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The neuroendocrine protein VGF is sorted into dense-core granules and is secreted apically by polarized rat thyroid epithelial cells

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Abstract

We have expressed the neuroendocrine VGF protein in FRT rat thyroid cells to study the molecular mechanisms of its sorting to the regulated and polarized pathways of secretion. By immunoelectron microscopy, we have demonstrated that VGF localizes in dense-core granules. Rapid secretion of VGF is induced by PMA stimulation. Moreover, human chromogranin B, a protein of the regulated pathway, co-localizes in the same granules with VGF. In confluent, FRT monolayers on filters protein secretion occur from the apical cell domain. VGF deletion mutants have been generated. By confocal microscopy, we have found that in transient transfection, all mutant proteins are sorted into granules and co-localize with the full-length VGF. They all retain the apical polarity of secretion. We also found that intracellular VGF and its deletion mutants are largely in an aggregated form. We conclude that FRT thyroid cells correctly decode the sorting information of VGF. The signals present on the protein to enter the granules and to be secreted apically cannot be separated from each other and are not in just one discrete portion of the protein. We propose that selective aggregation might represent the signal for sorting VGF to the regulated, apical route.
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Introduction

Endocrine cells deliver secretory proteins to the plasma membrane either by a constitutive or by a regulated pathway [1]. The constitutive pathway is part of the homeostatic maintenance of cells and is in fact common to all cell types. The regulated pathway is instead a specialized process by which proteins are packaged into long half-life granules that are stored within the cells and are secreted following specific extracellular signals. Segregation of proteins into secretory granules requires a molecular machinery that recognizes signal(s) on the regulated proteins and separates them from constitutive proteins. The sorting process occurs at the level of the TGN and it might continue in the immature secretory granules [2]. Morphological and biochemical evidences suggest that a key step for the sorting of regulated proteins at the TGN level is their selective aggregation, which appears to depend on mildly acidic pH and on millimolar Ca^{2+} concentrations [3–5]. Sorting signals, consisting of specific motifs in the protein sequence, also play a role in the process of segregation of proteins into secretory granules [6–13]. Individual endocrine cell types have apparently developed multiple mechanisms to sort a variety of regulated secretory proteins [14].

In endocrine cells, as well as in all epithelial cells, protein secretion besides being constitutive or regulated is also polarized and occurs through specific domains of the plasma membrane [15]. In epithelial cells, the apical plasma membrane is separated from the basolateral one and distinct groups of proteins are destined to either domain. To target proteins to one domain or the other of the plasma membrane, sorting signals on the protein itself are needed [16,17]. While some information is available on the sorting signals responsible for transmembrane protein targeting to the plasma membrane, little is known on the sorting determinants of soluble proteins. It has been proposed that N-glycans may act as signals to target proteins to the apical cell domain [18]. An alternative model, in which glycans only play an indirect structural role, has also been proposed [19].

Polarized cells should be able to recognize, in principle, a dual sorting information on regulated proteins: one to segregate them to secretory granules, another to sort them to the specific plasma membrane domain. To study both sorting mechanisms, an in vitro model system consisting of polarized cell able to express a regulated pathway of secretion might be useful. Several proteins of the regulated pathway have been expressed in MDCK-polarized epithelial cells [20]: they did not show formation of storage organelles nor polarized secretion. Moreover, the polarity of secretion of several regulated secretory proteins: the neurotrophins NT-3, BDNF and NGF, and of the chromogranin A (CgA) has been investigated in the same cell line. It has been demonstrated that while the neurotrophins have a basolateral polarity [21], CgA has an apical polarity of secretion that is reversed upon tunicamycin treatment [22]. In a different study, it has been demonstrated that CgA has the specific property to induce dense-core granule formation [23]. Another report indicates that von Willebrand factor, when expressed in MDCK cells, can be stored in secretory granules and can be secreted by a regulated pathway [24].

In the present study, we have investigated the sorting process of the VGF neuroendocrine protein in polarized thyroid follicular cells. VGF is a protein originally identified in the rat pheochromocytoma PC12 cell line where its transcription is induced by NGF [25] and its secretion is promoted by a variety of stimuli [26]. VGF is rich in charged, acidic amino acid residues, has many proline and glutamic acid clusters, has a low isoelectric point [26,27] and has a high heat-stability (Gentile et al., unpublished results). Based on these properties and on its tissue distribution, it appears to belong to the granin family of proteins [28]. VGF has been found by immunocytochemistry in various neuronal groups, including primary sensory and enteric neurons, and in endocrine cells of the adrenal medulla, adenohypophysis, gut and pancreatic islets [29]. In different cell types, the protein is processed in a post-Golgi compartment, is packaged in large dense-core vesicles, and follows a regulated pathway of secretion [30,31].

We have stably expressed VGF in the polarized FRT epithelial cell line derived from Fischer rat thyroid [32]. We demonstrate here that FRT thyroid cells recognize the sorting signals of the neuroendocrine VGF protein: they form dense-core granules and they secrete VGF with an apical polarity. We also show that VGF deletion mutants follow the same pathway as the full-length VGF protein suggesting that sorting signals to enter the secretory granules and to be sorted to the apical domain might be superimposed and not in just one discrete region of the protein.

Materials and methods

Cell culture

FRT cells and the generated stable clones expressing VGF and VGF mutants were cultured in Falcon tissue culture plastic dishes (Becton Dickinson Labware, Lincoln Park, NJ) at $37°C$, in humidified atmosphere containing 5% CO_2 and 95% air. The culture media used has been Coon's modified Ham's F12 (either Sigma Co. or Euro Clone ltd, UK). Penicillin, streptomycin (Gibco BRL Life Technologies, Paisley, UK) and, only for the transfected cells, 500 µg/ml of G418 (Gibco BRL Life Technologies) were added to the media. Cells cultured on filters in bicameral system were seeded at confluence (ca. 2×10^6 cells/dish) onto Costar Transwell 24 mm diameters (Corning Costar Corporation, Cambridge, MA). Transepithelial resistance was routinely measured with a Millicell ERS apparatus (Millipore Corp., Bedford, MA).

Antibodies

A rabbit antiserum against a C-terminal peptide VGF (573–617) [31] was used for most immunofluorescence (1:1500) and immunoblotting (1:3000) experiments. An N-terminal policlonal antiserum against the peptide VGF(4–240) was also used for some immunofluorescence experiments [33]. The mouse monoclonal antibody anti-human CgB (HCgB) has been described [34]; mAb anti-c-*myc*, 9E10, was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); mouse monoclonal antibody, ME 20.4, against the ectodomain of the human neurotrophin receptor p75^{NTR}, was a kind gift of André Le Bivic (Marseilles, France); the anti-bip antibody SpA-827 was from StressGene (Victoria, BC, Canada); mouse monoclonal antibody against TGN38 was from Affinity BioReagents, Inc. (Golden, CO, USA); policlonal goat anti-rab7 was from Santa Cruz Biotechnology, Inc.; mouse monoclonal antibody anti-EEA1 was from BD Transduction Laboratories (Lexington, KY); horse radish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from

Amersham (Buckinghamshire, UK); rhodamine or fluorescein-tagged goat anti-mouse or anti-rabbit secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

Plasmids and transfections

Full-length VGF expression vector, CMV-VGF, was obtained by subcloning VGF cDNA, a PvuII–XbaI fragment of the VGF8a genomic plasmid [26], downstream the CMV promoter in pcDNA.3 vector (Invitrogen, Groningen, the Netherlands). Expression vectors encoding VGF myc-tagged deletions were constructed based on the pSecTag2 plasmids (Invitrogen), using restriction sites present in VGF cDNA. The pSecTag2 vectors (frame A–B–C) provide an N-terminal leader sequence for the translocation into the RER and a C-terminal myc epitope. The signal peptide of VGF, which is predicted to consist of residues 1–22 of the protein [35], has not been inserted in the vectors. Each construct was named according to the residues of VGF that are fused in frame with the leader and the tag, respectively. For convenience, the PmlI site of VGF cDNA located 18 bp upstream the stop codon was converted into an *Eco*RI site. The following constructs were generated: VGF67-611myc: an *Eco*RI–*Eco*RI fragment of VGF cDNA (aa residues 67–611) subcloned in the *Eco*RI site of pSecTag2-B; VGF80-450myc: a BamHI fragment of VGF cDNA (aa residues 80–450) subcloned in the *Bam*HI site of pSecTag2-A; VGF80-285myc: an *Xho*I fragment deletion (aa residues 286–450) of VGF80-450myc vector, filled-in and re-ligated; VGF286-450myc: the *Xho*I fragment (aa residues 286–450) of VGF80-450myc subcloned in the *Xho*I site of pSecTag2-C; VGF450-611myc: a *Bam*HI–*Eco*RI fragment of VGF cDNA (aa residues 450–611) subcloned in *Bam*HI–*Eco*RI sites of pSecTag2-A. pRSV-NEO and pRSV-HYGRO have been previously described [36]. The vector expressing the HCgB gene has been described [37]. The vector expressing the secretory form of p75^{NTR} (p75^{NTR}sec) [38] was obtained from André Le Bivic. Plasmid DNA was purified by Qiagen cartridges (QIAGEN GmbH, Hilden, Germany).

Stable clones of FRT cells were obtained by transfections with the calcium phosphate technique as described [39]. Transient transfections were performed directly on glass coverslips (12-mm diameter) with 2 µg of DNA and 5 µg of Lipofectin (Gibco BRL Life Technologies) as described [40].

SDS PAGE and Western blot

Cultured cells were washed two times with PBS, lysed in 500 µl of ice-cold TBS (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 1% Triton-X100 and a mixture of protease inhibitors (leupeptin, antipain, pepstatin and apro-

tinin) for 40 min on ice, scraped and collected in Eppendorf tubes. Media were collected and stored on ice in Eppendorf tubes after addition of the same mixture of protease inhibitors. All the samples were centrifuged for 5 min at 4°C in an Eppendorf centrifuge at 14,000 rpm (20800 rcf). The pellets were discarded and the samples were transferred in new Eppendorf tubes. Equal volume of samples 80 µl (or 40 µl for mini gel) was solubilized in 5× Laemmli sample buffer, boiled for 5 min and analyzed on SDS 6–15% (or 10% for mini gel) PAGE.

SDS-polyacrylamide gels were blotted onto PVDF Immobilon P (Millipore Corporation) filters using a Bio-Rad apparatus according to manufacturer protocol (Bio-Rad Laboratories, Hercules, CA). The filters were blocked at room temperature for 1 h in 4% Non-Fat Dry Milk (NFDM, Bio-Rad Laboratories) in TBS (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) and, after two washes with TTBS (TBS containing 0.05% Tween 20) and with TBS, were incubated for 1 h at room temperature with the primary antibody diluted in 0.5% NFDM in TBS. The filters were washed extensively with TTBS and with TBS and incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Amersham) diluted in 0.5% NFDM in TBS. The filters were then washed six times with TTBS and once with TBS and developed using an ECL detection method (Amersham) according to manufacturer's directions.

Immunofluorescence

Immunofluorescence studies were performed on cells (ca. 1×10^5) seeded onto 12-mm-diameter glass coverslips. Cells were fixed for 20 min with 4% paraformaldehyde in PBS containing 0.9 mM calcium and 0.5 mM magnesium (PBS CM) at room temperature, washed twice in PBS CM containing 50 mM NH$_4$Cl and incubated two times (10 min each) with PBS CM containing 0.2% gelatin and 0.075% saponin. Gelatin and saponin were present in all solutions from here on, up to the final washes. Cells were then incubated for 1 h with the primary antibodies diluted in PBS CM. After washing three times (10 min each) with PBS CM, the cells were incubated for 20 min with the appropriate rhodamine or fluorescein-tagged goat anti-mouse or anti-rabbit secondary antibody diluted 1:50 in PBS CM. After final washes with PBS CM, the coverslips were mounted on a microscope slide using a 50% solution of glycerol in PBS CM and examined with a Zeiss Axiophot microscope or with laser scanning microscope Zeiss, Olympus or Bio-Rad.

Immunoelectronmicroscopy

For immunoelectronmicroscopy, 12% gelatin-embedded, 2.3 M sucrose-infused blocks of aldehyde-fixed growing FRT cells, transfected with VGF cDNA, were

Fig. 1. Expression of VGF in FRT cells. FRT cells were stably transfected with the full-length cDNA coding for VGF. Proteins in the cell lysates (C) and in the culture media (M) of a representative clone and of wild type FRT cells were separated on SDS/6–15% PAGE and analyzed by Western blot using an anti-VGF (C-terminal) antiserum diluted 1:3000. Molecular weight standards are indicated on the left. A major band of 90 kDa is detected in cell lysates (C) and culture media (M) of FRT-VGF cells but not in untransfected cells. In the culture media few other bands are present. Some of them possibly correspond to C-terminal peptides generated by extracellular proteolysis.

frozen in liquid nitrogen. Ultra thin cryo-sections were obtained with a Reichert-Jung Ultracut E with FC4E cryoattachment and collected on copper–formvar–carbon coated grids. Sections were immunostained with the rabbit antiserum directed against VGF, followed by 15 nm Protein A-gold (Slot, Utrecht, NL). In all control sections, very low levels of labeling were detected (not shown). Sections were examined with Zeiss EM 902 or EM10C electron microscopes.

Analysis of the state of aggregation of VGF and of VGF mutant proteins

Cells (two 15-cm dishes of subconfluent cells) were homogenized and a postnuclear supernatant (1.2 ml) was divided in two aliquots and diluted with an equal volume of either aggregative milieu (20 mM MES–NaOH, 20 mM $CaCl_2$, pH 6.4, 1.2 mM leupeptin and Triton X-100 2%) or nonaggregative milieu (20 mM MES–NaOH, 60 mM kCl, pH 7.4, 1.2 mM leupeptin and Triton X-100 2%) [41]. The samples were incubated for 10 min at 4°C under agitation and for 20 min at 0°C. Samples were then subjected to centrifugation (100.000 g for 15 min) and the pellet and the supernatant were analyzed by SDS-PAGE and Western blot. The media collected from cell cultures after incubation in serum-free medium for 14–16 h were analyzed by the same technique. This experiment should allow the separation of protein aggregates and of soluble proteins: protein aggregates are collected in the pellet while soluble proteins remain in the supernatant.
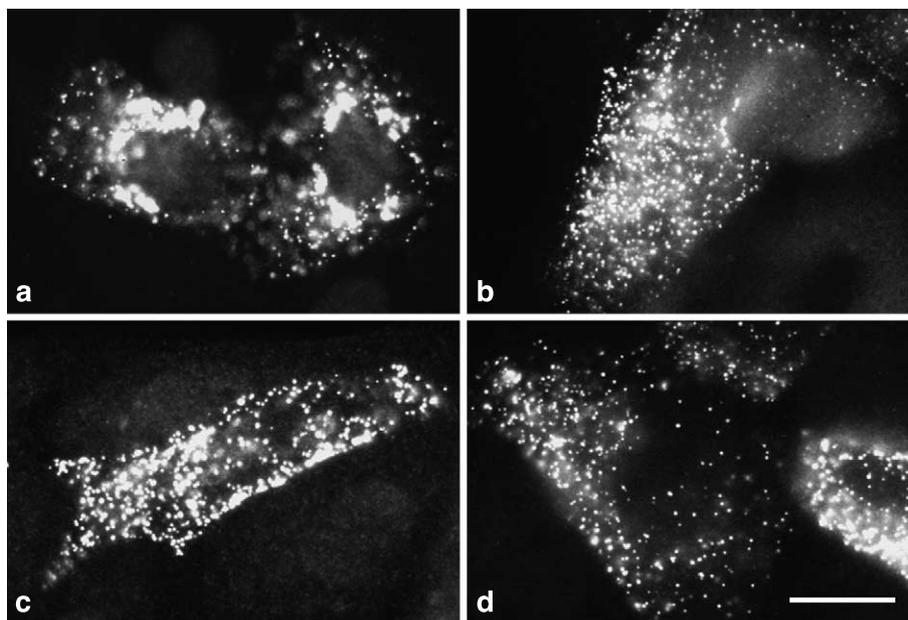

Fig. 2. VGF protein is segregated in a granular compartment. Stably transfected FRT-VGF cells were stained by indirect immunofluorescence using the anti-VGF (C-terminal) antiserum diluted 1:1500. A perinuclear and a prominent dot-like staining are observed (a). Cells were treated with 150 μg/μl of cycloheximide for 1 h (b), 3 h (c) and 5 h (d), fixed and stained by indirect immunofluorescence using the anti-VGF (C-terminal) antiserum. The perinuclear staining seen in (a) disappears after 1 h (b) of cycloheximide treatment and only granular staining is retained at 3 h (c) and 5h (d). Scale bar = 10 μm.
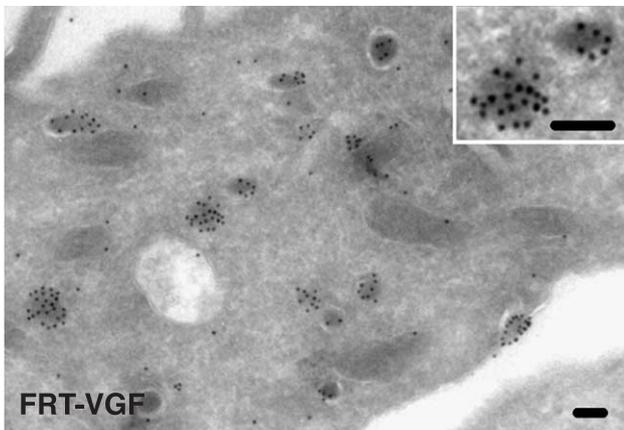
Fig. 3. VGF protein is stored in dense core granules. FRT-VGF cells were treated with cycloheximide for 1 h, they were aldehyde-fixed, removed from the culture dish with a scraper and embedded in gelatin-sucrose. Ultra thin cryo-sections were immunostained with the anti-VGF antiserum (C-terminal) and colloidal-gold conjugated protein A. Colloidal gold particles are mainly localized in granules of 70–200 nm diameter. A pair of granules is shown at higher magnification in the inset. Scale bar = 0.15 μm.

Chemicals

All chemicals were purchased from Sigma Co. (St. Louis, MO) unless otherwise indicated.

Results

Expression of VGF in FRT cells

FRT cells were co-transfected with the full-length cDNA coding for VGF and with the NEO gene that confers resistance to G-418. Stable clones were tested for VGF expression by immunofluorescence. Single clones and pools of positive clones were used thereafter. By using an antibody against the C-terminal portion of VGF and Western blot analysis, a major band of 90 kDa was observed in the cell lysate of all fluorescence positive clones but not in untransfected cells (Fig. 1) indicating that there is no endogenous expression of the protein. The observed band corresponds to the expected molecular weight of the mature VGF protein [26]. In the medium collected after 18 h of culture, the same 90-kDa band was detected, together with less intense bands of 80, 50/55, 10/12 kDa and some nonspecific bands (Fig. 1). The presence of these bands in the culture medium indicates that the protein is secreted and that extracellular proteolysis is occurring after its secretion. It is known that the protein has many potential proteolytic processing sites.

FRT-VGF clones were examined by immunofluorescence to determine the intracellular distribution of the
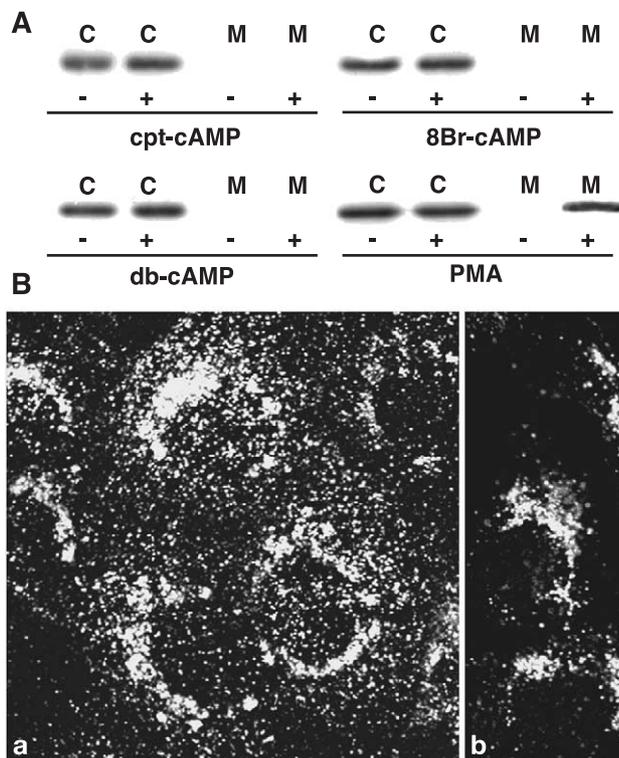

Fig. 4. PMA promotes VGF secretion. (A) Subconfluent FRT-VGF monolayers were washed three times with medium without serum and were incubated for 30 min at 37°C with one of the following secretagogues: 250 μM cpt-cAMP, 10^{-6} M 8Br-cAmp, 10^{-3} M db-cAmp, 300 nM PMA. Cell lysates (C) and culture media (M) of treated (+) and control (−) cell cultures were analyzed by Western blot using the anti-VGF antiserum (C-terminal) diluted 1:1500. Only PMA is able to induce significant VGF secretion into the culture medium. (B) FRT-VGF cells treated with PMA for 30 min were stained by indirect immunofluorescence using the anti-VGF antiserum. The number of VGF positive granules is dramatically reduced in treated cells (b) with respect to untreated cells (a). Scale bar = 10 μm.

protein. A prominent dot-like staining, suggestive of a secretory granule compartment, was observed (Fig. 2a). Some peri-nuclear staining, suggestive of a Golgi apparatus, was also evidenced. No staining was present in wild type FRT cells (data not shown). To evidence the presence of a secretory granule compartment, FRT-VGF cells were treated
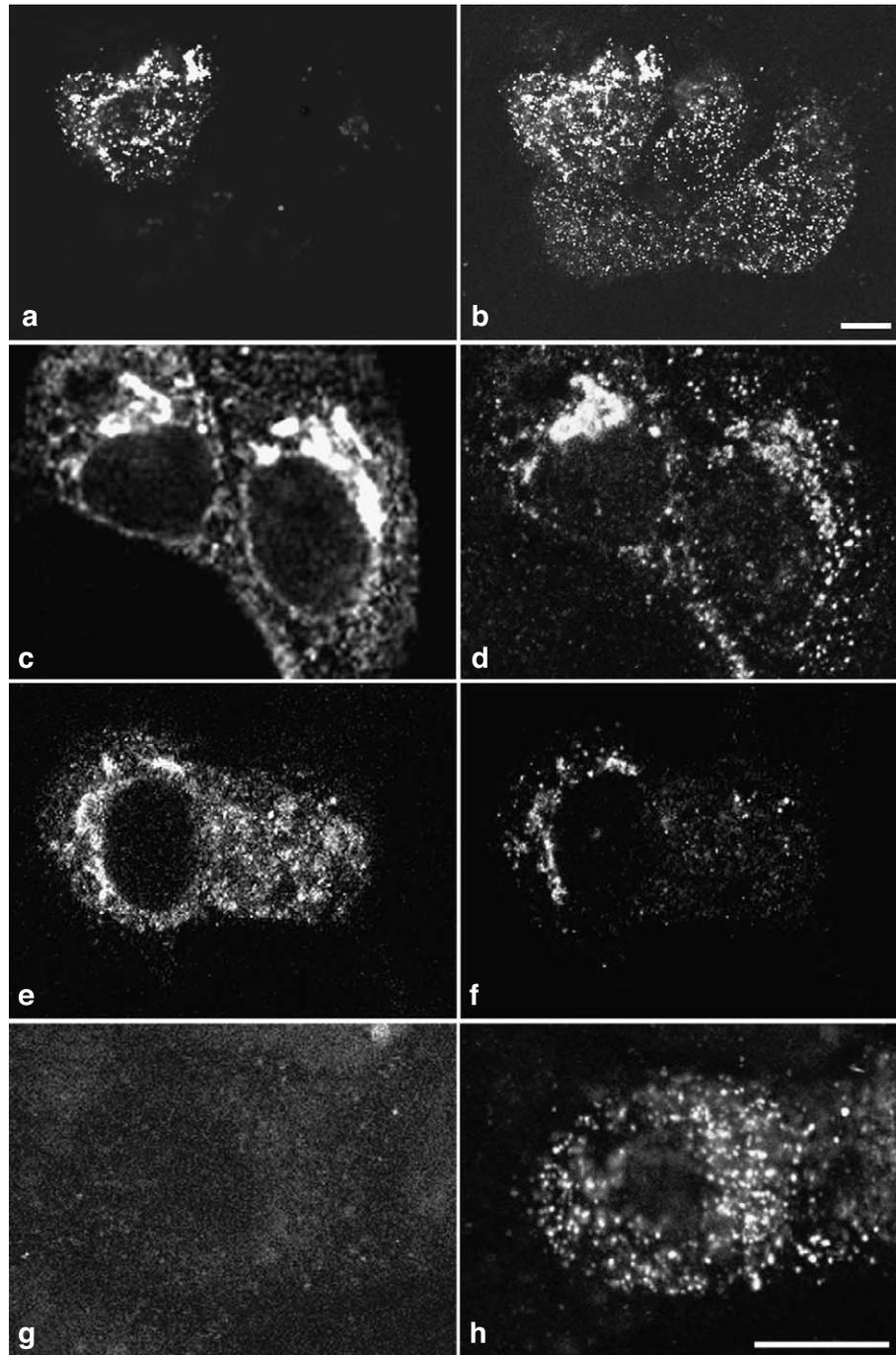

Fig. 5. VGF and HCgB, but not p75NTRsec, co-localize in secretory granules. FRT-VGF cells were transiently transfected with cDNA coding for HCgB and treated with 150 μg/μl of cycloheximide for 1 h. Cells were stained by double immunofluorescence with a monoclonal antibody against the HCgB (1:400) (a) and the polyclonal anti-VGF (C-terminal) antiserum (b). The cell that is stained in (a) is the only one in the cluster of VGF-positive cells (b) that co-expresses HCgB. VGF co-localizes with HCgB within secretory granules (a, b). Scale bar = 10 μm. FRT-p75NTRsec cells were transiently transfected with the cDNA coding for VGF and examined either untreated (c, d) or after 30 min of treatment with 300 nM PMA (e, f) or after 1 h of treatment with 150 μg/μl of cycloheximide (g, h). Cells were stained by double immunofluorescence with the mouse monoclonal antibody ME 20.4 (1:200), against the ectodomain of human neurotrophin receptor p75NTR (c, e, g) and the anti-VGF polyclonal antiserum (d, f, h). VGF and p75NTRsec co-localize in the perinuclear Golgi area but do not co-localize in the granules dispersed in the peripheral cytoplasm, which are only stained by the anti-VGF antibody. p75NTRsec is completely cleared from the cells by the cycloheximide treatment (g). Scale bar = 10 μm.

with cycloheximide for different times and analyzed by immunofluorescence. The perinuclear staining seen in untreated cells (Fig. 2a) disappeared during the treatment and by 1 h all the staining was in granules within the cytosol (Fig. 2b). This staining was still prominent after 3 and 5 h (Figs. 2c, d) and only after a longer time (> 6 h) it disappeared from most cells. These results suggested that VGF might reside in a compartment with the properties of secretory granules. VGF expressing cells were also double-stained with anti-VGF antibodies and with antibodies against proteins expressed by rough endoplasmic reticulum or TGN or endosomes (anti-bip, anti-TGN38, anti-EEA1 and anti-rab7, respectively). The dot-like staining of VGF did not co-localize with the staining of any of these antibodies (data not shown). To determine if the dot-like staining corresponded to dense-core granules, colloidal-gold immunoelectronmicroscopy analysis was then performed. When immunostained sections of FRT-VGF cells were observed, colloidal-gold particles were found to be mostly in electron-dense membrane-bound vesicles, of size ranging between 70 and 200 nm, resembling dense-core granules or large dense-core vesicles [21] (Fig. 3).

VGF secretion can be induced by PMA treatment

We determined if VGF secretion was dependent upon extracellular stimuli. FRT cells have been derived from thyroid follicular cells [32], which are known to respond to TSH stimulation. FRT cells, however, have lost many differentiated properties and they do not express the TSH receptor [42]. Since cAMP acts as second messenger in TSH stimulation, we decided to use cAMP derivatives to promote exocytosis. However, none of the cAMP analogs we tested, cpt-cAMP, 8Br-cAMP and db-cAMP, was able to induce VGF secretion (Fig. 4, panel A). It is known that the activation of the protein kinase C is a potent stimulus for protein secretion. It has been demonstrated that in the insulinoma-derived beta-cell line INS-1 a 6-fold increase in VGF secretion is seen after PMA treatment [31]. FRT-VGF cells were treated with PMA for 30 min. The amount of protein secreted during the PMA treatment was analyzed by Western blot. A great increase (10- to 15-folds) in the amount of VGF secreted in the medium of PMA treated cells with respect to untreated cells was observed (Fig. 4, panel A). As predicted, an abrupt decrease in the number of secretory granules could be observed in PMA-treated cells by indirect immunofluorescence (Fig. 4, panel B).

We also tested whether other proteins that are secreted by a regulated or by a constitutive pathway co-localized with VGF in the same granule compartment. We used HCgB as a regulated protein [28] and p75^{NTR}sec, the extracellular domain of the p75 neurotrophin receptor, as a constitutive protein [38]. FRT-VGF cells were transiently transfected with HCgB and it was found, by confocal microscopy analysis, that HCgB was localized in the same granule compartment as VGF (Fig. 5). In a similar experiment,

stable clones of FRT/p75^{NTR}sec [43] were transiently transfected with VGF and then analyzed by confocal microscopy either untreated or after PMA stimulation or after cycloheximide treatment (Fig. 5). No co-localization of VGF and p75^{NTR} sec was observed in granules dispersed in the peripheral cytoplasm, while a significant extent of co-localization was seen in the perinuclear Golgi area (Fig. 5). Although we have not studied this point in detail, we cannot exclude that some p75^{NTR} sec were present in immature secretory granules, as it has been described for alpha 1-antitrypsin [44].

VGF is secreted through the apical cell domain

It has been described that in hippocampal rat neurons in primary culture VGF has a polarized distribution, being segregated into the axon [45]. It has been proposed that similar mechanisms mediate polarized sorting of some, but not all, membrane proteins in both epithelial cells and neurons [46–48]. Little information is available on the sorting mechanisms of soluble proteins that are secreted by a regulated pathway in either cell type. A recent paper on CgA indicates that this protein has an apical, polarized secretion when expressed in MDCK cells. However, it has not been assessed whether in these cells CgA is segregated
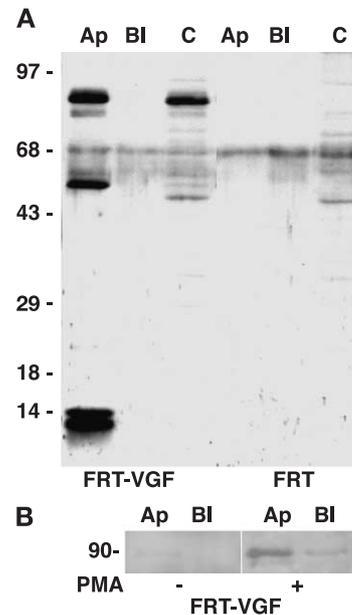

Fig. 6. VGF secretion by FRT cells is polarized. (A) FRT-VGF cells were grown to confluence on filters, in bicameral system, for 5 days and then incubated overnight in medium without serum. Apical and basolateral culture media, and cell lysates were run in SDS/6-15% PAGE and analyzed by Western blot. A major band of 90 kDa was detected in cell lysates (C) of FRT-VGF cells. A band of 90 kDa and few other bands were detected in the apical medium (Ap) but not in the basolateral medium (Bl) of the same cells. Untransfected FRT cells have only background immunoreactivity. (B) FRT-VGF cells were grown to confluence on filters, in bicameral system, for 5 days and then incubated for 30 min in medium w/o serum with or w/o 300 nM PMA. Apical (Ap) and basolateral (Bl) culture media were run in SDS/6–15% PAGE and analyzed by Western blot.
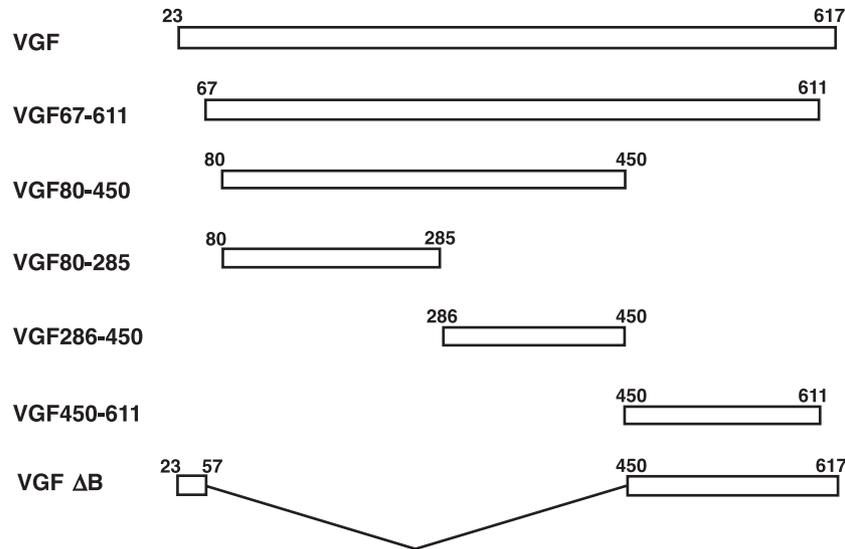
Fig. 7. VGF deletion mutants. Schematic representation of VGF constructs, wild-type and deletion mutants. Residues 1–22 of VGF, which are predicted to function as a signal peptide [35], have not been inserted in the vectors that have their own signal peptide.

into dense-core granules [22]. We have analyzed stable clones of FRT-VGF cells that were cultured to confluence on filters, in bicameral systems. By confocal microscopy, we found that most VGF secretory granules were located toward the apical cell domain and very few were at the basal pole (data not shown). In the same culture system, we also determined the sorting polarity of the VGF protein. FRT-VGF cells grown in bicameral systems were incubated overnight in medium without serum, the media from the apical and basolateral compartments were collected, separated on SDS/PAGE and analyzed by Western blot. The 90-kDa protein and few less represented peptides, recognized by anti-VGF (C-terminal) antibody, were predominantly found in the medium collected from the apical compartment (Fig. 6A). In similar experiments, we also assessed the polarity of secretion of VGF released during 30 min of PMA stimulation. A significant increase in the amount of secreted VGF was observed in treated samples and VGF retained the apical polarity of sorting (Fig. 6B). Moreover, up to 3 h of stimulation, we did not detect any significant leak of VGF into the basolateral compartment (data not shown). These data indicate that VGF secretion is polarized and occurs through the apical domain of FRT cells.

VGF truncated peptides retain sorting signals

To identify signals on the VGF protein that were responsible for segregation into secretory granules and/or for apical delivery, five deletion mutants were generated. They coded for the following truncated proteins: VGF67-611, VGF80-450, VGF80-285, VGF286-450, VGF450-611 (see Materials and methods and Fig. 7). All these proteins were fused to a myc tag at their C-terminus. Wild-type FRT cells were transiently transfected with either mutant and analyzed by immunofluorescence. A dot-like, granular signal, similar to the one observed with the full-length VGF, was observed (Fig. 8). To determine if the fluorescence signal of each truncated protein was coincident with the one generated by the entire VGF protein, FRT-VGF cells were transiently transfected with each mutant and analyzed by double
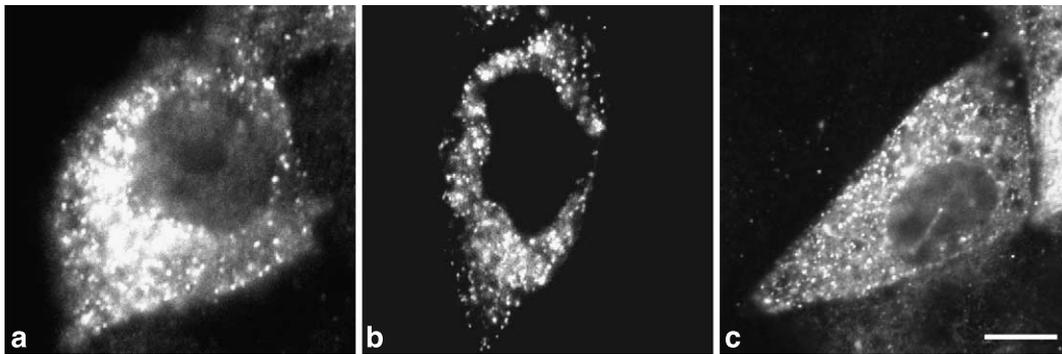

Fig. 8. VGF mutant proteins are segregated in granules. FRT-VGF cells were transiently transfected with DNAs coding for VGF deletion mutants fused to a myc-tag. The following mutants were transfected: VGF80-285myc (a); VGF286-450myc (b); VGF450-611myc (c). The mutated proteins were localized by immunofluorescence using a mouse monoclonal antibody against the myc-tag. A dot-like, granular staining is observed with all mutants. Scale bar = 10 μm.
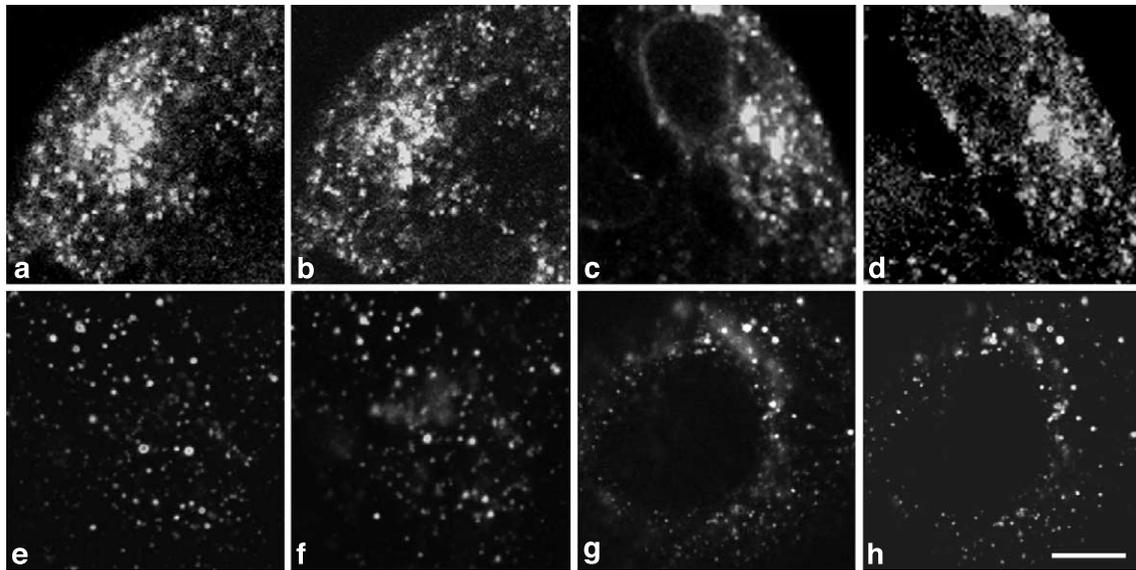
Fig. 9. VGF mutant proteins are segregated in the same granule compartment as VGF. FRT-VGF cells were transiently transfected with DNAs coding for VGF deletion mutants fused to a myc-tag. The following mutants were transfected: VGF80-450myc (a, b); VGF450-611myc (c, d); VGF80-285myc (e, f); VGF286-450myc (g, h). VGF protein and VGF deletion mutant were co-localized by immunofluorescence and confocal microscopy using a mouse monoclonal antibody against the myc-tag (a, c, e, g) and the VGF antiserum against the C-terminal peptide (b, f, h) or the N-terminal peptide (d). Scale bar = 5 μm.

immunofluorescence at a confocal microscope. The intact VGF protein was detected with either an anti-C- or an anti-N-terminal antibody, while the mutated proteins were detected by the anti-myc antibody. The analysis revealed that all truncated peptides co-localized with the intact VGF in the same granules. The co-localization of intact VGF with the truncated proteins VGF80-450, VGF80-285, VGF286-450, VGF450-611 is shown in Fig. 9.

We eventually tested whether the truncated proteins retained sorting polarity. Stable clones of FRT cells expressing each truncated protein were obtained. The polarity of secretion was determined by Western blot analysis of the culture media of confluent cells grown on filters in bicam-

eral system. Since we have not been able to detect the VGF450-611 peptide in the culture medium of filter-grown cells, we analyzed instead a different mutant, ΔB, which contains the VGF450-611 fragment (see Fig. 7). We observed that all truncated peptides were predominantly present in the apical culture medium (Fig. 10) indicating that they were preferentially secreted through the apical cell domain.

We finally asked the question of whether VGF shared with the granin family of proteins the property of selective aggregation in a mildly acidic milieu and in the presence of millimolar calcium ions [3–5]. FRT-VGF cell extracts were resuspended either in an aggregative (low pH, high Ca^{2+}) or
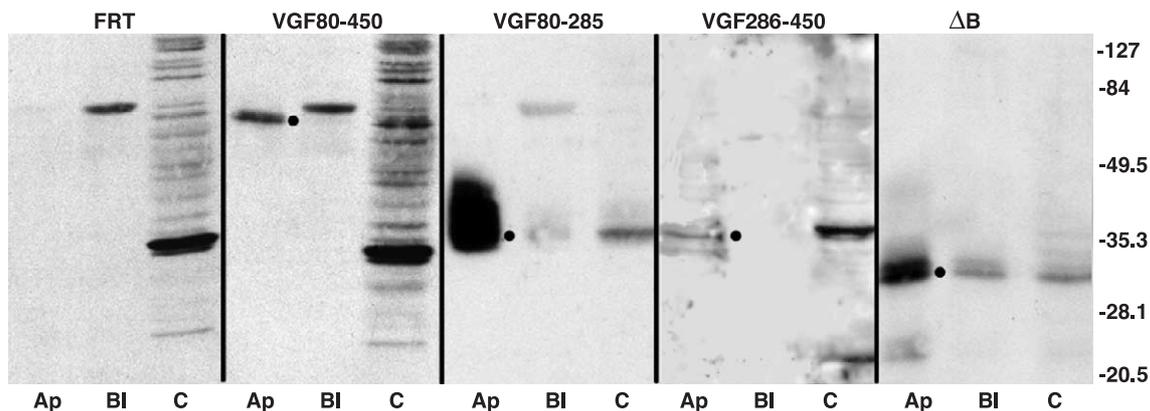

Fig. 10. VGF deletion mutant proteins retain signal(s) for apical secretion. Stable clones of FRT cells expressing VGF mutant proteins were grown to confluence on filters in bicameral systems. After 5 days, the monolayers were washed in medium without serum and incubated overnight in the same medium. Apical (Ap) and basolateral (Bl) media, and cell lysates (C) were run in SDS/6–15% PAGE and analyzed by Western blot using the mouse monoclonal antibody anti-human c-*myc* (1:500) for FRT, VGF80–450, VGF80–285, VGF286–450 cells, and the VGF antiserum against the C-terminal peptide (1:1500) for ΔB cells. The positions where the truncated peptides migrate in the apical medium (Ap) lanes are indicated by black dots. Note that the anti-c-*myc* antibody recognizes a non-specific band of about 60 kDa in the media of non-transfected FRT cells.
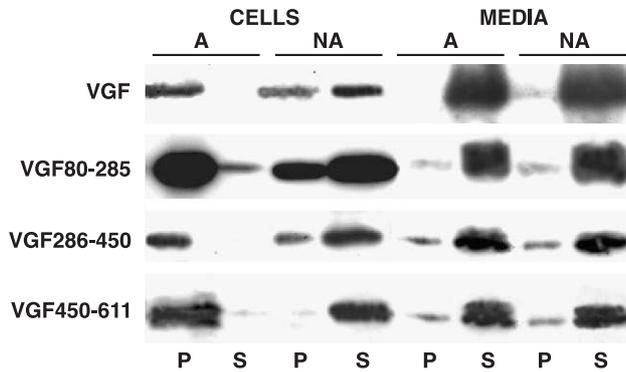
Fig. 11. VGF and VGF deletion mutant proteins are in an aggregated state along the secretory pathway. Stable clones of FRT cells expressing VGF or VGF mutant proteins (VGF80−285, VGF286−450, VGF450−611) were grown to subconfluence. Cells were homogenized and the postnuclear supernatant was divided in two aliquots and diluted with an equal volume of either aggregative (A) or nonaggregative (NA) milieu (see Materials and methods). The samples were incubated for 30 min in the cold and then subjected to centrifugation, and the pellet (P) and the supernatant (S) were analyzed by SDS-PAGE and Western blot. The media collected from cell cultures during 18 h in the absence of serum were analyzed by the same procedure.

in a nonaggregative (neutral pH, low Ca^{2+}) milieu and then centrifuged [41]. It was observed that about 90% of VGF was found in the pellet in the aggregative milieu while less than 40% was found in the pellet in the nonaggregative milieu (Fig. 11). On the contrary, VGF that had been secreted in the culture medium was almost completely recovered in the supernatant either when resuspended in aggregative either in nonaggregative milieu (Fig. 11). We then tested how truncated VGF proteins behaved in the same aggregation assay. We found that in the aggregative milieu, more than 75% of any of the three peptides examined (VGF80-285, VGF286-450 and VGF450-611) was in the pellet fraction while in the nonaggregative milieu the amount of each protein in the pellet fraction was less than 35% (Fig. 11). In all cases, the protein secreted in the culture medium was almost completely recovered in the supernatant fraction (Fig. 11).

Discussion

We demonstrate here that FRT rat thyroid cells are able to sort VGF into a compartment that resemble dense-core secretory granules and are able to secrete VGF through the apical cell domain. FRT cells are therefore able to recognize and to correctly decode the signals that are present on a neuroendocrine protein that they do not normally express. This represents a model system to study in vitro signals and mechanisms for the polarized secretion of regulated proteins in endocrine cells.

It is not known if the secretory granules in which VGF is segregated are already present in FRT cells or if they are induced by the transfection of the VGF protein. FRT cells

are derived from thyrocytes, which are known to form apical vesicles to deliver their main secretory product, thyroglobulin, to the follicle lumen [49]. However, FRT cells are poorly differentiated [32] and they do not synthesize nor secrete thyroglobulin although they have retained the polarized, epithelial phenotype and some property of thyrocytes [39]. It is not known if FRT cells sort VGF into a compartment where thyroglobulin would be segregated, neither it is known if they secrete any other protein by a regulated pathway. It should be considered that a regulated pathway might remain cryptic if the proteins that take that route are in small amounts. As a matter of fact, it has been suggested that regulated pathways are more widespread among different cell types than ever believed [50].

It has recently been provided evidence that dense-core secretory granule biogenesis can be promoted in both endocrine and nonendocrine cells by the granin CgA [23]. Granins are a widespread constituent of the secretory granules in neuroendocrine cells [28]. To the same family belong, among other members, CgA, CgB and secretogranins II, III and IV [4]. Granins have long been proposed to be involved in large dense-core vesicle formation in endocrine cells. However, only CgA and not other granins has been shown to induce dense-core secretory granule formation in transfected cells [23]. The data obtained with FRT cells are compatible with the hypothesis that the VGF granin might share with CgA the property to promote secretory granule formation.

The secretion of VGF in FRT transfected cells can be stimulated: it is, in fact, promoted by the activation of protein kinase C, which is known to enhance VGF secretion in the insulinoma cell line INS-1 [31]. We have also tested the ability of cAMP analogs to induce VGF secretion, since cAMP is the natural second messenger able to induce thyroglobulin secretion in thyroid cells. FRT cells, however, do not respond to cAMP stimulation. PMA, on the contrary, induces a dramatic increase in the release of VGF in the culture medium and a corresponding decrease in intracellular secretory granules. Although it is known that PMA might also stimulate some other export pathway [51], our results suggest that VGF secretion can occur, at least in part, by a regulated pathway. Our data (see Fig. 1) also indicate that some secretion of VGF spontaneously occurs under routine culture conditions. This could be either due to some constitutive secretion or to basal exocytosis of dense-core granules [52]. It is known that exogenous proteins are often over-expressed in the transfected cells and they may thus saturate specialized secretory routes. It is therefore possible that a fraction of the transfected VGF protein is secreted by the constitutive pathway and that another fraction is directed into the regulated route. We have not discriminated between these two possibilities also due to the lack of sulfated amino acids that makes it difficult to label the VGF protein.

To identify which signals on VGF are recognized by FRT cells in the sorting process, we have generated several VGF deletion mutants. We have found that all VGF mutants

retain sorting information both to the dense-core granules and to the apical route, although major and variable portions of the protein have been deleted. There might be, therefore, superimposition of the two putative types of signals in all mutants. This would put the issue of sorting signals for polarized delivery in a new perspective: endocrine epithelial cells might not need to recognize specific signals for apical delivery of proteins of the regulated pathway. As it has been already hypothesized, a 'targeting hierarchy' might exist such that properly packaged regulated secretory proteins are always targeted to the apical cell domain [49,53]. Our results support this hypothesis.

The retention of targeting information by all deletion mutants also indicates that signals for the regulated/apical secretion do not consist in determinants that are in just one discrete portion of the protein but might reside in a more general property of the entire protein. All VGF deletion mutants we tested are rich in acidic amino acids and have low isoelectric point. These structural features that the mutants share with the native VGF protein may enable a 'selective aggregation' in the TGN in the presence of adequate Ca^{2+} concentration and mildly acidic pH [4,41]. We have demonstrated that VGF protein and VGF deletion mutants are in an aggregated state when they are within the cell, along the secretory compartment, while they are almost completely soluble after they have been secreted into the culture medium. We propose that aggregation itself might be part of the mechanism not only for the sorting of VGF to the regulated route but also for its polarized delivery to the apical plasma membrane domain.

Acknowledgments

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