MINIREVIEW

Genetics of Pituitary Tumors: Focus on G-Protein Mutations

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In recent years the demonstration that human pituitary adenomas are monoclonal in origin has provided further evidence that pituitary neoplasia arise from the replication of a single mutated cell in which growth advantage results from either activation of proto-oncogenes or inactivation of tumor suppressor genes. While common oncogenes, such as Ras, are only exceptionally involved, the only mutations identified in a significant proportion of pituitary tumors, and particular in GH-secreting adenomas, occur in the Gsα gene (GNAS1) and cause constitutive activation of the cAMP pathway (*gsp* oncogene). Moreover, pituitary tumors overexpress hypothalamic releasing hormones, growth factors, and their receptors as well as cyclins involved in cell cycle progression. As far as the role of tumor suppressor genes in pituitary tumorigenesis is concerned, reduced expression of these genes seems to frequently occur in pituitary tumors as a consequence of abnormal methylation processes. Although the only mutational change so far identified in pituitary tumors is the *gsp* oncogene, this oncogene is not associated with a clear phenotype in patients bearing positive tumors. Mechanisms able to counteract the cAMP pathway, such as high sensitivity to somatostatin, and induction of genes with opposite actions, such as phosphodiesterases, CREB end ICER, or instability of mutant Gsα, have been proposed to account for the lack of genotype/phenotype relationships. Exp Biol Med 228:1004–1017, 2003

Key words: pituitary adenomas; cAMP; oncogene; tumor suppressor genes genes; *gsp*

Pituitary tumors are rare neoplasia that derive from excessive proliferation of each subtype of pituitary cells and present with specific endocrine syndromes (acromegaly, amenorrea-galactorrea, Cushing disease, TSH-induced hyperthyroidism, hypopituitarism) or mass symptoms or both. The genesis of pituitary tumors has been controversial for many years and the respective role and importance of intrinsic alterations of the pituicytes themselves, hypothalamic dysregulation, and locally produced growth factors is still under debate (1–4). The demonstration by X-chromosome inactivation analysis that the majority of pituitary adenomas is monoclonal in origin represents a milestone in this debate (5, 6). In fact, these data unequivocally indicate that pituitary neoplasia arise from the replication of a single mutated cell in which growth advantage results from either activation of protooncogenes or inactivation of tumor suppressor genes (Tables I and II). Accordingly, changes in the expression of these genes have been observed in pituitary tumors, although it is presently unknown whether these changes have a causative role or represent a secondary event. In particular, although pituitary tumors may be induced in the mice by overexpressing or knocking out specific protooncogenes or tumors suppressor genes, respectively, these animal models only partially recapitulate pituitary human tumorigenesis. In particular, contrary to what is observed in humans, these manipulations generally cause tumor formation almost exclusively in female animals and are preceded by a long-standing phase of cell hyperplasia (Table III).

Consistent with the general principles of tumorigenesis, several *in vivo* and *in vitro* evidence suggest that clonal expansion of the mutated pituitary cell requires a secondary event for tumor progression. The need for a second hit is indicated by the clinical observation that high resolution neuroradiological imaging "incidentally" detects pituitary microadenomas in about 20% of subjects without signs or

This work was supported in part by MURST Grant 2001068427 and Ricerca Corrente funds of Ospedale Maggiore IRCCS.

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1535-3702/03/2289-1004$15.00

Table I. Gain-of-Function Events in Pituitary Tumors

Gene	Defect	Human pituitary tumor
Cyclin E	Increased expression	ACTH-omas
Cyclin D1	Increased expression	Aggressive adenomas
Gsα	Somatic mutations	GH-omas
Gi2α	Somatic mutations	NFPA
Ras	Somatic mutations	Pituitary carcinomas metastases; aggressive prolactinoma
PKC	Somatic mutations	Invasive NFPA
PTTG	Increased expression	All types
FGFR4	Alternative transcription initiation	All types

Table II. Loss-of-Function Events in Sporadic Pituitary Tumors

Gene	Defect	Human pituitary tumor
RB	Promoter methylation	Aggressive adenomas
p16INK4a	Promoter methylation	All types
p27Kip1	Reduced expression	ACTH-omas
TRβ	Inactivating mutations	TSH-omas
GR	LOH	ACTH-omas
D2R	Reduced expression	Resistant PRL-omas
Sst2	Reduced expression	Resistant GH-omas

Table III. Proto-Oncogene Activation and Tumor Suppressor Gene Loss in Animal Models

Transgenic mice	Pituitary lesion
Cholera toxin	Somatotroph hyperplasia
GHRH	Somatotroph hyperplasia → somatotroph adenoma
bFGF	Lactotroph hyperplasia
NGF	Lactotroph hyperplasia
TGFα	Lactotroph hyperplasia, lactotroph adenoma (female only)
LIF	Corticotroph hyperplasia
FGF4R variant	Lactotroph adenoma
HMGA2	Lactotroph amd somatotroph adenoma
KO mice	**Pituitary lesion**
D2R	Lactotroph hyperplasia → Lactotroph adenoma
RB	Intermediate lobe hyperplasia → ACTH-secreting adenoma
p27Kip1	Intermediate lobe hyperplasia → ACTH-secreting adenoma

symptoms of pituitary disorders, a value about 1.000-fold higher than the clinical prevalence of the disease and approaching the incidence of pituitary adenomas found in unselected autopsies (7, 8). However, our understanding of the molecular mechanisms responsible for pituitary tumor progression, and particularly the relative importance of hypothalamic neuropeptides, steroids, growth factors, and their signaling pathways, is still incomplete.

In this review we summarize the different molecular alterations that have been proposed to be involved in pituitary tumorigenesis, with particular emphasis on pituitary specific events. Moreover, this review focuses on the only mutational change so far unequivocally identified in pituitary adenomas (i.e., gain of function mutations of the guanine nucleotide binding α-subunit 1 gene [GNAS1], termed *gsp* for Gs protein (9, 10), and its impact on the tumor phenotype).

Alterations of Common and Pituitary Specific Proto-oncogene Sequence and/or Expression in Pituitary Tumors

Common Proto-oncogenes. Pituitary tumors may originate from either gain of function mutations or overexpression of ubiquitously expressed proto-oncogenes that are components of common proliferative pathways. The common protooncogenes that have been extensively analyzed in pituitary tumors include proteins involved in signal transduction, growth factors and their receptors, and cell cycle proteins (Table I).

Proteins Involved in Signal Transduction. The family of RAS proto-oncogene encodes a 21-kD monomeric GDP/GTP binding protein mainly involved in the transduction of growth factor signalling. These proto-oncogenes may acquire mitogenic properties by point mutations that increase the affinity for GTP in the GTP-binding domain (codons 12 and 13) or prevent GTP-ase activity (codon 61). Mutations, particularly in codon 12 of the RAS gene, are present with relatively high frequency in human malignancies while they are uncommon in pituitary tumors. Indeed, a Gly12 to Val substitution has been observed in one single unusually aggressive prolactinoma resistant to dopaminergic inhibition that eventually was lethal (11). Subsequent

Table IV. Clinical and Biochemical Features of Acromegalic Patients with and without *gsp* Mutation

No differences in
Clinical features
Sex, age, GH levels, GH responses to OGTT, clinical outcome
Morphological features
Immunostaining ranking for GH, PRL, and αGSU, local invasiveness
In *gsp*+ tumors
gsp+ tumors smaller than *gsp*–
Better response to somatostatin analogue treatment
Ultrastructural features consistent with hypersecretory activity

studies on large series of functioning and nonfunctioning pituitary tumors failed to find RAS mutations (12). Consistent with the view that this mutational change is associated with unusual malignant feature and probably represents a late event, RAS mutations have been detected in metastases of 3 pituitary carcinomas, but not in the primitive tumors (13).

The stimulatory G protein (Gs) is a ubiquitously expressed protein, belonging to the family of heterotrimeric G proteins that are constituted by the specific α subunit and the common βγ subunits. Gs protein is required for the activation of adenylyl cyclase and generation of cAMP in pituitary target cells in response to several hormones, such as GH-releasing hormone (GHRH) and corticotroph-releasing hormone (CRH). The first mutational change associated with pituitary tumors was identified in the gene encoding the α subunit of Gs (GNAS1) that maps on chromosome 20q13. In particular, amino acid substitutions replacing either Arg 201 or, less frequently, Gln 227 were identified in a subset of GH-secreting adenomas characterized by extremely high adenylyl cyclase activity and cAMP levels not further stimulated by specific and aspecific agents (7). When transfected into S49 cyc-cells, mutant Gsα showed a 30-fold decrease in the rate of α subunit mediated hydrolysis of GTP to GDP, a mechanism inherent in all α subunits that is required for the reassembly of the αβγ heterotrimer and the turn-off of the activation (10) (Fig. 1). Since cAMP represents a mitogenic signal in somatotrophs, 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. The *in vivo* and *in vitro* phenotype resulting from the expression of *gsp* oncogene will be discussed subsequently.

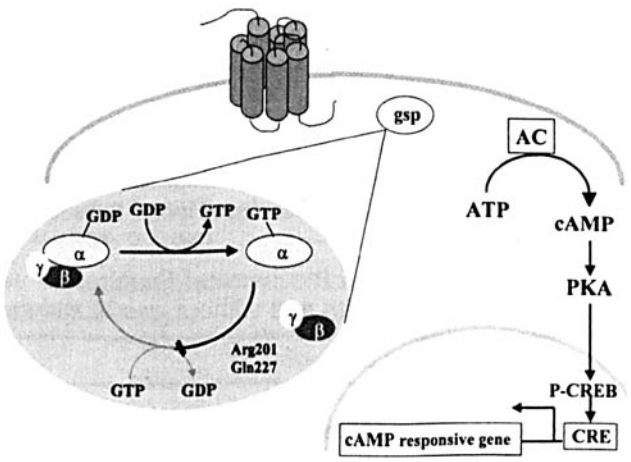

Figure 1. Schematic representation of G protein activation and signaling in the presence or in the absence of *gsp* oncogene. The receptor molecules cause the activation of G proteins by facilitating the exchange of GTP for GDP on the α subunit, which leads α subunit to dissociate from βγ dimer. The duration of subunit separation is timed by the rate of α subunit-mediated hydrolysis of GTP. The activating mutations of the Gsα gene (GNAS1) inhibit GTPase and thereby prevent the formation of the inactive αβγ complex. AC, adenylyl cyclase; PKA, proteinkinase A; P-CREB, phosphorylated cAMP response element binding protein; CRE, cAMP response element.

Following the first identification in GH-secreting adenomas, *gsp* mutations were found in other endocrine tumors, that is nonfunctioning (10%) and ACTH-secreting adenomas (<5%), in thyroid hyperfunctioning adenomas (5%–20%) and in rare cases of thyroid cold nodules and differentiated adenocarcinomas, in a significant proportion of ovarian and testicular stromal Leydig cell tumors, and in rare cases of nodular adrenal hyperplasia causing Cushing syndrome (14–18). However, the number of endocrine and nonendocrine tissues in which cAMP may act as a mitogenic signal has been expanded by the demonstration that the McCune-Albright syndrome, a sporadic disorder characterized by polyostotic fibrous dysplasia, cafè-au-lait skin hyperpigmentation, and autonomous hyperfunction of several endocrine glands, is due to *gsp* mutations occurring as an early postzygotic event (19).

Although 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, at present Gsα is the only G protein that has been identified as target for activating mutations that unequivocally cause endocrine diseases. In fact, data in the literature concerning mutations of Gi2α protein, a protein involved in the inhibition of adenylyl cyclase and calcium influx, are discordant (20–25). Previous screening studies reporting amino acids substitutions of Arg 179 (corresponding to Arg 201 of Gsα sequence) of Gi2α gene in ovarian sex cord stromal tumors and adrenal cortex tumors and a different mutation replacing Gln 205 (corresponding to Gln 227 of Gsα sequence) with Arg in a subset of pituitary nonfunctioning tumors (20, 21) have not been confirmed in subsequent reports (22–26).

Finally, no mutations have been so far identified in other G proteins, in particular Gq and G11, that are involved in the generation of Ca^{2+}/calmodulin and phospholipid-dependent protein kinase C (PKC), a large ubiquitous eukaryotic kinase family that participates in growth factor- and hormone-mediated transmembrane signaling and cell proliferation (26, 27).

Despite the absence of mutations in the Gq and G11 genes, some reports suggest an overactivity of PKC pathway in pituitary adenomas. In fact, the PKC α isoform appears overexpressed in pituitary tumors with respect to normal pituitaries, particularly in invasive tumors (28). Moreover, point mutation replacing Gly294 with Asp, a strategic region of this enzyme containing the calcium-binding site, has been observed in 4 invasive pituitary tumors (28), an observation not confirmed by subsequent studies (29). When expressed in cell line, activated PCK mutant shows abnormal perinuclear and nuclear translocation together with transforming properties (30). More recently, the same mutant has been detected in thyroid neoplasia (31).

Growth Factors and Receptors. The normal pituitary and pituitary tumors produce a wide number of substances with secretory, differentiating, and proliferative potentials and express specific receptors. Transforming growth factor-α (TGF-α), epidermal growth factor (EGF), and their common tyrosin kinase receptor (EGF-R) are overexpressed in pituitary adenomas, particularly in those with high aggressiveness (32–35). Moreover, in gonadotroph adenomas activin/inhibin subunits appear highly expressed together with the specific type I and type II receptors while follistatin, which prevents activin action by binding this subunit, is reduced (36–38). Among the members of the fibroblast growth factor (FGF) and receptor family, particular attention has been paid to FGF-2 and FGF-4, both showing mitogenic and angiogenic activity (4, 39–42). More recently, it has been demonstrated that FGF expression is induced by the pituitary tumor transforming gene (PTTG), an estrogen-inducible gene with high transforming properties found in several human neoplasia, including pituitary adenomas (43, 44).

Among the 4 receptors mediating FGF signalling, the aberrant expression of an N-terminally truncated FGF receptor-4 that is constitutively phosphorylated and causes transformation *in vitro* and *in vivo* has been reported in about 40% of pituitary adenomas (45). Interestingly, in contrast to previous models of pituitary tumorigenesis, the expression of the truncated receptor in the pituitary of transgenic mice results in tumor formation in the absence of massive hyperplasia, a phenomenon similar to that observed in human pituitary adenomas (45, 46).

Proteins Involved in Cell Cycle Progression. Pituitary adenomas express the early immediate genes JUN, FOS, and MYC at variable levels and without clear correlation with cell proliferation index Ki-67, hormone profile, tumor size, and aggressiveness (47, 48). More recent studies are molecules that importantly regulate cell progression through G1 of the cell cycle have been carried out to evaluate the expression of cyclins in pituitary tumors. In a recent study carried out on 95 pituitaries (including normal pituitaries, secreting and nonsecreting pituitary adenomas, and pituitary carcinomas) cyclin D1 resulted overexpressed in aggressive and nonfunctioning pituitary tumors, while cyclin E was preferentially detected in corticotroph adenomas (49). The high levels of cyclin E might be related to the low levels of p27 protein reported in this tumor type. Moreover, using a frequent polymorphism in cyclin D1 (CCND1) gene, allelic imbalance indicative of gene amplification has been found in about 25% of pituitary tumors, despite the absence of a clear increase of cyclin D1 protein (50).

All pituitary adenoma subtypes, and particularly invasive hormone-secreting adenomas, express PTTG (51, 52). Structural characterization has identified PTGG as a member of the securin family that regulates the separation of sister chromatids during mitosis (44). Moreover, securin proteins participate in cellular responses to DNA damage, since it has been demonstrated that securin is a downstream target of the tumor suppressor gene p53 (53). Therefore, due to the critical role of PTTG in maintaining genomic stability, it is likely that PTTG overexpression may be, at least in part, responsible for the aneuploidism frequently observed in pituitary tumors (42, 54).

Overexpression of high-mobility group A nonhistone chromosomal proteins (HMGA) that play a role in determining chromatin structure is characteristic of rapidly dividing cells in embryonic tissues and in tumors. Overexpression of HMGA2 causes GH- and PRL-secreting adenomas in 80% of female transgenic mice by 6 months of age while the transgenic males develop the same phenotype with a lower penetrance and a longer latency period (55). Consistent with these observations, high levels of HMGA2 protein have been detected in pituitary adenomas, suggesting a possible role for these proteins in pituitary tumorigenesis (56).

Pituitary Specific Proto-oncogenes. Pituitary function is under the control of hypothalamic neurohormones that are required for pituitary cell commitment and growth as well as hormone synthesis and release. Therefore, they may be considered pituitary specific growth factors. Indeed, it is a common clinical observation that the excess of releasing hormones, such as GHRH or CRH, produced by neuroendocrine tumors, may result in the formation of GH- and ACTH-secreting adenomas, respectively (57–60). These clinical observations have been at least partially confirmed by the phenotype of transgenic mice, although in these models a prolonged hyperplasia generally precedes tumor formation.

Mutations of Genes Encoding Stimulatory Hormone Receptors. Studies carried out on large series of functioning and nonfunctioning adenomas indicate that genes encoding receptors for stimulatory hormones are normal in almost all tumors. In particular, TRH receptor gene has been found unaltered in a large series of secreting and nonsecreting adenomas (61) while prolactinomas generally express a truncated TRH receptor variant. Moreover, GnRH, vasopressin V3, and CRH receptors are normal in gonadotropin and ACTH-secreting adenomas, respectively (62–64). As far as the structure of the GHRH receptor gene is concerned, homozygous or heterozygous nucleotide substitutions have been found in about 20% of GH-secreting adenomas (65). However, none of these variant receptors were associated with high cAMP levels in basal conditions or in response to GHRH (65). Moreover, the Ala57 to Thr substitution in GHRH gene that is associated with increased responsiveness to GHRH seems to be a frequent polymorphism in the general population, probably accounting for the different responsiveness to GHRH observed in healthy subjects (66).

Taken together these data indicate that gain of function mutations of receptors mediating the action of hypothalamic-releasing hormones do not represent a mechanism of growth advantage operating in pituitary cells.

Overexpression of Stimulatory Hormone Receptors. In addition to mutations in the coding sequence, stimulatory signals may be amplified by increased receptor expression. Indeed, overexpression of TRH, V3, and CRH receptor genes has been detected in prolactinomas and ACTH-secreting adenomas, respectively (64, 67). These data are consistent with the so-called "paradoxical" responses to nonspecific releasing hormones that occur in a proportion of patients with pituitary adenomas and in almost all cultured tumors (68, 69). Although it is difficult to ascertain whether these receptors are ectopically or eutopically expressed in the tumors due to the cell heterogeneity of the normal pituitary, the wide spectrum of receptors highly expressed in pituitary tumors may represent an additional proliferative mechanism.

Alterations of Tumor Suppressor Gene Sequence and/or Expression in Pituitary Tumors

Pituitary cell proliferation may result from the inactivation of either common tumor suppressor genes or specific inhibitors of pituitary cell function and growth (Tables II and III). Contrary to oncogenes that cause the tumoral phenotype also when present in only 1 allele, tumor suppressor genes are recessive and the inactivation of both alleles is believed to be required to cause the loss of antitumoral action (70, 71).

Common Tumor Suppressor Genes. The role of loss of tumor suppressor genes in causing pituitary tumors has been clearly demonstrated in rodents. In fact, the knock out mice for retinoblastoma gene (RB) as well as the mice lacking p27Kip1, a cyclin-dependent kinase inhibitor that induces G1 arrest by RB hypophosphorylation, develop hyperplasia of pituitary intermediate lobe and ACTH-secreting adenoma by a few months of age (72–74). Several studies carried out on pituitary functioning and nonfunctioning adenomas indicate that loss of heterozygosity (LOH) on chromosome 13q, where RB gene is located, is a relatively frequent event (75, 76). In particular, deletion of 1 RB allele is observed in most highly invasive or malignant pituitary tumors and their metastases, although these tissues do not carry mutations in the retained allele (75–77). Considering possible defects occurring at the RNA or protein level, some immunohistochemical studies reported low Rb protein levels, probably due to RB gene promoter methylation, while other studies did not confirm these data (76, 77).

As far as the loss of p27Kip1 is considered, recent studies carried out on a large number of pituitary tumors failed to detect mutational changes and LOH in the region of this gene (78, 79). The low expression of p27Kip1 protein found in ACTH-secreting adenomas, recurrent pituitary tumors, and pituitary carcinomas by immunohistochemistry and not by mRNA analysis is indicative of protein degradation rather than reduced transcription (79, 80). A similar reduced expression, probably depending on methylation within the exon 1 CpG island, affects p16INK4a, another cyclin-dependent kinase inhibitor that prevents RB phosphorylation (81, 82).

No mutation in the tumor suppressor p53 gene has been ever found in human pituitary tumors (83, 84). Although mutant p53 has a longer half-life than the normal protein, the significance of p53 overexpression detected by immunohistochemistry in invasive nonfunctioning pituitary tumors and corticotropinomas remains elusive (85).

Since pituitary tumor is part of the multiple endocrine neoplasia type I (MEN-I) syndrome, the gene responsible for the disease has been thought to be implicated also in the genesis of sporadic pituitary adenomas (86). Although LOH in the region 11q13, the region where MEN1 gene is located, is present in 10% to 20% of sporadic pituitary adenomas (84, 87) subsequent studies failed to find either mutations in MEN 1 gene in the retained allele or reduced mRNA levels in most pituitary tumors (88–91). The LOH in several other loci in addition to 11q13 and 13q, such as 10q26, 11p, 22q13, suggest the involvement of other still unknown tumor suppressor genes in the genesis of human pituitary adenomas (2).

In addition to molecules that are directly involved in cell cycle regulation, molecules that participate in the transduction of extracellular signals may have inhibitory function. In particular, molecules that are involved in the negative control of the cAMP cascade may be considered as putative tumor suppressor genes in tissues where cAMP is mitogenic, such as the pituitary. Recently, particular attention has been paid to gene encoding the type 1 α regulatory subunit of protein kinase A (PRKAR1) that is crucial for intracellular protection against unrestrained catalytic subunit activity. In fact, inactivating mutations of this gene that render the catalytic subunit more susceptible to activation by cAMP have been identified in the Carney complex, an autosomal dominant multiple neoplasia syndrome featuring cardiac, endocrine, cutaneous, and neural tumors as well as a variety of pigmented lesions of skin and mucosa (92, 93). Subsequent sequence analyses of this gene indicate the absence of mutations in the PRKAR1 gene and normal levels of PRKA1 transcripts in a series of functioning and nonfunctioning pituitary adenomas, suggesting a minor role, if any, of this tumor suppressor gene in pituitary tumorigenesis (94, 95).

Pituitary Specific Tumor Suppressor Genes. Genes coding for membrane and nuclear receptors that physiologically inhibit pituitary hormone secretion may be considered as possible targets for inactivating mutations leading to hormone hypersecretion and eventually tumor growth. The best candidates among the G protein-coupled receptors are the dopaminergic D2 receptor (D2R) and the somatostatin receptor (sst) type 1-3 and 5. D2R-deficient mice develop massive lactotroph hyperplasia and subsequently lactotroph adenomas (96, 97). Although this model indicates that loss of dopamine inhibition induces neoplastic transformation, its relevance to the human situation is questionable. Indeed, several pieces of evidence suggest that the central dopaminergic tone is not reduced but even increased, probably in response to hyperprolactinemia, in patients with

PRL-secreting adenomas. Accordingly, no mutation in the D2R gene has been so far reported, although it is worth noting that the number of screened tumors is low since patients with this tumor type rarely undergo pituitary surgery (98). Conversely, resistant prolactinomas frequently show a reduction of D2R mRNA, and particularly of the shortest isoform that is more efficiently coupled to phospholipase C (99). Interestingly, the expression of D2R in resistant PRL-secreting adenomas may be induced by the *in vitro* treatment with nerve growth factor (100, 101).

In addition to the defect in D2R splicing and expression, the absence of D2R protein despite the retention of D2 transcript has been observed in metastases of a malignant prolactinoma resistant to different dopamine agonists (102), suggesting that alterations in protein stability or degradation may contribute to the failure of medical therapy and eventually to lactotroph growth.

Several studies suggest that the different responsiveness to somatostatin analog observed in acromegalic patients is probably related to a low expression of sst2 while the role of sst5 is still controversial (103–106). As far as mutational changes of the ssts genes are concerned, these events seem to occur exceptionally. In fact, only 1 mutation in the sst5 gene has been reported so far. In particular, a point mutation replacing Arg240 with Trp in the third intracellular loop of sst5 gene has been identified in one octreotide-resistant acromegalic patient (107). This region represents the cytoplasmic extension of the α-elix 6 and is a consensus sequence identifying a potential site for coupling to G proteins. The expression of the mutant receptor in CHOK1 cells causes a high rate of cell proliferation as well as the abrogation of the antiproliferative action of somatostatin (107).

Another regulatory mechanism frequently lost in pituitary tumors is the negative feedback. It is temping to speculate that the poor sensitivity of ACTH- and TSH-secreting adenomas to dexamethazone and T3, respectively, may be due to defective action of glucocorticoid or thyroid hormone receptors in the tumors. However, no mutations in the glucocorticoid receptor (GR) have been detected in ACTH-secreting adenomas, with the only exception of 1 macroadenoma from a patient who developed Nelson syndrome after bilateral adrenalectomy for Cushing disease (108). Similarly, there is no evidence for a substantial modification in the relative expression of the ligand binding GRα isoform and the dominant negative GRβ splice variant in pituitary tumors (109, 110). Finally, LOH at the GR gene locus, possibly resulting in low mRNA levels, has been found in about a third of ACTH-secreting adenomas (111).

The other pituitary adenoma that is typically resistant to the negative feedback of peripheral hormones is the TSH-secreting adenoma. In one of these rare tumors a somatic mutation in the ligand binding domain of the thyroid hormone receptor β isoform (TRβ) has been identified (112). Moreover, a 135-bd deletion due to alternative splicing of TRβ2 mRNA and causing lack of T3 binding domain has been reported in another TSH-secreting adenoma, supporting the view that posttrascriptional mechanism may generate abnormal receptors in tumors in which no mutations are detected in genomic DNA (113).

gsp Oncogene and Its Impact on Tumor Phenotype

The extensive search for mutational changes conferring growth advantage to pituitary cells failed to identify the initial pathogentic event in most tumors. Accordingly, the only mutational changes so far unequivocally found are point mutations in the GNAS1 gene that constitutively activates adenylyl cyclase by inhibiting the GTPase activity of the mutant Gsα (*gsp* oncogene) in about 30% to 40% of GH-secreting pituitary adenomas. The activation of the cAMP pathway induces a series of downstream processes that importantly cooperate in determining the tumoral phenotype. In particular, the discrepancy between the mitogenic action of the mutant Gsα observed in transfected cell lines and the poor growth rate of GH-secreting adenomas naturally expressing the same mutation strongly suggest the presence of events *in vivo* counteracting the putative growth advantage conferred by the mutant Gsα.

Functional Studies in Cell Lines Expressing Mutant Gsα. The expression of mutant Gsα causes the transcription of a variety of common early immediate genes in several endocrine and nonendocrine cell lines while in cells from the pituitary lineages such as GH3, this approach results in the induction of prolactin and GH gene transcription, probably via the specific transcription factor Pit-1 (114–116). Moreover, the expression of mutant Gsα confers growth advantage to cells of different origin. Pituitary GH3 cells possessing Gln227Leu substitution have a higher proliferation rate, associated with higher cAMP levels (116). Similarly, the expression of mutant Gsα in FRTL-5 thyroid cells, a cell line that depends on TSH and cAMP for growth (117), is sufficient to bypass the requirement for TSH, particularly in the presence of phosphodiesterase inhibitors. The mitogenic effect of mutant Gsα is not restricted to endocrine cells since Swiss 3T3 fibroblasts carrying the mutant subunit show a high growth rate that is particularly evident after cAMP hydrolysis blockade (118). These data indicate that the expression of constitutively active Gsα promotes transcription of common and specific genes as well as autonomous proliferation of endocrine and not endocrine cell types, this effect being amplified by pharmacologically preventing cAMP hydrolysis.

***In vivo* Characteristics of Patients with Tumors Expressing *gsp* Oncogene.** Screening studies carried out on large series of GH-secreting adenomas indicate the absence of clinical differences between patients bearing tumors with and without *gsp* oncogene (119–122) (Table IV). In particular, no difference in tumor growth or recurrence rate has been reported, in agreement with the poor evidence of cell replication detected by morphological analysis (119). Despite the lack of significant differences in circulating GH

levels in basal conditions and in response to stimulatory agents, patients with *gsp* positive tumors develop full manifestations of the disease in the presence of very small adenomas, suggesting a secretory hyperactivity. This view is consistent with the ultrastructure morphology of the tumors, which are generally constituted of densely granulated cells with a well-developed secretory apparatus (119). Due to the lack of growth advantage observed in tumors with mutant Gsα, the existence of possible counteracting mechanisms has been recently reconsidered in our laboratory and here briefly reviewed.

***In Vivo* and *In Vitro* Sensitivity to Somatostatin of Tumors Expressing *gsp* Oncogene.** Tumors expressing the *gsp* oncogene are highly sensitive to somatostatin action. The strong inhibition of GH secretion induced by the peptide is detectable both *in vivo* and *in vitro* (119). In fact, it has been demonstrated that acute administration as well as chronic treatment with the somatostatin analogue octreotide causes a reduction of serum GH levels that is particularly relevant in patients with *gsp* positive tumors (121–123). These results are consistent with *in vitro* studies showing increased sensitivity to somatostatin analogues in cells obtained from tumors with *gsp* oncogene and the occurrence of resistance to somatostatin only among the negative adenomas (120, 122). Taking into account the presence of a cAMP responsive element (CRE) in sst2 promoter, it is tempting to speculate that the constitutive activation of adenylyl cyclase induced by mutant Gsα might result in increased somatostatin receptor expression and therefore increased sensibility to the peptide (124). However, until now there is no evidence for a relationship between *gsp* mutations and sst2 and sst5 mRNA induction (104, 106).

Effect of *gsp* Oncogene on Phosphodiesterase Activity and Expression. Phosphodiesterases (PDE) represent a superfamily of enzymes involved in the hydrolysis of cyclic nucleotides (i.e., cAMP and cGMP) acting, along with phosphatases, as negative regulators in the cyclic nucleotide signaling cascades. Until now, 11 families of PDEs, which encompasses distinct genes encoding multiple proteins by alternative splicing and/or the use of multiple promoters, have been characterized. Increases in cAMP levels upregulate the activity of some PDE isoforms, particularly the cAMP-specific, rolipram-sensitive, cAMP-PDE (PDE4) by activating PKA-dependent phosphorylation processes and/or by inducing PDE gene transcription (125). Accordingly, the expression of constitutively active Gsα in FRTL-5 thyroid cells has been reported to increase PDE4 activity and expression (126). A similar phenotype is also present in pituitary tumors with *gsp* oncogene. Indeed, *gsp* positive adenomas showed up to 7-fold increase of PDE activity, particularly PDE4, that was largely due to increased transcription of the PDE4C and D genes (127, 128) (Fig. 2). The PDE induction has a relevant impact on tumor phenotype. In fact, although *gsp* oncogene constitutively activates adenylyl cyclase by inhibiting the intrinsic GTPase activity, differences in intracellular cAMP levels between

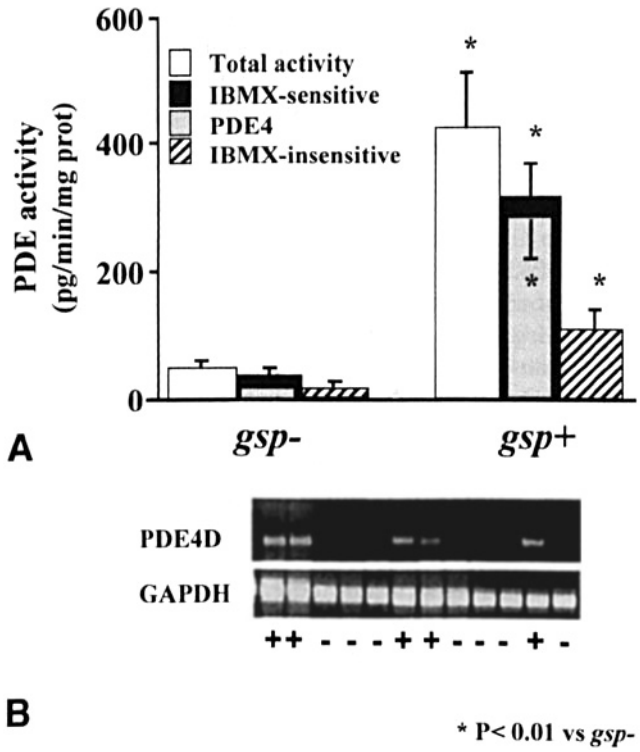

Figure 2. (A) Pharmacological characterization of cAMP-hydrolyzing activity in tissue homogenates evaluated by means of nonselective (IBMX, 1 mM) or selective inhibitors (rolipram, 10 μM, for cAMP-specific PDE4) demonstrated a dramatic increase in PDE activity in *gsp*+ tumors that was mainly due to PDE4. * $P < 0.05$ vs. the *gsp* tumors and normal pituitary. (B) Agarose gel stained with ethidium bromide of RT-PCR products obtained using oligonucleotides specific for the amplification of PDE4D and GAPDH mRNAs in 12 adenomas. All *gsp* positive tumors have an increased expression of PDE4D if compared with *gsp* negative adenomas. +, *gsp*+ GH-omas; –, *gsp*– GH-omas.

tumors with and without *gsp* mutations are detectable only when PDE activity is blockade by nonselective and PDE4 selective inhibitors. Moreover, while in basal conditions *gsp* positive and negative tumors showed similar levels of PKA-dependent phosphorylation of the cAMP-responsive element binding protein (CREB) (128, 129), the blockade of endogenous PDEs in *gsp* positive tumors is sufficient to dramatically increase CREB phosphorylation (128).

Effects of *gsp* Oncogene on cAMP-Responsive Element Binding Protein (CREB) and Inducible cAMP Early Repressor (ICER) Expression. The transcription factors CREB and ICER are final targets of cAMP-dependent pathway and are positively regulated by cAMP signaling. Phosphorylated CREB and CREM (cAMP Responsive Element Modulator) bind as dimers to palindromic cAMP response element (CRE) sequences, thus modulating the expression of cAMP-dependent genes. The CREB and CREM genes encode different isoforms that act as either activators or repressors of gene transcription. The increase in cAMP levels induces the expression of CREB and ICER since both the promoter of CREB and the internal promoter of CREM that regulates ICER expression contain CREs. By investigating the expression of CREB and ICER

mRNA in GH-secreting adenomas it appears that the levels of both transcripts are higher in *gsp* positive tumors in comparison with negative tissues (130). Therefore, the expression of mutant Gsα results in the transcription of factors with opposite action, since ICER competes with the binding of CREB to CRE, thus efficiently counteracting the stimulatory effects of CREB on gene transcription. Moreover, the observation that relatively high levels of CREB or ICER are present in few tumors not harboring the *gsp* oncogene suggest that beside Gsα mutants, different and partially unknown molecules may be active in *gsp* negative tumors (130).

Effect of *gsp* Oncogene on Gsα Expression Tumors with and without *gsp* Mutations are Markedly Different in Their Content of Gsα Protein. In fact, while *gsp* negative tumors show high levels of the two Gsα isoforms, in *gsp* positive tumors the protein is barely detectable (131) (Fig. 3). This difference concerns only Gsα protein, since both types of tumors express similar amounts of Gqα, Gi1-3α, and G0α. The low levels of mutant Gsα are not due to reduced mRNA synthesis or stability but rather to increased protein degradation (Fig. 3). Accordingly, cholera toxin, a toxin known to mimic *gsp* mutations by covalently modifying Arg 201 and to constitutively activate adenylyl cyclase, is able to dramatically reduce Gsα protein levels in *gsp* negative tumors (131) (Fig. 3). Taking into account that both cholera toxin and *gsp* mutations activate adenylyl cyclase by inhibiting GTPase and thereby preventing the formation of the inactive complex, it is conceivable that Gsα protein becomes highly susceptible to degradation, when it is in the activated free state.

While the instability of mutant Gsα is likely responsible for the low levels of Gsα in *gsp* positive tumors, the high levels of Gsα in *gsp* negative tumors has been recently attributed to the relaxation of GNAS1 gene imprinting occurring in these adenomas (132).

Effect of *gsp* Oncogene on ERK1/2 Activity. The mitogen-activated protein kinases (MAPK) are serine/threonine-specific protein kinases involved in the control of cell differentiation and proliferation that include the extracellular signal-regulated kinases ERK1 and ERK2, the cJun NH2-terminal kinases, and the p38-MAP kinases (133). Although in the past MAPK pathway was thought to represent the intracellular cascade specifically activated by growth factors, it is now well established that several G protein coupled receptors are able to stimulate ERK1/2 by different mechanisms (134). It has been recently demonstrated that in pituitary tumors ERK1/2 activation may be induced by peptides that classically operate through the cAMP-dependent pathway such as GHRH, this effect being mainly due to PKC activation (135). Consistent with the marginal involvement of the cAMP pathway on this pathway, no differences in basal ERK1/2 activity and in ERK1/2 responsiveness to GHRH were observed in GH-omas with or without the *gsp* mutation (Fig. 4) (135).

GNAS1 Imprinting in GH-Secreting Adenomas. Recent studies have investigated the role of Gsα gene imprinting in the pathogenesis of pituitary adenomas (132, 136). Genomic imprinting is an epigenetic phenomenon affecting a small number of genes by which 1 allele undergoes a partial or total loss of expression, either during the embryogenesis or in the postnatal life (137). GNAS1 maps on human chromosome 20q13 and on mouse distal chromosome 2. This locus is under complex imprinting control, with multiple maternally, paternally, and biallelically alternatively spliced transcripts encoding multiple products, (i.e., the Gsα, the extra large αs-like protein (XLαs), the neuroendocrine secretory protein NESP55, a nontranslated transcript deriving from Exon 1A, and a poly-adenylated antisense transcript) (138–146) (Fig. 5). Heterozygous loss of function mutations of GNAS1 lead to Albright's Hereditary Osteodistrophy (AHO). Interestingly, when the mutations are inherited from the mother, AHO is associated with end organ resistance to the action of hormones that activate

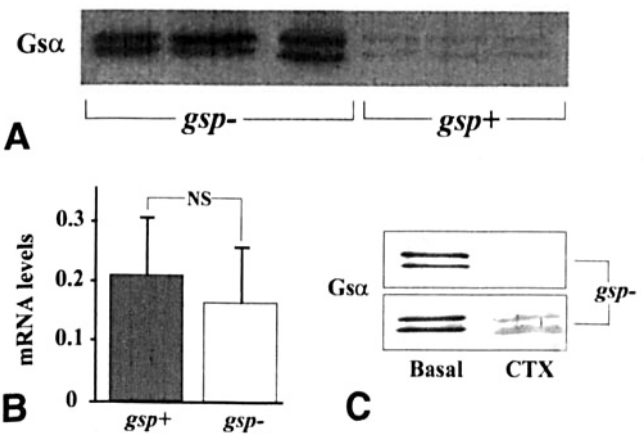

Figure 3. (A) Representative immunoblotting performed with antiserum to Gsα, showing different amounts of protein in *gsp*+ and *gsp*– tumors. While Gsα is expressed at high levels in all *gsp* negative adenomas, it is undetectable or barely detectable in blots obtained from tumors with *gsp* oncogene. (B) Semiquantitative RT-PCR analysis shows that Gsα mRNA levels are similar in wild-type and mutant tissues levels. (C) Treatment of cells obtained from three *gsp* negative tumors with cholera toxin (1 μg/mL) for 8 h causes a marked reduction in Gsα levels. CTX, cholera toxin.

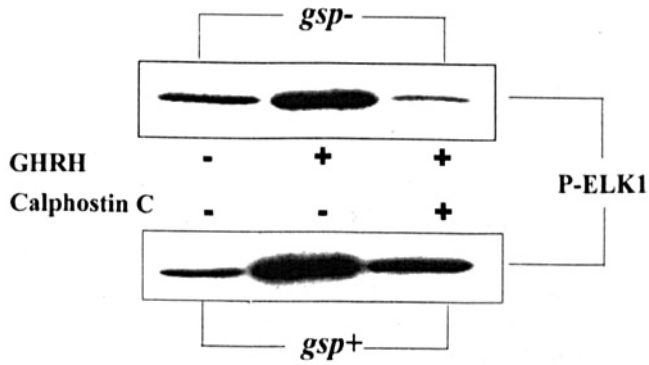

Figure 4. Representative immunoblotting showing the increase of ERK1/2 activity after GHRH incubation in human adenomatous somatotrophs with or without *gsp* mutation. PKC blockade by calphostin C induced the almost complete abolishment of GHRH-induced ERK1/2 activation both in *gsp*– and *gsp*+ tumors.

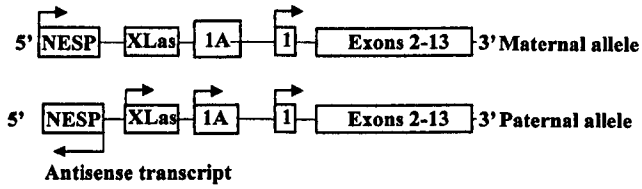

Figure 5. Genomic organization of human GNAS1 locus. The figure shows four alternative first exons that splice into exon 2, generating four different transcripts: Gsα (exon 1), NESP55, XLαs, and an untranslated gene product from exon 1A. Both the maternal and the paternal allele are represented: arrows designate transcription start sites. While NESP55, Xlαs, and exon 1A are monoallelically expressed either from the maternal or the paternal allele, the Gsα gene is biallelically expressed in most human tissues and prevalently maternally expressed in specific organs such as the pituitary, the thyroid, and the gonad.

receptors coupled to Gs, primarily PTH, TSH, and gonadotropins (pseudohypoparathyroidism, PHP). When the same mutations are inherited from the father, patients show the physical abnormalities of AHO, without hormone resistance (pseudo-pseudohypoparathyroidism, PPHP) (147, 148). This pattern of inheritance is consistent with tissue-specific imprinting of GNAS1 paternal allele. Indeed, in contrast with previous expression studies on various human fetal tissues (149, 150), 3 different reports recently demonstrated that in specific endocrine tissues GNAS1 transcription mainly derives from the maternal allele (132, 136, 151). In particular, a predominant, though not exclusive, maternal origin of Gsα was observed in adult human thyroid (136, 151), gonad (136), and pituitary (132, 136). These data strongly support the hypothesis that imprinting of GNAS1 is the potential mechanism responsible for occurrence of variable resistance to hormone action in patients with GNAS1 mutations.

According with the paternal imprinting of GNAS1 gene, almost all *gsp* positive tumors show mutations on the maternal allele (132). Moreover, it has been reported that independently from Gsα mutations GH-secreting adenomas have a partial loss of GNAS1 imprinting, raising the possibility that this phenomenon may be responsible for Gsα overexpression observed in *gsp* negative tumors (132). However, data from our lab showing a striking predominance of the maternal allele over the paternal one in *gsp* positive as well as negative tumors, do not support this hypothesis (136) (Fig. 6).

Conclusion

In the past years several candidate factors have been implicated in the genesis and progression of pituitary adenomas. To date, GNAS1 is the only gene that has been identified as target for activating mutations that unequivocally cause cell proliferation in about 30% to 40% of GH-secreting adenomas. Conversely, screening studies carried out on tumors negative for this mutation failed to identify disease genes. However, abnormalities in the expression of membrane and nuclear receptors, growth factors, transcription factors, and their signalling proteins have been proposed to play a relevant role in cell transformation

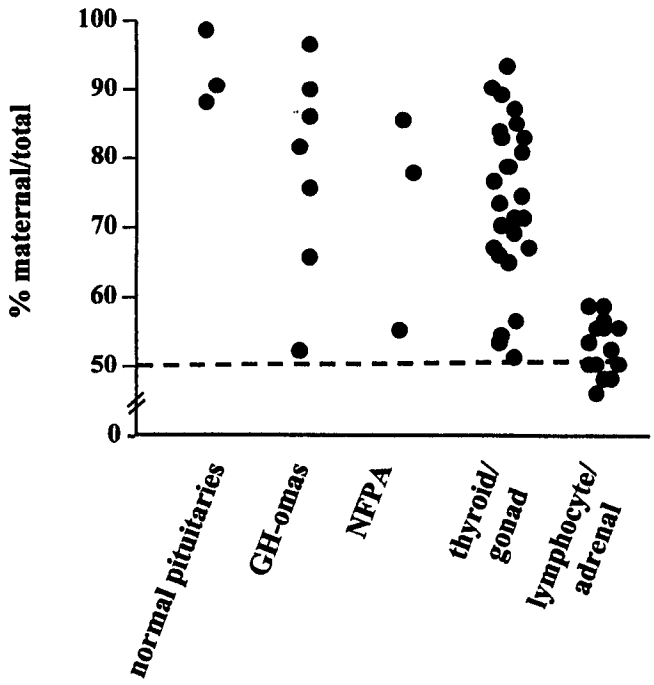

Figure 6. The diagram shows the estimated percentage of maternally derived transcripts in 12 pituitary glands (3 normal ones, 7 somatotroph adenomas, and 3 non-functioning adenomas), 14 thyroid glands, 10 gonads (granulosa cells), 11 lymphocyte samples, and 3 adrenal glands.

and/or clonal expansion. Similarly, the low expression of tumor suppressor genes has been implicated in pituitary tumorigenesis.

Several questions arise when studying the genotype/phenotype relationships of GNAS1 mutations in GH-secreting adenomas. These activating mutations 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, in contrast with the results obtained in cell lines expressing the mutant Gsα, patients bearing tumors with *gsp* oncogene do not show differences in tumor growth and recurrence rate, suggesting the existence of mechanisms able to counteract the activation of the cAMP pathway, that have been only partially identified. 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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