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INVITED REVIEW

G protein mutations in endocrine diseases

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Abstract

This review summarizes the pathogenetic role of naturally occurring mutations of G protein genes in endocrine diseases. Although *in vitro* mutagenesis and transfection assays indicate that several G proteins have mitogenic potential, to date only two G proteins have been identified which harbor naturally occurring mutations, Gsα, the activator of adenylyl cyclase and Gi2α, which is involved in several functions, including adenylyl cyclase inhibition and ion channel modulation. The gene encoding Gsα (GNAS1) may be altered by loss or gain of function mutations. Indeed, heterozygous inactivating germ line mutations in this gene cause pseudohypoparathyroidism type Ia, in which physical features of Albright hereditary osteodystrophy (AHO) are associated with resistance to several hormones, i.e. PTH, TSH and gonadotropins, that activate Gs-coupled receptors or pseudopseudohypoparathyroidism in which AHO is the only clinical manifestation. Evidence suggests that the variable and tissue-specific hormone resistance observed in PHP Ia may result from tissue-specific imprinting of the GNAS1 gene, although the Gsα knockout model only in part reproduces the human AHO phenotype. Activating somatic Gsα mutations leading to cell proliferation have been identified in endocrine tumors constituted by cells in which cAMP is a mitogenic signal, i.e. GH-secreting pituitary adenomas, hyperfunctioning thyroid adenomas and Leydig cell tumors. When the same mutations occur very early in embryogenesis they cause McCune–Albright syndrome. Although these mutations would in principle confer growth advantage, studies failed to detect differences in the clinical and hormonal phenotypes, suggesting the existence of mechanisms able to counteract the activation of the cAMP pathway. Activating mutations of Gi2α have been identified in a subset of ovarian, adrenal and pituitary tumors, but their prevalence and significance are still controversial. Finally, although Gα subunits are the only components of the heterotrimeric GTP binding proteins which harbor known mutations, β/γ subunits should be considered possible targets of genetic alterations as suggested by the frequent presence of β3 subunit variants in patients with essential hypertension.

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Introduction

The majority of polypeptide hormones, all monoamine neurotransmitters, prostaglandins and even ions, such as Ca^{2+}, signal their target cells through membrane receptors belonging to a superfamily that share a common structural and functional motif, i.e. a single polypeptide with seven membrane-spanning domains, and a common transduction mechanism, i.e. coupling to G proteins. Therefore, G proteins play a key role in relaying signals from the plasma membrane to intracellular effectors. In the past few years, defects in G protein-coupled signal transduction have been identified as the cause of endocrine disorders (1–6). In particular, several G protein-coupled receptors have been demonstrated to be altered by loss or gain of function mutations, leading to the clinical phenotype of hormone defect or excess, respectively. Conversely, while a number of mutant G proteins cause cell transformation, as demonstrated by *in vitro* mutagenesis and transfection assays, to date only two G protein genes have been identified which harbor naturally occurring mutations in endocrine disorders. Moreover, mutations of the effector molecules seem to occur even more infrequently in human diseases. This review will briefly describe how G proteins activate signal transduction and how mutations of these proteins cause endocrine diseases.

G protein structure and function

Heterotrimeric guanine nucleotide binding proteins, known as G proteins, form the superfamily of proteins involved in the signal transduction from seven transmembrane receptors to intracellular effectors. They are

heterotrimers composed of three distinct subunits, α, β and γ, the functional specificity of each G protein depending on the α subunit, which differs from one G protein to another (7–9). The α subunit contains high affinity binding sites for guanine nucleotide and has intrinsic GTPase activity. The α subunit guanine nucleotide pocket consists of five distinct, highly conserved stretches (G1–G5). The G1, G4 and G5 regions are important for the binding of GTP while the G2 and G3 regions determine the intrinsic GTPase activity of the α subunit. The GDP-bound form binds tightly to βγ and is inactive, whereas the GTP-bound form dissociates from βγ and serves as a regulator of effector proteins. The receptor molecules cause the activation of G proteins by affecting several steps of the GTP cycle, resulting in the facilitation of the exchange of GTP for GDP on the α subunit. The duration of subunit separation is timed by the rate of α subunit mediated hydrolysis of GTP (Fig. 1). However, deactivation of G protein signaling pathways *in vivo* can occur 10- to 100-fold faster than the rate of GTP hydrolysis *in vitro*, suggesting the existence of GTPase activating proteins able to deactivate the α subunit. In fact, a family of GTPase activating proteins termed RGS (regulators of G protein signaling), that deactivates several G proteins by allowing inactive heterotrimers to reform, has been identified (10–12). It is worth noting that a RGS protein able to deactivate the stimulatory regulatory protein of adenylyl cyclase has not yet been identified (12).

Although the Gα subunit family includes proteins with different functions, unequivocal assignment of one

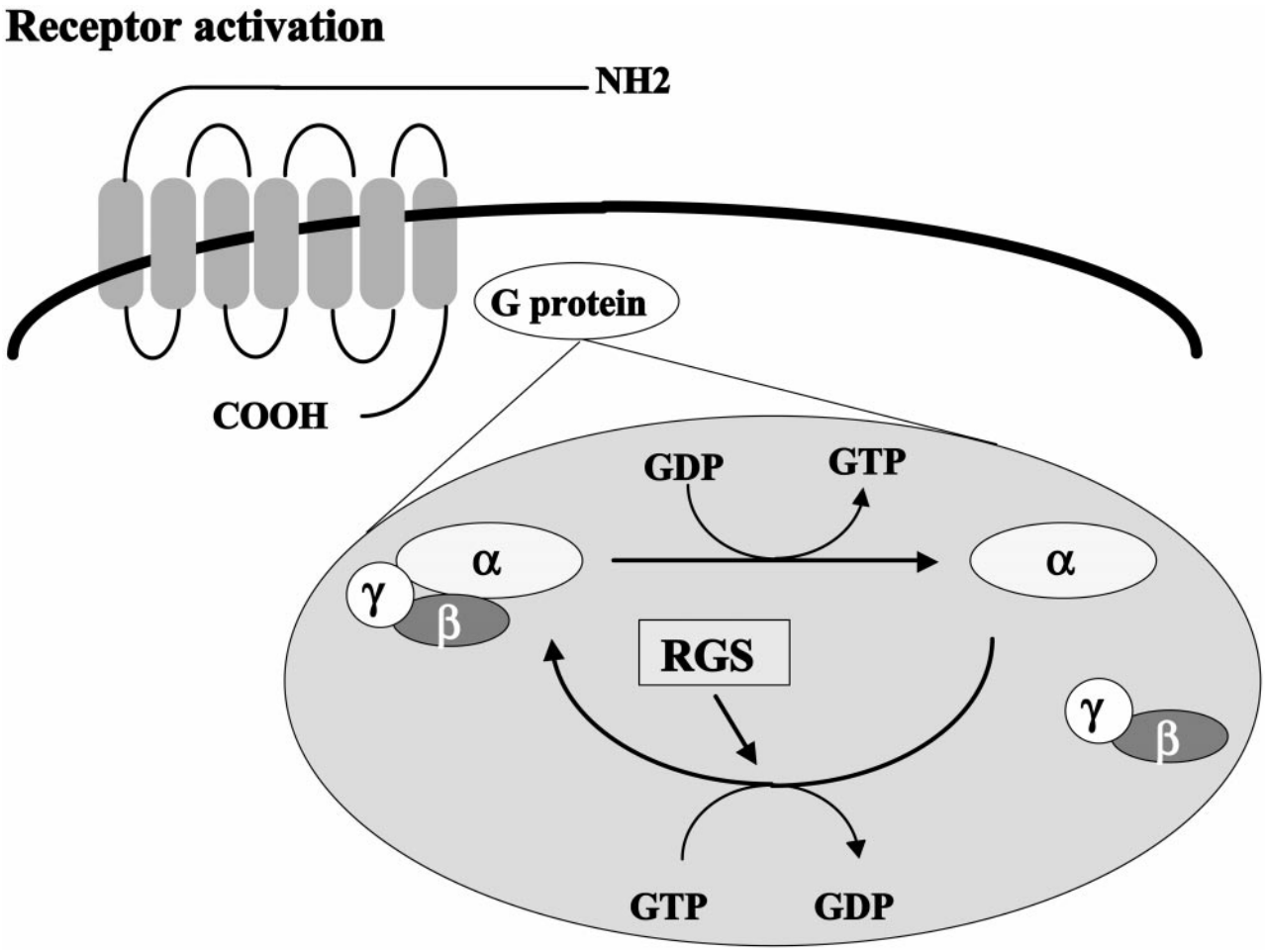

Figure 1 Schematic representation of G protein activation and signaling. Heterotrimeric G proteins are composed of three distinct subunits α, β and γ, the functional specificity of each G protein depending on the α subunit. The α subunit contains high affinity binding sites for guanine nucleotides and have intrinsic GTPase activity. The GDP-bound form binds tightly to βγ and is inactive, whereas the GTP-bound form dissociates from βγ and serves as a regulator of effector proteins. The receptor molecules cause the activation of G proteins by facilitating the exchange of GTP for GDP on the α subunit. The duration of subunit separation is timed by the rate of α subunit mediated hydrolysis of GTP. Finally, a family of GTPase activating proteins termed RGS (regulators of G protein signaling) are able to deactivate several G proteins by allowing inactive heterotrimers to reform.

Table 1 G protein signaling.

Gα subtype	Downstream signal
Gsα	Increased AC activity Ca^{2+} L-channel opening K^+-channel closing
$Gi_{1-3}\alpha$, Goα	Reduced AC activity Ca^{2+} L-channel closing K^+-channel opening
Gq/11α	Increased PLCβ activity
$G_{13}\alpha$	Cytoskeleton rearrangement Na^+/H^+ antiporter

AC, adenylyl cylase; PLCβ, phospholipase Cβ.

G protein to a single effector molecule has been only achieved for some G proteins (13–15). To date, about 20 distinct α subunits have been cloned. According to homologies in sequence and function, they can be divided into four major subfamilies represented by Gsα, Giα, Gqα and G12α. Proteins of the Gs class have been defined as ubiquitous activators of all adenylyl cyclase isoforms, whereas their effects on ion channel activity are restricted to selected cell types. Members of the Gi class, which includes several protein substrates for pertussis toxin ADP ribosylation such as Gi1–3 and Go, are involved in adenylyl cyclase inhibition, ion channel modulation and phosphatase activation. Subunits of the Gq/11 class are putative mediators of phospholipase C activation, whereas the current knowledge about Gα12, 13 and Z is sparse (Table 1).

Five β subunits and 12 γ subunits have so far been identified. Until recently the G protein α subunit alone was thought to activate intracellular effectors, newer evidence indicates that β and/or γ subunits also play a part in signal transduction. In fact, it has been demonstrated that these subunits may activate specific isozymes of both phospholipase C and adenylyl cyclase (16). This additional mechanism of action of G proteins seems to occur with high selectivity. For instance, whereas βγ inhibits type I adenylyl cyclase activity, this complex greatly potentiates the stimulatory effect of Gs on both type II and IV adenylyl cyclase and is ineffective on the other isozymes (17, 18). Moreover, among the different β and γ subunits so far cloned, the βγ of the Gi family seems to be frequently involved in the modulation of intracellular effectors involved in cell proliferation (19–21).

Abnormalities of G protein signaling pathways

It has long been known that proteins involved in signaling pathways are possible targets for mutations. As previously demonstrated for nuclear hormone receptors and growth factor receptors, it has been proposed that components of G protein signaling pathways may potentially be involved in the development of neoplastic and non-neoplastic human diseases (1–6, 22, 23). In fact, it has been demonstrated that mutations in the genes encoding these proteins are responsible for several human diseases presenting with the clinical phenotype of hormone excess or defect. The abnormal transduction may be due to mutations in the genes encoding either G protein-coupled receptors or G proteins or effectors. In Table 2 human diseases due to G protein alterations are summarized. By contrast, mutations of effector molecules seem to occur very infrequently in human diseases. In particular, the presence in invasive pituitary tumors of point mutations of protein kinase C-α, an enzyme known to importantly regulates cell growth and differentiation, has been previously reported, but not confirmed by subsequent screening studies (24, 25). Very recently, a mutation in the regulatory subunit of the protein kinase A gene has been identified in patients affected with Carney complex type 1 (26) suggesting the possible involvement of this kinase in the pathogenesis of sporadic endocrine neoplasms, such as pituitary tumors, that are included in this syndrome.

Table 2 Endocrine diseases resulting from G protein alterations.

Gsα		
Loss of function		
Pseudohypoparathyroidism type Ia	Point mutations, deletions, insertions impairing any Gsα functional domain	Germline
Pseudohypoparathyroidism type Ib	Alterations of GNAS1 locus imprinting probably leading to reduced Gsα expression; uncoupling GNAS1 mutation	Germline
Gain of function		
Pituitary or thyroid adenomas	Point mutation of Arg 201 or Gln 227, inhibition of GTP hydrolysis	Somatic
Leydig cell tumors	Point mutation of Arg 201, inhibition of GTP hydrolysis	Somatic
McCune–Albright syndrome	Point mutation of Arg 201, inhibition of GTP hydrolysis	Somatic
Gain or loss of function		
Testotoxicosis with pseudohypoparathyroidism type Ia	Point mutation of Arg385Ser, acceleration of GDP release and signal activation at 34 °C (testis); inactivation of Gsα at 37 °C (parathyroid)	Germline
Gi2α		
Gain of function		
Pituitary adenomas	Point mutation of Arg 179 or Gln 205, inhibition of GTP hydrolysis	Somatic
Adrenal cortex and ovary tumors	Point mutation of Arg 179, inhibition of GTP hydrolysis	Somatic

The first indication that alterations in the structure of G proteins could lead to development of disease was suggested by the observation that the *Vibrio cholerae* toxin possesses an ADP-ribosyl transferase activity, the target amino acid for this reaction being Arg 201 in the Gsα subunit. The ADP ribosylation of this residue and the subsequent blockade of the intrinsic GTP-ase activity induces the constitutive activation of Gsα, leading to maintained and hormone-independent activation of adenylyl cyclase. The constitutive activation of adenylyl cyclase in intestinal epithelial cells results in increased secretion of electrolytes into the bowel lumen and the subsequent watery diarrhea. *In vitro* mutagenesis experiments confirmed that Arg 201 is a key component of the regulatory turn-off mechanism of Gsα and a similar role is played by Arg residues at equivalent positions in other G protein α subunits. The pathogenetic toxin of *Bordetella pertussis* causes ADP ribosylation of a cystein residue located in the C-terminal tails of G proteins belonging to the Giα family, resulting in reduced responsiveness to receptor activation (15).

In the past few years, molecular biological approaches have provided important insights into the pathogenetic role of naturally occurring mutations in G protein genes with consequent altered signal transduction. The phenotypic expression of these mutations depend on several determinants; in particular, mutations may occur as germ-line mutations, affecting every cell in which the gene is expressed *vs* somatic mutations that lead to focal manifestations of the disease. Moreover, G protein mutations may cause either loss or gain of function, by inactivating or activating signal transduction, leading to the clinical phenotype of hormone defect or excess, respectively.

Inactivating mutations of the Gsα gene (GNAS1)

Albright hereditary osteodystrophy and pseudohypoparathyroidism

In 1942 Albright *et al.* described the first hormone resistance syndrome, which they termed pseudohypoparathyroidism (PHP) (27). They reported patients with normal renal function, in which hypocalcemia and hyperphosphatemia were associated with elevation of serum PTH levels. These patients also showed a reduced calcemic and phosphaturic response to injected bovine parathyroid extract compared with patients with primary hypoparathyroidism, leading to the hypothesis of a resistance to PTH action. Moreover, these patients displayed a constellation of physical features including short stature, centripetal obesity, rounded face, short neck and brachydactyly which is now referred to as Albright hereditary osteodystrophy (AHO). In subsequent reports subcutaneous ossifications and mental retardation were also found to accompany the majority of cases of AHO (28, 29). In contrast, patients showing the physical features of AHO without any evidence of PTH resistance were described by Albright *et al.* ten years after their first report of PHP (30). This new syndrome, which was termed pseudopseudohypoparathyroidism (PPHP) may be present either in kindreds in which PHP is present or as an isolated defect. As more and more cases were described it appeared that the majority of familial PHPs were inherited in an autosomal dominant manner (31–33).

The identification of the PTH receptor and its signal transduction pathway (35, 36) has lead us to a better understanding of PHP pathophysiology. Since the PTH receptor is coupled to Gs and therefore activates cAMP formation, measurement of serum and urinary cAMP levels after the injection of bovine PTH permitted the differentiation of PHP type I, in which a blunted cAMP response is observed, from PHP type II in which the cAMP response to PTH is conserved but a deficient phosphaturic response indicates a defect distal to cAMP generation in target cells (Table 3). Moreover, PHP type I now refers to a heterogeneous group of disorders with AHO clinical manifestations that can be differentiated by the presence (PHP Ia and PHP Ic) (37–41) or absence (PHP Ib) (39, 42) of resistance to hormones other than PTH that act via Gs coupled receptors, such as TSH and gonadotropins. Patients with PHP Ia have a partial deficiency (about 50%) of Gs activity in the membranes of various cell types (erythrocytes, fibroblasts, platelets, etc.), due to a reduction in mRNA and protein levels (38–40) whereas this defect is absent in patients with PHP Ic (42–44) (Table 3). Patients with PPHP generally coexist with PHP Ia in the same family (43) and have the same ~50% deficiency of Gs activity in cell membranes (44, 45). However, in contrast to their relatives with PHP Ia, patients with PPHP show a normal response of urinary cAMP to exogenous PTH (44). Clinical features of PPHP can also be found in families in which PHP Ia is absent, thus presenting as an isolated defect. In these cases diagnosis of PPHP is particularly difficult as many features of AHO are quite unspecific or are present in other disorders, some of which ascribed to specific chromosomal defects, as for the small terminal deletions on chromosome 2 in AHO-like syndrome (46–48).

Table 3 Classification of pseudohypoparathyroidism.

	AHO	Hormone resistance	GNASI defect
PHP Ia	Yes	Multiple	Yes
PPHP	Yes	None	Yes
PHP Ib	No	PTH	Yes
PHP Ic	Yes	Multiple	No
PHP II	No	PTH	No

PHP, pseudohypoparathyroidism; PPHP, pseudopseudohypoparathyroidism.

Genetic analysis of GNAS1 gene

PHP type Ia and PPHP

In 1990 Pattern *et al.* (49) detected and described the first heterozygous inactivating mutation in the gene encoding the Gsα (GNAS1), responsible for PHP type Ia in one family. The genetic defect in the majority of patients with PHP Ia and in their relatives with PPHP has been then confirmed by the identification of multiple heterozygous loss of function mutations within this gene (50–65).

The human GNAS1 gene maps to 20q13 (66) and contains 13 exons, its cDNA spanning a region of about 1.2 kb. Figure 2 shows the functional domains encoded by the gene. Mutations have been localized in the entire coding region of the gene, each mutation being usually associated to a single kindred. All exons can be affected by loss of function alterations, with the exception of exon 3, where no mutations have been detected to date. This is not surprising given the alternative splicing patterns observed and the lack of conservation with other α subunits, suggesting that mutations within exon 3 might have little or no clinical consequence. On the other hand, mutations in exon 1 are probably underestimated in the literature, as the extremely GC-rich nature of the flanking sequences has precluded its analysis by many authors. Considering the type of mutations, small insertions/deletions and amino acid substitutions predominate, but nonsense mutations and point mutations that lead to altered translation initiation or aberrant mRNA splicing have also been documented.

An intriguing missense mutation (54, 67) localized within the highly conserved G5 region of the Gsα, has been identified in two unrelated males who presented with AHO, PTH resistance and testotoxicosis (54). This substitution (A366S) leads to constitutive activation of adenylyl cyclase by causing accelerated release of GDP, thus increasing the fraction of active GTP-bound Gsα. However, while this mutant protein is stable at the reduced temperature of the testis, it is thermolabile at 37 °C, resulting in reduced Gsα activity in almost tissues and AHO phenotype. In females, where it has never been detected, it would be expected to only give rise to AHO.

Although each mutation is usually associated to a single kindred, a mutational hot-spot involving 20% of all mutations so far described has been identified within exon 7 (51, 63–65, 68, 69). It is a 4 bp deletion which coincides with a defined consensus sequence for arrest of DNA polymerase α, a region known to be prone to sporadic deletion mutations (69, 70). In most cases it has been found as a *de novo* mutation, thus representing a recurring new mutation rather than a founder effect. Moreover, four families have been found to carry mutations within exon 5, affecting prolines 115 and 116 (58, 63–65), while three different insertion/deletions have been found to be clustered at nucleotides 1106–1108 in exon 13 (65). Alterations in exon 5 are predicted to disrupt the highly conserved domain of Gsα that interacts with adenylate cyclase, while exon 13 is responsible for the interaction with the receptor (71). Given the relatively small number of kindreds with PHP Ia described in the literature (about 60), these particular regions seem to undergo mutational changes with a significant frequency, probably representing two new potential mutational hot-spots in GNAS1.

In families in which PHP Ia and PPHP coexist, mutations in GNAS1 can be detected in all the affected members, i.e. members affected with either PHP Ia or PPHP. On the contrary, no mutation in the GNAS1 coding sequence has ever been found in families in whom sporadic or familial PPHP was the only clinical manifestation (48, 63, 64). These results support the view that PHP Ia and isolated PPHP may represent two genetically distinct entities, even if the possibility that a defect may exist in the promoter region or in other regulatory intronic sequences of GNAS1 cannot be completely excluded.

PHP Ib

PHP Ib refers to a condition characterized by renal resistance to PTH in the absence of other endocrine or

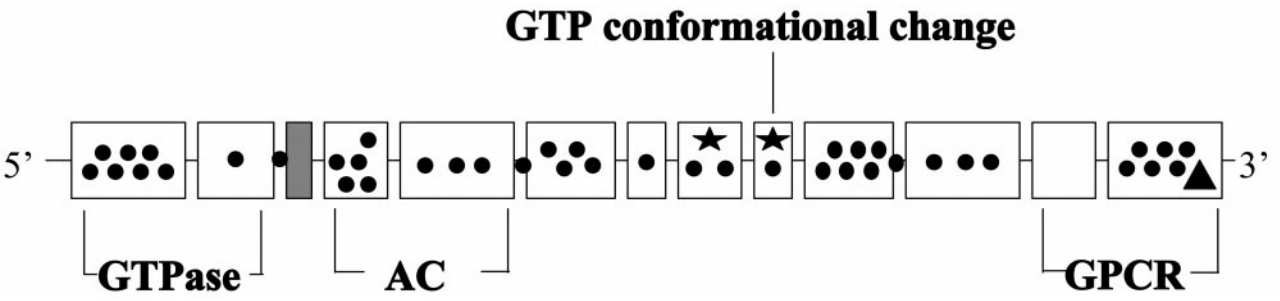

Figure 2 Schematic representation of the 13 exons composing the Gsα gene, together with its functional domains (AC: adenylyl cyclase activity domain; GPCR: G-protein coupled receptor interaction domain). Exon 3, which is shaded in the figure, is subjected to alternative splicing. The figure also shows the inactivating (circles) and activating (stars) mutations detected up to now in this gene; the triangle in exon 13 represents an inactivating mutation which has been recently described in a patient with PHP type Ib.

physical abnormalities and in the presence of a normal Gsα activity in cells that can be easily sampled (39, 41, 72, 73). The defect is usually sporadic but occasionally is familial, with a pattern of transmission consistent with an autosomal dominant one (72). The urinary cAMP response to exogenous PTH is blunted (39), implicating a defect in the signaling pathway proximal to cAMP generation. Selective resistance of target tissues to PTH and normal Gsα activity had pointed at mutations in the PTH receptor type 1 gene as possible candidates to explain the disease. However, molecular studies failed to detect genetic alterations in the coding exons and promoter region of the gene, as well as its mRNA (74–76). Moreover, neither in humans nor in mice does inactivation of one PTH1 receptor allele does end in PTH resistance (77, 78).

Linkage analysis has recently (79) mapped the genetic locus for PHP Ib to a small region of chromosome 20q13.3 in four unrelated families. Since GNAS1 is located in this region, the possibility that some patients with PHP Ib have inherited a GNAS1 mutation that leads to a selective defect in PTH-dependent signaling has been put forward. Alternatively, another gene very close to GNAS1 could be responsible for the disease. Both these hypotheses have been confirmed by recent studies. A unique mutation in exon 13 of the GNAS1 gene, the exon responsible for the interaction with the receptor, that caused autosomal dominant PTH resistance in three brothers with PHP Ib, but which was clinically silent in their mother and maternal grandfather, has been described (79). This mutant Gsα (ΔIle 382), when expressed *in vitro*, was unable to couple to the PTH 1b receptor but was able to interact normally with other Gs-coupled receptors such as LH, TSH and β-adrenergic receptors, thus explaining the phenotype of the patients. However, the prevalence of GNAS1 mutations as a cause of PHP Ib is unlikely to be high, since screening studies on several families with this disorder failed to find mutations in the coding sequence of this gene (81).

Another pathogenetic mechanism as a cause of PHP Ib has been proposed by Liu *et al.* (82). They identified a region upstream of the Gsα promoter, which is normally methylated on the maternal allele and unmethylated on the paternal allele (see below), which was unmethylated on both alleles in all 13 patients with PHP Ib studied. Unmethylation allows an alternative exon 1 (exon 1A), normally expressed only from the paternal allele, to be expressed biallelically in PHP Ib patients. Therefore, PHP Ib would be associated with an abnormal expression of exon 1A, leading to a decreased Gsα expression in renal proximal tubules, that normally express Gsα only from the maternal allele. Little or no effect is seen in other tissues, where Gsα is expressed from both parental alleles. How an alteration in the imprinting of exon 1A could alter the expression of GNAS1 remains to be explained.

PHP Ic

This term refers to a small subset of patients with all the clinical and biochemical features of PHP Ia (generalized hormone resistance and AHO), without evidence of reduced Gsα activity (38–39). The molecular defect responsible for this disease, that may involve any component of the proximal cAMP pathway (adenylyl cyclase, Gi, phosphodiesterases), has not been established yet.

PHP II

Patients affected with PHP II show clinical evidence of PTH resistance with a normal urinary cAMP response to the injection of exogenous PTH but a blunted phosphaturic response to the same hormone (83), thus indicating a defect distal to cAMP production in the PTH-mediated transduction pathway. To date, there is no evidence of the specific alterations responsible for this disorder.

It has also been hypothesized that in most cases PHP II may be an acquired defect secondary to vitamin D deficiency (84), as suggested by the observation that calcium and vitamin D replacement is able to normalize the phosphaturic response to PTH in these patients (81, 84).

GNAS1 and imprinting

Two questions arise when studying families whose members are affected by PHP Ia and PPHP. Firstly, why apparently identical Gsα deficiency associated to the same GNAS1 mutation can lead to variable phenotypic expression, in particular in terms of presence or absence of generalized hormone resistance in PHP Ia and PPHP, respectively. Secondly, why PHP Ia patients display a resistance to some (PTH, TSH and gonadotropins) but not all hormones that activate the Gs-coupled pathway.

Genomic imprinting of the GNAS1 gene has been proposed as a potential mechanism to explain the occurrence of PHP Ia and PPHP in patients with GNAS 1 mutations since it is now clear that, with one exception (85), only maternal transmission of GNAS1 mutations leads to the complete expression of the disease (PHP Ia), while paternal transmission of the same mutations is associated with PPHP in the offspring (86, 87). Moreover, genomic imprinting would be limited mainly to tissues in which there is a parent-of-origin specific difference in hormone responsiveness, such as the renal proximal tubule and the thyroid. Genomic imprinting is an epigenetic phenomenon affecting a small number of genes by which one allele (maternal or paternal) undergoes, either during the embryogenesis or in the post-natal period, a partial or total loss of expression (88). DNA methylation is the critical phenomenon for both the initiation and the

maintenance of imprinting and virtually all imprinted genes known to date have regions in which CpG dinucleotides are differentially methylated between the paternal and maternal alleles. Through the creation of uniparental disomies and partial disomies (89) at least 11 imprinted regions in the mouse genome have been identified. Indeed, GNAS1 in the mouse (Gnas1) maps within a region on distal chromosome 2 presumed to have more than one imprinted genes, as indicated by the distinct and opposite phenotypes resulting from maternal and paternal uniparental disomies of this region (90). Generation of mice with a null allele of Gnas1 (91) gave strength to this hypothesis. In fact, while homozygous Gsα deficiency is embryonically lethal, heterozygotes with maternal (m−/+) or paternal (+/p−) inheritance of the Gnas1 null allele have distinct phenotypes: m−/+, but not +/p−, show resistance to PTH, while both have a normal maximal physiological response to vasopressin. Moreover, Gsα expression studies demonstrated a reduced expression in the renal cortex, but not in the renal inner medulla (site of action of vasopressin) in m−/+ mice, as expected on the basis of clinical observations in PHP Ia. More recently, a study demonstrated the exclusive maternal expression of this gene in the renal proximal tubule, but not in other segments of the nephron (92). Interestingly, expression studies gave evidence of paternal imprinting also in brown and white adipose tissue, suggesting that the obesity observed in m−/+ mice, as well as in humans with AHO, may be the consequence of markedly reduced Gsα expression in adipose tissue. Moreover, m−/+ newborns have wide, square-shaped bodies, subcutaneous edema and higher birth weights; at 6–21 days after birth, most of these mice develop ataxia, tremor imbalance and difficulties in breathing, probably due to a delayed development of the cerebellar cortex, and then die. Surprisingly, +/p− mice also show an abnormal phenotype, characterized by lower birth weight and decreased fat mass, failure to suckle milk, severe hypoglycemia resulting in early lethality (93).

These observations provide evidence that the variable and tissue-specific hormone resistance observed in PHP Ia may result from tissue-specific imprinting of the GNAS1 gene, although the Gsα knockout model is only in part an analogue of the human AHO phenotype. In addition, even if the inheritance pattern of AHO is consistent with imprinting of the GNAS1 paternal allele, expression studies on RNA from various human fetal tissues have failed to demonstrate monoallelic expression of this gene (94). However, these negative results obtained in fetal tissues may at least in part be due to the fact that imprinting of the paternal allele might be a process beginning and evolving in postnatal life (87). This is in line with the recent observation that Gsα is monoallelically expressed in human normal pituitary (95).

Recent studies on the GNAS1 locus indicate that this region is extremely complex, with multiple alternatively spliced transcripts encoding multiple protein products in man as well as in mouse (Fig. 3). By using alternative promoters and first exons, the GNAS1 locus gives rise not only to the Gsα gene, but to at least three other gene products, i.e. XLαs (extra large αs-like protein), a Golgi-specific isoform of Gsα, and NESP55 (neuroendocrine secretory protein 55), that are oppositely imprinted (96, 97). While the former is expressed from the paternal allele, with its promoter methylated on the maternal one, the latter is expressed from the maternal allele, its promoter being methylated on the paternal one. Both proteins have been found primarily expressed in neuroendocrine tissues and their function is largely unknown (100, 101). A third alternative promoter and first exon (exon 1A) is located

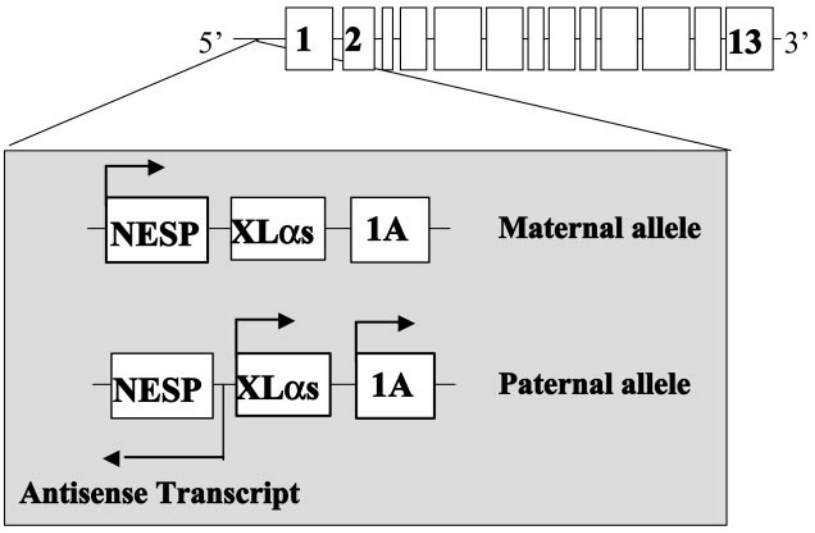

Figure 3 Genomic organization of human GNAS1 locus. The figure shows four alternative first exons which splice into exon 2, generating four different transcripts: Gsα, NESP55, XLαs and an unknown gene product from exon 1A. Exons 2–13 are common to all transcripts, even if they are not translated in NESP55. The shaded box indicates also the different expression of transcripts in maternal rather than paternal allele (arrows stay for presence of transcription). Finally, an antisense transcript has been recently described and demonstrated to be imprinted in the maternal allele, thus being expressed only in the paternal one.

2.5 kb upstream of Gsα exon 1 and probably generates untranslated transcripts of unknown function with a pattern of expression similar to that of Gsα (101–103). In the mouse, differential methylation in this region is established during gametogenesis, being present in oocytes and absent in spermatozoa and it is then maintained throughout pre- and postimplantation development, thus constituting a methylation imprint mark, which may possibly be important for the tissue-specific imprinting of Gsα, whose promoter is, on the contrary, unmethylated (104). Finally, the maternally methylated region upstream of the Xlαs exon gives rise to a spliced poly-adenylated antisense transcript, which spans the upstream NESP55 region (105). This antisense transcript is imprinted and expressed only from the paternal allele, thus suggesting that it may have a specific role in suppressing *in cis* the activity of the paternal NESP55, as it has been already described for other imprinted loci, such as the murine *Igf2r* locus (106).

In conclusion, the organization and regulation of the GNAS1 locus, as well as the clinical significance of the different transcripts originating from it, are still insufficiently understood and specifically targeted knockout mice lacking one or more of these alternative first exons will help to solve some of these questions.

Activating mutations of G proteins

Mutations of the Gsα gene (GNAS1): gsp oncogene

The first clue to the possible existence of activating mutations of G protein genes as a cause of human neoplasia arose from the identification of a subset of GH-secreting pituitary adenomas characterized by high levels of *in vitro* GH release, intracellular cAMP accumulation and membrane adenylyl cyclase activity (107). The presence of GNAS1 mutations leading to the constitutive activation of Gsα was hypothesized on the basis of high adenylyl cyclase levels in basal conditions which were not further stimulated either by agents known to directly activate Gsα such as GTP and

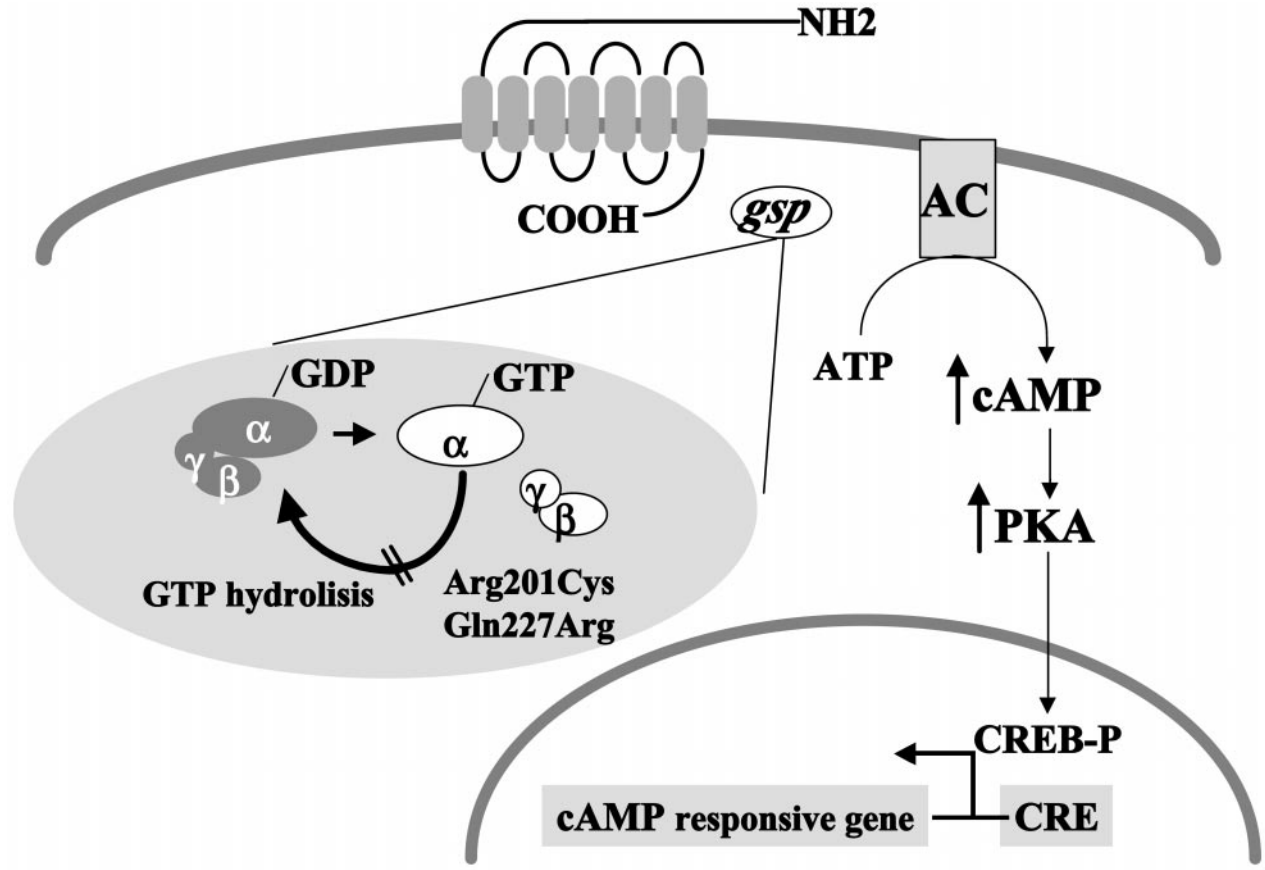

Figure 4 Schematic representation of *gsp* oncogene. Somatic mutations of GNAS1 gene replacing either Arg 201 with Cys or His or Ser, or Gln 227 with Arg or Leu have been identified in a subset of endocrine tumors. By inhibiting the intrinsic GTPase activity, these mutations cause constitutive activation of adenylyl cyclase (AC), leading to increased cAMP formation and proteinkinase A activation. The proteinkinase A (PKA) induced phosphorylation of the cAMP responsive element binding protein CREB facilitates its traslocation to the nucleus and activation of cAMP-responsive gene transcription.

fluoride, or by peptides operating through Gs-coupled receptors, such as GHRH (107). The subsequent analysis of DNA from these tumors revealed amino acid substitutions in exons 8 and 9, replacing either Arg 201 with Cys, His or Ser, or Gln 227 with Arg or Leu (108–110) (Fig. 4). Although *in vitro* mutagenesis studies have documented a number of possible activating substitutions in GNAS1 gene, these two residues are the only location for mutations so far identified. When transfected into S49 cyc-cells, mutant Gsα showed a 30-fold decrease in intrinsic GTPase activity. Indeed both residues are known to be important in GTP hydrolysis (108). Arg 201 is the residue that is ADP-ribosylated by cholera toxin, this covalent modification resulting in hormone-independent activation of adenylyl cyclase due to GTPase inhibition. Similarly, *in vitro* mutagenesis experiments confirmed that the Gln 227 residue is involved GTP hydrolysis. Therefore, these two mutations cause constituitive activation of cAMP formation by inhibiting the turn-off mechanism of Gsα. Since somatotrophs belong to a set of cells that recognizes cAMP as a mitogenic signal, Gsα may be considered the product of a proto-oncogene that is converted into an oncogene, designated *gsp* (for Gs protein) in selected cell types.

Functional studies of gsp oncogene

Studies on cell lines transfected with mutant Gsα yielded important insights into the series of events resulting from the activation of cAMP cascade. Indeed, at variance with the phenotype induced by the activation of the classical oncogenes, the specific pathways activated by cAMP stimulate both growth and specialized functions. Indeed, the transcription of a variety of common cAMP-responsive genes, including the immediate early genes such as c-*fos*, c-*jun* and *jun* B, are enhanced by the expression of mutant Gsα (111). Moreover, mutant Gsα stimulates GH and PRL promoter activity in GH3 cells expressing this protein (112). As far as the mitogenic effect of *gsp* mutations is concerned, the introduction of mutant Gsα results in enhanced function and growth of selected cell types in which the cAMP cascade activates proliferation processes. In particular, Swiss 3T3 fibroblasts carrying mutant Gsα show a mitogenic activity higher than that of wild-type cells, as indicated by the low serum concentration required for growth (113). The introduction of the Gln227Leu mutation in FRTL-5 thyroid cells is sufficient to induce a TSH-independent proliferation (114). Similarly GH3 cells expressing this mutation show enhanced proliferation and GH and PRL secretion (115). Although these results suggest that the expression of mutationally activated Gsα is sufficient to bypass the requirement for the specific growth factor and promotes autonomous cell growth of specific cell types, most of these effects were observed only when cAMP hydrolysis was blocked by phosphodiesterase (PDE) inhibitors (113, 114). Indeed, the presence of mutant Gsα is accompanied by a concomitant increase in PDE activity and expression, likely as a result of the feedback mechanism by which cAMP controls the expression of its own degrading enzymes (113, 114, 116, 117). The impact of cAMP hydrolysis on the phenotype produced by the expression of mutant Gsα is indicated by the observation that phosphodiesterase blockade results in a further stimulation of both cAMP levels and proliferation in different cell systems (116–118).

gsp Oncogene in pituitary adenomas

Several screening studies confirmed that approximately 30–40% of GH-secreting adenomas is associated with *gsp* mutations, that most frequently replace Arg 201 (wild-type codon TGC) with Cys (mutant codon TGT). Some ethnic differences in the occurrence of these mutations seem to exist due to the considerably low prevalence (5–10%) reported in Japanese acromegalic patients (119–123). The presence of Gsα mutations is not exclusive to GH-secreting adenomas, although its frequency in the other pituitary adenomas is definitely low. Indeed, the *gsp* oncogene has been observed in 0–13% of non-functioning pituitary adenomas (124, 125) while a single study reports its presence in 5% of ACTH-secreting adenomas (126). These mutations are somatic in origin as indicated by the presence of wild-type Gsα in the peripheral blood leukocytes from affected patients and dominant, as indicated by the presence of both mutant and wild-type Gsα in genomic DNA from the tumor.

Several *in vivo* studies indicate no difference in age, sex, clinical features, duration of the disease or cure rate in patients with or without *gsp* mutations (119–121, 123, 127). However, tumors expressing *gsp* mutations are most frequently very small in size, consistent with the hypersecretory activity of tumoral somatotrophs. Due to the constitutive activation of cAMP formation, patients with *gsp*-positive tumors do not increase plasma GH levels after GHRH whereas they respond to agents acting via a cAMP-independent pathway. Moreover, these patients show a high sensitivity to the inhibitory action of long-acting somatostatin analogues, an effect not associated with increased expression of somatostatin receptor sst2 and sst5 in the tumor (127–129).

Since *gsp* mutations would in principle confer growth advantage, the low growth rate of tumors with these mutations probably reflects the existence of mechanisms able to counteract the activation of the cAMP pathway. Over the last few years, some of these mechanisms have been unveiled. Recent studies demonstrated that, in analogy with the upregulation of PDE in FRTL5 expressing mutant Gsα, in *gsp*-positive tumors PDE activity is about 7-fold higher than that observed in wild-type tissues, this effect being mainly due to the increased expression of cAMP-specific PDE4

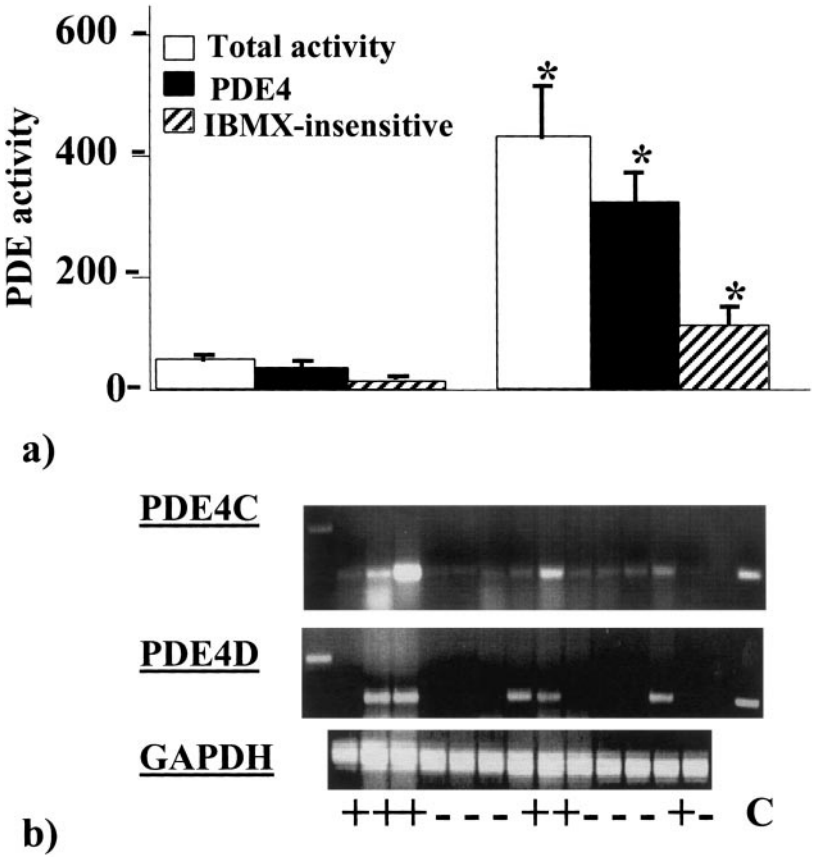

Figure 5 Phosphodiesterase hyperactivity and overexpression in GH-secreting tumors with *gsp* oncogene. a) Tumors with *gsp* oncogene (on the right) show significantly higher activity of phosphodiesterases (PDE), particularly the cAMP-specific PDE4 isoform, than tumors without this alteration (on the left). b) By RT-PCR analysis it appears that the increased PDE activity observed in *gsp* positive tumors (+) is mainly due to the induction of cAMP specific PDE4C and PDE4D genes transcription. Therefore, the presence of mutant Gsα is accompanied by a concomitant increase in PDE activity and expression, as a result of the feedback mechanism by which cAMP controls the expression of its own degrading enzymes.

(Fig. 5) (117, 130). Moreover, *gsp*-positive tumors highly express two nuclear transcription factors that are final targets of the cAMP-dependent pathway and are positively regulated by cAMP signaling, i.e. the cAMP-responsive element binding protein (CREB) and the inducible cAMP early repressor (ICER). The increased expression of the repressor transcription factor ICER, that competes with the binding of CREB to CREs, may inhibit the transcription of several cAMP responsive genes, including CREB itself (131). This counteracting mechanism is consistent with a previous report indicating elevated levels of phosphorylated, hence activated, CREB in GH-secreting adenomas, independently of the presence or absence of *gsp* mutations (132). Finally, although no differences in Gsα mRNA levels have been reported in tumors with or without *gsp* mutations, the mutant protein is present in very low amounts, probably because of the increased rate of degradation of the instable dissociated α subunit (128, 133; Fig. 6). The recent report that the monoallelic expression of Gsα from the maternal allele found in the normal pituitary (95) is relaxed in somatotroph tumors with and without *gsp* mutations suggests the existence of another mechanism, i.e. loss of GNAS1 imprinting, able to amplify the cAMP pathway independently of the presence or not of *gsp* mutations.

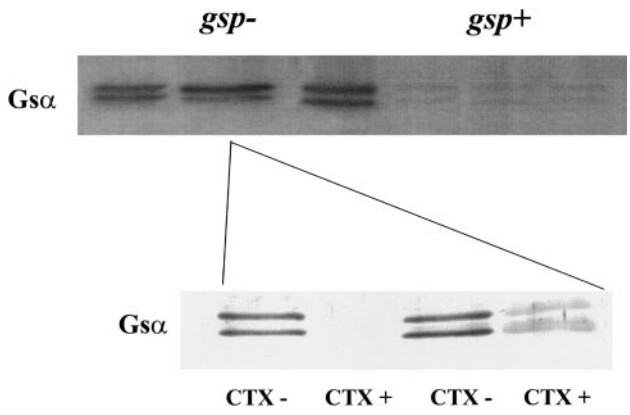

Figure 6 Representative immunoblotting with antibodies to Gsα in *gsp*-positive and -negative tumors. Tumors with *gsp* oncogene are characterized by a reduced or undetectable expression of Gsα protein. This is probably due to the increased rate of degradation of the instable dissociated α subunit, since the same reduction is observed when *gsp* negative cells are treated with cholera toxin, an agent known to block GTPase activity and to induce constitutive activation of adenylyl cyclase, by ADP-ribosylating Arg 201 in Gsα.

gsp Oncogene in thyroid neoplasms

Following the identification of *gsp* mutations in GH-secreting adenomas, mutations involving the same

two hot-spots in the GNAS1 gene have been identified in hyperfunctioning thyroid adenomas (134). This finding is consistent with the key role of the cAMP pathway in mediating TSH action on both thyroid hormone secretion and thyrocyte proliferation. The frequency of *gsp* mutations in thyroid hot nodules is variable from one series to another, ranging from 5 to 30%, and is definitely lower than that of TSH receptor gene mutations (135, 136). Therefore, the main alterations that constitutively activate the cAMP pathway in thyrocytes are mutations in the TSH receptor while in somatotrophs there are mutations in the Gsα gene. As occurs in GH-secreting adenomas with *gsp* mutations, the phenotype of thyroid adenomas carrying mutant Gsα or TSH receptor is different from that predicted on the basis of *in vitro* models. In fact, it has been observed that in tumors with these mutations the expression of the activated, phosphorylated form of CREB is not increased when compared with that of the paired normal thyroid tissue, but decreased (137). It is likely that the increase in PDE activity and expression that occur in these adenomas may participate in determining the loss of activation of the cAMP dependent signaling (118). Mutant Gsα may also be present with low frequency (<10%) in hypofunctioning thyroid adenomas (cold nodules) as well as in differentiated thyroid adenocarcinomas (138). In particular, *gsp* mutations were detected in a subset of papillary and follicular carcinomas selected on the basis of high adenylyl cyclase activity in basal conditions not further stimulated by TSH (139). No *gsp* mutations have been detected in anaplastic carcinoma. The data collected from the different studies indicate that whereas *gsp* oncogene may be considered as an initiator for a minority of hyperfunctioning thyroid adenomas, its role in thyroid tumorigenesis is much less certain.

gsp Oncogene in McCune–Albright syndrome

The identification of activating mutations in the GNAS1 gene in patients with McCune–Albright syndrome (MAS) has provided clear evidence that the activation of the cAMP pathway is associated with hyperfunction and hyperplasia of cells other than pituicytes and thyrocytes. This syndrome is a sporadic disorder characterized by polyostotic fibrous dysplasia, café-au-lait skin hyperpigmentation and autonomous hyperfunction of several endocrine glands, such as gonads, pituitary, thyroid and adrenal cortex, i.e. glands sensitive to trophic agents acting through the cAMP-dependent pathway. Mutations of the Gsα gene have been detected in all affected subjects and Arg 201 is the only location so far reported. Mutant Gsα is expressed in the affected endocrine organs as well as in tissues not classically involved in MAS, the highest proportion of mutant alleles being found in regions of abnormal proliferation (140, 141). This mosaic distribution is consistent with the hypothesis that this syndrome is due to a somatic mutation in Gsα gene occurring as an early postzygotic event. Therefore, the time of occurrence of GNAS1 mutations seems to be an important factor in determining the nature of the disease. Due to the ubiquitous expression of Gsα, late occurring mutations cause focal disease such as acromegaly and toxic thyroid adenomas, while when the same mutations occur very early in embryogenesis they cause disorders with widespread manifestations, such as McCune–Albright syndrome (MAS). It is tempting to speculate that activating germ-line mutations of Gsα would be incompatible with life (23).

Recent studies have provided insights into the pathological role of mutant Gsα in non-endocrine organs involved in MAS. It has been shown that melanocytes from the café-au-lait spots of MAS patients have high mRNA levels of tyrosinase gene, probably responsible for alteration in skin pigmentation (142). As far as fibrous dysplasia is concerned, high levels of c-fos expression, presumably a consequence of increased adenylyl cyclase activity, have been detected in bone lesions from all MAS patients studied, consistent with the bone disorders present in transgenic mice overexpressing c-fos proto-oncogene. Moreover, transplantation of skeletal progenitor cells obtained from fibrous dysplastic marrow of patients with MAS into immunocompromised mice caused abnormal ossicle formation, resembling human fibrous dysplasia (143, 144). Interestingly, lesion development required the coexistence of normal cells and cells with a mutant allele, thus reproducing the mosaic distribution of Gsα mutations that characterizes the syndrome. Finally, substitutions at Arg 201 of GNAS1 gene have also been found in isolated fibrous dysplasia occurring outside of the context of typical MAS (144).

gsp Oncogene in other endocrine disorders

Other endocrine organs have been screened for *gsp* mutations since they contain cell types in which cAMP is a positive growth stimulus, such as the endocrine pancreas, the parathyroid, the adrenal gland and the gonads. No Gsα mutation has so far been identified in hyperfunctioning neoplasia from the pancreas, the parathyroid or the adrenal glands, the only exception has been an Arg 201 to Cys substitution in the genomic DNA from nodular adrenal hyperplasia in an infant with Cushing's syndrome (145, 146). By contrast, Arg 201 to Cys changes were found in a significant proportion (4 of 6) of ovarian and testicular stromal Leydig cell tumors, that had caused hormonal hypersecretion resulting in virilization and gynecomastia in female and male patients, respectively (147). As reported for other endocrine neoplasias with *gsp* mutations, there was no evidence of clinical or hormonal differences between patients with *gsp*-positive and -negative tumors (147).

Mutations of the Gi2α gene

Taking into account that all G proteins have a common mechanism of binding and hydrolyzing GTP and share highly conserved primary structures in regions corresponding to Arg 201 and Gln 227 of Gsα, it was predicted that other G proteins would be converted into oncogenes by GTPase-inhibiting mutations. At present, the Gsα gene is the only gene that has been identified as a target for activating or inactivating mutations that unequivocally cause endocrine diseases (Table 2). In fact, neither activating nor inactivating mutations of Gq, the G protein involved in the activation of the Ca^{2+}–calmodulin protein kinase C-dependent pathway have been identified yet (148). Conversely, discordant data on mutations of Gi2 protein are present in the literature. In fact, screening studies of human tumors for mutations of the Gi2α gene revealed aminoacids substitutions of Arg 179 (corresponding to the Arg 201 of the Gsα gene) to His in ovarian sex cord stromal tumors and adrenal cortex tumors. In particular, the mutant Gi2α (*gip 2* oncogene) was detected in two granulosa cell tumors and one thecoma from 10 ovarian tumors. However, this data was not confirmed by subsequent screening studies (149). Similarly, two subsequent studies failed to detect Gi2α mutations in adrenal cortex adenomas and carcinomas (150, 151). Finally, a different mutation replacing Gln 205 (corresponding to the Gln 227 of the Gsα gene) with Arg was reported in three of 22 non-functioning pituitary adenomas. Interestingly, two of these tumors also had concomitant *gsp* mutations, with a paradoxical result in terms of cAMP generation considering that Gsα and Gi2α genes have opposing effects on adenylyl cyclase activity (Table 1).

The possible oncogenic potential of the constitutive activation of Gi2α gene is difficult to ascertain since Gi2α is involved in the activation of multiple and probably not fully understood intracellular pathways. It has been demonstrated that the mutant Gi2a (*gip 2* oncogene) induces the constitutive inhibition of adenylyl cyclase and reduction of cytosolic calcium in transfected cells (152). However, the gip2 oncogene may affect pathways other than the cAMP or the Ca^{2+}–calmodulin cascades. In fact, it has been demonstrated that Gi-coupled receptors are able to activate the MAP kinase pathway (21, 153) and that, in certain cell systems, the expression of constitutively active Giα causes cell transformation (154, 155). The mechanism through which gip2 induces cell proliferation is probably mediated by MAPK activation (153–155).

G-protein β3 variant in essential hypertension

The observation of an increased activity of a Na^+/H^+ exchanger in blood cells of a subgroup of hypertensive patients that was consistent with abnormal signaling by Gi proteins resulted in the identification of substitution in the gene encoding the G protein β3 subunit (GNB3) (156). This substitution causes an aberrant splicing of β3 mRNA and production of a small subunit that lacks 41 residues in the middle of the amino acid sequence and is constitutively active. Although the mechanisms by which the small β3 increases Gi-dependent hormone responses are uncertain, this variant seems to be present in 53% of patients with hypertension and 44% of normotensive subjects, suggesting that the variant allele substantially increases the risk of hypertension (156). If future studies confirm that the G protein β3 variant contributes to the incidence of hypertension, this will have important implications for understanding pathogenesis of the disease and for improving its treatment (157).

Conclusions

It is well established that proteins involved in signal transduction are targets for naturally occurring mutations resulting in human diseases. Admittedly, defects in G proteins almost always result in endocrine disorders, the only exception being inactivating mutations of Gtα, that mediate rod-cell responses to photons in inherited congenital night blindness (158). Since much evidence indicates that several G proteins are involved in cell growth regulation, it is likely that additional endocrine disorders will be found to be caused by G protein defects. To date, however, GNAS1 is the only gene encoding a G protein that has been identified as a target for mutations that unequivocally cause endocrine diseases. Indeed, inactivating germ line mutations of this gene cause AHO and pseudohypoparathyroidism while activating somatic mutations lead to the proliferation of endocrine cells in which cAMP is a mitogenic signal. Although in the recent years screening studies have detected the presence of new inactivating or activating mutations of GNAS1 gene and established their prevalence in the different diseases, several questions arise when studying the genotype–phenotype relationships. In particular, why apparently identical Gsα deficiency associated with the same GNAS1 mutation can lead to the presence or absence of generalized hormone resistance, and why the resistance is limited to some hormones, i.e. PTH, TSH and gonadotropins, while others that equally activate the Gs-coupled pathway are unaffected. Although evidence suggests that the variable and tissue-specific hormone resistance observed in PHP Ia may result from tissue-specific imprinting of the GNAS1 gene, the Gsα knockout model is only in part analogous to the human AHO phenotype and other studies are needed to understand the molecular basis of this disorder. Activating mutations of GNAS1 gene would in principle confer growth advantage in the selected cell types in which cAMP acts as a mitogenic signal, and on this basis these mutations were

referred to as *gsp* oncogene. However, studies carried out on several neoplasias carrying this oncogene, i.e. GH-secreting pituitary adenomas, hyperfunctioning thyroid adenomas and Leydig cell tumors, failed to detect differences in the clinical and hormonal phenotypes. Therefore, the low growth rate of tumors with these mutations probably reflects the existence of mechanisms able to counteract the activation of the cAMP pathway, that are still insufficiently understood. Similarly, the prevalence and significance of Gi2α mutations are still controversial. Moreover, although this review has focused on Gα subunits because they have been well studied and harbor known mutations, studies on β/γ subunits should be particularly fruitful, due to the increasing appreciation of the importance of these components in signal transduction. In this respect, it has been reported that patients with essential hypertension frequently have a β3 subunit variant which is constitutively active. Although the impact of the short β3 on hormone signaling is not known, it has been suggested that this polymorphism may contribute to hypertension. Finally, the identification of naturally occurring mutations of G proteins has already had major implications for understanding the structure and function of these signaling proteins. Unfortunately, the implications of identifying G protein mutations for diagnosis and treatment of endocrine disorders are, as yet, rather limited.

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