Rab18 Is Reduced in Pituitary Tumors Causing Acromegaly and Its Overexpression Reverts Growth Hormone Hypersecretion

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Context: Rab proteins regulate the sequential steps of intracellular membrane transport. Alterations of these GTPases and their associated proteins are emerging as the underlying cause for several human diseases involving dysregulated secretory activities.

Objective: Herein we investigated the role of Rab18, which negatively regulates hormone secretion by interacting with secretory granules, in relation to the altered functioning of tumoral pituitary somatotropes causing acromegaly.

Patients: A total of 18 patients diagnosed with pituitary tumors causing acromegaly (nine patients) or nonfunctioning adenomas (nine patients) underwent endoscopic transsphenoidal surgery. Adenomas were subsequently processed to evaluate Rab18 production in relation to GH secretion.

Results: We found that somatotropinoma cells are characterized by a high secretory activity concomitantly with a remarkably reduced Rab18 expression (15%) and protein content levels (30%), as compared with cells from nonfunctioning pituitary adenomas derived from patients with normal or reduced GH plasma levels (100%). Furthermore, immunoelectron microscopy revealed that Rab18 association with the surface of GH-containing secretory granules was significantly lower in somatotropes from acromegalies than nonfunctioning pituitary adenomas. Finally, we provide evidence that modulation of Rab18 gene expression can revert substantially the hypersecretory activity of cells because Rab18 overexpression reduced by 40% the capacity of cells from acromegalies to respond to GHRH stimulation.

Conclusion: These results suggest that molecular alterations affecting individual components of the secretory granule traffic machinery can contribute to maintain a high level of GH in plasma. Accordingly, Rab18 constitutes a valuable target as a diagnostic, prognostic, and/or therapeutic tool for human acromegaly. (***J Clin Endocrinol Metab* 93: 2269–2276, 2008**)

Hormone hypersecretion is the origin of a number of endocrine diseases, such as those produced by pituitary tumors that overproduce prolactin, GH, and ACTH, which cause hyperprolactinemia, acromegaly, and Cushing's disease, respectively. However, the molecular mechanisms underlying this hormone excess are not well understood. Acromegaly generally

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Abbreviations: GFP, Green fluorescent protein; NFPA, nonfunctioning pituitary adenoma; SN, secretoneurin.

arises from a pituitary somatotrope adenoma characterized by GH hypersecretion, which, in turn, stimulates hepatic IGF-I production (1). It has an estimated prevalence of 53.2 cases per million people and an average incidence of 3.06 new cases per million people per year (2, 3), representing the 13.2% of total pituitary adenomas (4). Somatic effects caused by acromegaly are derived from direct GH metabolic actions on their target tissues and indirect consequences as result of elevated levels of IGF-I in plasma (1). Therefore, GH and IGF-I levels were originally chosen as biochemical markers of the disease (5), and they are still widely used as such. Moreover, these biochemical parameters are also targets of pharmacological therapies aimed at alleviating acromegaly symptoms and normalization of GH and/or IGF-I secretion, which considerably reduces the risk of mortality (3, 6–9). In particular, drugs developed against acromegaly are, so far, directed to regulatory agents of somatotrope function, such as somatostatin analogs (10), dopamine agonists (11, 12), and GH receptor antagonists (13–15).

Currently little information is available on the molecular features characterizing acromegaly or other pituitary hypersecretory adenomas at a cellular level, especially with respect to the different intracellular components participating in the secretory pathway that may affect the secretory rate. In this context, the family of Rab GTPases represents an attractive group of proteins potentially involved in the pathophysiology of secretory diseases, as they coordinate the sequential steps of intracellular transport, such as vesicle formation, motility, and membrane fusion along the secretory pathway (16). In fact, loss-of-function mutations in Rab proteins or Rab regulatory proteins are known to be implicated in causing secretory diseases (17–21) and certain cancers (22–25). In this scenario, the Rab protein Rab18 may be of special interest because: 1) it is expressed in neuroendocrine cells, wherein it decreases hormone release by interacting with secretory granules (26), 2) its expression is down-regulated in cells displaying high secreting phenotype (27), and 3) its overexpression in neuroendocrine cells results in inhibition of secretory activity (26). Accordingly, in the present work, we analyzed the secretory activity of somatotrope cells derived from pituitary adenomas causing acromegaly and have examined its relationship with the level of Rab18 expression and protein content.

Patients and Methods

The study was carried out in accordance with the Declaration of Helsinki and approved by the Reina Sofia University Hospital (Cordoba, Spain), Virgen del Rocio Hospital (Sevilla, Spain), and Sant Pau Hospital (Barcelona, Spain) Ethics Committees. Written informed consent was obtained from each patient.

Patients

This study included analysis of pituitary samples from nine patients with acromegaly and nine patients bearing nonfunctioning pituitary adenomas (NFPA). Patients chosen for this study were consecutive subjects from three different hospital centers. Patient profiles are shown in Table 1. All nine patients with acromegaly were subjected to 30 mg octreotide treatment, alone (AC1, AC8, and AC9) or in combination with 120 mg lanreotide (AC2, AC3, AC4, AC5, and AC6) or 15 mg pegvisomant (AC7), once monthly during 3–9 months prior to surgical intervention.

Reagents

The following materials were obtained from the indicated sources: anti-Rab18 antibody was from Calbiochem (Barcelona, Spain); anti-GH antibody was from MorphoSys (Martinsried, Germany); PowerScript reverse transcriptase from CLONTECH (Palo Alto, CA); SYBR Greentag DNA polymerase from Bio-Rad (Madrid, Spain); normal human pituitary total RNA samples from CLONTECH (Palo Alto, CA) and Clinisciences (Montrouge, France); phrGFP-N1 reporter plasmid from Stratagene (La Jolla, CA); FM5–95 from Molecular Probes (Barcelona, Spain); Lipofectamine 2000 reagent, OPTI-MEM, and TRIZOL reagent from Invitrogen (Barcelona, Spain); and Araldite Luft 502 from Poly-

TABLE 1. Preoperative features, tumor description, and immunocytochemistry of nine pituitary samples from patients suffering acromegaly (AC) and nine patients with nonfunctioning adenomas (NF)

	Serum GH levels (μg/liter)	Serum IGF-I levels (mg/liter)	Tumor description	Tumor diameter (mm)	Immuno-histochemistry GH
AC1	4.4	748	Macroadenoma	15	97.2%
AC2	28.5	1120	Macroadenoma	10	90.5%
AC3	3.2	638	Macroadenoma (infrasellar expansion)		ND
AC4	28.1	>600	Microadenoma	8	ND
AC5	7.1	651	Macroadenoma	15	ND
AC6	8	>600	Microadenoma	7	98.0%
AC7	75.9	1357	Macroadenoma	25	97.7%
AC8	72	999	Macroadenoma	15	98.9%
AC9	36	349	Macroadenoma	15	97.3%
NF1	0.32	308	Macroadenoma (suprasellar expansion)	30	0.6%
NF2	<0.1	31	Macroadenoma (suprasellar expansion)	37	5.8%
NF3	0.17	72	Macroadenoma	48	0.7%
NF4	0.2	157	Macroadenoma (suprasellar expansion)	20	0.7%
NF5	0.2	101	Macroadenoma (suprasellar expansion)	25	1.0%
NF6	<0.1	118	Macroadenoma (suprasellar expansion)		ND
NF7	<0.1	138	Macroadenoma	14	0.7%
NF8	0.2	342	Macroadenoma (suprasellar expansion)	30	ND
NF9	0.1	135	Macroadenoma (suprasellar and parasellar expansion)		ND

ND, Not determined.

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sciences (Northampton, UK). All other reagents were purchased from Sigma-Aldrich Corp. (Madrid, Spain).

Cell dispersion and culture

Pituitary adenomas were removed by endoscopic trans-sphenoidal resection and either snap frozen in liquid nitrogen immediately after removal or transferred to sterile cold (4 C) culture medium (DMEM) containing 0.1% BSA, 2 mM L-glutamine, 100 U/ml penicillin, and 24 mM HEPES. Those samples kept in culture medium were minced under sterile conditions into 1- to 2-mm^3 pieces, which were either snap frozen for total RNA isolation or fixed and processed for subsequent electron microscopy studies as described below. Remaining tissue samples underwent an enzymatic and mechanical dispersion procedure and dispersed cells were cultured as described elsewhere (28). Cell viability was always higher than 92%.

PCR amplification

RNA isolation was carried out by using the TRIZOL reagent following the manufacturer's recommendations. Three-microgram total RNA was used for RT-PCR with a PowerScript reverse transcriptase kit and 150 ng random primers. Subsequent real-time PCR was performed using an iCycler IQ PCR detection system (Bio-Rad) and quantifying SYBR Green-tagging. The specific primers used for human Rab18 were 5′-CCCTGAAGATCCTCATCATCGG-3′ sense and 5′-CCTCTCTTGACCAGCAGTATCCCA-3′ antisense, which amplify a 185-bp fragment. As endogenous reference gene, the hypoxanthine phosphoribosyl transferase gene was amplified in parallel to Rab18 with the primer set 5′-AGGCCATCACATCGTAGCCCTCT-3′ sense and 5′-TCGCCCGTTGACTGGTCATTACA-3′ antisense, yielding an amplification product of 165 bp. Amplification profiles were analyzed off-line with the iCycler IQ Optical System software 3.1 (Bio-Rad).

Immunoelectron microscopy

Samples were processed as previously described (28). In brief, tissues were fixed with 1% glutaraldehyde/2% paraformaldehyde in 0.1 M cacodylate buffer for 2 h at 4 C and postfixed with 1% OsO_4 in 0.1 M cacodylate buffer (1 h, 4 C). Samples were then dehydrated and embedded overnight in Araldite Luft 502 (Polysciences). Ultrathin sections of specimens were prepared and incubated with anti-Rab18 at 1:500 and a 10-nm gold-conjugated antirabbit IgG at 1:100, and, on the other side of the nickel grid, anti-GH at 1:1000 and a 6-nm gold-conjugated antirabbit IgG at 1:100. Samples were examined under a CM-10 electron microscope (Philips, Madrid, Spain).

Immunofluorescence microscopy

Dispersed cells were plated on poly-L-lysine-coated, 25-mm coverslips, fixed with 4% paraformaldehyde in phosphate buffer (PBS) (pH 7.4) for 15 min, washed thoroughly, and incubated in PBS containing 0.3% Triton X-100 and 1% BSA for 1 h. Then cells were exposed to rabbit antirat Rab18 antiserum (1:1000) overnight at 4 C and Alexa594-conjugated goat antirabbit secondary antibody (1:500) for 2 h at room temperature.

Fluorescence intensity in single cells was monitored using a fluorescence microscope (Zeiss, Göttingen, Germany) fitted with a Plan-Fluar ×100 objective. The slides were epiilluminated at 540 nm for 2 sec. Image acquisition was controlled using a CoolSNAP charge-coupled device camera (Roper Scientific, Tucson, AZ) and acquisition parameters kept strictly constant for all images captured. Quantification of fluorescence intensity in single cells was carried out using ImageJ 1.32 software (National Institutes of Health, Bethesda, MD). Regions of the same field devoid of cells were selected for monitoring of the background.

Evaluation of single-cell secretory activity

For immunoblot experiments, we followed the protocol previously reported (29) with slight modifications. Briefly, 50-μl DMEM droplets containing approximately 5000 dispersed pituitary cells were applied to pieces of polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were incubated 24 h at 37 C in a 5% CO_2 atmosphere and then fixed for 1 h with Bouin's solution and thoroughly rinsed with PBS. Thereafter membranes were incubated in the anti-GH antibody at 1:2000 followed by the rabbit ExtrAvidin peroxidase staining kit (Sigma-Aldrich) as indicated by the manufacturer. Specificity of the immunoreaction was tested by: 1) omission of the specific antibody and 2) preadsorption of the primary antibody with 2×10^{-6} M human GH.

Staining intensity and area of the halo formed around single cells were monitored using an inverted microscope (Eclipse TE2000-E; Nikon, Tokyo, Japan) fitted with a Plan-Fluar ×60 objective. Membranes were epiilluminated for 3 sec and random images captured using a charge-coupled device camera running in one-binning mode. Image acquisition parameters were kept constant using MetaMorph PC software (Universal Imaging Corp., West Chester, PA). Before measuring each set of preparations, the condenser was positioned in the optimal conditions to ensure that the illumination of the object plane was even and homogeneous. Quantification of halo intensity and area was assessed by ImageJ software and expressed as the OD per pixel (2). Regions of the membrane devoid of cells were randomly chosen in each image to estimate background levels. To avoid small variations on the OD due to factors such as illumination or focusing, each set of membranes was measured during the same session.

Rab18 transfection experiments

Cells were plated onto poly-L-lysine-coated coverslips, transfected with a vector coding for green fluorescent protein (GFP)-Rab18 by adding 0.8–1 μg of the reporter construct and 3 μl Lipofectamine 2000 per plate and incubated in OPTI-MEM at 37 C, 5% CO_2 for 24 h. Then cells were rinsed with DMEM supplemented with 20 mM $NaHCO_3$ and 1 mM HEPES and exposed to the fluorescent dye FM5–95 (2 μM) for 5 min at 37 C. Coverslips were placed on the temperature-controlled stage of the fluorescence microscope fitted with a Plan-Fluar ×60 oil immersion objective. Fields containing transfected and nontransfected cells were selected. Cells were epiilluminated at 540 nm for 100 msec every 1 min for 30 min. Images were acquired with a charge-coupled device camera (ORCA-BT-1024G; Hamamatsu, Hamamatsu City, Japan) controlled by Metafluor, and fluorescence emissions were monitored over time. Regions of the same field devoid of cells were selected for continuous monitoring of the background, which was subtracted from the specific signal profile off-line.

To study the possible relationship between Rab18 expression level and the secretory granule pool size, dispersed somatotrope adenoma cells were transfected with the vector coding for GFP-Rab18. Preparations were then subjected to the fluorescence immunostaining protocol explained above using an antibody against the secretory granule marker secretoneurin (SN; 1:1000). Subsequently samples were placed under the fluorescence microscope and observed with a Plan-Fluar ×60 objective; a dichroic with an exciter filter at 495 nm; a bandpass emitter filter comprised between 525 and 550 nm for GFP visualization; and a dichroic with an exciter filter at 565 nm and a long-pass emitter filter at 640 nm (Chroma Technology Corp., Rockingham, VT). This filter setup together with the corresponding emission signal obtained with the two filters from cells either expressing GFP or immunostained with Alexa 594 warrant no overlapping of one channel over the other. Cells expressing GFP-Rab18 were randomly chosen and emission signals from GFP and Alexa594 recorded after excitation for 800 msec. Image acquisition was controlled by MetaMorph (Universal Imaging). Images were stored and fluorescence intensity calculated off-line with ImageJ 1.32 (National Institutes of Health).

Results

Pituitary adenomas from patients with acromegaly exhibit reduced Rab18 mRNA levels and protein content

First, using quantitative RT-PCR, we evaluated the amount of Rab18 transcripts in tumors from patients with acromegaly,

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which, as revealed by immunocytochemistry, are mainly composed of GH-immunopositive cells (96.01 ± 1.52%; n = 6 cases), and compared it with that observed in NFPA, which show a percentage of GH-immunopositive cells more variable, ranging from 0.55 to 5.80% (mean 1.57 ± 0.93%; n = 6 cases) and comprise a significant proportion of α-subunit immunoreactive cells (data not shown). As an additional control, normal human pituitary mRNA extracts from two commercial suppliers were used. We found that, individually, all acromegalic tissue samples analyzed exhibited Rab18 gene expression levels clearly below those found in normal pituitary samples (Fig. 1A, *top panel*). Quantitatively, Rab18 mRNA levels in somatotropinomas were 60–95% lower than in the normal pituitary samples. On average, the value obtained for tumor samples from acromegalic patients was one third of the Rab18 expression level measured in normal human pituitary (Fig. 1A, *top panel*, *inset*). Contrarily, in NFPA from patients with normal or even reduced plasma GH levels (see Table 1), Rab18 expression was always above control values, except in one case (*i.e.* NF5), in which Rab18 gene expression was 19% lower (Fig. 1A, *bottom panel*). When the data from the different NFPA analyzed were pooled together, the average Rab18 mRNA expression value was 50% higher than in nontumoral pituitary tissue extracts, although no statistical difference was observed (Fig. 1A, *bottom panel*, *inset*).

Rab18 protein content was evaluated by densitometric quantification of Rab18-immunostained cells from these adenomas. As shown in Fig. 1B, densitometric quantification of Rab18 immunolabeling in single cells revealed that somatotropinoma cells contain approximately 3-fold less signal than nonfunctioning adenomatous cells ($P < 0.001$; n = 79 and 57 cells from somatotropinoma and NFPA, respectively).

A comparative analysis of Rab18 mRNA levels in somatotropinoma cells in relation to GH plasma levels from each individual acromegalic patient revealed that Rab18 expression in pituitary tissues is inversely correlated to GH levels in plasma (Pearson = −0.529). Indeed, tissues from acromegalic patients exhibiting highest concentrations of GH in serum presented lowest Rab18 expression levels and vice versa (Fig. 1C).

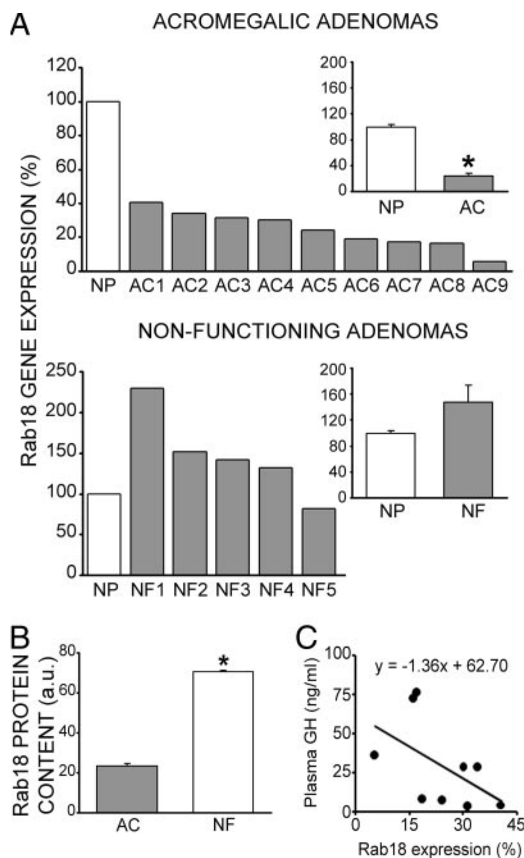

FIG. 1. Rab18 gene expression and protein content in tissues from acromegalic patients. A, Quantitative RT-PCR analysis of Rab18 mRNA levels in somatotropinoma samples (*top panels*) and NFPAs (*bottom panels*) from the patients (*gray bars*), compared with those obtained from commercial samples of normal human pituitary (*open bars*). The hypoxanthine phosphoribosyl transferase gene was used as endogenous reference gene and amplified in parallel to Rab18. *Insets* illustrate the average values of all individual tumor samples analyzed. B, Comparison of Rab18 protein content in single cells from somatotropinoma and nonfunctioning pituitaries as estimated by immunofluorescence staining. C, Correlation analysis of Rab18 mRNA level obtained from each acromegalic tumor and GH plasma concentration exhibited by the corresponding patient. NP, Normal pituitary; AC, acromegaly; NF, nonfunctioning pituitary tumor. *, $P < 0.001$ *vs.* Rab18 gene expression in NP (A) or Rab18 protein content in AC (B).

Rab18 localizes to the membrane of secretory granules in somatotropes

Electron microscopy revealed that all the somatotropinoma tissues analyzed, but one, are composed of densely granulated cells with a highly developed rough endoplasmic reticulum (data not shown). In these cells, as well as in cells from nonfunctioning adenomas, Rab18 immunoreactivity was detected in close apposition to the surface of a discrete population of GH-containing granules (Fig 2, A and B, respectively), which represented 29.4 ± 2.8 and 58.0 ± 5.2% (from 78 and 63 cells, respectively) of the total granule content per cell in somatotropinomas and NFPAs, respectively (Fig. 2C).

Secretory activity of individual somatotropes from pituitary samples of acromegalic patients is enhanced, compared with that of somatotropes from NFPA

Figure 3A depicts representative examples of nitrocellulose immunoblot membranes with pituitary cells from a patient with acromegaly (*top panel*) or NFPA (*bottom panel*) immunostained with the anti-GH antiserum. As shown, both staining intensity of the halo and the magnitude of the halo area were clearly higher in the former than in the latter. Likewise, the percentage of GH-immunopositive cells is also appreciably larger in the sample from acromegaly than from NFPA. No immunoreaction for GH was observed when the primary antibody was omitted or preadsorbed with human GH (data not shown).

Densitometric analysis confirmed that GH immunoreactivity of the halo area in single somatotrope cells from each one of the acromegalies analyzed in this study was significantly higher than that shown by somatotrope cells from all the NFPA investigated. On average, both staining intensity of the halo and halo area were 2.9- and 1.6-fold higher in somatotrope cells from acromegalic patients than in somatotrope cells from nonfunctioning adenomas, respectively (Fig. 3, B and C). These results indicate that, under basal conditions, the secretory activity of single so-

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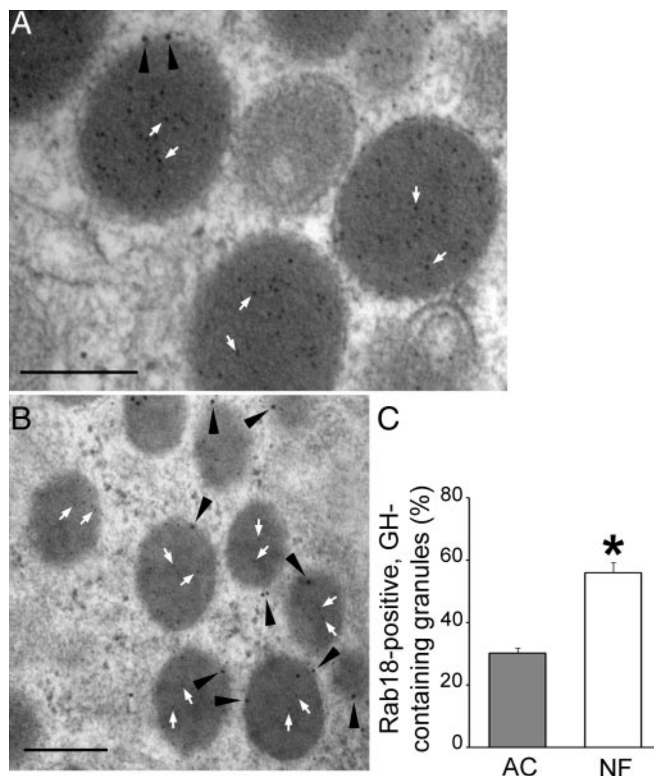

FIG. 2. Rab18 intracellular localization in somatotrope cells from acromegalies and NFPA. (A and B) Representative immunoelectron micrographs of somatotrope cells from an acromegalic patient (A) and a patient with NFPA (B) immunolabeled for Rab18 (visualized with 10 nm gold particles; *black arrows*) and GH (revealed with 6 nm gold particles; *white arrows*). Rab18 immunoreactivity appeared associated to the membrane of a subset of GH-containing secretory granules. *Scale bars*, 100 nm. C, Percentage of Rab18-immunopositive, GH-containing secretory granules in cells from GH-secreting acromegalic (*gray bar*) and NFPAs (*open bar*). AC, Acromegaly; NF, nonfunctioning pituitary tumor. *, $P < 0.001$ *vs.* percentage of Rab18-immunopositive secretory granules in AC.

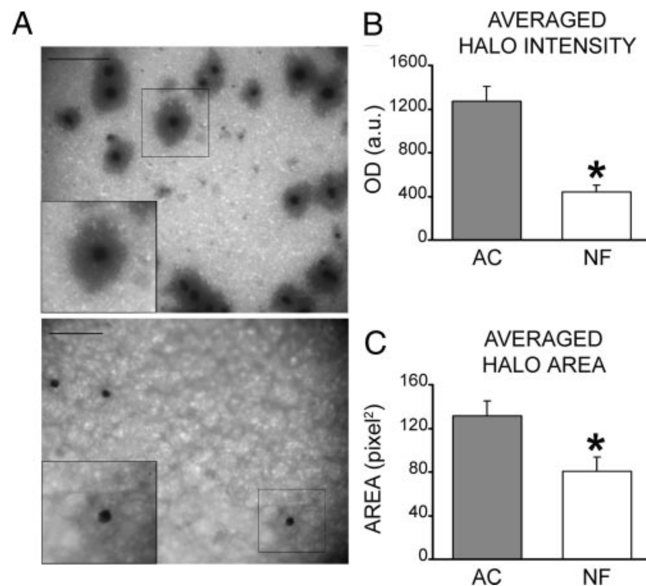

FIG. 3. Comparison of the GH secretory rate exhibited by individual somatotropes from acromagalic patients and NFPAs. A, Representative images of dispersed cells from somatotropinoma (*top panel*) and nonfunctioning pituitary tissues (*bottom panel*) cultured on nitrocellulose membranes and immunostained for GH. *Scale bars*, 200 μm. B and C, Averaged halo immunostaining intensity (B) and halo area (C) of somatotrope cells from acromegalic (*gray bars*) and nonfunctioning pituitary tumors (*open bars*). Both parameters were significantly higher in somatotrope cells from acromegalies than from NFPAs. AC, Acromegaly; NF, nonfunctioning pituitary tumor. *, $P < 0.001$ *vs.* AC.

matotropes from tumors causing acromegaly is higher than that displayed by individual somatotrope cells from NF adenomas.

Overexpression of Rab18 reduces the secretory activity of single somatotropinoma cells

Having established that somatotropes from tumors causing acromegaly display a clear hyperactivity in terms of hormone secretion at the individual cell level, we investigated whether manipulation of Rab18 gene expression in these cells could revert or modulate their hypersecretory state. To answer this question, we carried out a series of experiments aimed at monitoring in real time the basal and GHRH-stimulated secretory activity of individual somatotropinoma cells previously transfected with the vector coding for the GFP-Rab18 protein, and compared this effect with that induced in nontransfected cells. For these experiments, we used a highly lipophilic, fluorescent marker (*i.e.* FM5–95), which has been extensively used for evaluating the secretory dynamics of single cells over time (for review see Ref. 30). Using this methodological approach, we found that somatotropinoma cells expressing GFP-Rab18 showed reduced incorporation of FM5–95 in response to 10^{-8} M GHRH, compared with the fluorescence incorporation profile displayed by nontransfected cells selected within the same microscopic field (Fig. 4A). Quantitatively, the rate of FM5–95 uptake calculated as the area under the curve that encompasses from the beginning of the GHRH pulse to the last time point recorded, resulted 40% lower in GFP-Rab18-expressing cells than in nontransfected cells (Fig. 4A, *inset*; n = 44 and 23 nontransfected and GFP-Rab18-transfected cells, respectively, obtained from seven different patients). As control, we used rat GHRH-R-expressing GH3 cells mock transfected with a vector coding for GFP alone following the same experimental protocol that for somatotropinoma cells. These experiments revealed that GFP-Rab18-transfected GH3 cells show a reduced secretory capacity in response to 10^{-8} M GHRH (100.00 ± 14.43 *vs.* 75.11 ± 8.01; n = 17 and 10 nontransfected and GFP-Rab18 transfected cells, respectively, $P < 0.05$), whereas secretory activity of GFP-transfected cells was unaltered, compared with nontransfected GH3 cells (100.00 ± 4.67 *vs.* 108.88 ± 10.53; n = 19 and 7 nontransfected and GFP-transfected cells, respectively, $P > 0.05$), thus indicating that this effect on the secretory response is not due to a side effect of the transfection procedure. In summary, these data are in accordance with those previously reported on the inhibitory effect that Rab18 exhibits on the secretory activity of neuroendocrine cells (26).

In line with this observation, overexpression of Rab18 in somatotrope cells from acromegalic patients also induced an accumulation of secretory granules within the cells. More specifically, combined analysis of Rab18 content (expressed as GFP fluorescence intensity in single cells) and the intracellular concentration of the secretory granule marker SN (expressed as the intensity of SN immunoreactivity in those cells overexpressing GFP-Rab18) (31, 32) yielded a direct correlation (Fig 4B; n = 56

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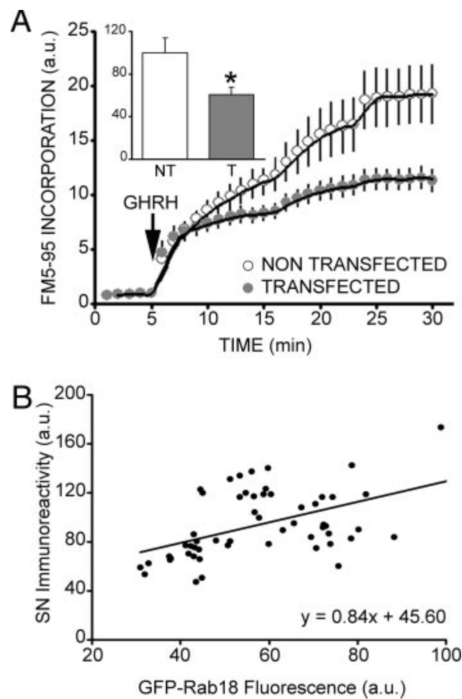

FIG. 4. Effect of Rab18 overexpression on the secretory activity of individual somatotropinoma cells. A, Time-course incorporation of FM5–95 in cells from acromegalic patients. FM5–95 uptake was estimated every 1 min for a total of 30 min. After collecting a 5-min baseline, cells were exposed to 10^{-8} M GHRH. After 25 min in the presence of the stimulatory factor, the secretory response was 40% lower in Rab18-overexpressing cells (○) than nontransfected cells (●). *Panel inset* corresponds to the averaged area under the curve of FM5–95 incorporation after treatment in nontransfected cells (NT) and GFP-Rab18-transfected cells (T). *, $P < 0.001$ *vs.* nontransfected cells. B, Correlation analysis of Rab18 overexpression rate (estimated as GFP fluorescence intensity) and secretory granule pool size (estimated as SN immunoreactivity) in individual GFP-Rab18-transfected somatotropinoma cells.

cells; Pearson = 0.512). Thus, those cells exhibiting higher expression of Rab18 also showed a more abundant secretory granule pool size. This finding further supports the inhibitory role of Rab18 on secretion in somatotropinoma cells because its overexpression causes secretory granule accumulation within the cells.

Discussion

It is well known that acromegaly is associated to high circulating levels of GH as the result of somatotrope hyperplasia (1). However, to date it remains unclear whether individual somatotropinoma cells also exhibit an altered secretory activity that would contribute to the GH hypersecretory tone. In this scenario, other and our electron microscopy studies have demonstrated that, in those cases in which somatotropinoma cells from acromegalic patients are densely granulated, these cells display morphological features consistent with a hypersecretory state (*i.e.* prominent rough endoplasmic reticulum and Golgi complex) (33). Accordingly, in the present work, we also demonstrated that individual somatotropinoma cells from acromegalic patients do exhibit GH secretory rate significantly higher than that displayed by single GH-immunopositive cells from pituitary samples obtained from patients with normal or even low concentrations of circulating GH and bearing NFPA. These data suggest that the pathological alterations leading to GH hypersecretion may also have a molecular component intrinsic to each individual somatotropinoma cell. This could arise from dysfunctions in critical points of control within intracellular machineries such as signal transduction, gene expression, and hormone synthesis and/or transport.

Rab GTPase proteins have been considered as intracellular membrane organizers because they regulate vesicle trafficking pathways by behaving as membrane-bound molecular switches (16). Specifically, Rab18 is a scarcely explored member of the Rab family of proteins, which was previously identified in virtue of its preferential expression in endocrine cells exhibiting a low-secreting phenotype. In human, Rab18 expression has been reported in all the normal tissues analyzed, such as testis (34), endothelium, heart, kidney, liver, or brain (35) as well as in tumoral tissues, such as medulloblastoma (36). In the current work, we show that Rab18 is also expressed in both normal and tumoral human pituitary, thus supporting a role for this small GTPase in the functioning of this gland. Furthermore, our double immunocytochemical studies revealed that pituitary somatotropes do contain Rab18, which extends previous results on the expression of Rab18 in other pituitary cell types from other species, including both primary cells and cell lines (26). In these cell models, Rab18 was found in association to the surface of a discrete population of secretory granules (26), an observation that has been proven to be also the case in human somatotropes. Interestingly, the proportion of Rab18-immunolabeled secretory granules was consistently lower in somatotropes from acromegalic patients than in those from patients with nonfunctioning adenomas with normal GH circulating levels. This is in accordance with the lower Rab18 protein content observed by immunofluorescence in single pituitary cells from the former patients. Furthermore, Rab18 mRNA levels were also lower in pituitary adenomas from acromegalic patients, compared with those from either normal pituitary tissues or NFPA.

In line with these findings, association of abnormal Rab levels to human pathologies has been already reported. For example, mutations in Rab27A result in Griscelli's syndrome, caused by defects in melanosome transport in melanocytes and loss of cytotoxic killing activity in T cells (18). We do not know yet the mechanisms responsible of the low Rab18 levels found in acromegaly. In other cell models, such as human umbilical vein endothelial cells (35) and ACTH-producing AtT20 cells (26), regulatory factors such as histamine or CRH have been shown to up-regulate Rab18 mRNA expression in a rapid and specific manner. In these scenarios, it is tempting to propose that hypothalamic factors regulating GH secretion such as somatostatin, and thus, its synthetic analogs commonly used for the treatment of acromegaly (37) could act, among other levels, to modulate Rab18 gene expression in these cells. Future studies will be aimed at elucidating the specific extracellular regulation to which Rab18 gene expression and function is subjected in human somatotrope cells under both physiological and pathological conditions.

Previously we have shown that Rab18 mRNA and protein

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levels are inversely correlated to the secretory activity of neuroendocrine cells. In these cells, Rab18 functions as a molecular brake of secretory granule traffic, thus provoking accumulation of secretory granules and reducing the secretory activity of individual cells (26). In the current work, we have shown that highly secretory somatropes exhibit lower levels of Rab18, which supports the view that lack of this GTPase may be responsible, at least in part, for the hypersecretory activity exhibited by somatotropinoma cells. In accordance with this, our data show that Rab18 expression levels in samples from individual acromegalic patients and the amount of GH circulating in plasma from the same corresponding patients were inversely correlated. Thus, pituitary samples from those patients exhibiting higher levels of GH in plasma presented lower Rab18 mRNA content and vice versa. Furthermore, our results on individual somatotropinoma cells indicate that the hypersecretory activity shown by these cells can be reverted substantially by overexpression of Rab18, thereby suggesting that modulation of Rab18 production may represent an important point of control within the process of hormone secretion. These results, coupled with our finding that overexpression of Rab18 in somatotropinoma cells induces accumulation of secretory granules, invite speculation that low Rab18 content in these cells results in the partial loss of control on the molecular mechanism that retains a particular subset of the secretory granules from being released under basal conditions and/or on stimulatory inputs.

In the current medical management of acromegaly, pharmacotherapeutic agents commonly used (*i.e.* somatostatin analogs octreotide, lanreotide, *etc.*) are based on their ability to activate surface receptors in somatotropes that ultimately results in the normalization of GH secretion rate. Notwithstanding this, the molecular mechanisms underlying a sustained reduction of GH secretion remain to be elucidated. In the present work, we provide compelling evidence supporting that Rab18 participates in the intrinsic control of the secretory activity of somatotropinoma cells. To date, there is still little information on the molecular alterations that affect the secretory activity of cells from hypersecretory diseases. In this context, the current study could pave the way for exploring new therapeutic approaches aimed at controlling GH hormone hypersecretion in acromegaly from a molecular point of view. Future studies will be aimed at elucidating the effects that drugs used in the treatment of acromegaly might elicit on Rab18 and how this translates into GH hypersecretion.

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