml PRP was pelleted by centrifugation to prepare platelet protein extracts. PRP from EDTA anticoagulated blood was used for platelet RNA extraction. Informed consent was obtained from all participants and/or their legal representatives. The Institutional Review Board approved this study.

Platelet aggregation-inhibition test

Platelet aggregation was performed within 3 h after blood collection on two dual-channel Chrono-Log Aggregometers (Chronolog Corp., Havertown, PA) as described (29, 30). Aggregation inhibition studies involved dose-response curves upon platelet stimulation with Gsα agonists: prostaglandin E1 (Prostin, 0–1 μg/ml; GE Healthcare Life Sciences, Uppsala, Sweden), or the stable prostacyclin analog Iloprost (Ilomédine, 0–5 ng/ml; Schering-Plough, Kenilworth, NY). These were added to PRP 1 min before induction of aggregation by collagen (2 μg/ml). The IC_{50} with SD was calculated for each Gsα agonist from the patient's dose-response curve and compared with the IC_{50} for the same Gsα agonist of a control person, performed simultaneously. The intraassay and interassay coefficients of variation are, respectively, 12.5 and 13.9% for prostaglandin E1 (n = 5) and 7 and 9.3% for prostacyclin (n = 5).

GNAS mutation detection

Total RNA was extracted from platelets using the TRIzol (Invitrogen) reagent, according to the manufacturer's protocol. The *GNAS* gene was amplified from platelet RNA to detect potential alternative splicing

TABLE 1. Clinical and biochemical features of the patient cohort

Patient no., gender, age at diagnosis (yr)	Family history	Clinical classification	AHO features						PTH resistance			TSH resistance
			RF	BM	SCC	MR	SS	O	Calcium (8.9–10.5) mg/dl	Phosphate (2.3–4.7) mg/dl	PTH (3–40) ng/liter	TSH (0.15–4.6) mIU/liter
1, F, 16	No	PHPIa	+	+	−	+	+	+	5.4	8.1	430	22.9
2, F, 18	No	PPHIa	+	+	+	+	+	+	[a]	[a]	[a]	[a]
3, F, 7	No	PHPIa	+	+	+	+	+	+	[a]	[a]	[a]	5.79
4, M, 13	No	PHPIa	+	+	+	+	+	+	5.2	11.2	206	8.7
5, M, 4	Yes	PHPIa	+	+	+	+	+	+	8.55	4.34	187	6.77
6, F, 5	No	PPHP	−	+	−	+	+	+	10.1	5.20	36.7	3,62
7, F, 20	No	PHPIb	−	−	−	−	−	−	4.8	9.3	210.4	2.4
8, F, 29	No	PHPIb	−	−	−	−	−	+	6.34	5.01	68.6	5.3
9, F, 25	No	PHPIb	−	−	−	−	−	−	8.73	4.40	239.8	2.8
10, M, 13	No	PHPIb	−	−	−	+	−	−	7.1	7	158	NA
11, F, 21	No	PHPIb	−	−	−	−	−	−	9.58	3.35	162	1.8
12, M, 9	No	PHPIb	−	−	−	−	−	−	[a]	[a]	[a]	
13, M, 14	NA	PHPIb	−	−	−	−	−	−	6.6	3.53	102	11.6
14, M, 9	Yes	PHPIb	−	−	−	−	−	−	6.31	6.05	100.6	3.10
	Mother	normal	−	−	−	−	−	−	9.24	3.92	18.5	2.39
	Sister	normal	−	−	−	−	−	−	9.4	4.5	33.1	NA
15, M, 32	Yes	PHPIb	−	−	−	−	−	−	7.8	3.2	201	Normal

Presence or absence of a symptom is indicated by a plus or minus sign. The normal values for all biochemical parameters are indicated between *parentheses*. RF, Round face; BM, brachymetacarpy; SCC, sc calcifications; MR, mental retardation; SS, short stature; O, obesity; F, female; M, male; NA, not available.

[a] Biochemical values before treatment with 1-hydroxy vitamin D_3 are not known.

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TABLE 2. Molecular findings and platelet Gsα functional data for all patients

				Functional platelet Gsα test			
Patient no.	Clinical classification	*GNAS* mutation	Gsα function	IC_{50} Prostaglandin E1, ng/ml (59.20 ± 24.49) (n = 24)	Mean ± SD	IC_{50} Prostacyclin, ng/ml (1.06 ± 0.37) (n = 24)	Mean ± SD
1	PHPIa	D173N	Hypofunction	366[a]		2.1[a]	
2	PPHIa	Deletion of 1bp (codon 328)	Hypofunction	>1000[a]		>2.5[a]	
3	PHPIa	Insertion 21bp (codon110)	Hypofunction	>1000[a]		>2.5[a]	
4	PHPIa	Codons 24 and 25 are replaced by 14 bp	Hypofunction	520[a]		2.4[a]	
5	PHPIa	Q35X	Hypofunction	>1000[a]		>2.5[a]	
Mother	NA	Q35X	NA	NA		NA	
6	PPHP	R231C	Hypofunction	490[a]		1.63[a]	
7	PHPIb	Abnormal methylation in NESP, XL, and 1A	Mild hypofunction	145[a]		1.65[a]	
8	PHPIb	Abnormal methylation in NESP, XL, and 1A	Mild hypofunction	140[a]		1.9[a]	
9	PHPIb	Abnormal methylation in NESP, XL, and 1A	Mild hypofunction	78[a]	95.3 ± 41.9 (n = 6)	1.85[a]	1.68 ± 0.16[a] (n = 6)
10	PHPIb	Abnormal methylation in NESP, XL, and 1A	Mild hypofunction	42		1.55[a]	
11	PHPIb	Abnormal methylation in NESP, XL, and 1A	Mild hypofunction	62		1.6[a]	
12	PHPIb	Abnormal methylation in NESP, XL, and 1A	Mild hypofunction	105[a]		1.52[a]	
13	PHPIb	Abnormal methylation in 1A, *STX16* deletion	Normal	52		0.5	
14	PHPIb	Abnormal methylation in 1A, *STX16* deletion	Normal	35		0.85	
Mother	Mild PHPIb	Normal methylation in 1A, *STX16* deletion	Normal	34	40.6 ± 9.8 (n = 3)	1.1	0.71 ± 0.19 (n = 3)
Sister	Normal	Normal methylation, No *STX16* deletion	Normal	85		0.9	
15	PHPIb	Abnormal methylation in 1A, *STX16* deletion	Normal	35		0.8	

The numbering of the mutations refers to the codon number as described by Kozasa *et al.* (1). The concentration of Gsα agonist to inhibit the collagen-induced platelet aggregation by 50% (IC_{50}) is indicated between *parentheses* for 24 normal controls. A Gsα hypofunction is defined as requiring a significantly higher IC_{50} value. NA, Not available.

[a] *P* values <0.05.

defects. Primers for the *GNAS* amplification were GSF3 (5′-GGCTGC-CTCGGGAACAGTAAG-3′) and GS6R (5′-TAATCATGCCCTATG-GTGGGTG-3′). Exons 1 to 13 of *GNAS* including intron/exon boundaries were amplified from leukocyte gDNA in 13 PCR fragments. Part of the *GNAS* promoter and exon 1 were amplified using 7-deaza-dGTP (GE Healthcare) instead of dGTP during the PCR. Sequencing was performed using the BigDye terminator chemistry on an ABI310 sequencer (Applied Biosystems, Foster City, CA). All primers and PCR conditions are available upon request.

STX16 and NESP deletion analysis

Leukocyte gDNA samples from PHPIb patients were screened for the presence of the 3-kb deletion mutation within *STX16* by PCR as previously described (31). PHPIb patients with an overall *GNAS* methylation defect and negative for the *STX16* deletion were screened for NESP deletions by Southern blot analysis and PCR (32).

Methylation analysis of the GNAS cluster by Southern blot analysis

Southern blot analysis was done after double digestion of gDNA (15 μg) with different combinations of methylation-insensitive (*Pst*I, *Bgl*II, and *Sac*I) and methylation-sensitive (*Asc*I, *Ngom*IV, and *Not*I) enzymes as previously described (30). Radioactive labeled exon 1A, exon XL, or exon NESP*55*-specific PCR fragments were used as genomic probes.

Methylation analysis of the GNAS cluster by bisulfite conversion of DNA

The bisulfite reaction was carried out on 500 ng of gDNA with the MethylDetector bisulfite modification kit (Active Motif, Carlsbad, CA). A conversion reaction was performed in a 140-μl mixture containing 13 μl of gDNA, 7 μl of hydroquinone, and 120 μl of conversion buffer, as described by the manufacturer. DNA was denaturated with an initial melt at 94 C for 3 min, and then a conversion at 50 C for 9 h was

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performed in a PTC-200 Peltier Thermal Cycler (MJ Research, Reno, NV). An on-column desulfonation of converted DNA was performed, and DNA was eluted as indicated by the manufacturer. Control nested PCRs were performed on 40 ng of both unconverted and converted DNA to verify the DNA conversion using primers and reagents provided with the kit. Different nested PCRs were performed to amplify the NESP55, XLαs, and exon 1A regions using 5 μl bisulfite-treated gDNA, 0.5 μM primers, 1.5 mM $MgCl_2$, 1× buffer [20 mM Tris (pH 8.4), 50 mM KCl], and 0.2 μl (5 U/μl) Taq DNA polymerase (Invitrogen). All primer sequences and PCR conditions are available in supplementary Table 1 (published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Amplified PCR products were further digested with restriction enzymes that only recognize the nonconverted (methylated) DNA sequence: *Ssi*I (Fermentas, St. Leon-Rot, Germany) for NESP55, *Sal*I (Fermentas) for XL, and *Hinf*I (Fermentas) for exon 1A. Digested PCR fragments were separated on a 2% agarose gel.

Platelet immunoblot analysis

Platelets isolated from citrated blood were directly lysed in ice-cold PBS containing 1% igepal CA-630 (Sigma Chemical, St. Louis, MO), 2 mmol/liter Na_3VO_4, 1 mmol/liter EDTA, 1 mmol/liter phenylmethylsulfonyl fluoride, 2 mmol/liter dithioerythreitol, 1% aprotinin, and 2 mmol/liter NaF, and incubated on ice for 60 min. Lysates were cleared of insoluble debris by centrifugation at 14,000 × *g* for 20 min at 4 C. Platelet soluble cytosol protein fractions (12.5 μg for Gsα and β tubulin and 25 μg for XLαs) were mixed with Laemmli sample buffer (5% SDS reducing buffer), resolved by SDS/PAGE on 10% acrylamide gels, and transferred to Hybond ECL-nitro-cellulose membrane (GE Healthcare). Blots were blocked for 1 h at room temperature in Tris-buffered saline with Tween (0.1% Tween-20) supplemented with 5% nonfat dry milk. Incubation with primary (overnight at 4 C) and secondary (2 to 3 h at room temperature) antibody was performed in Tris-buffered saline with Tween with 5% nonfat milk. Blots were revealed with a polyclonal anti-Gsα antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), a monoclonal anti-XLαs antibody (11F7) (28) or a monoclonal anti-β tubulin antibody (Santa Cruz). Bands were quantified using the Java image processing program ImageJ 1.34 g (NIH Image software).

Statistical analysis

We used GraphPad InStat 3.01 software (GraphPad Software Inc., San Diego, CA). Continuous variables with small to mild skewness were summarized as mean ± SD and compared by the Student *t* test for unpaired data. Two-sided *P* values <0.05 were considered significant.

Results

Patients

Table 1 summarizes the clinical data of 16 index cases, comprising six patients with multiple AHO features with or without endocrinopathies and nine unrelated patients with PHPIb.

Novel mutations in GNAS and imprinting analysis

We screened the classical *GNAS* gene for mutations using platelet RNA for RT-PCR with primers encompassing exons 1 to 13 and leukocyte gDNA for sequencing all exons, including exon/intron boundaries. Six different heterozygous mutations, scattered throughout the *GNAS* gene, were found in AHO patients 1 to 6; these are not listed in the Human Genome Mutation Database for *GNAS* mutations except for the R231C mutation (Table 2). The *GNAS* mutations in patients 1, 4, and 6 are *de novo* mutations, whereas patient 5 inherited the Q35X mutation from her mother. Parental DNA samples were not available for patients 2 and 3.

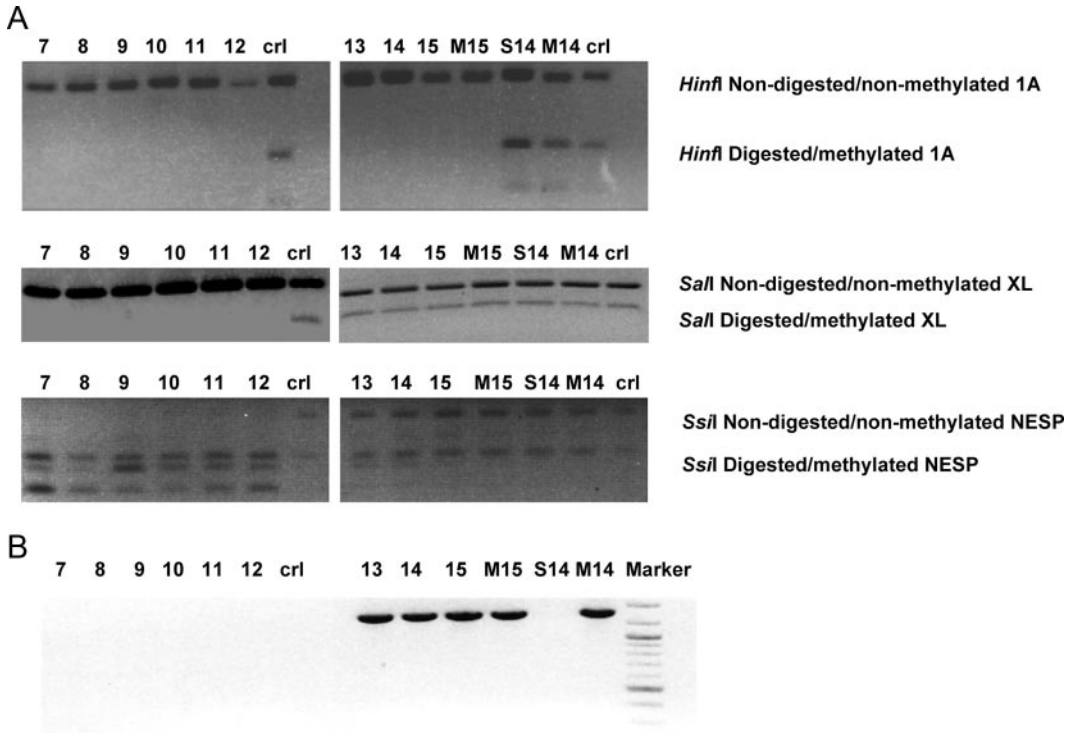

FIG. 2. *GNAS* methylation and *STX16* deletion analysis in PHPIb patients. A, Methylation analysis of exon 1A, exon XL, and exon NESP55 by PCR and restriction digestion (*Hinf*I, *Sal*I, and *Ssi*I, respectively) of bisulfite-treated DNA from the indicated individuals and unrelated normal controls (crl). The mother (M14) and sister (S14) of patient 14 and the mother of patient 15 (M15) were also studied. B, Patients 13 to 15, M14, and M15 are carriers of the *STX16* deletion as detected by PCR using primers, flanking the deletion.

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The methylation status of the exon 1A, NESP, and XL region of the *GNAS* cluster was also studied in nine PHPIb patients with PTH resistance without an AHO phenotype (Table 2). We developed a novel, easy, and reliable method to study *GNAS* methylation, based on bisulfite treatment of leukocyte gDNA, followed by PCR and restriction digestion with enzymes that only recognize the nonconverted methylated allele. All data could be confirmed by Southern blot analysis (data not shown). PHPIb patients 7 to 12 have overall imprinting defects with the loss of the *Hinf*I and *Sal*I restriction digestion sites in the exon 1A and XL regions, respectively, and a gain of the *Ssi*I digestion site in the NESP region (Fig. 2A). As expected, the 3-kb *STX16* deletion could not be detected in the PHPIb patients with a broad epigenetic *GNAS* defect (Fig. 2B). Recently, two unrelated PHPIb patients were described with an overall *GNAS* methylation defect, without a *STX16* deletion, but having deletions involving NESP and two of the antisense exons (32). We could not detect a deletion by Southern blot and PCR in the NESP region in patients 7 to 12 (data not shown). In line with previous evidence (23), patients 13, 14, and 15, with an autosomal dominant form of PHPIb, have an exon 1A-only epigenetic defect with only a loss of exon 1A methylation (Fig. 2A) and have the 3-kb *STX16* deletion (Fig. 2B). The mother of patient 14 with normal PTH levels carried the *STX16* deletion but had a normal exon 1A methylation, as studied by PCR and Southern blot analysis of leukocyte DNA. The parental origin of the STX16 deletion could not be determined in this subject.

Platelet Gsα screening assay

We have previously used the platelet aggregation-inhibition test to demonstrate Gsα hyperactivity (28). Now, we performed the platelet aggregation-inhibition test in 24 healthy controls and compared their mean IC_{50} values ± SD, determined for two different Gsα agonists, with the IC_{50} values for the PHPIa, PPHP, and PHPIb patients (Table 2). When platelet aggregation was induced with collagen in AHO patients 1 to 6, after preincubation with either prostaglandin E1 (Prostin) or a stable prostacyclin analog (Iloprost), significantly higher concentrations of both Gsα agonists were required to achieve 50% inhibition of platelet aggregation (IC_{50}), compared with the healthy controls. Figure 3 illustrates the platelet aggregation-inhibition test for patient 1, carrying GNAS missense mutation D173N, and patient 3, with a 21-bp insertion, *vs.* two unrelated normal controls. Further studies with increased patient numbers are needed to define whether there are differences in platelet functional Gsα activity between PHPIa and PPHP patients.

PHPIb patients 7 to 12 had mild Gsα hypofunction, in association with an overall *GNAS* imprinting defect, as we already described for patient 7 (30). Platelet Gsα activity was normal in PHPIb patients with an exon 1A-only methylation defect and a STX16 deletion (Table 2).

Sporadic PHPIb *vs.* autosomal dominant PHPIb in platelets

To rationalize the differences in functional platelet Gsα activity in patients with an overall *vs.* an exon 1A-only methylation defect, platelet Gsα and XLαs protein levels were quantified.

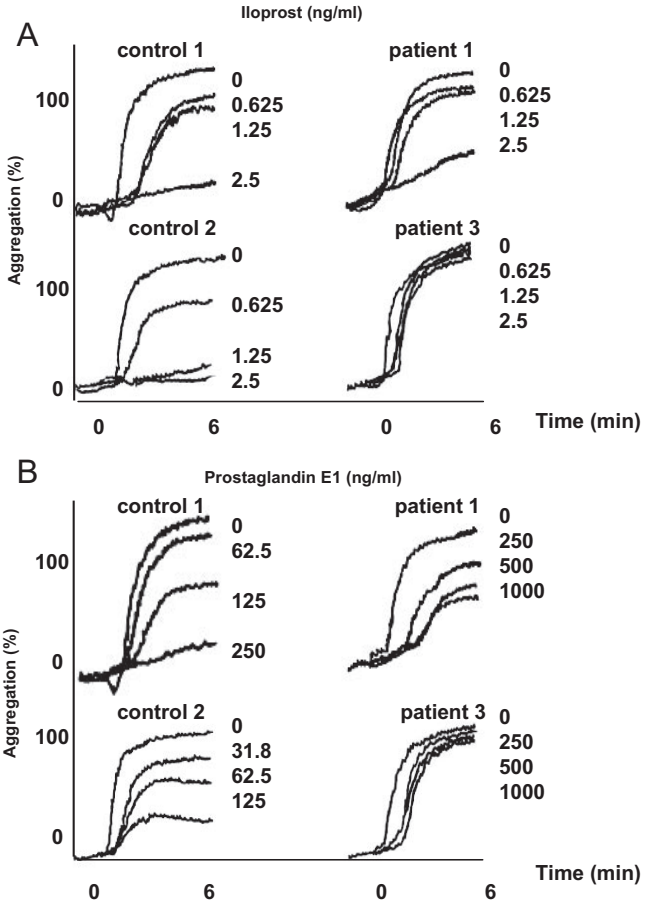

FIG. 3. Platelet Gsα aggregation-inhibition test. Dose-dependent inhibition of collagen-induced platelet aggregation (2 μg/ml) by the indicated concentrations of two Gsα-coupled receptor agonists: prostacyclin (Iloprost, ng/ml) (A) and prostaglandin E1 (ng/ml) (B) for patient 1 and a control and for patient 3 and a control.

Both PHPIb patient groups express lower levels of Gsα in the platelet soluble cytosol fraction compared with unrelated normal controls, but remarkably, no differences were seen in Gsα expression between the two different PHPIb groups (Fig. 4). Gsα expression was normal in patient 1 with the D173N GNAS missense mutation and was decreased about 60% in PHPIa patients 2 and 3, with an out-of-frame GNAS mutation (supplementary Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). As we described before (30), platelet cytosol fractions from PHPIb patients with an overall methylation defect, including an XL loss of methylation, express higher levels of XLαs compared with control platelets. This difference in XLαs expression was not observed in platelets from PHPIb patients with an exon 1A-only methylation defect or in the PHPIa patients (Fig. 4). No differences were seen in the expression levels of the widely expressed β tubulin between the PHPIb patients and the controls.

Discussion

Platelets are easily accessible cells and have been used to study the coupling between membrane receptors, heterotrimeric G pro-

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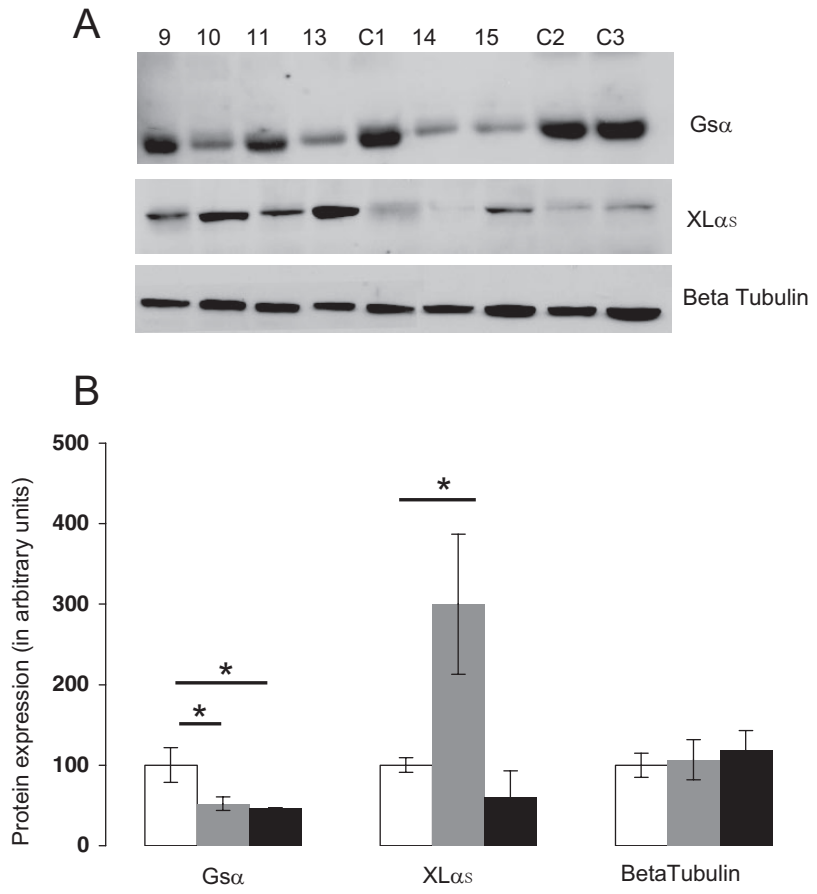

FIG. 4. Gsα and XLαs expression in PHPIb platelets. Immunoblot analysis (A) and densitometric scanning (B) of Gsα, XLαs, and β tubulin proteins in platelet lysates from PHPIb patients with an overall methylation defect (patients 9, 10, 11, and 13 or *gray bars*; n = 4), PHPIb patients with an exon 1A-only methylation defect (patients 14 and 15 or *black bars*; n = 2), and normal controls (*white bars*; n = 3). The results are expressed as percentage of controls (taken as 100%). Values are means ± SD. *, *P* values <0.05.

teins, and intracellular signaling events (33). The role of different G proteins in platelet function is extensively studied in mouse models for Gqα, Gα12, Gα13, Gαz, Giα2, and Gαi3 deficiencies (34), but the role of Gsα in platelet signaling has not been studied in Gsα heterozygous knockout mice (13, 35) or in humans with either PHPIa or PPHP. We hypothesized that an individual's Gsα activity could easily be estimated by the platelet aggregation-inhibition test, based on the inhibition of platelet aggregation by cAMP, induced after preincubation with different specific Gsα agonists. In this study, platelet Gsα signaling was evaluated in patients with PHPIa, PPHP, or PHPIb.

When platelet aggregation from six patients with PHPIa or PPHP was induced with collagen, after preincubation with either prostaglandin E1 (Prostin) or a stable prostacyclin analog (Iloprost), significantly higher concentrations of both Gsα agonists were required to cause platelet aggregation inhibition, compared with simultaneously studied normal controls. This platelet-based Gsα test helps to confirm the clinical diagnosis of AHO, which can be difficult if it is simply based on the presence of nonspecific but common phenotypic features such as short stature, brachydactyly, mental retardation, and other less frequent symptoms such as cataract and deafness (26). The severity of the AHO phenotype varies considerably among patients, and some patients with inactivating GNAS mutations have either few or no features of the syndrome (26, 36, 37). Moreover, some features of AHO are also present in other disorders, such as the AHO-like syndrome caused by small terminal deletions on chromosome 2 (38, 39). We have studied platelet Gsα signaling in two previously described patients (40) with a 2q37.3 deletion and found normal Gsα platelet activity (data not shown). This makes the diagnosis of PPHP, especially in families without endocrinopathies, extremely difficult. Here we showed that Gsα function in a platelet aggregation-inhibition test is an easy and reliable diagnostic test, not only for patients with PHPIa but also for patients with PPHP. Analysis of more patients is needed to compare the efficiency of the platelet-based assay with the more generally used erythrocyte test, especially because it was recently shown that PHPIa and PPHP patients with an abnormal erythrocyte Gsα activity do not carry a *GNAS* mutation in 36 and 77% of all cases, respectively, and also because *GNAS* mutations were found in patients with a normal Gsα activity (27).

Erythrocytes and platelets of patients with PHPIb have previously been shown to have normal Gsα activity using the membrane complementation assay (26, 41). But it was also shown that platelets from patients with PHPIb accumulate less cAMP in response to prostacyclin than do platelets from control subjects (41). Our platelet-based Gsα assay can be used to detect mild Gsα hypofunction in sporadic PHPIb patients with overall *GNAS* methylation defects, but platelet Gsα activity was normal in PHPIb patients with an exon 1A-only methylation defect and a *STX16* deletion. A recent study showed no clinical or laboratory differences in PHPIb patients with an overall or exon 1A-only methylation defect (42). Mild platelet Gsα hypofunction was previously described in PHPIb patient 7 with an overall *GNAS* imprinting defect (30). In this paper, we studied the platelet Gsα activity in five additional PHPIb patients with an overall imprinting defect and could confirm the presence of a mild platelet Gsα hypofunction. Similar to the findings for PHPIb patient 8, platelets from four patients with an overall methylation defect showed decreased Gsα and increased XLαs protein levels, compared with control platelets. In contrast, three PHPIb patients with an exon 1A-only methylation defect presented with normal platelet Gsα activity. Platelets from two of these patients showed decreased Gsα but normal XLαs protein levels. The nine PHPIb patients share the exon 1A hypomethylation defect, suggesting that the disturbed transcriptional regulation between exon 1A and exon 1 of the *GNAS* gene could account for the decreased platelet Gsα expression in all PHPIb patients, but it could not be responsible for the altered platelet Gsα function in only a subgroup of the PHPIb patients. We have previously shown that patient 8 with an

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overall methylation defect also had an XL hypomethylation with increased XLαs platelet protein expression (30). In agreement, platelets from four other PHPIb patients with overall methylation defects have increased XLαs protein levels, whereas XLαs was normally expressed in platelets from PHPIb patients with an exon 1A-only methylation defect. Further studies are needed to find out whether similar expression differences are seen in the insoluble platelet membrane fractions and whether XLαs upregulation in platelets is directly responsible for the Gsα hypofunction in the six PHPIb patients with the overall imprinting defects. Normally, XLαs can substitute for Gsα, but this has not yet been studied for platelets (43). Compatible with this observation is the opposite phenotype that patients with a bleeding tendency and a 36-bp insertion in XLαs showed increased Gsα levels and platelet Gsα hyperactivity (29, 44). The six PHPIb patients with the overall imprinting defects did not have deletions in the NESP55 region, as recently described for two PHPIb patients (32), which is suggestive of the existence of a third not yet known imprinting control domain for *GNAS*. The six PHPIb patients with the overall imprinting defects have no family history of the disease, which also raises the possibility of an autosomal recessive form of PHPIb in these subjects.

Because platelets are anucleated cells, it is impossible to study their GNAS methylation status. Megakaryocytes or platelet progenitor cells have a polyploid DNA content. To our knowledge, there are no studies describing the imprinting of the GNAS cluster during megakaryopoiesis or platelet production or in mature platelets. In a previous study, we found that exon 1A was hypermethylated in a human megakaryocytic cell line MEG01 (30). Therefore, it would be interesting to study the GNAS imprinting during megakaryocyte development from hematopoietic stem cells of PHPIb patients and normal individuals.

In conclusion, the platelet aggregation-inhibition test can be rapidly performed and reflects the behavior of the Gsα pathway *in vivo*. Moreover, in contrast to the erythrocyte membrane complementation assay, this assay uses the complete signaling system of the patient, thereby providing the opportunity to study the Gsα defect in more functional detail. One blood sampling per patient was sufficient to study the platelet Gsα activity, the platelet *GNAS* gene sequence, leukocyte gDNA sequence, and *GNAS* methylation profile, and finally the platelet Gsα protein levels. Platelet studies can be used to unravel pathogenic mechanisms in patients having more than a platelet dysfunction (45). By studying easily accessible cells such as platelets, we could detect defective Gsα signaling in PHPIa, PPHP, and PHPIb patients with broad epigenetic GNAS defects.

Acknowledgments

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