Immunodetection of G proteins in human pituitary adenomas:
evidence for a low expression of proteins of the Gi subfamily

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Abstract

G proteins mediate signal transduction in a variety of cell systems. As the expression of these proteins has not yet been investigated in detail in human pituitary tumors, we evaluated the presence of G proteins in a series of tumors including six non-functioning adenomas, five GH-secreting adenomas, three prolactinomas and one TSH-secreting adenoma, using immunoblotting and immunohistochemistry. By immunoblotting, Gi\textsubscript{1/2}\textgreek{a} was undetectable in six and barely detectable in nine tumors. A similar pattern of expression was observed by probing with the antibody to Gi\textsubscript{3}\textgreek{a}, which detected a very weak band in 11 tumors and no protein in four. In contrast, using large amounts of membrane proteins (40 \textmu g), both Gi\textsubscript{1/2}\textgreek{a} and Gi\textsubscript{3}\textgreek{a} were detected, although at very low levels, in the negative tumors. The low expression of these proteins appeared to be specific to tumoral tissues, as both Gi\textsubscript{1/2}\textgreek{a} and Gi\textsubscript{3}\textgreek{a} were abundant in normal human and rat pituitary. In all tumors, Go\textgreek{a}, the two Gs\textgreek{a} forms, Gq/11 and G\textsubscript{b} were present in significant amounts. Semiquantitative analysis indicated that Gs\textgreek{a} was clearly detected when 2.5 \textmu g loaded proteins were used, whereas Gi\textsubscript{1/2}\textgreek{a} and Gi\textsubscript{3}\textgreek{a} were barely detected with 5 \textmu g. By immunofluorescence, all tumors studied were markedly positive for Gs\textgreek{a} that was immunolocalized at the cell periphery, whereas they showed a weak positivity for Gi\textsubscript{1/2}\textgreek{a} and Gi\textsubscript{3}\textgreek{a}. The study is the first to provide evidence for a low expression of Gi proteins, which are involved in the transduction of inhibitory signals, in pituitary adenomas.

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Introduction

G proteins belong to the large superfamily of GTP-binding proteins that function as transducers of information across the cell membrane by coupling diverse receptors to intracellular effectors (1–6). By definition, all G proteins are heterotrimers consisting of \textalpha-, \textbeta- and \textgamma-subunits, each the product of distinct genes. Although there is evidence that the \textbeta\textgamma complex could modulate certain intracellular effectors, the \textalpha-subunit has been shown to have the critical role in the regulation of several effectors by their corresponding G proteins (7, 8). To date, about 20 mammalian \textalpha-subunit genes have been cloned and divided into four major subfamilies on the basis of the degree of amino acid identity: Gs, Gi, Gq and G12 (4, 9). Gs and Gq mediate stimulatory processes, such as hormone secretion, by activating adenyl cyclase and phospholipase C, respectively, and members of the Gi subfamily generally transduce inhibitory signals by reducing adenyl cyclase activity and intracellular Ca\textsuperscript{2+} levels via K\textsuperscript{+} channel activation and Ca\textsuperscript{2+} channel blockade. The physiological role of the G12 subfamily is still under investigation (10, 11).

Pituitary cells are sensitive to hypothalamic neurohormones that exert either stimulatory or inhibitory effects on hormone secretion and cell proliferation. All receptors for hypothalamic hormones belong to the superfamily of G-protein-coupled receptors, sharing the common structural and functional motif characterized by seven transmembrane domains (12, 13). It is therefore evident that the quality or quantity of G proteins available for receptor activation may be relevant in the transduction of stimulatory and inhibitory signals in the pituitary. As far as G proteins expressed in pituitary tumors are concerned, mutations of the Gs\textalpha-subunit gene leading to the constitutive activation of adenyl cyclase have been reported in about 35% and 10% of growth hormone (GH)-secreting adenomas and non-functioning pituitary adenomas (NFPAs) respectively (14–16). In contrast, to date the distribution and levels of expression of the individual G proteins have been poorly investigated in human pituitary adenomas, the data available deriving largely from animal models (17–19).

The aim of the present study was to identify both stimulatory and inhibitory G proteins in a series of
functioning and non-functioning human pituitary adenomas, using immunoblotting and immunohistochemistry. The study demonstrates the differential expression of the individual G proteins in pituitary adenomas and provides evidence for a low expression of members of the Gi subfamily in these tumors.

Materials and methods

Patients and tumors

The study was carried out using 15 pituitary tumors removed from patients requiring adenomectomy (Table 1). The series includes six non-functioning pituitary adenomas, five GH-secreting adenomas, three prolactinomas and one thyroid hormone-stimulating hormone (TSH)-secreting adenoma. The diagnosis of secreting and non-secreting tumors was made on the basis of clinical features and standard hormonal criteria and confirmed by immunofluorescence studies on tumor specimens. Patients with prolactinomas were operated on because of resistance to dopaminergic drugs. In these patients, bromocriptine in concentrations up to 20 mg/day induced neither a >50% reduction in serum prolactin (PRL) concentrations, nor tumor shrinkage. Bromocriptine was withdrawn at least 2 months before surgery. In four patients with acromegaly, octreotide (100 μg) was given s.c. and a decrease in serum GH to more than 50% below basal values was considered a positive response (Table 1). Patients with diabetes mellitus were not included in the study. Patients did not receive and were not addicted to drugs known to influence G protein expression, in particular ethanol, opiates and lithium (20–22). No patient had previously undergone pituitary irradiation. The adenomas were surgically removed by the transphenoidal route and tissue fragments were either quickly frozen in liquid nitrogen and stored at −80°C until required for immunoblotting, or fixed for immunohistochemistry. Local ethics committee approval was obtained for all studies.

Immunoblotting of G protein α-subunits

Immunoblotting analysis was performed on pituitary tissues as previously described (23). Briefly, tissues were homogenized in a glass Teflon Potter homogenizer in 10 mmol Hepes pH 7.3, 150 mmol NaCl, 1 mmol phenyl methyl sulfonyl fluoride (PMSF), 4 μg/ml pepsatin and 4 μg/ml aprotinin. Cell debris was removed by centrifugation at 3000 g for 15 min at 4°C. Supernatants were centrifuged at 40 000 g for 30 min at 4°C and crude membranes were resuspended in solubilization buffer. Protein concentrations were determined using the bicinchoninic (BCA) protein assay. G protein α-subunits were determined on 20 μg protein fractions, separated by 10% SDS–PAGE and transferred electrophoretically to nitrocellulose membranes, using molecular weight standards as reference. Proteins on membranes were detected by staining with Ponceau S.
The membranes were incubated overnight with blocking buffer (8% non-fat dry milk in PBS) at 4 °C. Subsequently, blots were probed with polyclonal rabbit antibodies to the G protein α-subunits at 1:250 dilution for 3 h at room temperature. The commercial antibodies used were raised against highly conserved sequences from the C termini of Gi1/2α (antibody AS/7), Gi3α (antibody EC/2), Gsα (antibody RM/1), Gq/11α (antibody QL), Gβ (antibody MS/1) and from the N-terminal sequence of Goα (antibody GC/2) and are cross-reactive with different species (mouse, rat, dog and human). For Gi1/2α, antibody against amino acids 93–112 reactive with Gi1/2α and Gi3α was also used in two tumors (Nos 1 and 15). After incubation, membranes were extensively washed, incubated with 125I-labeled protein A (4–5 × 10^5 c.p.m./ml) for 45 min at room temperature and finally subjected to autoradiography with X-Omat radiographic film. The regions of the blot corresponding to the bands and equivalent sized areas of nitrocellulose that did not contain immunoreactive proteins were excised and the amount of radioactivity was quantitated by γ counter (Packard A5550). To evaluate the specificity of the reaction, membranes were incubated with preimmune serum and negative controls for each antiserum were obtained by immunosorption with control peptides. Positive controls for Gi1/2α and Gi3α were obtained by loading control peptides (1–5 μg) and probing with corresponding antisera (1/250). In each experiment, normal human liver tissue obtained at surgery from an extratumoral area were used as positive control. Normal pituitary tissues surrounding microadenomas (two microprolactinomas) were pooled and used as normal control for antibodies to Gi1/2α and Gi3α. Anterior pituitaries obtained from adult male and female Sprague–Dawley rats were used as controls for normal pituitary of different species.

**Immunohistochemistry**

Immunohistochemistry was carried out using an indirect immunofluorescence technique according to Rosa et al. (24). After fixation, tissues were passed through a series of increasing concentrations of sucrose solution, mounted on specimen holders, frozen in isopentane and finally stored in liquid nitrogen. Frozen sections (0.5 μm thickness) obtained by an Ultra-cut E microtome (Reid, Vienna, Austria) equipped with an FC4 cryochamber were mounted on gelatine-coated slides, washed in PBS 0.01 mol/l for 30 min at room temperature, incubated in avidin-blocking solution for 15 min, washed in PBS and finally incubated in biotin-blocking solution to reduce any aspecific labeling attributable to avidin and biotin reagents. Subsequently, the sections were incubated with antibodies to either Gi1/2α or Gsα (1:250) at 37 °C for 1 h, then with an anti-rabbit biotinylated donkey serum at 20 °C for 1 h and finally with streptavidin–sulfurodamine at 20 °C for 1 h in the dark. Subsequently, the slices were washed with 0.01 mol/l PBS pH 7.2, mounted in 95% glycerol–5% PBS and examined under a Zeiss Photomicroscope III equipped with epifluorescence optics and Planapo objective (Carl Zeiss, Oberkochen, Germany). To
evaluate the specificity of the reaction, sections were incubated with antibodies at working dilution and control peptide at different concentrations: anti-Gsα (1:250) anti-Gi1/2 (1:250) and anti-Gi3α (1:250) were incubated with control peptide corresponding to amino acids 377–394 of Gsα (0.8–0.008 μg/ml). In these experimental conditions, staining with anti-Gsα was completely blocked, whereas that with anti-Gi1/2 and anti-Gi3α was unchanged.

Materials

Pepstatin, aprotinin and PMSF were obtained from Sigma (St Louis, MO, USA). The BCA protein assay was purchased from Pierce (Rockford, IL, USA). Nitrocellulose membrane and molecular weight standards were obtained from Bio-Rad (Hercules, CA, USA). Polyclonal rabbit anti-G protein α-subunit antibodies and 125I-labeled protein A were purchased from NEN-Dupont (Boston, MA, USA). Two additional anti-Gi1/2α sera were obtained from Calbiochem Novabiochem (San Diego, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Control Gsα was from Santa Cruz Biotechnology. X-Omat radiographic film was obtained from Eastman Kodak (Rochester, NY, USA). Anti-rabbit immunoglobulin, biotinylated species-specific whole antibody and streptavidin Texas red were from Amersham International (Aylesbury, UK).

Results

Immunoblotting of G protein α-subunits

Figure 1 summarizes the data of immunoblots obtained with antisera to different G proteins in membranes from secreting and non-secreting adenomas. In the different adenomas, the amounts of G protein subunits were very variable. In particular, the antibody AS/7 that reacts with both αi1 and αi2 recognized one band of approximately 42 kDa in membrane fractions from four tumors (two NFP A and two GH-secreting adenomas), whereas the protein was barely detectable in five and undetectable in six (three prolactinomas, two NFP A and one TSH-secreting adenoma) (Figs 1 and 2). In order to confirm the minimal presence of αi1 and αi2, experiments with an antibody against amino acids 93–112 were performed in two tumors (Nos 1 and 15) and gave the same results as were obtained with the antibody against the C-terminal sequence (data not shown). On probing with the antibody EC/2 to Gi3α, three tumors showed a detectable band of 42 kDa that was very weak in another eight and absent in pituitary adenoma.
four (three prolactinomas and one NFPA) (Figs 1 and 2). With the exception of tumors Nos 5 and 10, all tumors were tested with antisera to Gi1/2α and Gi3α at least twice, giving similar results. The possibility that, in these tumors, Gi1/2α and Gi3α could be localized in the cytosol rather than in the membrane fractions was excluded by the inability of the antisera to detect G proteins in the supernatant resulting from the second centrifugation and representing cytosolic fractions (data not shown). In contrast, using large amounts of membrane proteins (40 μg) obtained from tumors (Nos 6 and 15) negative in standard experimental conditions (20 μg protein), both Gi1/2α and Gi3α were detected, although in very low concentrations (Fig. 3 and data not shown). The low expression of these proteins appeared to be specific of tumoral pituitary tissues, as antisera to Gi1/2α and Gi3α detected high amounts of proteins in the normal human pituitary (Fig. 4). Similarly, in male and female rat pituitary, representing normal tissues enriched in somatotrophs and lactotrophs respectively, both Gi1/2α and G3α were clearly detected (Fig. 5).

When membrane proteins were probed with antibody GC/2 specific for Goα, a 39 kDa protein was detected in all tumors in significant amounts (Figs 1 and 2). Although Goα was expressed in variable concentrations in the membrane preparations obtained from this series of tumors, no major differences between the different types of functioning and non-functioning adenomas were observed (Figs 1 and 2).

In all pituitary adenomas, antibody RM/1 recognized the two distinct isoforms of αs, that is, the 47 kDa corresponding to the more slowly migrating long form (Gsα-L) and the 46 kDa corresponding to the more rapidly migrating short form (Gsα-S), arising from alternative splicing of αs mRNA transcripts (Figs 1 and 6). Both Gsα isoforms were generally expressed in high concentrations (Figs 1 and 6); in four NFPA, one GH-secreting adenoma and one prolactinoma, Gsα-L was more abundant than Gsα-S, and in the remaining tumors the two forms were present in similar amounts (Fig. 6 and data not shown). No significant differences were found in the amounts of Gsα present in functioning and non-functioning adenomas. Comparison between the quantity of Gsα and that of Gi1/2α was made taking advantage of the linear relationship between the amount of protein loaded and the intensity of the signal from the immunoblots (Fig. 7). Using the same tissue preparations obtained from tumors positive for Gi1/2α and Gi3α in standard experimental conditions (20 μg of loaded proteins), Gsα was clearly detected when amounts of proteins as low as 2.5 μg were loaded, whereas Gi1/2α and Gi3α were barely detected with 5 μg (Fig. 7 and data not shown). Probing with antisera specific for the common C-terminal sequence of αq and α11 detected one single band of approximately 44 kDa, the gel system we used not allowing the accurate identification of the two proteins. In membrane fractions from pituitary adenomas, Gq/11α was expressed in high concentrations, although in the majority of tumors it was less abundant than Gsα (Figs 1 and 6). Finally, using antisera specific for the β-subunit common to G proteins, almost all tumors showed large amounts of this protein (Figs 1 and 6).

**Immunodetection of G protein α-subunits**

The distribution of Gi1/2α, Gi3α and Gsα was investigated in semithin cryosections of five pituitary adenomas (Nos 4–7 and 9), using immunofluorescence. The intensity of fluorescence obtained differed greatly between the different antisera, reflecting the amount of the different G proteins present in the cell (Fig. 8 and data not shown). In all tumors, almost all cells were heavily stained with the antibody to Gsα and the fluorescence was only found in the cell periphery, consistent with labeling of the plasma membrane. The immunopositivity for Gsα was clearly stronger than...
that for Gi1/2a and Gi3α; indeed, the immunofluorescence data obtained with antisera specific for Gi1/2a indicated a weak positivity for the protein in tumors Nos 7 and 9, and no labeling in tumors Nos 4–6 (Fig. 7 and data not shown). Similar immunofluorescence data were obtained using antibody to Gi3α (Fig. 7).

Discussion

This study is the first to show that G proteins are differentially expressed in human pituitary adenomas. The pattern of G protein distribution was characterized by very low levels of proteins belonging to Gi subfamily. Indeed, in experimental conditions appropriate to unequivocal identification of the other G proteins, if present, Gi1/2α and Gi3α were undetectable or barely detectable in about 67% and 50% of tumors respectively. The absence of Gi1/2α and Gi3α observed in a consistent number of tumors, and confirmed by the use of different antibodies, probably reflected a very low expression of these proteins, probably below the limit of detection of the immunoblotting system used in these negative tumors. Gi1/2α and Gi3α were subsequently revealed by doubling the amounts of loaded proteins. The low levels of both Gi1/2α and Gi3α were confirmed by the immunofluorescence analysis, which showed a very low immunopositivity for these proteins. Several processes, including either reduced gene expression or instability of the proteins or their corresponding mRNAs, may be responsible for the low expression of Gi1/2α and Gi3α – an aspect deserving further investigation. Whatever the mechanism responsible for the low expression of these G proteins, this phenomenon appeared to be specific to tumoral pituitary tissues, as both Gi1/2α and Gi3α were detected in normal human and rat pituitary in high amounts. The results obtained in normal tissues are consistent with the notion that proteins of the Gi subfamily are abundant within the cell (25).

Very low levels of Gi1/2α and Gi3α were observed in pituitary adenomas, regardless of the nature of the tumor. In particular, the three prolactinomas of the series that were surgically removed because of poor responsiveness to dopaminergic treatment showed undetectable amounts of the two proteins. Although ethical considerations preclude the removal of pituitary tissues from patients fully responsive to dopaminergic agents for purposes of comparison, it is tempting to speculate that the low levels of Gi proteins may contribute to the resistance of these tumors to the action of dopamine (26). In this respect, it of interest that a selective reduction of Gi2α mRNA has recently been documented in prolactinomas removed from patients resistant to dopaminergic drugs (27).

Abnormal expression of G proteins in different target tissues has been described in numerous pathophysiological states (28, 29). However, from the data available, it appears that disorders frequently associated with pituitary tumors, such as hypothyroidism, hypoadrenalism and hypogonadism, may cause a reduction in Gs and an increase in Gi expression (17, 30, 31). As this pattern of expression is the opposite of that observed in pituitary tumors, it is unlikely that the low expression of Gi1/2α and Gi3α in pituitary adenomas might result from a coexistent partial or total hypopituitarism. Because several groups have observed impaired function and expression of Gi in different tissues from diabetic animals, patients with that disorder were not included in the present study (32).

The presence of Goα, Gsα and Gq/11α was clearly detected in all pituitary tumors. Although these proteins were present in variable amounts in different tumors, they were clearly more abundant than Gi1/2α and Gi3α. As the expression of different G proteins in
endocrine tumors has not yet been examined in detail, it is not possible to establish whether this altered ratio between G\(\alpha\)i and G\(\alpha\)s is specific to pituitary tumors. However, it has recently been reported that, in toxic thyroid adenomas the pattern of G protein expression seems to be different, high levels of G\(\alpha\)s being accompanied by increased expression of G\(\alpha\)i (33).

Several pieces of evidence indicate that both dopamine and somatostatin receptors couple to G\(\alpha i/2\alpha\) to inhibit adenyl cyclase and cAMP formation, whereas G\(\alpha i3\) and G\(\alpha o\) are associated with K\(^+\) and Ca\(^{2+}\) channels, respectively, though some discrepant data on the specific G protein associated with ion channels remain (34–37). Moreover, experimental models in which cloned receptors have been transfected in different cell types consistently indicate that the quantity or quality of G proteins available for receptor activation may qualify the generation of intracellular effectors (35, 38). It is tempting, therefore, to speculate that, although cells probably require relatively few G proteins for transducing hormonal signals, the pattern of G protein expressed in pituitary tumors favors the reduction of Ca\(^{2+}\) currents (by G\(\alpha q\)), the activation of adenyl cyclase (by G\(\alpha s\)) and the activation of phospholipase C-\(\beta\) (by G\(\alpha o\)). Although decreasing the Ca\(^{2+}\) concentrations in tumoral cells via G\(\alpha q\) is probably sufficient to reduce hormone hypersecretion (39), the low expression of G\(\alpha i1/2\alpha\) and G\(\alpha i3\) proteins may be responsible for a poor capacity of inhibitory neurohormones to reduce the production of cAMP, which in pituitary cells represents a proliferative signal (14, 15, 40).

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