Positional dissociation between the genetic mutation responsible for pseudohypoparathyroidism type Ib and the associated methylation defect at exon A/B: evidence for a long-range regulatory element within the imprinted GNAS1 locus


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Pseudohypoparathyroidism type Ib (PHP-Ib) is a paternally imprinted disorder which maps to a region on chromosome 20q13.3 that comprises GNAS1 at its telomeric boundary. Exon A/B of this gene was recently shown to display a loss of methylation in several PHP-Ib patients. In nine unrelated PHP-Ib kindreds, in whom haplotype analysis and mode of inheritance provided no evidence against linkage to this chromosomal region, we confirmed lack of exon A/B methylation for affected individuals, while unaffected carriers showed no epigenetic abnormality at this locus. However, affected individuals in one kindred (Y2) displayed additional methylation defects involving exons NESP55, AS and XL, and unaffected carriers in this family showed an abnormal methylation at exon NESP55, but not at other exons. Taken together, current evidence thus suggests that distinct mutations within or close to GNAS1 can lead to PHP-Ib and the associated epigenetic changes. To further delineate the telomeric boundary of the PHP-Ib locus, the previously reported kindred F, in which patient F-V/51 is recombinant within GNAS1, was investigated with several new markers and direct nucleotide sequence analysis. These studies revealed that F-V/51 remains recombinant at a single nucleotide polymorphism (SNP) located 1.2 kb upstream of XL. No heterozygous mutation was identified between exon XL and an SNP ∼8 kb upstream of NESP55, where this affected individual becomes linked, suggesting that the genetic defect responsible for parathyroid hormone resistance in kindred F, and probably other PHP-Ib patients, is located ≥56 kb centromeric of the abnormally methylated exon A/B. A region upstream of the known coding exons of GNAS1 is therefore predicted to exert, presumably through imprinting of exon A/B, long-range effects on G_{s,\alpha} expression.

INTRODUCTION

Pseudohypoparathyroidism (PHP; MIM 300800) is characterized by parathyroid hormone (PTH)-resistant hypocalcemia and hyperphosphatemia. Patients with PHP are classified according to the presence or absence of additional endocrine abnormalities, such as resistance to thyroid-stimulating hormone (TSH) and gonadotropins, and the dysmorphic features of Albright’s hereditary osteodystrophy (AHO), which may include short stature, obesity, brachydactyly, heterotopic ossifications and mental retardation (1–3). Individuals with AHO and resistance to PTH, TSH, and often additional hormones, are referred to as having PHP-Ia. These
patients typically carry heterozygous inactivating mutations in one of the thirteen GNAS1 exons encoding the α-subunit of the stimulatory G protein (G\(\alpha\),α), which lead to an \(-50\%\) reduction in \(G\alpha\) activity and protein. Germline mutations of GNAS1, along with a similar reduction in \(G\alpha\) activity and protein, are also found in patients with pseudo-pseudohyoparathyroidism (PPHP), who have the same physical appearance as patients with PHP-Ia (i.e. AHO), but lack any hormonal resistance. PHP-Ia and PPHP are typically found within the same kindreds, but never within the same sibships, a conundrum explained by paternal imprinting of the hormonal resistance. Accordingly, PHP-Ia occurs if the defective gene is inherited from a female affected by either PHP-Ia or PPHP, whereas PPHP develops if the abnormal gene is inherited from a male affected by either of the two disorders (4–6).

PTH resistance is also observed in some patients who lack AHO and typically show no evidence for other hormonal abnormalities. In this form of PHP, referred to as PHP-Ib, resistance to PTH appears to be confined to the proximal renal tubules, as these patients show no evidence for impaired PTH-dependent calcium reabsorption in the distal renal tubules (7) and frequently develop hyperparathyroid bone disease (8). Unlike patients with PHP-Ia and PPHP, \(G\alpha\) protein and activity are normal in circulating blood cells and fibroblasts from PHP-Ib patients, and the genetic mutation responsible for this disorder presently remains unknown. In a genome-wide scan, however, we have previously revealed linkage of the PHP-Ib gene to a chromosomal region that comprises GNAS1 (20q13.3), and have furthermore demonstrated that the mode of inheritance for the hormonal resistance in PHP-Ib is identical to that observed in PHP-Ia, i.e. the PTH resistance occurs only if the defect is inherited from a female carrier of the disease gene (9). Taken together, these findings suggested that a mutation located within GNAS1, but not in those exons encoding \(G\alpha\), can be responsible for PHP-Ib.

GNAS1 exemplifies an imprinted gene locus with multiple sense and antisense (AS) transcripts which exhibit maternal, paternal or bi-allelic expression. The sense exons XL (10,11) and A/B (also referred to as exon 1A or 1') (12–14) are methylated on the maternal allele and are transcribed only from the paternal allele. Likewise, the promoter region for the putatively non-coding AS transcript is methylated on the maternal allele and its expression occurs exclusively from the paternal allele (15). Conversely, the exon encoding the chromogranin-like secretory protein NESP55 (16) shows methylation on the paternal allele, and this transcript is derived only from the maternally inherited GNAS1 allele (11,13,15,17–19). In contrast, the promoter for \(G\alpha\) transcripts is not methylated and expression takes place in most tissues from both parental alleles (11). Nonetheless, evidence from PHP-Ia patients (1,3), and from mice in which the paternal or maternal copy of Gnas exon 2 is disrupted (20), strongly suggest that the \(G\alpha\) protein is derived in the proximal renal tubular cells, adipocytes, and possibly other tissues from the maternal allele alone.

In 11 sporadic and two familial cases of PHP-Ib, Liu et al. (21) have recently demonstrated various GNAS1 methylation defects. In that study, five of the investigated affected individuals had epigenetic defects at two or more GNAS1 exons. Common to all, however, was a loss of methylation at the differentially methylated region (DMR) comprising exon A/B. In contrast, none of the investigated healthy controls or unaffected family members (19 in total), and none of the investigated patients with AHO (PHP-Ia or PPHP), showed an abnormal methylation at exon A/B, indicating that exon A/B and its epigenetic regulation is involved in the molecular pathogenesis of most PHP-Ib cases. Furthermore, these findings suggested that the genetic mutation responsible for this disorder resides within the ‘PHP-Ib’ locus (9) which comprises the imprinted GNAS1 gene. We now show that the reported methylation defect at exon A/B is present in affected individuals from the four PHP-Ib kindreds that were used to establish linkage to chromosome 20q13.3 (9), as well as in patients from five additional kindreds that appear to map to this genetic locus. However, the further genetic and mutational analysis of the most informative of these PHP-Ib kindreds indicated that the disorder and, presumably, the methylation abnormalities at exon A/B, are caused by a mutation located 256 kb upstream of this exon.

RESULTS

Affected individuals in nine unrelated PHP-Ib kindreds show a loss of methylation at GNAS1 exon A/B

Using four large kindreds, we had previously mapped the genetic defect leading to an autosomal dominant form of PHP-Ib to an \(-9\) cM genetic interval comprising GNAS1 (9). Subsequently, a defect in the parent-specific methylation pattern of exon A/B was reported in 11 sporadic and two familial cases of PHP-Ib, suggesting that mutations in this portion of the GNAS1 gene may be responsible for the disorder (21). To determine whether a similar epigenetic abnormality is present in our cohort of familial cases, we investigated the four PHP-Ib kindreds initially studied (9) and several recently diagnosed kindreds with this disorder (Fig. 1).

To exclude linkage discordance to chromosome 20q13.3, we first performed genetic analyses of the new kindreds using previously described (9) and novel markers at the PHP-Ib locus (Table 1) (22,23). In kindreds S1, Y1, W, E and Y2, affected individuals and unaffected carriers of the disease gene shared the same haplotype throughout the linked region (Fig. 1). Note that, consistent with the previously established paternal imprinting for PHP-Ib (9), none of the unaffected individuals who carried the disease-associated haplotype (kindreds S1, W and Y2) (Fig. 1A, C and E) was an offspring of a female obligate gene carrier. The mode of inheritance and the haplotypes thus provided no evidence against linkage to 20q13.3 in these five new PHP-Ib kindreds. In fact, LOD scores for kindreds S1 and W, calculated by taking paternal imprinting into consideration and thus excluding the offspring of male obligate gene carriers (9), provided confirmation for linkage of PHP-Ib to this chromosomal region (combined LOD score = 3.72 with D20S171, \(\theta = 0\)). In two additional kindreds with at least two affected siblings, however, we observed linkage discordance between the disease gene and the markers in this chromosomal region, suggesting locus heterogeneity (data not shown).

We proceeded with the analysis of GNAS1 exon A/B methylation in one or more affected individuals from each of the nine PHP-Ib kindreds that showed, or did not argue against, linkage to 20q13.3 (Fig. 1 and the kindreds reported in ref. 9). Southern analysis of genomic DNA digested with
EcoRV/EagI, SacI/AscI or BamHI/NrdI, followed by hybridization to a probe specific for exon A/B, revealed that all the investigated affected individuals from the nine kindreds show a loss of methylation throughout exon A/B (Table 2). Assessment of methylation in the three remaining DMRs, exon NESP55, exon XL and the region upstream of AS exon 1, did not indicate abnormalities in these individuals, except for the affected individuals from kindred Y2. Both affected brothers showed, in addition to the defect in exon A/B, a loss of methylation in exon XL and AS exon 1, and a gain of methylation in exon NESP55. The unaffected female Y2-I/3 (mother of the two affected individuals) and her unaffected sister shared, between markers D20S25 and D20S93, the same haplotype as the affected individuals Y2-II/1 and Y2-II/2 (Fig. 1E). Interestingly, both unaffected, presumed obligate gene carriers showed a loss of methylation at the exon NESP55 DMR without additional epigenetic changes (Fig. 2). In contrast, none of the investigated unaffected individuals or unrelated spouses from other kindreds (18 individuals from kindreds F, S1, Y2, E and W), including those who are carriers of the disease gene, showed epigenetic abnormalities in exon A/B or other DMRs within GNAS1 (Table 2). These results corroborated the previous observations by Liu et al. (21), and indicated that loss of methylation at GNAS1 exon A/B is present only in individuals affected by PHP-Ib.

The genetic mutation responsible for PHP-Ib is located ≥56 kb upstream of the abnormally methylated exon A/B.
cases of PHP-Ib (ref. 21 and findings described above) strongly suggested that the mutation responsible for the disease resides within or close to \textit{GNAS1}. A portion of this gene is positioned outside the candidate region for PHP-Ib; however, as the affected individual F-V/51, a member of the largest previously analyzed kindred, had been demonstrated to be recombinant at a marker located in \textit{GNAS1} intron 3 (9). Recently, contig Chr_20ctg125 (assembled by the Sanger Centre) (24) has revealed that the 5’ end of \textit{GNAS1} (for the sense transcripts) is positioned toward the centromere. These mapping data, combined with the genetic data from kindred F, indicated that the genomic region comprising exon N1 and exons 4–13 is excluded as a positional candidate for PHP-Ib (Fig. 3).

The affected members of the most informative branch of kindred F, including individual F-V/51, also revealed the
methylation defect that appears to be specific for PHP-Ib (Fig. 4A). In the Southern blot analysis of genomic DNA digested with EcoRV and EagI (methylation sensitive), the 6.2 kb fragment representing the methylated allele could not be detected in F-V/51 (and her affected mother F-IV/47), whereas the 4.3 kb fragment representing the unmethylated allele was present (Fig. 4B); note that the smaller EagI fragments (627, 330, 370 and 520 bp) were run off the gel. Both methylated and unmethylated DNA fragments were visualized for the healthy family members, including F-III/31 and F-III/34, who are unaffected obligate carriers of the disease gene based on having affected offspring, and having the same haplotype throughout the linked region as the affected individuals (Fig. 4C) (9). These results indicated that the mutation responsible for PTH resistance affects GNAS1 methylation also in this kindred, even though a part of this gene had been excluded from the PHP-Ib locus (9).

To redefine the telomeric boundary of the linked interval, and to thereby exclude additional portions of GNAS1, we identified additional polymorphisms in this genomic region. Further analysis of this branch of kindred F with the newly identified markers revealed two intragenic polymorphisms; a 5 bp repeat polymorphism within exon A/B (309F20-GGCGC) and a C→G single nucleotide polymorphism (SNP) located...
678 bp upstream of this exon (309F20-28551) (Table 1), which proved informative when analyzing the haplotypes of F-V/51, her unaffected brother F-V/50, and her affected mother F-IV/47. All three individuals were heterozygous for both polymorphisms. Note that the father of the two children is deceased, and could not be analyzed (Fig. 4C). Because the two children inherited the same paternal allele throughout the linked region, these genotypes indicated that F-V/51 remained recombinant at these two loci. We also identified a fully informative C→A SNP (806M20-98760) located 7967 bp upstream of NESP55 with respect to the translational initiation codon (GenBank accession no. AJ251760). Direct sequencing of PCR-amplified genomic DNA (Fig. 5A), as well as restriction digest of the product with MwoI (Fig. 5B), whose recognition sequence is introduced by the cytosine nucleotide, revealed that the affected individual F-IV/47 is heterozygous (A/C). Both of her children, the affected daughter F-V/51 and the unaffected son F-V/50, are homozygous for the ‘C’ allele (C/C) (Fig. 5A). Furthermore, the two carriers of the disease gene, F-III/31 and F-III/34, are homozygous for the ‘A’ allele, and the unaffected, non-carrier F-III/32 is homozygous for the ‘C’ allele (Fig. 5B). These findings thus indicated that F-V/51, who did not inherit the disease-associated ‘A’ allele from her affected mother, is recombinant also at this marker.

The next informative marker centromeric of 806M20-98760 was a C→A SNP (806M20-98760) located 7967 bp upstream of NESP55 with respect to the translational initiation codon (GenBank accession no. AJ251760). Although DNA from the father of F-V/50 and F-V/51 could not be investigated, analysis of genotypes at this locus indicated that the affected F-V/51 and her unaffected brother F-V/50 inherited different alleles from their affected mother F-IV/47 (Fig. 4C). Since the ‘A’ allele inherited by F-V/51 is associated with the disease, these findings suggested that marker 806M20-98760 is linked to the disease-causing allele. All other polymorphic markers
identified within the ∼21 kb genomic interval between 806M20-98760 and 806M20-119516 were uninformative. Taken together, these results redefined the telomeric boundary of the PHP-Ib locus, and excluded genetically exons XL and A/B, and all exons encoding Gsα. Remarkably, the DMR that comprises exon A/B, which is unmethylated in patients from kindred F and all other PHP-Ib patients investigated thus far, resides within the excluded portion of GNAS1 (Fig. 6).

Based on these genetic data, the exons giving rise to the AS transcript and NESP55 remained positional candidates. To search for mutations in these regions, we performed direct sequence analysis of both strands using genomic DNA from F-IV/47 (the affected mother of F-V/51). No heterozygous mutation, which would be expected for an autosomal dominant disorder, was detected in this individual between SNP 806M20-119516 and SNP 806M20-98760 (however, nucleotides 112750–113320, i.e. 570 bp, could not yet be amplified and remain to be analyzed). However, as evidence for the quality of the sequence analysis, several novel or known SNPs were identified for which F-IV/47 was homozygous (data not shown).

Through direct sequence analysis, mutations in genomic regions encoding exon NESP55, AS exon 1 and exon XL, and their flanking intronic regions, were furthermore excluded for one affected member of kindreds D, P, T and Y2. Also, Southern blot analysis using genomic DNA from more than 20 sporadic and familial PHP-Ib patients, including an affected member of kindred F (F-III/37), did not provide any evidence for large deletions or rearrangements between XL and NESP55 (data not shown).

DISCUSSION

In the current study, we analyzed the methylation pattern of GNAS1 in nine unrelated PHP-Ib kindreds which all map to the previously established locus within the chromosomal region 20q13.3 (9). Consistent with a molecular defect within or close to GNAS1, affected, but not healthy individuals from these kindreds showed a methylation defect at exon A/B similar to that identified previously in 11 sporadic and two familial cases of PHP-Ib (21). Abnormal methylation of exon A/B thus appears to be the most consistent finding in patients affected by this disorder. However, the further genetic analysis of the largest of the investigated PHP-Ib kindreds, kindred F, and direct nucleotide sequence of an affected individual from this family, revealed that the mutation leading to the disease and, presumably, to the associated methylation defects at exon A/B, most likely resides in untranscribed GNAS1 sequences upstream of exon NESP55.

A substantial body of evidence from human and animal studies suggests that the PTH resistance in PHP-Ib, which appears to be limited to the proximal renal tubules, is caused by a specific loss of Gα expression in that portion of the kidney (2,20,21). The demonstration of a loss-of-methylation defect within GNAS1 further implicates this signaling protein in the molecular pathogenesis of most cases of PHP-Ib, and indicates
that the exon A/B DMR, which is unmethylated in all the PHP-Ib patients investigated thus far, plays a pivotal role in regulating parent-specific expression of G_\alpha in the renal proximal tubule. In fact, the mutation leading to PHP-Ib and the associated loss of methylation at exon A/B likely disrupt a putative cis-acting element with long-range effects, particularly affecting the establishment and/or maintenance of the maternal imprint at exon A/B.

Heterozygous mutations affecting one of the GNAS1 exons encoding G_\alpha are likely to represent only a very rare cause of PHP-Ib. One such mutation, which affects GNAS1 exon 13 and leads to the deletion of isoleucine 382, appears to uncouple G_\alpha from the PTH/PTHrP receptor without impairing signal transduction through other G_\alpha-coupled receptors (25). If these findings can be confirmed in other more rigorously controlled in vitro systems, and if the advanced bone age documented for two of the affected children proves to be unrelated to the documented isolated PTH resistance, the findings observed in this kindred may indeed represent an unusual variant of PHP-Ib.

Regulatory regions that control the methylation imprint at several neighboring loci have been identified for the locus of the Prader–Willi/Angelman syndrome on human chromosome 15 (26), for the H19-Igf2 locus on mouse chromosome 7 (27,28), and for the Igf2 receptor gene on mouse chromosome 17 (29). A high percentage of CpG di-nucleotides and the presence of parent-specific methylation appear to be the common features of these cis-acting elements. The GNAS1 locus contains three CpG islands. Two of these islands, the one that encompasses exon NESP55 and the one that includes XL and the promoter of AS, show complete allele-specific methylation (11,15,17). The most telomeric of these CpG islands comprises exon A/B and exon 1, of which only the region around the former shows differential methylation (13). Based on our genetic data, only a small portion of the differentially methylated CpG island which comprises XL and the promoter of AS transcripts, and that encompassing exon NESP55, remained to be positional candidates. Mutations in these regions, however, were excluded through direct sequence analysis, suggesting that the defective regulatory element in PHP-Ib is likely to be located further centromeric. One can speculate that, due to the potential distance between the mutated regulatory element and the imprinting defect in PHP-Ib, other imprinted genes located within or adjacent to the linked interval may also be affected. Nonetheless, GNAS1 and its murine homolog appear to be the only imprinted gene thus far identified in this region of the human and mouse genome (30,31).

The AS transcript, which is presumed to be non-coding, has been suggested to regulate the imprinted expression from GNAS1 (15). Paternal expression of the AS transcript may serve to silence, from the same allele, the expression of NESP55 and of the renal cortex-specific G_\alpha transcript. It would then be possible that a partial or total loss of silencing of AS on the maternal GNAS1 allele results in a complete lack of G_\alpha expression in the renal cortex, thereby accounting for the PTH resistance in PHP-Ib. Nevertheless, exclusion of mutations in the promoter and exonic sequences of AS, and demonstration of a normal epigenotype at the differentially methylated promoter region of this AS transcript in genomic DNA from most affected individuals, strongly argue against this hypothesis.

Our methylation analysis of GNAS1 in the nine unrelated PHP-Ib kindreds described above revealed that unaffected carriers bearing the mutation on their paternal allele do not display any methylation abnormality at the A/B locus. Consistent with the findings by Liu et al. (21), PTH resistance and abnormal methylation at this locus are thus present concomitantly and occur only if the defect is located on the maternal allele. While the presence of epigenetic abnormalities at the exon A/B DMR appears to be the indicator of PHP-Ib, the lack of methylation changes in unaffected carriers unfortunately makes it impossible to predict, without detailed haplotype analysis, whether an unaffected individual in a given PHP-Ib kindred carries the disease gene.

In kindred Y2, however, where the maternal mutation causes a maternal-to-paternal switch in methylation of the entire GNAS1 region, unaffected carriers also exhibited a methylation defect (Fig. 2). Two of the 13 PHP-Ib patients investigated by Liu et al. (21) also had a broad pattern of methylation abnormality similar to that in the affected individuals of kindred Y2, but these cases were sporadic and therefore no information on the methylation status of family members was provided. Our findings in kindred Y2 suggest that the paternal transmission of the mutation in this family (presumably through the father of Y2-I/3 and Y2-I/4) leads to a methylation defect also in unaffected disease gene carriers. This abnormality, however, is confined to the NESP55 DMR. The presence or absence of a methylation defect at the NESP55 locus may thus provide, at least in the few PHP-Ib families where these broader methylation

Figure 6. The imprinted GNAS1 locus and respective positions of the informative polymorphisms employed in the genetic analysis of kindred F. Plus and minus signs indicate methylated and unmethylated alleles, respectively; plus sign in parentheses indicates loss of methylation, which has been consistently observed in all PHP-Ib patients investigated thus far. Note that the affected individual F-V/51 is recombinant at markers GNAS, 309F20-GGCCGC, 309F20-28551 and 806M20-119516 (underlined), and linked at marker 806M20-98760 (Fig. 4C); the genetic defect leading to PHP-Ib thus resides in kindred F and possibly in other PHP-Ib kindreds, upstream of the methylation abnormality observed at exon A/B (Fig. 4B).
abnormalities occur, a means to identify potential carriers of the disease gene without the need for detailed haplotype analysis of the 20q13.3 region. Furthermore, if a methylation abnormality of NESP55 alone can be a reliable predictor of the mutation leading to PHP-Ib in this kindred may reside at an imprinted control element that is located either between AS and Y1, not Y1-I/6. The latter was diagnosed with PHP-Ib in his twenties, but details of his disorder and genomic DNA were not available. In kindred S1, Y1-I/1, Y1-II/4, S1-II/1, S1-II/2, S1-II/3, S1-II/5 and S1-II/6 were all tested by PTH infusion and showed no increase in urinary cAMP and phosphate excretion. Note that each of the affected males, S1-II/1 and S1-II/2, has two unaffected children. In kindred Y1 (Fig. 1B), the proband (Y1-I/1) was diagnosed with PHP-Ib in 1981 at the age of 13, after complaining of paresthesias in both hands. Her parents were said to be healthy. One of her daughters, Y1-I/1, was diagnosed in 1997 at the age of 7 years; she was asymptomatic. Y1-I/1 (the proband) and Y1-I/4 had no increase in urinary cAMP and phosphate after PTH infusion. The proband has an unaffected (Y1-I/5) and an affected (Y1-I/6) brother. The latter was diagnosed with PHP-Ib in his twenties, but details of his disorder and genomic DNA were not available. In kindred W (Fig. 1C), the proband (W-III/3) was diagnosed when blood and urinary studies were performed to evaluate recurrent microscopic hematuria at 8 years of age. He had experienced episodes of abdominal pain for several years with occasional diarrhea, which improved with a lactose-free diet. He also had mild fatigue. A dual energy X-ray absorptiometry study was normal. Treatment was begun with calcitriol (0.25 µg bid) and 1000 mg of calcium carbonate daily. Normalization of serum calcium concentration was associated with increased stamina and decreased abdominal pain. After about 1.5 years, the dose of calcitriol was decreased to 0.25 µg once daily without supplemental calcium. Patient W-III/8 began to have ‘absence seizures’ at the age of 5 years, which increased in frequency and subsequently included spastic movements. She was treated for seizure disorder with higher than usual doses of medication, but continued to have breakthrough seizures and increase in spastic movements with a sudden movement or running. More than a year after the onset of the seizures, she was referred to a pediatric endocrinologist because low blood calcium was noticed upon further laboratory evaluation. By this time, she was having episodes of carpal-pedal spasm. Once treated with calcitriol, she was able to stop anti-convulsive medication and previous abnormalities in the electroencephalogram resolved. Patient W-III/7, 2 years older than W-III/8, with no symptoms of hypocalcemia, was diagnosed at the time of her sister’s evaluation. Both girls are currently receiving 0.25 µg of calcitriol daily and calcium carbonate tablets.

**MATERIALS AND METHODS**

**PHP-Ib kindreds**

Several of us are involved in the long-term medical care of the investigated PHP-Ib kindreds (T.S., K.T., M.K., Y.A., K.K., A.L.R. and H.K.). The kindreds from North America (W) and Germany (E) are Caucasian; three other kindreds (S1, Y1 and Y2) are from Japan; kindred F was described previously (9). Genomic DNA was extracted from peripheral blood leukocytes as described previously (36); the study was approved by the Subcommittee on Human Studies of the Massachusetts General Hospital (GenBank accession no. 92-7338).

In each of the new PHP-Ib kindreds at least two family members showed evidence for PTH resistance, i.e. hypocalcemia and hyperphosphatemia despite elevated PTH, but had no clinical and radiological features of AHO. In kindreds S1, Y1 and E, PTH resistance was further documented through an Ellsworth–Howard test. As noted previously (2,9), the severity of the disease was variable, even within a single kindred; several individuals were not diagnosed until evaluated for the present study.

In kindred S1 (Fig. 1A), three of the affected individuals, S1-II/1, S1-II/2 and S1-III/13, complained of paresthesias at the ages of 27, 30 and 20 years, respectively, and S1-II/3 had convulsions at the age of 35 years. All other affected members of this kindred were asymptomatic when evaluated for the present study. S1-II/1, S1-II/2, S1-II/3, S1-II/5 and S1-II/6 were all tested by PTH infusion and showed no increase in urinary cAMP and phosphate excretion. Note that each of the affected males, S1-II/1 and S1-II/2, has two unaffected children.

In kindred Y1 (Fig. 1B), the proband (Y1-I/1) was diagnosed with PHP-Ib in 1981 at the age of 13, after complaining of paresthesias in both hands. Her parents were said to be healthy. One of her daughters, Y1-I/1, was diagnosed in 1997 at the age of 7 years; she was asymptomatic. Y1-I/1 (the proband) and Y1-I/4 had no increase in urinary cAMP and phosphate after PTH infusion. The proband has an unaffected (Y1-I/5) and an affected (Y1-I/6) brother. The latter was diagnosed with PHP-Ib in his twenties, but details of his disorder and genomic DNA were not available.

In kindred W (Fig. 1C), the proband (W-III/3) was diagnosed when blood and urinary studies were performed to evaluate recurrent microscopic hematuria at 8 years of age. He had experienced episodes of abdominal pain for several years with occasional diarrhea, which improved with a lactose-free diet. He also had mild fatigue. A dual energy X-ray absorptiometry study was normal. Treatment was begun with calcitriol (0.25 µg bid) and 1000 mg of calcium carbonate daily. Normalization of serum calcium concentration was associated with increased stamina and decreased abdominal pain. After about 1.5 years, the dose of calcitriol was decreased to 0.25 µg once daily without supplemental calcium. Patient W-III/8 began to have ‘absence seizures’ at the age of 5 years, which increased in frequency and subsequently included spastic movements. She was treated for seizure disorder with higher than usual doses of medication, but continued to have breakthrough seizures and increase in spastic movements with a sudden movement or running. More than a year after the onset of the seizures, she was referred to a pediatric endocrinologist because low blood calcium was noticed upon further laboratory evaluation. By this time, she was having episodes of carpal-pedal spasm. Once treated with calcitriol, she was able to stop anti-convulsive medication and previous abnormalities in the electroencephalogram resolved. Patient W-III/7, 2 years older than W-III/8, with no symptoms of hypocalcemia, was diagnosed at the time of her sister’s evaluation. Both girls are currently receiving 0.25 µg of calcitriol daily and calcium carbonate tablets.
In kindred E (Fig. 1D), the proband (E-II/3) had undergone surgery for epiphysiodesis of both femoral heads at age 11 years; growth, and psychomotor and puberty development had been normal. At the age of 12.5 years, she was hospitalized with hypocalcemic convulsions. Infusion of PTH failed to increase cAMP in plasma and urine, and there was no clinical or radiological evidence for AHO. Treatment with calcitriol was begun. cAMP in plasma and urine, and there was no clinical or radiological evidence for AHO. Treatment with calcitriol was begun. In addition, T3 and T4 were slightly below the normal range with an increase of TSH (5.6 μU/ml; l-thyroxine (75 μg/day) was initiated. The patient’s mother (E-I/1) had mild hypocalcemia, hyperphosphatemia and secondary hyperparathyroidism at the age of 6 years, but the diagnosis of PHP-Ib was not made until the age of 25 years when the PTH-stimulation test showed no increase in urinary cAMP excretion. Treatment with calcium and vitamin D3 was initiated. Psychomotor development had been normal and there was no evidence for abnormal thyroid function or AHO. The patient’s brother (E-II/4) was diagnosed with PHP-Ib at the age of 8 years and 10 months. He was treated with 1 μg of calcitriol daily. Like his sister, he is also treated with thyroid hormone.

In kindred Y2 (Fig. 1E), the propositus (Y2-II/1) was initially thought to have epilepsy when he complained, in 1980 at the age of 11 years, of muscle weakness and fainting spells. He was started on anti-epileptic medication, which failed to improve his condition, and at the age of 16 years he was diagnosed with PHP. His younger brother (Y2-II/2) had recurrent convulsions, which were initially thought to be caused by epilepsy and were treated accordingly. However, as for Y2-II/1, his symptoms did not improve and at age 13 he was also diagnosed as having PHP. Both brothers showed remarkable calcifications of the basal ganglia, thalamus, pineal body and choroid plexus, but no features of AHO. Of note, the two patients had elevated TSH accompanied by low to low-normal free T4. Their parents and the mother’s sister, as well as two maternal cousins, are healthy and showed no laboratory or clinical abnormalities.

Methylation analysis of GNAS1

Southern blot analysis was performed after double-digestion of genomic DNA with different combinations of methylation-insensitive and -sensitive restriction enzymes as described previously (11,15,17,34). For methylation analysis of GNAS1 exon A/B, genomic DNA was digested with EcoRV and the methylation-sensitive enzyme EagI. Methylation of this region was also assessed using BamHI/NruI or SacI/AciI. For methylation analysis of NNAI, genomic DNA was double-digested with BamHI/NruI or EcoRV/ExpI. After separation on a 0.8% agarose gel and transfer onto nitrocellulose, the blots were probe with different DNA fragments of GNAS1, which were 3P-labeled by random priming as previously described (36); these probes included nucleotides 317–1705 of NESP55 (GenBank accession no. AJ009849), nucleotides 80–1693 of XLtts (accession no. AJ224868) and nucleotides 11646–13156 of AS (accession no. AJ251760); the A/B probe included nucleotides 28580–31035 of PAC clone 309F20 (accession no. AL121917).

PAC/BAC contig covering the linked region

Sequence-tagged site mapping and fingerprinting of the BAC and PAC clones were carried out at the Sanger Centre as part of the Human Genome Project (Chr_20ctg125) (24). These clones are available from BACPAC Resources. Sequencing of the clones at the telomeric end of the PHP-Ib locus (261P9, 806M20, 309F20 and 543J19) have been recently finished (37), and the data are currently available at GenBank non-redundant nucleotide sequences database (accession nos AL139349, AL132655, AL121917 and AL109840, respectively).

Search for GNAS1 mutations

Individual exons and intronic sequences were amplified from genomic DNA using PCR. Products were purified from unincorporated nucleotides and primers using the Qiaquick PCR purification kit from Qiagen. Direct sequencing of the purified PCR products was performed at the Massachusetts General Hospital DNA Sequencing Core Facility using Applied Biosystems Taq DyeDeoxy Terminator cycle sequencing kit. Southern blot analysis was performed using genomic DNA digested with BamHI, EcoRI, XbaI or XhoI. After separation on an 0.8% agarose gel and transfer onto nitrocellulose, individual blots were hybridized to PCR-generated genomic probes corresponding to exon NESP55, AS exon 1 and exon XL for the nucleotide positions of the probes, see ‘Methylation analysis of GNAS1’ (Materials and Methods).

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