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Cushing's Syndrome due to a Gastric Inhibitory Polypeptide-Dependent Adrenal Adenoma: Insights into Hormonal Control of Adrenocortical Tumorigenesis*

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ABSTRACT

We studied a patient with food-induced, ACTH-independent, Cushing's syndrome and a unilateral adrenocortical adenoma. *In vivo* cortisol secretion was stimulated by mixed, glucidic, lipidic, or proteic meals. Plasma ACTH levels were undetectable, but iv injection of ACTH stimulated cortisol secretion. Unilateral adrenalectomy was followed by hypocortisolism with loss of steroidogenic responses to both food and ACTH. *In vitro*, cortisol secretion by isolated tumor cells was stimulated by the gut hormone gastric inhibitory polypeptide (GIP) and ACTH, but not by another gut hormone, glucagon-like peptide-1 (GLP-1). Both peptides stimulated the production of cAMP but not of inositol 1,4,5-trisphosphate. In quiescent cells, GIP and ACTH stimulated [^{3}H]thymidine incorporation and p42-p44 mitogen-activated protein kinase activity. GIP receptor messenger ribonucleic acid (RNA), assessed by RT-PCR, was highly expressed in the tumor, whereas it was undetectable in the adjacent hypotrophic adrenal tissue, in two adrenal tumors responsible for food-independent Cushing's syndrome, and in two hyperplastic adrenals associated with ACTH hypersecretion. *In situ* hybridization demonstrated that expression of GIP receptor RNA was confined to the adrenocortical tumor cells. Low levels of ACTH receptor messenger RNA were also detectable in the tumor. We conclude that abnormal expression of the GIP receptor allows adrenocortical cells to respond to food intake with an increase in cAMP that may participate in the stimulation of both cortisol secretion and proliferation of the tumor cells. (*J Clin Endocrinol Metab* **83:** 3134–3143, 1998)

ADRENOCORTICAL tumors responsible for Cushing's syndrome have the capacity to secrete cortisol in the absence of ACTH. The cellular mechanisms responsible for this property are largely unknown. However, it has recently been recognized that in some cases, secretion of cortisol by bilateral adrenal hyperplasia (1–4) or, more rarely, by an adrenal adenoma (5, 6) is controlled by hormones other than ACTH, such as gastric inhibitory peptide (GIP) (2, 3, 5), vasopressin (4, 7, 8), or catecholamines (1). In three cases, the abnormal regulation of cortisol secretion could be related to the expression of the receptors of these hormones in the pathological adrenal cells (5, 7, 9). These observations led to the hypothesis that ACTH-independent cortisol secretion by tumoral or hyperplastic cells can be related to the activation of ectopic or overexpressed receptors.

However, the roles of these ectopic receptors in adrenocortical tumorigenesis remains unclear. In the normal adrenal cortex, ACTH regulates both cortisol secretion and trophicity. It acts through activation of a G protein-coupled receptor that stimulates adenylate cyclase (10). It has thus been assumed that activation of ectopic receptors could have similar effects on the adrenocortical cells and could be responsible for both hypercortisolism and tumorigenesis. To date, however, no data have been provided on the potential effect of these receptors on adrenocortical cell proliferation. Temporary suppression of the activation of hormone receptors ectopically expressed in adrenals has been attempted by different means: inhibition of GIP secretion by somatostatin (2, 3, 5) or β_2-adrenergic receptor blockade by propanolol (1). These treatments resulted in the inhibition of cortisol secretion, but did not induce any measurable regression of adrenal hyperplasia or tumors. Thus, the relationship between ectopic expression of hormone receptors and the development of adrenal hyperplasia or tumors remains to be addressed.

We report here the study of a patient suffering from a food-dependent, ACTH-independent, Cushing's syndrome related to a single adrenocortical adenoma. *In vivo* cortisol secretion was stimulated by any type of food intake, but not by insulin, iv glucose, or orthostatism. *In vitro* cortisol secretion by the dispersed tumor cells was stimulated by GIP, but not by glucagon-like peptide 1 (GLP-1). The tumor cells also responded to ACTH both *in vivo* and *in vitro*, suggesting that they had retained functional ACTH receptors despite suppression of ACTH secretion by hypercortisolism. Thus, the tumor cells provided a unique model to compare the

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mechanism of action of an unconventional stimulator, GIP, to that of the physiological regulator of adrenal cortex secretion and proliferation, ACTH.

Materials and Methods

All studies were performed according to the rules of the hospital medical ethics committee. The patient gave informed consent.

Case report

A 32-yr-old woman was admitted for suspicion of hypercortisolism because of a 10-kg weight gain in the upper part of the body, rounding of the face, hirsutism, acnea, secondary amenorrhea, and high blood pressure (160/90 mm Hg). All signs had developed during the previous year. A standard 1-mg dexamethasone overnight suppression test had showed a plasma cortisol value of 121 nmol/L at 08 h. However, because of the severe clinical features, further exploration was decided. Free urinary cortisol excretion was 1700 nmol/24 h (normal, 120–250), and circadian variations in serum cortisol were remarkable because of low values in the morning and peaks always after meals (Fig. 1A). Plasma ACTH was below or very close to the detection limit (0.5 pmol/L) at all times, and the standard 2-mg dexamethasone suppression test did not prevent the peaks of plasma cortisol or diminish free urinary cortisol excretion. The ACTH-independent stimulation of cortisol secretion followed any kind of meal: mixed, oral glucose (100 g), fat-based (490 Cal; 82% fat, 16% carbohydrate, and 2% protein), and protein-based (490 Cal; 87% protein, 8% carbohydrate, and 5% fat) meals. When an overnight fast was performed for 19 h, the morning and afternoon peaks of cortisol secretion were suppressed (Fig. 1B). Intravenous injection of glucose (100 g/3 h) had no effect on cortisol secretion. Injection of 10 IU insulin induced hypoglycemia, but no stimulation of cortisol or ACTH secretion. Measurements of plasma GIP concentrations showed that preoperative values of plasma cortisol and GIP were correlated (r = 0.9; Fig. 1, A and B). Subcutaneous injection of 500 μg of the somatostatin analog octreotide 45 min before a meal blunted the postprandial stimulation of GIP (160% *vs.* 400% without octreotide) and cortisol (134% *vs.* 280%). An iv GIP stimulation test was proposed to the patient, but she refused consent.

Abdominal tomodensitometry revealed a unilateral 3-cm right adrenal mass, whereas the left adrenal was not hypertrophic and showed no nodules. A right adrenalectomy was performed by laparoscopic surgery. Pathological examination revealed a 13-g cortical adenoma, with hypotrophic adjacent nonadenomatous adrenocortical tissue. Substitution with hydrocortisone was started during surgical procedure and was stopped 5 days after surgery to allow new testing of the patient. Cortisol secretion by the remaining controlateral adrenal was very low despite normal postprandial GIP levels (Fig. 1C). Four months after surgery, ACTH secretion was restored, and cortisol levels were correlated to ACTH (r = 0.93), but not to GIP (r = −0.38) levels (Fig. 1D).

Standard 1-h ACTH stimulation tests with iv injection of 250 μg ACTH-(1–24) (Cosyntropin) were also performed 8 h preoperatively and 5 days or 4 months postoperatively. They showed that 79% of the cortisol response to ACTH was lost after removal of the tumor. Four months after surgery, basal and ACTH-stimulated cortisol secretion of the controlateral gland had increased (Table 1). Eight months after surgery another test was performed at 11 h (2 h after a normal breakfast), and cortisol values were 174 and 334 nmol/L before and after ACTH injection. Maximal stimulation of cortisol remains lower than normal (>580 nmol/L), indicating persistent hypotrophy of the remaining adrenal gland.

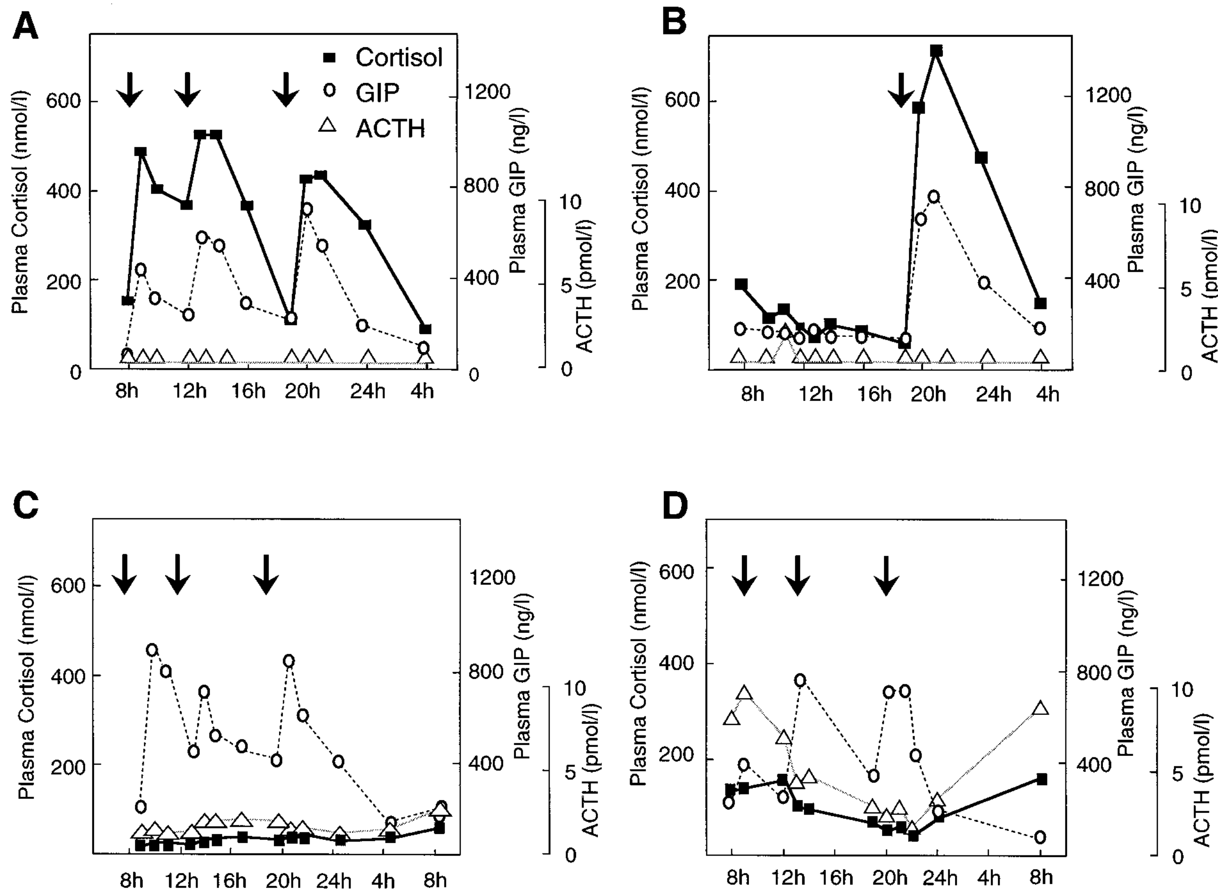

FIG. 1. Food-induced and ACTH-independent hypercortisolism related to a single adrenocortical adenoma. Plasma cortisol, ACTH, and GIP were measured before surgery (A and B) or 5 days (C) or 4 months (D) after surgical resection of a right adrenocortical adenoma. Food intake consisted of three meals except in B, where the overnight fast was pursued for 19 h. The *arrows* show the moment of food intake. Meals were regular mixed meals except for a 100-g oral glucose load at 8 h in C.

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TABLE 1. Pre- and postoperative responses to the 1-h ACTH stimulation test

Time (min)	Cortisol (nmol/L)		
	Pre-op	5 days post-op	4 months post-op
0	246	54	165
60	703	149	239

Reagents and kits

ACTH-(1–24) (Cosyntropin, Synacthene) was obtained from Ciba (Basel, Switzerland). Human GIP and human GLP-1-(7–36) amide (GLP-1) were purchased from Bachem Biochimie (France); carbamylcholine chloride (carbachol) and forskolin were obtained from Sigma (St. Louis, MO). Plasma ACTH-(1–39) was measured by immunoradiometric assay, and GIP by RIA with commercial kits [Nichols Institute (San Juan Capistrano, CA) and Peninsula Laboratories (Belmont, CA), respectively]; cortisol was determined by RIA using antiserum from Endocrine Sciences (Calabasa, CA); cAMP was measured by RIA with a cAMP antiserum given to us by Dr. José Saez (Lyon, France); inositol 1,4,5-trisphosphate was determined by RIA using [^{3}H]D-*myo*-inositol 1,4,5,-trisphosphate (IP3; Amersham, Les Ullis, France).

Cell isolation and culture

Six grams of fresh human tumor were freed of fat and sliced (0.5-mm section) with a Steady-Riggs microtome. The slices were washed three times in medium A [Ham's F-12-DMEM (1:1) containing 10 mmol/L HEPES, 14 mmol/L $NaHCO_3$, and antibiotics (20 U/mL penicillin, 50 μg/mL streptomycin, and 20 U/mL nystatin)]. The tissue was supplemented with 50 mL medium A containing 3 mg/mL collagenase A (Boehringer Mannheim, Indianapolis, IN) and 0.1 mg/mL deoxyribonuclease (Sigma) and incubated for 30 min at 37 C with stirring. The suspension was then filtered on sterile gauze, and the volume was completed to 50 mL with medium A containing 10% horse serum and 2.5% FCS before centrifugation for 10 min at 400 $\times$ *g*. The cell pellets were washed with the same medium and further purified on a Percoll (Pharmacia, Uppsala, Sweden) gradient (50% Percoll in Ham's F-12 medium) preformed by centrifugation during 30 min at 62,000 $\times$ *g*. The cells were layered on this gradient, then centrifuged for 15 min at 2,000 $\times$ *g*. Three zones appeared on the gradient: the top one contained cellular fragments, the bottom one contained erythrocytes, and the middle one contained adrenal cells. This latter fraction was aspirated and washed twice with medium A containing serum. It contained 40 $\times$ 10^6 cells; 10 $\times$ 10^6 cells were used in suspension, and 30 $\times$ 10^6 cells were seeded in multiwell dishes and cultured for 24 h in Ham's F-12-DMEM (1:1) containing 10% FCS with antibiotics, 10 μg/mL transferrin, 10 μg/mL insulin, and 10^{-4} mol/L vitamin C. After 24 h of culture, the medium was changed to serum-free Ham's F-12-DMEM. Normal human adrenocortical cells were prepared with the same procedure using a fragment of adrenal removed for pheochromocytoma.

Cortisol production

Freshly dispersed human cells (10^5 cells in 1 mL) were incubated for 90 min in Ham's F-12 with various hormones. Then, cortisol secreted into the medium was measured by RIA. Cultured cells were seeded in 16-mm wells at a density of 80 $\times$ 10^3 cells/well. Twenty-four hours later, the medium was removed and replaced by Ham's F-12 medium during 2 h. Cortisol production was measured by RIA.

cAMP production

Freshly dispersed cells (10^5 cells in 1 mL) were incubated in KRGH medium (120 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 2.5 mmol/L $CaCl_2$, 20 mmol/L HEPES, and 2 g/L glucose, pH 7.4) containing 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX). Cells were incubated for 20 min with various effectors, and the reaction was stopped by the addition of 2 mL ethanol. After centrifugation, the supernatant was evaporated, sodium acetate buffer (50 mmol/L; pH 5.6) was added, and cAMP was measured by RIA. Cultured cells were seeded in 16-mm wells at a density of 80 $\times$ 10^3 cells/well. On day 1, medium was removed and replaced by KRGH containing 1 mmol/L IBMX, and a 20-min incubation with the various effectors was performed. The reaction was stopped by the addition of 2 mL ethanol. cAMP was measured by RIA.

IP3 determination

Freshly dispersed cells were incubated in KRGH at 37 C for 10 or 30 s with different effectors. The reaction was stopped by the addition of 0.2 mL perchloric acid. After neutralization with KOH, IP3 was measured in the supernatant by RIA.

DNA synthesis

DNA synthesis in human adrenocortical tumor cells or in bovine adrenocortical fasciculata-reticularis cells (BAC cells) was assessed in triplicate wells by measurement of [^{3}H]thymidine incorporation. Cells were seeded in 16-mm wells at a density of 80 $\times$ 10^3 cells/well. After 24 h of culture in serum-supplemented medium, the cells were serum starved for 3 days in Ham's F-12 containing 0.1% albumin and antibiotics. Quiescent cells were then incubated in fresh Ham's F-12 containing 0.1% albumin and various hormones for the indicated periods of time. Then, 0.25 μCi [^{3}H]thymidine (SA, 87 Ci/mmol) was added to each well during the last 3 h of incubation. Radioactivity incorporated into trichloroacetic acid- insoluble material was measured by scintillation counting.

Mitogen-activated protein (MAP) kinase assay

This assay was performed as previously described (11). Briefly, 24 h after seeding, the cells were serum starved for 48 h in F-12 medium containing 0.1% BSA before stimulation with various hormones. After stimulation, the medium was removed, and the cells were scrapped off and homogenized in a lysis buffer. The cell extract was centrifuged, and the supernatant was analyzed on a MonoQ Sepharose (Pharmacia, Piscataway, NJ) microcolumn with stepwise elution by increasing salt concentration. Western blotting analysis was performed on the elution fractions using a rabbit anti-p42^{mapk}-p44^{mapk} antiserum directed against a synthetic peptide from the C-terminus of rat p44^{mapk} (gift from Dr. Jacques Pouyssegur, Nice, France), and phosphorylation of myelin basic protein was measured in the fractions containing p42^{mapk}-p44^{mapk} kinase immunoreactivity.

RT-PCR

Ribonucleic acid (RNA) from adrenal tumors or from other tissues was purified by a modification of the method of Chomczynski (12) using the total RNA isolation system from Promega (Charbonnieres, France). RNA (5 μg) was treated for 30 min at 37 C and for 5 min at 90 C with RQI deoxyribonuclease (Promega), and first strand complementary DNAs (cDNAs) were generated using 200 U reverse transcriptase (Superscript II, Life Technologies, Grand Island, NY) and 0.2 μg random hexamer DNA primers for 50 min at 37 C and for 15 min at 75 C. Control reactions without reverse transcriptase were performed for each RNA sample. cDNA (0.5 μg) was then PCR amplified in a final volume of 25 μL containing 2.5 U *Taq* DNA polymerase (Promega), 0.2% dimethylsulfoxide, and 14 pmol of each oligonucleotide primer. The amplification parameters were 94 C (2 min), then 35 or 42 cycles at 94 C (1 min), 55 C (1 min), and 72 C (2 min). For the human GIP receptor, two pairs of primers were designed, based on published sequences (13): sense 1 (nucleotides 99–123), TCACGATGACTACCTCTCCGATCC; antisense 1 (nucleotides 571–594), CGCCTGAACAAA-CTCAAGATGAGC; sense 2 (nucleotides 546–564), TCTCTCGCCACACTGCTGC; and antisense 2 (nucleotides 1008–1027), CAAGATGGTCATGAGGATGG.

For the human ACTH receptor, one pair of primers was designed, based on published sequence EMBL X65633: sense (nucleotides 753–774), GACTGTCCTCGTGTGGTTTTGC, and antisense (nucleotides 1012–990), ATGATGTCATCGGCTGTGGTTTC. The amplification parameters were 94 C (2 min), then 25 or 30 cycles at 94 C (1 min), 55 C (1 min), and 72 C (2 min).

To ensure semiquantitative results, the number of PCR cycles for each set of primers and probes was determined to be in the linear range of amplification. In addition, all cDNA samples were adjusted to yield equal amplification of a fragment of ribosomal protein L27 cDNA (14)

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as internal standard. Amplified products were separated by agarose gel electrophoresis (2%).

Origin of human tissues

Human tissues used as controls for GIP receptor or ACTH receptor RNA expression have the following origin. Cerebral tissue, collected from epileptic surgery, was a piece of temporal cortex outside epileptic foci. Neuropathological examination clearly indicated the absence of any tumoral process. Spleen tissue, collected from splenectomy for lymphoma, was a sample exempt of tumoral process. Adrenal tissues considered normal were adrenal cortex adjacent to a pheochromocytoma (one sporadic and one related to a germinal 634 mutation of the *ret* protooncogene); adrenocortical tumors were collected from unilateral adrenalectomy for Cushing's syndrome (food independent), and adrenocortical hyperplasia tissues were collected from bilateral adrenalectomy for paraneoplastic ACTH-dependent Cushing's syndrome. All tissues originate from patients who underwent surgery in the University Hospital of Grenoble, France.

Hybridization with labeled internal oligonucleotidic probe

Hybridization was performed with a 20-mer oligonucleotide probe located in exon 9 of GIP receptor messenger RNA (mRNA; position 910–929 bp) specifically labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (Life Technologies). Agarose gel was transferred under vacuum onto a Hybond N membrane (Amersham) in 4 N NaOH for 1 h, and the membrane was washed twice with 1 × SSC (0.15 mol/L NaCl and 15 mmol/L sodium citrate). Hybridization with the labeled probe was performed overnight at 42 C in 5 × SSC, 0.1% SDS, and 5 × Denhardt's solution [prepared as described previously (15)]. The blots were washed twice at room temperature in 2 × SSC, followed by two washes at 42 C with 2 × SSC and 0.1% SDS. Radiolabeled bands were visualized on a β-imager (PhosphorImager, Molecular Dynamics, Sunnyvale, CA).

In situ hybridization

In situ hybridization was performed using a 48-mer oligonucleotide probe located in exon 4 of the GIP receptor (position 276–323) and with a 43-mer oligonucleotide probe located in exon 5 (position 387–430). The oligonucleotides were labeled using terminal deoxynucleotide transferase (Boehringer Mannheim) and [α-^{35}S]deoxy-ATP (New England Nuclear-DuPont, Boston, MA; 1250 Ci/mmol). The tissue sections were incubated with labeled probe in the presence or absence of a 100-fold excess of unlabeled probe as previously described (16). Slides were exposed for 28 days at 4 C before revelation and counterstaining with toluidine blue.

Statistics

Data are reported as the mean of triplicate determinations ± SD. All experiments in BAC cells were performed at least three times in an independent fashion. Statistical analysis of the raw data was performed by ANOVA followed by appropriate *post-hoc* tests (Student's *t* test and Scheffe's F test). Unless otherwise indicated, values are taken as significant for $P < 0.05$.

Results

In vitro studies on dispersed and cultured tumor cells

Tumor cells, normal human adrenocortical cells, and bovine adrenocortical cells were obtained by enzymatic dispersion and put in suspension or maintained in primary culture for 72 h. Measurements of cortisol and second messenger synthesis were performed on both cell suspension and primary culture, whereas measurement of [^{3}H]thymidine incorporation and MAP kinase activity were performed only on primary cultures.

Cortisol secretion and second messenger synthesis

In tumor cells, cortisol secretion was stimulated by ACTH, forskolin, and GIP, whereas GLP-1 had no effect. Stimulation by GIP was maximal at the lowest concentration (0.1 nmol/L) tested in cell suspension (Fig. 2A) and showed a dose-response curve with a maximal response at 1 nmol/L in primary culture (Table 2). In normal cells either in suspension or in culture, cortisol production was stimulated by 1 nmol/L ACTH (6- or 3-fold, respectively), whereas no stimulation was elicited by 1 or 10 nmol/L GIP (data not shown). Stimulation of cortisol secretion by GIP was correlated with a stimulation of cAMP production similar to that obtained by ACTH or forskolin, whereas GLP-1 had no effect (Fig. 2B and Table 2). IP3 production was stimulated by carbamylcholine (carbachol), an agonist for the acetylcholine m1-muscarinic receptor, but by neither GIP nor ACTH (Table 3).

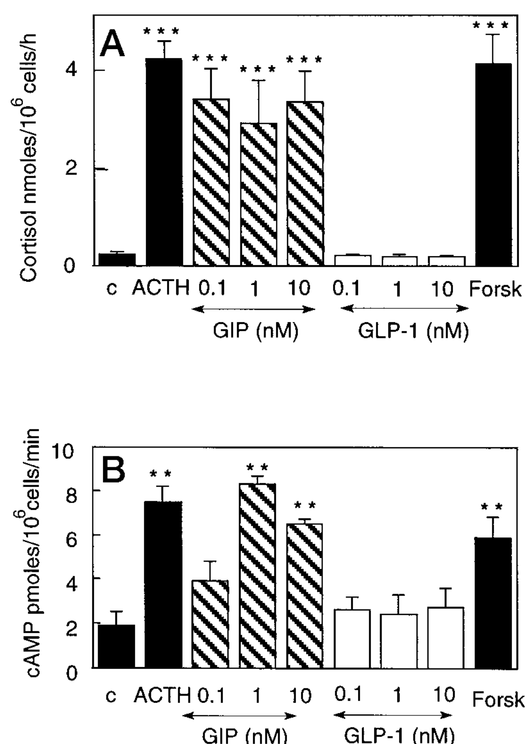

FIG. 2. Cortisol secretion and cAMP production by dispersed tumor cells in suspension. A, Freshly dispersed cells (10^5 cells in 1 mL medium) were incubated with medium alone (c), ACTH (1 nmol/L), GIP (0.1–10 nmol/L), GLP-1 (0.1–10 nmol/L), or forskolin (10 μmol/L). After 90 min of incubation, centrifugation was performed, and cortisol production was measured in the medium by RIA. Values represent the mean ± SE of triplicate determinations. Statistical analysis indicates that ACTH, GIP (0.1–10 nmol/L), and forskolin are significantly different from the control value ($P < 0.01$) and from GLP-1 ($P < 0.01$), and that GLP-1 (0.1–10) is not different from the control value ($P > 0.05$). B, Freshly dispersed cells (10^5 cells in 1 mL buffer containing 1 mmol/L IBMX) were incubated for 30 min with medium alone (c), ACTH (1 nmol/L), GIP (0.1–10 nmol/L), GLP-1 (0.1–10 nmol/L), or forskolin (10 μmol/L). Then, ethanol (2 mL) was added, and cAMP production was measured by RIA. Values represent the mean ± SE of triplicate determinations. Statistical analysis indicates that ACTH, GIP (1–10 nmol/L), and forskolin are significantly different from the control value ($P < 0.02$) and from GLP-1 ($P < 0.02$), and that GLP-1 (0.1–10) is not different from the control value ($P > 0.05$). ***, $P < 0.01$; **, $P < 0.02$.

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TABLE 2. Cortisol and cAMP production of tumor cells in culture (day 1)

	Cortisol (nmol/10^6 cells·min)	cAMP (nmol/10^6 cells·min)
Control	0.54 ± 0.03	0.84 ± 0.19
ACTH	1.13 ± 0.14[a]	4.86 ± 0.85[b]
GIP (0.01 nmol/L)	0.78 ± 0.18	ND
GIP (0.1 nmol/L)	0.88 ± 0.26	1.91 ± 0.07[a]
GIP (1 nmol/L)	1.63 ± 0.20[b]	11.19 ± 0.73[b]
GIP (10 nmol/L)	1.56 ± 0.03[b]	12.5 ± 0.50[b]
GLP-1 (10 nmol/L)	0.53 ± 0.03	0.88 ± 0.09

For measurement of cortisol production, cultured cells (80 × 10^3 cells/16-mm well) were incubated on day 1 with medium alone (control), ACTH (1 nmol/L), GIP (0.01–10 nmol/L), or GLP-1 (10 nmol/L) for 2 h. Cortisol was quantified in the medium by RIA. Values represent the mean ± SE of triplicate determinations. Statistical analysis indicates that ACTH and GIP (1–10 nmol/L) are significantly different from control and GLP-1. GLP-1 is not different from control ($P > 0.05$). For measurement of cAMP production, cultured cells (80 × 10^3 cells/16-mm well) were incubated for 30 min in buffer containing 1 mmol/L IBMX alone (control) or ACTH (1 nmol/L), GIP (0.1–10 nmol/L), and GLP-1 (10 nmol/L). Values represent the mean ± SE of triplicate determinations. Statistical analysis indicates that ACTH and GIP (0.1–10 nmol/L) are significantly different from control and GLP-1 ($P < 0.01$) and that GLP-1 is not different from control ($P > 0.05$).

[a] $P < 0.05$.
[b] $P < 0.01$.

TABLE 3. Production of IP3 by dispersed tumor cells

	IP3 (pmol/10^6 cells)	
Time (s)	10	30
Control	3.1 ± 0.05	2.8 ± 0.3
ACTH (1 nmol/L)	3.6 ± 1	2.4 ± 0.1
GIP (10 nmol/L)	3.0 ± 0.2	2.6 ± 0.6
Carbachol (200 μmol/L)	7.5 ± 0.8	13.5 ± 2.5

IP3 (picomoles per 10^6 cells) was measured by RIA after stimulation of freshly dispersed tumor cells with the different hormones for 10 or 30 s. Results represent the average of duplicate measurements in the same experiment (±SE).

DNA synthesis

Quiescent tumor cells were treated with serum, ACTH, GIP, or GLP-1, and incorporation of [^{3}H]thymidine was assessed 24, 30, and 36 h later. We found that serum induced an 8- to 9-fold stimulation of [^{3}H]thymidine incorporation. Both ACTH and GIP induced a 3-fold stimulation at 36 h, whereas GLP-1 had no significant effect (Fig. 3). When BAC cells and normal human adrenocortical cells were tested under the same experimental conditions as the human tumor cells, GIP had no effect, whereas ACTH stimulated [^{3}H]thymidine 2- and 3.3-fold, respectively (data not shown).

MAP kinase activity

MAP kinases are serine/threonine protein kinases whose activity is essential to the control of cell proliferation (17). They can be activated by signals originating from either tyrosine kinase or G protein-coupled receptors (18). This prompted us to investigate the MAP kinase responses to ACTH and GIP in the adrenal tumor cells. Quiescent tumor cells were stimulated by serum, ACTH, or GIP. Activation of p42-p44 MAP kinases was measured 8 min (acute response) and 120 min (sustained response) after addition of the stimulus. Serum appeared to stimulate MAP kinase activity 7-fold after 8 min and 4.5-fold after 120 min, whereas both ACTH and GIP induced 5- and 4-fold stimulations after 8 min and 120 min, respectively (Fig. 4). When tested under the same conditions, BAC cells also showed a significant activation of MAP kinase by ACTH (2-fold; data not shown).

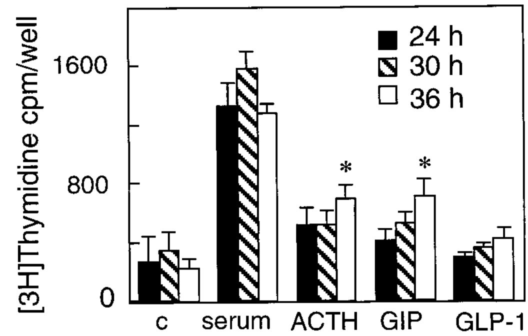

FIG. 3. [^{3}H]thymidine incorporation in tumor cells. Quiescent tumor cells were incubated for 24, 30, and 36 h in Ham's F-12 medium with 0.1% BSA alone (C) or in the presence of 10% FCS (serum), ACTH (10 nmol/L), GIP (10 nmol/L), and GLP-1 (10 nmol/L). [^{3}H]Thymidine incorporation was allowed for the last 3 h of incubation and assayed as described in *Materials and Methods*. Measurements were performed in triplicate in the same experiment. Statistical analysis indicates that at 36 h, ACTH and GIP are significantly different from the control value ($P < 0.05$) and from GLP-1 ($P < 0.05$), and GLP-1 is not different from the control value ($P > 0.05$). *, $P < 0.05$.

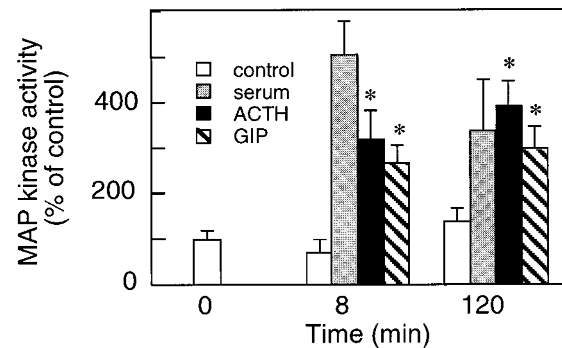

FIG. 4. MAP kinase activities of tumor cells. Quiescent tumor cells were incubated with 10% FCS, ACTH (1 nmol/L), and GIP (10 nmol/L). MAP kinase activities were assayed as described in *Materials and Methods*. Each value is the mean ± SD of normalized data. All measurements were performed in triplicate in the same experiment. Statistical analysis indicates that at 8 and 120 min, ACTH and GIP are significantly different from the control value ($P < 0.05$). *, $P < 0.05$.

Expression of GIP receptor and ACTH receptor mRNAs in the tumor and other tissues

GIP and ACTH receptor mRNA expressions were assessed by semiquantitative RT-PCR of tumor RNA. For GIP receptor, two pairs of primers were used. The first pair (S1-AS1) allows amplification of a fragment that includes part of exon 2, exons 3–5, and a part of exon 6. This fragment encodes for the predicted extracellular domain of the receptor, transmembrane domain I, and part of intracellular loop I. Amplification of tumoral cDNA with S1-AS1 showed three bands (Fig. 5A), one of the expected size (495 bp) and two smaller bands of approximately 390 and 290 bp. Sequencing

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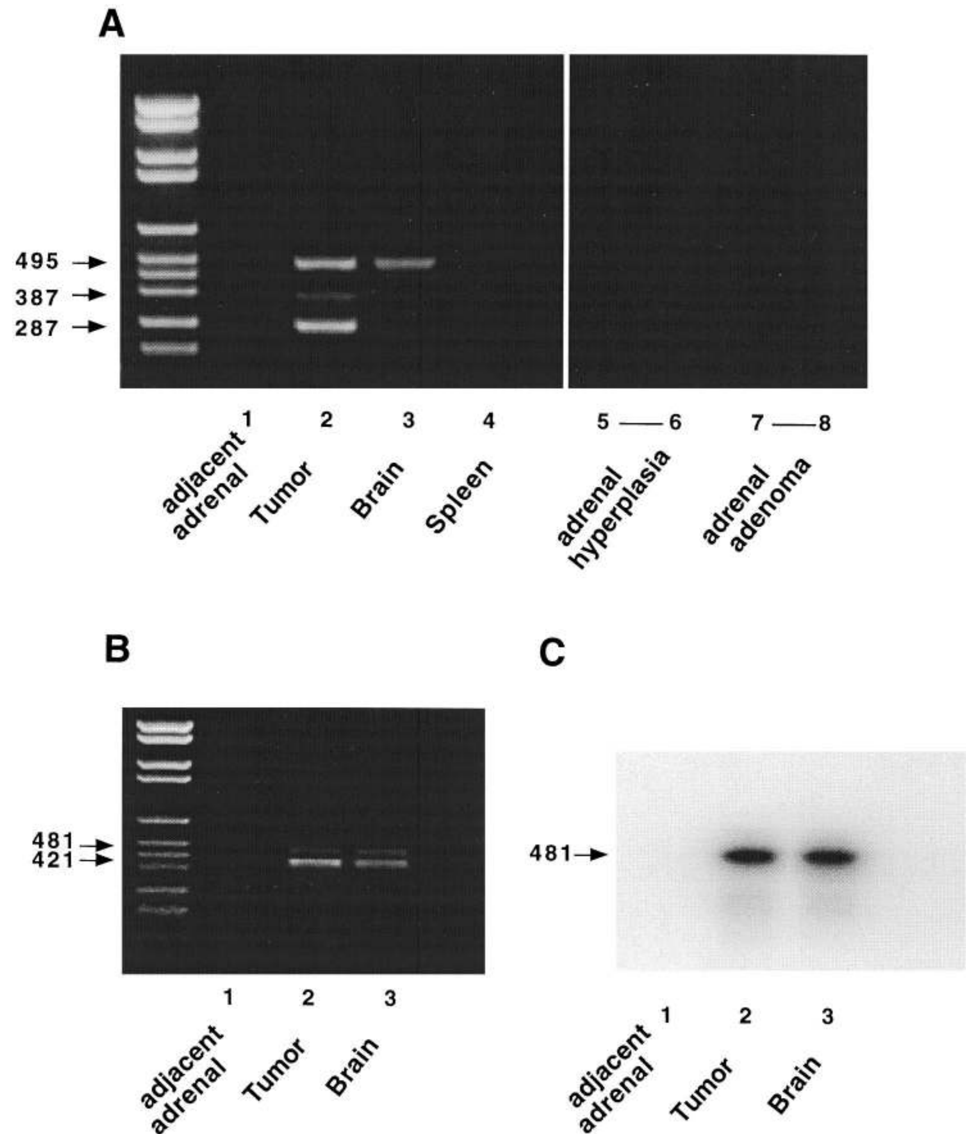

FIG. 5. RT-PCR analysis of GIP receptor expression in adrenal tissues. RNA from adrenal tissue adjacent to the tumor (1) or from the tumor (2), normal brain (3), normal spleen (4), hyperplastic adrenals originating form two patients with paraneoplastic ACTH-dependent Cushing's syndrome (5, 6), adrenal adenoma from two patients with ACTH-independent and food-independent Cushing's syndrome (7, 8) were reverse transcribed in cDNA and amplified by PCR in A with primers S1-AS1 and in B with primers S2-AS2 of GIP receptor cDNA sequence. A and B are ethidium bromide-stained agarose gels (2%), whereas C is an autoradiogram of a blot hybridized with internal oligonucleotide located in exon 9 (primers S2-AS2).

of the amplification products revealed that all three bands contained GIP receptor cDNA sequences; the larger one was the expected entire fragment, whereas the smaller bands were lacking exon 4 or both exons 4 and 3. The second pair (S2-AS2) allowed amplification of a fragment that includes part of exon 6 and exons 7–10. It encodes the region of the receptor comprised between transmembrane segments I and V. Amplification of tumoral cDNA with S2-AS2 showed a weak band of the expected size (481 bp) and a smaller, but intense, band around 420 bp (Fig. 5B). Sequencing of the amplification products showed that the smaller band was constituted of exons 6, 7, 8, and 10, but lacked exon 9. The presence of exon 9 in the larger band was confirmed by specific hybridization (Fig. 5C).

Using the same PCR conditions (35 cycles), amplification of RNA from human brain tissue also showed expression of GIP receptor DNA, whereas no bands were detected from the adjacent hypotrophic adrenal tissue, or 2 adrenocortical adenomas responsible for ACTH-independent and food-independent Cushing's syndrome, or 2 hyperplastic adrenals associated with ACTH-dependent Cushing's syndrome (Fig. 5, A and B). No bands were detected from normal adrenal cortex, even after 42 cycles of amplification (data not shown).

For the ACTH receptor, a fragment of 259 bp, which encodes the transmembrane domains I and II and the first intracellular loop, was amplified by RT-PCR. After 25 cycles of amplification, ACTH receptor cDNA was detected in normal adrenal, but not in the tumor or in the adjacent hypotrophic adrenal tissue (data not shown). After 30 cycles of amplification, ACTH receptor RNA was detected in the tumor but at a lower level than in a normal adrenal or pathological adrenals responsible for ACTH-dependent Cushing's syndrome or ACTH-independent and food-independent Cushing's syndrome (Fig. 6).

Identification of the tumor cells expressing GIP receptor RNA

In situ hybridization was performed on tumor slices using a radiolabeled GIP receptor cDNA probe. Hybridization sig-

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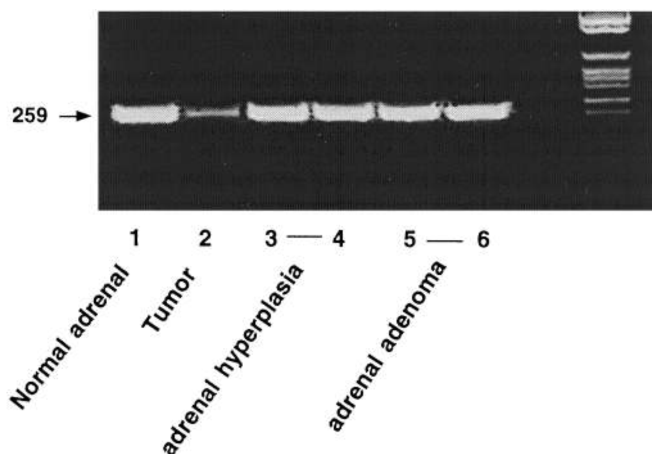

FIG. 6. RT-PCR analysis of ACTH receptor expression in adrenal tissues. RNA from normal adrenal tissue (no. 1), tumor (no. 2), hyperplastic adrenals originating form two patients with paraneoplastic ACTH-dependent Cushing's syndrome (no. 3 and 4), adrenal adenoma from two patients with ACTH-independent and food-independent Cushing's syndrome (no. 5 and 6), were reverse transcribed in cDNA and amplified by PCR with primers specific for the human ACTH receptor (sequences 753–774 and 1012–990).

nal was clearly detected on adrenocortical tumor cells, but was not observed in lymphocytic aggregates or in lipomatous metaplasia (Fig. 7).

Discussion

The adrenal adenoma responsible for Cushing's syndrome represents an interesting model of adrenocortical tumorigenesis. The main characteristic of this well differentiated benign tumor is that both its hormonal secretion and its cellular proliferation are independent of ACTH, the trophic hormone that regulates both functions in the normal adrenal cortex. The understanding of the molecular mechanisms leading to transformation of normal adrenocortical tissue into such an adenoma should point to important steps of adrenocortical tumor development. A comparison can be made between adrenal Cushing's adenoma and thyroid toxic adenoma, as this latter tumor is independent of TSH, which regulates both secretion and proliferation of thyroid cells. In the thyroid, TSH stimulates adenylate cyclase through a receptor coupled to the GTP-binding protein G_s; the subsequent rise in cAMP stimulates secretion and proliferation of thyroid cells (19). It is now known that thyroid toxic adenomas are commonly related to activating mutations of the TSH receptor (20) or more rarely to activating mutations of $G_s\alpha$ (21). Both types of mutations mimic a constant stimulation of the thyroid cells by TSH and thus provide a good molecular explanation for the TSH independence of thyroid toxic adenoma cells. Similarly, as ACTH receptor also activates adenylate cyclase (10) through coupling to G_s, one could expect to observe activating mutations of the ACTH receptor or $G_s\alpha$ in adrenal Cushing's adenoma. However, despite extensive search, no activating mutation of the ACTH receptor has been reported in adrenal tumors (22, 23). $G_s\alpha$ mutations are also generally not found in human adrenocortical neoplasms (24) and were reported in only two cases of Cushing's syndrome: an adrenal adenoma in a patient carrying the rare McCune-Albright syndrome (25) and adrenal hyperplasia in an infant (26). Mutations of another G protein α-subunit, $G_{i2}\alpha$, which, on the contrary, drives inhibition of adenylate cyclase, had originally been reported in 3 of 8 adrenocortical tumors (21). However, this finding was not confirmed by all subsequently published work on the topic, with no mutations of $G_{i2}\alpha$ found in a total of 85 adrenal tumors (24, 27, 28). Therefore, the molecular mechanisms leading to adrenal Cushing's adenoma remain largely unknown, even if some cases (25, 26) are consistent with the hypothesis that activation of the adenylate cyclase pathway may be involved.

In adrenal Cushing's syndrome, ACTH-independent cortisol secretion is usually very constant over time, consistent with the hypothesis that the adrenal tumor or hyperplasia responsible for hypercortisolism could be related to a molecular event mimicking a constant ACTH stimulation. However, the observation, in two patients, that cortisol secretion varied throughout the day as a function of food intake led to the recognition that in some cases, cortisol secretion was not truly autonomous but was controlled by a hormone other than ACTH, such as the gut hormone GIP (2, 3). This concept could later be extended to other hormones, such as vasopressin (4, 7, 8), and catecholamines (1). In some of these cases, it could be demonstrated that the mechanism underlying the abnormal hormonal control of cortisol secretion was ectopic expression (or overexpression) of the corresponding receptor in the pathological adrenal (1, 5, 9). Except for three cases (5–7), all patients reported with adrenal Cushing's syndrome linked to ectopic receptor expression had bilateral adrenal hyperplasia, which suggests that abnormal expression of the receptor was present in all adrenal cells.

Our patient presented a severe ACTH-independent hypercortisolism and a right unilateral adrenal mass. Surprisingly, cortisol levels were low in the morning and showed a diurnal variation coincident with food intake. A causal relationship between food intake and cortisol secretion was suggested by the observation that fasting prevented cortisol secretion. All subsequent *in vivo* explorations (including the lack of effect of iv glucose, insulin, or orthostatism; the positive effect of glucidic, lipidic, proteic, or mixed meals; and the prevention of food-induced cortisol secretion by somatostatin pretreatment) pointed to an abnormal control of cortisol secretion by a stimulus linked to food intake, whatever its type. There are only two known gut hormones whose secretion is stimulated by food intake: GIP and GLP-1. Both hormones are incretins, as their main function is to stimulate insulin secretion by the pancreatic islet β-cells. In our patient, serum GIP levels were highly correlated with serum cortisol, which made this hormone a likely candidate for controlling adrenal tumor cortisol secretion. However, for ethical reasons (refusal of the patient), it was not possible to test the *in vivo* effect of GIP or GLP-1. Besides food, cortisol secretion could also be stimulated by synthetic ACTH-(1–24) (Cosyntropin). It should be stressed, however, that this sensitivity to ACTH could not be responsible for hypercortisolism, as endogenous secretion of ACTH was totally suppressed.

The relationship between the adrenal mass and the food-induced hypercortisolism was demonstrated by surgery; right adrenalectomy was followed by cure of hypercorti-

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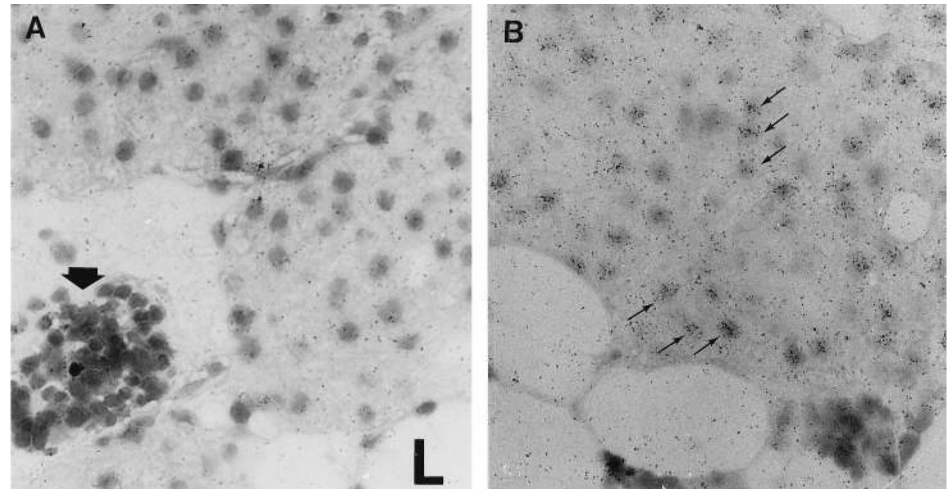

FIG. 7. GIP receptor mRNA distribution in human adrenal tumor. Tumor tissues sections were hybridized with a labeled 48-mer oligonucleotide specific for the GIP receptor mRNA without (B) or with (A) a 100-fold excess of unlabeled probe, which shows nonspecific hybridization. Specific hybridization is seen in B in the perinuclear region of adrenocortical tumor cells (*thin arrows*). Lymphocytic aggregates (*large arrow*) or lipomatous metaplasia (L) are shown in A.

solism, which was replaced by profound hypocortisolism, with loss of the responses to both food and ACTH stimulation. Pathological examination showed a single adrenal adenoma, with hypotrophic adrenal cortex tissue adjacent to it. This suggested that the adenoma was solely responsible for both food-induced hypercortisolism and the cortisol response to ACTH-(1–24). Four months after surgery, ACTH secretion had recovered, and cortisol secretion, still insufficient, was strictly correlated to ACTH levels, but no longer to GIP levels. This confirmed that the controlateral adrenal gland was not sensible to food intake, and that it had reacted normally first to suppression, and then to restoration of ACTH secretion. Thus, the conclusion of the *in vivo* observations is that the adrenal adenoma cells were sensitive both to a factor linked to food intake, presumably GIP or GLP-1, and to ACTH.

The *in vitro* experiments were designed to study the potential mechanisms of action of GIP and GLP-1 on the adenoma cells, with ACTH as a positive control. Cortisol secretion by cells in suspension proved to be sensitive to stimulation by GIP and ACTH, but not by GLP-1, an observation that was reproduced in plated cells. The response to GIP seemed more sensitive in suspended cells, but was observed in both conditions at 0.1 nmol/L, which is in the range of GIP serum concentrations in the patient and normal subjects (3). This result allowed to compare the mechanisms of action of GIP and ACTH in the tumor cells, with GLP-1 as a negative control. We show that GIP, like ACTH, stimulates the production of cAMP. This is consistent with the observation that GIP stimulates cAMP production in GIP-sensitive tissues (16) and in cells transfected with the GIP receptor (13, 16, 29). The rat GIP receptor was also shown to stimulate intracellular Ca^{2+} when transfected in HEK-293 cells (16) or COS-7 cells (29). In the latter report it was shown that Ca^{2+} was released from intracellular stores, presumably after liberation of IP3. This suggests that the GIP receptor can stimulate both adenylate cyclase and phospholipase C, like other G protein-coupled receptors from the same subfamily (30, 31). However, this is not the case in human adrenal adenoma cells, as production of IP3 could be detected after stimulation by carbachol, but not by GIP. Such differences in coupling between transfected cells and tissues has been observed with other G protein-coupled receptors and could result from differences in the levels of expression of G proteins (32) or other components of the signal transduction pathway.

We sought, then, to evaluate the potential effects of GIP on tumor cell proliferation. We found that in human adenoma cells, ACTH also had a significant stimulatory effect on DNA synthesis, although weaker than the effect of serum, and that this effect was reproduced by GIP. In BAC cells and normal human adrenocortical cells, GIP had no effect, whereas the stimulatory effect of ACTH was observed. This correlates well with the trophic effect of ACTH on normal adrenal cortex *in vivo* (33, 34) and suggests that GIP may also have a trophic effect on the tumor cells *in vivo*. It must be stressed that under different experimental conditions, ACTH was first reported to have a paradoxical inhibitory effect *in vitro* on BAC cell DNA synthesis and proliferation (35–37). However, a stimulatory effect has been observed under specific conditions (38), indicating that the *in vitro* effect of ACTH on adrenocortical cell DNA synthesis is dependent on the experimental protocol. Due to the limited amount of tumor material we could not test whether stimulation of DNA synthesis by GIP was correlated with an increase in tumor cell number. However, this was the case for BAC and normal human adrenocortical cells treated with ACTH under the same protocol; we measured increases in the cell number of, respectively, 40% and 30% after 96 h, compared to a 100% increase with serum (data not shown). Such an increase in cell number should represent the sum of the stimulation of cell proliferation and the inhibition of apoptosis (37).

cAMP has tissue-specific effects on growth, differentiation, and gene expression. In most cell types, cAMP inhibits cell proliferation and MAP kinases. By contrast, in some cells, it is stimulatory (39–41). Calleja *et al.* have shown that the effect of cAMP on MAP kinase stimulation depends on the cell type (42). In BAC cells, the effect of ACTH or cAMP on MAP kinase depends on the culture conditions: inhibitory in the conditions we had previously described (11) and stimulatory in the conditions used here. When we measured MAP kinase activity in the human adenoma cells under the same conditions, a weak stimulation by ACTH and GIP was observed. The correlation between ACTH or GIP effects on DNA synthesis and MAP kinase activity does not demon-

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strate that activation of MAP kinase is a necessary step in the stimulation of DNA synthesis in the human adenoma cells. However, it does provide other evidence that GIP has an effect similar to ACTH on these cells.

These data pointed to the presence of functional ACTH and GIP receptors in the tumor cells. The genes of rat (16) and human (13) GIP receptor have been cloned. In the rat, GIP receptor RNA expression is not limited to β-cells of pancreatic islets; it is highly expressed in the brain and more weakly in different peripheral organs, including adrenal cortex, but it is not expressed in the spleen (16). RT-PCR amplification of GIP receptor mRNA demonstrated the presence of transcripts of this gene in the tumor, whereas the adjacent hypotrophic adrenal tissue and the pathological adrenals, either responsible for autonomous cortisol hypersecretion or linked to ACTH-dependent cortisol hypersecretion, did not appear to express this gene. GIP receptor RNA expression was also detected in normal human brain, but not in the spleen. Remarkably, different transcripts for the GIP receptor were detected in the tumor: the full-length transcript and smaller transcripts lacking exon 3, 4, or 9. This finding, which is similar to that made by Lacroix and collaborators (9), shows that in the tumor, the abnormal expression of the GIP receptor gene is associated with abnormal splicing of its mRNA. However the biological significance of the short transcripts is probably limited, at least concerning the transcript lacking exon 9, as this exon codes for a significant part of the putative transmembrane domain IV; the disruption of this domain is expected to profoundly alter the receptor function. *In situ* hybridization experiments confirmed that, as expected, the expression of the GIP receptor was restricted to the tumor adenoma cells. ACTH receptor gene was also expressed in the tumor cells, as shown by RT-PCR of ACTH receptor mRNA, although to a lesser degree than in the adjacent normal tissue. Such a low level of expression should, however, be sufficient to explain the response to pharmacological doses of 1–24 ACTH.

In conclusion, we have demonstrated that an adrenal adenoma responsible for food-dependent Cushing's syndrome is characterized by a high level of expression of functional GIP receptors, and we provide several results suggesting that expression of this receptor may play a role in the development of the tumor. First, the expression of the GIP receptor gene was restricted to the adenoma cells; it was not detected in the adjacent adrenal tissue, and it should also be absent in the remaining controlateral gland, which is not sensitive to GIP. Therefore, unlike in food-dependent Cushing's syndrome associated with bilateral hyperplasia (9), the genetic abnormality is not present in all of the adrenal cells of the patient and must result from a postzygotic event. The nature of this event remains to be defined, but it could result from a somatic mutation in a segment of DNA controlling GIP receptor gene expression. Secondly, GIP receptor expression can fully account for the peculiar food-dependent cortisol secretion of the tumor. Thirdly, stimulation of tumor cell DNA synthesis by GIP suggests that GIP receptor may also play a role in tumor cell development. Finally, all measured effects of GIP on the tumor cells were similar to the effects of ACTH. In particular, GIP induced an increase in cAMP production. This is in favor of an important role for activation of the ACTH signal transduction pathway in adrenal Cushing's adenoma development.

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