A Prohormone Convertase Cleavage Site within a Predicted \( \alpha \)-Helix Mediates Sorting of the Neuronal and Endocrine Polypeptide VGF into the Regulated Secretory Pathway*

Received for publication, August 18, 2005, and in revised form, October 11, 2005. Published, JBC Papers in Press, October 12, 2005, DOI 10.1074/jbc.M509122200

Angelo L. Garcia1,2, Shan-Kuo Han1,11, William G. Janssen1, Zin Z. Khaing1, Timothy Ito1, Marc J. Glucksman1, Deanna L. Benson1, and Stephen R. Salton1,11

From the 1Fishberg Department of Neuroscience, 2Kastor Neurobiology of Aging Laboratories, and the 3Department of Geriatrics, Mount Sinai School of Medicine, New York, New York 10029 and the 4Midwest Proteome Center and Department of Biochemistry and Molecular Biology, Rosalind Franklin University of Medicine and Science/Chicago Medical School, North Chicago, Illinois 60064

Distinct intracellular pathways are involved in regulated and constitutive protein secretion from neuronal and endocrine cells, yet the peptide signals and molecular mechanisms responsible for targeting and retention of soluble proteins in secretory granules are incompletely understood. By using confocal microscopy and subcellular fractionation, we examined trafficking of the neuronal and endocrine peptide precursor VGF that is stored in large dense core vesicles and undergoes regulated secretion. VGF cofractionated with secretory vesicle membranes but was not detected in detergent-resistant lipid rafts. Deletional analysis using epitope-tagged VGF suggested that the C-terminal 73-amino acid fragment of VGF, containing two predicted \( \alpha \)-helical loops and four potential prohormone convertase (PC) cleavage sites, was necessary and sufficient with an N-terminal signal peptide-containing domain, for large dense core vesicle sorting and regulated secretion from PC12 and INS-1 cells. Further transfection analysis identified the sorting sequence as a compact C-terminal \( \alpha \)-helix and embedded 564RRR566 PC cleavage site; mutation of the 564RRR566 PC site in VGF-(1–65): GFP:VGF-(545–617) blocked regulated secretion, whereas disruption of the \( \alpha \)-helix had no effect. Mutation of the adjacent 565HFFH570 motif, a charged region that might enhance PC cleavage in acidic environments, also blocked regulated release. Finally, inhibition of PC cleavage in PC12 cells using the membrane-permeable synthetic peptide chloromethyl ketone (decanoyl-RVKR-CMK) blocked regulated secretion of VGF. Our studies define a critical RRR-containing C-terminal domain that targets VGF into the regulated pathway in neuronal PC12 and endocrine INS-1 cells, providing additional support for the proposed role that PCs and their cleavage sites play in regulated peptide secretion.

Regulated secretion of polypeptides from neuronal and endocrine cells, which relies on packaging of proteins into the appropriate vesicle pools within the cell, is a critical control point for regulating peptide levels and ultimately function. Polypeptide motifs that target cell surface proteins to specific vesicle populations or to discrete locations within the cell, such as the basolateral or apical surface of a polarized epithelial cell, have been relatively well characterized (1). Protein domains that are responsible for targeting soluble proteins into the regulated release pathway appear to be more heterogeneous, and thus generalized sorting domains and consensus motifs have been more difficult to identify. Perhaps as a consequence, several models that explain targeting into the regulated secretory pathway by selective sorting and/or selective retention have been proposed (2–5). Following signal sequence-directed translocation into the lumen of the endoplasmic reticulum, segregation of proteins into the constitutive or regulated pathways is suspected to occur in the trans-Golgi network (TGN). Constitutive secretory vesicles transit directly from the TGN to the plasma membrane where their protein contents are discharged. Secretory granules destined for regulated release undergo a process of maturation that redirects select proteins into smaller non-clathrin-coated vesicles of the constitutive pathway, and these are rapidly shuttled toward the plasma membrane. The remaining proteins that will undergo regulated release are often selectively aggregated, most likely in the mildly acidic, calcium-rich conditions of the TGN lumen.

VGF (non- acronymic) is a neuronal and neuroendocrine polypeptide that is stored in large dense core vesicles (LDCVs) (8) and is secreted through the regulated release pathway in neuroendocrine PC12 and endocrine INS-1 cells (9, 10). vgf gene expression is regulated in the brain in a region-specific manner by neuronal activity, brain-derived neurotrophic factor, seizure, injury, long term potentiation, circadian rhythm, and fasting (11, 12). Functional studies based on the analysis of VGF mutant mice indicate that the encoded protein plays a critical role in the control over feeding and energy expenditure (13, 14). VGF peptides have been identified in several endocrine organs including the pituitary and pancreas, as well as in the brain and cerebrospinal fluid (11). Recent studies have further demonstrated that expression of the vgf gene is induced in response to hippocampus-dependent learning paradigms, and that the C-terminal VGF peptides regulate hippocampal neuronal excitability (15).

The abbreviations used are: TGN, trans-Golgi network; LDCV, large dense core vesicle; PC, prohormone convertase; CPE, carboxypeptidase E; GFP, enhanced green fluorescent protein; HA, hemagglutinin; PC2, prohormone convertase 2; PC1, prohormone convertase 1; NPY, neuropeptide Y; POMC, proopiomelanocortin; dec, decanoyl; CMK, chloromethyl ketone; CHX, cycloheximide; MES, 4-morpholineethanesulfonic acid; DOC, doxycyclinic acid; PMA, phorbol 12-myristate 13-acetate.

1 Both authors contributed equally to this work.
2 Supported in part by an American Psychological Association Diversity Program in Neuroscience predoctoral fellowship.
3 To whom correspondence should be addressed: Fishberg Dept. of Neuroscience, Box 1065, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. Tel.: 212-659-5901; Fax: 212-996-9785; E-mail: stephen.salton@mssm.edu.
VOLUME 280 • NUMBER 50 • DECEMBER 16, 2005

VGF Sorting and Regulated Secretion

The ~68-kDa VGF polypeptide is relatively rich in proline and glutamic acid, contains a number of paired (di)basic residues, and is differentially processed in a cell type-specific manner in neuronal, neuroendocrine, and endocrine cells (11). This coupled with its localization in dense core secretory vesicles has suggested a similarity to the chromogranin/secretogranin family despite a lack of obvious similarities between their primary amino acid sequences. VGF, like chromogranin A, is not targeted to the regulated release pathway by the carboxypeptidase E (CPE) sorting receptor,5 so to identify potential intracellular targeting motifs, we transfected a series of plasmid constructs encoding epitope-tagged VGF, various VGF deletions, and VGF-enhanced green fluorescent protein (GFP) fusion proteins into neuroendocrine PC12 and endocrine INS-1 cells. Our studies suggest that both an N-terminal signal peptide-containing domain and the VGF 73-amino acid C-terminal domain, the latter with a predicted α-helical loop that contains a critical RRR566 prohormone convertase (PC) cleavage site, are required and sufficient for sorting and/or retention of VGF in LDCVs and secretion via the regulated release pathway.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The parental hemagglutinin (HA)-tagged VGF was created by inserting the peptide sequence SYPDVDPYAGT between Gly27 and Arg28 of the rat VGF polypeptide. Additional full-length VGF constructs were created by subcloning the rat VGF coding sequence, with the translation stop codon replaced by a BglII site, into Kpn-Bam-cut pcDNA3.1/MycHisB (Invitrogen) or pEGFP-N1 (Clontech). To create the Myc deletion series shown in Fig. 1, restriction fragments of the rat VGF coding sequence were subcloned in-frame into the appropriate pcDNA3.1/MycHisB vector. A related series of constructs was made by truncating the full-length HA-tagged VGF using PCR primers to introduce termination codons following amino acids 177, 282, 367, 480, and 543 of rat VGF. The VGF::GFP constructs, which directly fuse VGF and GFP sequences in the vector pcDNA3 (Invitrogen) at the indicated sites, were created by PCR using pEGFP-N1 and the rat VGF cDNA (7, 16) as templates. Sequences of all PCR-generated fragments were verified. GFP constructs with lacz chain leader sequences were created by subcloning the appropriate fragments into pSecTag2/Hygro (Invitrogen).

Transfection Analysis—PC12 cells (generously provided by Drs. L. A. Greene and R. Nishi) (17) and INS-1 cells (generously provided by Drs. K. Wilhelm) (18) were grown as described previously and were transfected with Lipofectamine (Invitrogen) and plasmid DNA (ratio 8:1, respectively) for 7 h. Cells were re-fed, and selection in G418 (500 μg/ml) or hygromycin (100 μg/ml) was initiated 48–72 h after transfection. Mixed stable transfectants were selected in approximately 3 weeks, and aliquots were frozen; and cells, passaged for an additional one to five generations under antibiotic selection, were employed for the release and subcellular fractionation experiments. A series of constructs was analyzed by transient transfection of PC12 cells using an Amaxa Nucleoporator. For these experiments, aliquots of 5–6 × 106 cells were nucleoporated with 5–6 μg of plasmid DNA, according to manufacturer’s instructions, and the cells were evenly divided into two 35-mm wells for measurement of basal and stimulated secretion 24–48 h post-transfection.

Two different release experiments were carried out. For measurement of regulated release, “sister cultures” grown in parallel were rinsed and fed with serum-free medium. Medium was collected after 3 h (basal release), and cultures were then re-fed with either serum-free medium (basal release) or release was stimulated with serum-free media containing 50 mM KCl (PC12 cells), PMA (INS-1), or 8-bromo-cAMP (INS-1), and these were collected after 3 h and the cells saved for analysis. Equal volumes of media were precipitated with 0.1 volume of 100% trichloroacetic acid, 0.4% deoxycholic acid (DOC) for 1 h at 4 °C and centrifuged, and the pellets were washed with acetone, dried, and resuspended in 2× Laemmli buffer. For select transfectants, to investigate whether the pattern of basal secretion was constitutive or “burst release,” cells were incubated in 10 ml of serum-free medium, and 1-ml aliquots were removed after 30, 120, 210, and 300 min of incubation, essentially as described (19). Media samples were precipitated with trichloroacetic acid/DOC as described above and analyzed by Western blotting.

Inhibition of Basic Amino Acid Cleavage in PC12 Cells—PC12 cells were plated on collagen and grown for 2–4 days in RPMI 1640 containing 10% horse serum (Gemini), 5% fetal calf serum (Gemini), and 100 μM decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (dec-RVKR-CMK, 10 μM stock in Me2SO) (Bachem), a membrane-permeable, synthetic PC inhibitor (20, 21). Growth medium was changed daily. After 2 or 4 days, PC12 cells were rinsed with serum-free medium and treated with serum-free medium (with or without 100 μM dec-RVKR-CMK) for 3 h (basal release) and finally with serum-free medium containing 50 mM KCl (with or without 100 μM dec-RVKR-CMK) for 3 h (stimulated release).

Equilibrium Density Fractionation—PC12 cell post-nuclear supernatants, prepared by Dounce homogenization (type B pestle) as described (22), were loaded on continuous 11 ml of 0.6–1.6 M (20–46%) sucrose gradients buffered with 20 mM HEPES, pH 7 (23). Gradients were centrifuged 274,000 × g for 3 h at 4 °C in a Sorvall TH641 rotor and were fractionated from the top. Each of the 11-ml fractions was precipitated with trichloroacetic acid/DOC as described above and resuspended in 2× Laemmli buffer (24).

For raft analysis (25), post-nuclear supernatants (22) were extracted in cold 1% Triton X-100 in TNE (50 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 7.4) for 30 min on ice. Lysates were made up to 1.2 M sucrose (3.9 ml), loaded in the bottom of a centrifuge tube, and overlaid with 5.4 ml of 1.1 M sucrose and 2.7 ml 0.5 M sucrose. Following centrifugation at 29,000 rpm in a Sorvall TH641 rotor for 15 h at 4 °C, 1-ml fractions were collected from the top, diluted with an equal volume of TNE, precipitated at 4 °C by addition of 100% trichloroacetic acid to a final concentration of 20% trichloroacetic acid, and were analyzed by SDS-PAGE and Western analysis as described above.

Preparation of LDCV Membranes and Protein Aggregation Analysis—PC12 post-nuclear supernatants were fractionated as described above, and VGF-containing LDCV fractions 5–10 were pooled. An aliquot was saved for later analysis, and the remainder was diluted in 20 mM HEPES, pH 7, and centrifuged at 274,000 × g for 1 h at 4 °C in a Sorvall TH641 rotor. Pellets were resuspended in the presence of 0.1% saponin under aggregating (10 mM MES, pH 6.4, 10 mM CaCl2, 1.2 mM leupeptin) or non-aggregating (10 mM MES, pH 7.4, 30 mM KCl, 5 mM EDTA, 1.2 mM leupeptin) conditions (26), were incubated for 30 min at 4 °C, and then centrifuged at 130,000 × g for 30 min. Pellets and supernatants were precipitated with trichloroacetic acid/DOC and subjected to Western analysis using anti-VGF and anti-GFP antisera.

Western Analysis—SDS-PAGE and Western blotting were carried out as described previously (13) by using the following primary antisera: rabbit anti-VGF (78–340) (27) and rabbit anti-VGF (588–617) (gift of Dr. H. Yan), rabbit anti-chromogranin B (gift of Dr. R. Fischer-Colbrie), mouse anti-GFP (Abcam, Clontech), rabbit anti-GFP (Clontech), mouse anti-HA (12CA5), mouse anti-Myc (9E10, ATCC), rabbit anti-synaptophysin (gift of Dr. P. DeCamilli), mouse anti-flotillin-1 (BD Bio-

5 E. Normant, S. R. J. Salton, and Y. P. Loh, unpublished observations.
sciences), mouse anti-transferrin receptor (gift of Dr. B. Winkler), and mouse anti-syntaxin-1 (Santa Cruz Biotechnology).

**Immunostaining and Confocal Microscopy**—Cultures were fixed in 4% paraformaldehyde, 4% sucrose in phosphate-buffered saline and permeabilized with 0.25% Triton X-100. Immunostaining was carried out as described previously (28) using the primary antibodies described above. Antibody binding was visualized either by incubating cells with a biotinylated secondary antibody followed by fluorescein-labeled streptavidin (both from Vector Laboratories, Burlingame, CA) or with Texas Red- (Vector Laboratories) or Alexa-labeled (Molecular Probes) secondary antibodies. For all studies where two antibodies were used simultaneously, staining was compared with that obtained in cultures that were incubated with a single primary antibody and with cultures incubated with different combinations of secondary antibodies.

Immunocytochemically identified proteins were localized using a Zeiss LSM 410 and an /H11003/100, 1.4 N.A. oil immersion objective, an Ar/Kr laser, a red-reflecting dichroic beam splitter, and filters /H9261/575–640 and /H9261/515–540. Brightness and contrast settings for each label in double-labeled preparations were kept within close range of one another. Single optical sections were used to compare localization of two labels. Data obtained from two channels simultaneously using a dichroic beam splitter were compared with data obtained sequentially using one laser line to ensure that emission spectra were clearly separated.

The extent of colocalization was determined by measuring fluorescence intensity along lines, 5–10 /H9262/m in length, drawn through the overlaid red and green images using MetaMorph (Universal Imaging) (29). Data were exported into Excel, and coincidence of intensity peaks (rather than levels of fluorescence) was assayed by determining correlation coefficients using Statview.

**Secondary Structure Analysis**—For sequence analysis and structure prediction, we employed a suite of programs that are obtainable at cubic.bioc.columbia.edu/predictprotein/predictprotein.html. Included within this suite is a neural network algorithm, PHDsec, a secondary structure prediction paradigm with a multiple alignment-based neural net system that is cross-validated by 720 unique proteins. Predictions were also generated using a program, employing a "tandem" neural network containing two simulated networks, that has a higher reliability in detecting correct structures.6 Results were additionally validated using AGADIR and JPRED at the University of Dundee.

**RESULTS**

**VGF and Epitope-tagged VGF Are Localized in LDCVs and Secreted via the Regulated Release Pathway**—VGF can be differentially processed, and the C terminus is rich in paired basic amino acids (7, 9, 16). Additionally, N-terminal signal peptide sequences and loop structures have been implicated in sorting, so two series of plasmids that contained either N- or C-terminal modifications were constructed. Sequence encoding rat VGF was modified to remove the stop codon and to fuse a Myc epitope to the C terminus (Fig. 1), and a series of deletion mutants with a C-terminal Myc epitope was generated as shown. In the other group of constructs, the N terminus of VGF was tagged with an HA epitope immediately C-terminal to the predicted signal peptide cleavage site, and a related series of deletion mutants with a C-terminal Myc epitope was generated as shown. In the other group of constructs, the N terminus of VGF was tagged with an HA epitope immediately C-terminal to the predicted signal peptide cleavage site, and a related series of deletion mutants was generated using the indicated restriction sites (Fig. 1). Plasmid constructs were transiently transfected into PC12 cells, and individual G418-resistant clones or stable transfectants were selected with the appropriate antibiotic.

6 M. J. Glucksman, unpublished data.
VGF Sorting and Regulated Secrecion

FIGURE 2. The HA-tagged full-length VGF fusion protein is secreted through the regulated release pathway and fractionated by equilibrium density centrifugation with VGF and chromogranin B. Panel A, stable PC12 cell lines or mixed stable populations, transfected with pcDNA3 encoding HA-tagged VGF, were generated. Growth medium was collected from replicate cultures under basal release conditions for 3 h (3 h med), then exchanged and replaced with medium (3 h med) or medium supplemented with 50 mM KCl (3 h KCl). Conditioned media were analyzed by Western blotting with anti-HA and anti-VGF antisera. Panel B, sucrose density centrifugation was employed to analyze post-nuclear supernatants from PC12 cell transfectants. HA-tagged VGF was found in a vesicle population that overlapped most closely those containing either native VGF or the LDCV marker chromogranin B but less so the synaptic vesicle protein synaptophysin.

mented, primarily in dense fractions that correspond to presumptive LDCVs. Synaptophysin, a marker of small clear vesicles, was found in fractions of lower density (Fig. 2, panel B). VGF and chromogranin B were also identified at the top of the gradient in soluble fractions corresponding to cytosol and possibly fractured vesicles, essentially as noted previously for VGF and for chromogranins A and B in similar density centrifugation experiments (9, 23, 30).

Both N- and C-terminal VGF Sequences Are Required for Sorting into LDCV-like Particles by Confocal Microscopy—We used indirect immunofluorescence and confocal microscopy to examine PC12 cells that had been transfected with plasmid constructs encoding the various tagged, truncated forms of VGF. Full-length VGF, tagged either with Myc or with HA, was found to sort into LDCV-like particles, based on particle size and distribution, and lack of overlap with immunostaining for synaptophysin, a protein restricted to small, clear or synaptic vesicles (Fig. 3, panel D). Progressive deletion of C-terminal VGF sequences changed the overall appearance of the VGF-Myc labeling pattern, with particles becoming less granular and more difficult to resolve (Fig. 3, panels A and B, right column, and data not shown), suggesting that N-terminal sequences alone, including the signal sequence, were insufficient to allow efficient sorting and/or retention in LDCV-like particles. None of the truncated VGF proteins appeared to codistribute with synaptophysin, suggesting that these polypeptides might be targeted to a vesicle population distinct from synaptic-like vesicles (Fig. 3 and data not shown). In contrast, a protein in which the N-terminal 80 amino acids of VGF were fused in-frame with the C-terminal 163 amino acids of VGF and a Myc tag was localized in a granular distribution that was strikingly reminiscent of intact VGF (Fig. 3, panel C, VGF1–80:VGF452–617), consistent with a possible requirement for both N- and C-terminal VGF sequences in the sorting and/or retention of these proteins in LDCV-like particles.

N- and C-terminal VGF Domains Are Necessary and Sufficient to Target the Cyttoplasmic Protein GFP into LDCV-like Particles—Because our results using confocal microscopy suggested that both N- and C-terminal VGF domains were required for targeting into LDCV-like vesicles, we investigated whether addition of VGF sequences to enhanced GFP, a cytosolic protein with no known sorting motifs, would be sufficient to similarly target the resultant fusion proteins. Plasmids encoding a series of VGF-GFP fusion proteins (see Fig. 1) were transferred into PC12 cells, and protein localization was examined by confocal microscopy and compared with native VGF using an antibody that recognizes an epitope contained within the region deleted in the GFP fusion proteins, or to chromogranin B which is also concentrated in LDCVs (31). The extent of codistribution was assessed by determining the correlation coefficient for the distribution of the two labels along a line drawn through the cell (see “Experimental Procedures” and TABLE ONE). The granular appearance of GFP labeling was most similar to that of VGF, and the extent of colocalization was highest when GFP was
FIGURE 4. Confocal images showing coimmunolocalization of native VGF and VGF:GFP fusion proteins in PC12 cells indicate that both an N-terminal signal peptide-containing domain and C-terminal VGF sequence are necessary and sufficient to target GFP into a vesicular pattern that resembles endogenous VGF. PC12 cells were transfected with constructs encoding truncation and deletion mutants of VGF fused to enhanced green fluorescent protein (GFP) (see Fig. 1), and in mixed stable cell transfectants, the distribution of native VGF (red, left-hand column) and GFP (green, center column) was compared in image overlays (right-hand column) and is shown for representative constructs (rows A–D). Yellow regions are indicative of colocalization, which would be expected to be highest for constructs distributed like the native protein. Quantitative analysis of the extent of colocalization of select GFP constructs with native VGF and chromogranin B appears in TABLE ONE. Magnification bar = 2.35 μm.

TABLE ONE

| Construct                                      | Correlation coefficient for native VGF vs. GFP | Correlation coefficient for chromogranin B vs. GFP |
|------------------------------------------------|-----------------------------------------------|--------------------------------------------------|
| VGF-(1–65):GFP:VGF-(452–617)                   | 0.68                                          | 0.71                                             |
| VGF-(1–65):GFP:VGF-(452–544)                   | 0.50                                          | 0.77                                             |
| VGF-(1–65):GFP                                 | 0.26                                          | 0.48                                             |
| lge signal:GFP:VGF-(452–617)                   | 0.59                                          | 0.75                                             |
| lge signal:GFP:VGF-(452–544)                   | 0.16                                          | 0.64                                             |
flanked by the VGF N-terminal sequence (amino acids 1–65) and either VGF C-terminal sequence 452–617 (TABLE ONE) or 452–544 (Fig. 4, panel A, and TABLE ONE). Colocalization was nearly absent when the C terminus was deleted altogether (VGF-(1–65):GFP) (Fig. 4, panel B and TABLE ONE). To determine whether the VGF N-terminal sequence conveyed unique targeting information or whether an unrelated leader sequence would be sufficient, the VGF signal peptide and N-terminal sequence (amino acids 1–65) were replaced by the Ig/H9260 signal peptide. As analyzed by confocal microscopy, the Ig/H9260 leader sequence behaved similarly to the native VGF N-terminal sequence. When Ig/H9260 was fused to GFP alone (Fig. 4, panel D), a fine granular distribution was obtained, slightly more diffuse than that seen with VGF-(1–65):GFP (Fig. 4, panel B), whereas fusion of Ig to GFP:VGF-(452–617) resulted in a vesicular pattern similar to native VGF (Fig. 4, panel C, and TABLE ONE).

Cellular fractionation and equilibrium density centrifugation were employed to better characterize the vesicle populations into which VGF:GFP fusion proteins were targeted. VGF-(1–617):GFP and VGF-(1–65):GFP:VGF-(452–617) were sorted into similar LDCV fractions as VGF (Fig. 5, upper and lower panels), suggesting that both N- and C-terminal domains are required for LDCV targeting. Consistent with this, VGF-(1–65):GFP, IgG:GFP, GFP, GFP:VGF-(452–617), and GFP:VGF-(452–544) were not found in LDCV fractions but were all concentrated at the top of the gradient in fractions corresponding to cytosol (fractions positive for lactate dehydrogenase as shown in Fig. 11). Removal of C-terminal VGF residues 545–617 resulted in less efficient sorting or retention of VGF-(1–65):GFP:VGF-(452–544) in LDCVs, with the bulk immunolocalized at the top of the gradient. Similarly, IgG:GFP:VGF-(452–617) was sorted into VGF-containing LDCV fractions, and by comparison, IgG:GFP:VGF-(452–544) was less efficiently sorted into or retained in LDCVs (Fig. 5). These data are consistent with a requirement for an N-terminal signal peptide-containing domain (either VGF or IgG) and the C-terminal VGF-(545–617) domain for sorting into and retention in PC12 cell LDCVs.

**N- and C-terminal VGF Domains Are Necessary and Sufficient to Target the Cytoplasmic Protein GFP into the Regulated Release Pathway in PC12 Cells**—Basal and KCl-stimulated release were measured to determine whether VGF:GFP fusion proteins were sorted into the constitutive or regulated secretory pathways. In these experiments, mixed populations of stable PC12 cell transfectants were analyzed. We noted that GFP was primarily cytosolic, essentially as described previously (for examples see Refs. 19 and 32–34), but was also inefficiently released from stable PC12 cell transfectants through a pathway that appeared to involve constitutive release rather than burst release as was demonstrated previously in AtT20 cells (data not shown; see Ref. 19). Fusions to GFP of several different fragments of the VGF N terminus (VGF-(1–23), VGF-(1–53), VGF-(1–65), and VGF-(1–80)), each containing the VGF signal sequence, were not sufficient to target these fusion proteins into the regulated release pathway (Fig. 6 and data not shown). Similarly, the IgG chain signal peptide fused to GFP was not sorted into the regulated release pathway (Fig. 6). However, fusion of VGF C-terminal sequence, from amino acids 452–617, to GFP, together with either the...
N-terminal VGF-(1–65) domain or the Igκ signal peptide, was sufficient to sort GFP into the regulated secretory pathway in PC12 cells (Fig. 6). Finally, deletion of C-terminal VGF amino acids 545–617, yielding fusion proteins VGF-(1–65):GFP:VGF-(452–544) and Igκ:GFP:VGF-(452–544), resulted in constitutive release (Fig. 6). After measuring intracellular levels of VGF and VGF:GFP fusion protein by Western analysis, basal and stimulated release were quantified as a percentage of total cellular protein (see TABLE TWO). These data are consistent with a requirement for an N-terminal signal peptide-containing domain (either VGF or Igκ) and the C-terminal VGF-(545–617) domain for sorting into the regulated secretory pathway in PC12 cells.

GFP Fusion Proteins That Contain Both N- and C-terminal VGF Domains Are Released via a Cycloheximide-resistant Release Pathway in PC12 Cells—Treatment with protein synthesis inhibitors blocks constitutive protein secretion (35–37) but has less effect on the regulated pathway, most likely a result of the comparatively larger pool of proteins that are stored in secretory granules destined for regulated release and the more rapid transit and release of proteins via the constitutive pathway. Stable PC12 cell transfectants were pretreated with cycloheximide (CHX) (1–10 μg/ml) for 2 h, after which basal release was measured for 3 h and then cells were incubated for 3 h in serum-free media with or without 40 mM KCl. Release of the same VGF:GFP fusion proteins (VGF-(1–65):GFP:VGF-(454–617) and Igκ:GFP:VGF-(454–617)) that were found to be targeted to the regulated pathway was stimulated by KCl in CHX-treated PC12 cells (Fig. 7). In contrast, CHX treatment blocked release of all fusion proteins that lacked the C-terminal VGF-(452–617) domain (e.g. VGF-(1–65):GFP, VGF-(1–65):Igκ:GFP:VGF-(454–544), and Igκ:GFP) (Fig. 7), further indicating that these proteins are sorted into the constitutive pathway.

N- and C-terminal VGF Domains Target GFP to the Regulated Secretory Pathway in Endocrine INS-1 Cells—Previous studies indicate that VGF is processed and secreted through the regulated pathway in INS-1 insulinoma cells (9, 10). Because targeting signals for several secreted polypeptides have been shown previously to be cell type-specific (37), we investigated whether VGF fusion proteins that are targeted to the regulated pathway in neuroendocrine PC12 cells are similarly targeted in endocrine INS-1 cells. We compared GFP fusion protein release to that of endogenous VGF (90 kDa) and VGF peptides of 18 and 10 kDa
that were visualized using antisera raised against the VGF C-terminal 30-amino acid peptide. Basal release of VGF and GFP fusion proteins, and release stimulated by PMA and 8-bromo-cAMP (10), measured as described under “Experimental Procedures”, indicated that the C-terminal VGF-(545–617) domain was required for regulated release, as was either the VGF N-terminal domain or Ig leader sequence (Fig. 8). These data are quantified in TABLE TWO where release is expressed as a fraction of the total cellular pool of VGF or GFP fusion protein.

**TABLE TWO**

| Construct                  | PC12 (A) 3-h basal | PC12 (A) 3-h KCl | PC12 (B) 3-h basal | PC12 (B) 3-h KCl | INS-1 3-h basal | INS-1 3-h cAMP | INS-1 3-h PMA |
|----------------------------|--------------------|------------------|--------------------|------------------|----------------|----------------|---------------|
| VGF-(1–65):GFP             | 29.8               | 30.7             | 30.8               | 28.5             | 28.9           | 31.3           | 24.2          |
| VGF-(1–65):GFP:VGF-(452–617)| 2.2               | 2.0              | 2.4               | 2.4              | 3.7            | 18.3           | 7.2           |
| VGF-(1–65):GFP:VGF-(452–544)| 25.5              | 19.7             | 25.7              | 19.9             | 40.6           | 45.9           | 36.7          |
| GFP:VGF-(452–617)         | 3.1                | 1.4              | 3.2               | 2.4              | ND             | ND             | ND            |
| GFP:VGF-(452–544)         | 5.2                | 0.9              | 6.1               | 2.6              | ND             | ND             | ND            |
| Igk:GFP                   | 14.0               | 14.7             | 14.4              | 16.6             | 5.5            | 6.5            | 6.1           |
| Igk:GFP:VGF-(452–617)     | 3.5                | 3.8              | 3.7               | 13.2             | 7.3            | 17.0           | 12.7          |
| Igk:GFP:VGF-(452–544)     | 8.0                | 5.0              | 10.5              | 9.3              | 27.7           | 29.2           | 32.1          |
| VGF 90 kDa (endogenous)   | 2.4                | 3.9              | 2.0               | 22.7             | 6.9            | 17.1           | 10.8          |
| VGF 18 kDa (endogenous)   | ND                 | ND               | ND                | ND               | 6.7            | 21.5           | 17.3          |

**FIGURE 7.** Cycloheximide treatment blocks secretion of VGF:GFP fusion proteins that lack the C-terminal VGF-(545–617) domain from transfected PC12 cells. PC12 cells were incubated in growth medium or pretreated with cycloheximide (1 μg/ml) for 2 h; basal release from each was then measured for 3 h in the presence or absence of cycloheximide. KCl-stimulated release in the presence or absence of cycloheximide was then determined. Aliquots of media were trichloroacetic acid-precipitated and analyzed by SDS-PAGE and Western blotting with anti-VGF and anti-GFP antisera, as indicated.

**FIGURE 8.** An N-terminal signal peptide-containing domain and the C-terminal VGF-(545–617) domain are required for regulated secretion of VGF:GFP fusion proteins from transfected INS-1 cells. Basal and stimulated release from mixed stable INS-1 cell transfectants was measured by Western analysis using anti-VGF-(588–617) C-terminal antibody and anti-GFP antisera as indicated. Growth medium was collected from replicate cultures under basal release conditions for 3 h (3 h basal) (A), stimulated with 8-bromo-cAMP for 3 h (B), or stimulated with PMA for 3 h (C). Aliquots of media were trichloroacetic acid-precipitated, and cell pellets were solubilized in 2× Laemmli buffer, and each was subjected to SDS-PAGE and Western analysis.

**Removal of the C-terminal VGF Domain Affects VGF Aggregation**—Regulated protein secretion has been hypothesized to depend on both sorting into and retention in the regulated pathway. For a number of proteins found within the LDCV lumen, including members of the secretogranin/chromogranin family, retention may depend on pH- and calcium-dependent aggregation. We therefore investigated whether native VGF and select VGF:GFP fusion proteins are soluble or whether they aggregate at mildly acidic pH in millimolar calcium, conditions that are found in the lumens of TGN vesicles. PC12 cell post-nuclear supernatants were fractionated by equilibrium density centrifugation, and the LDCV fractions were pooled, pelleted, and permeabilized in 0.1% sapon-

41602  JOURNAL OF BIOLOGICAL CHEMISTRY
nin buffer, either under aggregating (10 mM Ca\(^{2+}\), pH 6.4) or non-aggregating (pH 7.4 without Ca\(^{2+}\)) conditions (26). After incubation and centrifugation, both secretory vesicle membrane pellet and supernatant were subjected to gel electrophoresis and Western blotting using antisera directed against VGF or GFP. As is shown in Fig. 9, the VGF-(1–65):GFP:VGF-(454–617) fusion protein was found almost exclusively in the pellet under aggregating conditions and primarily in the pellet in non-aggregating buffer. The VGF-(1–65):GFP:VGF-(454–617) fusion protein is therefore less soluble than native VGF under aggregating or non-aggregating conditions. By comparison, the truncated fusion protein VGF-(1–65):GFP:VGF-(454–544) was detected exclusively in the supernatant under non-aggregating conditions, whereas ~65% was found in the membrane pellet under aggregating conditions (Fig. 9 and TABLE THREE). Therefore, removal of the C-terminal domain (amino acids 545–617) decreases Ca\(^{2+}\)/pH-dependent aggregation. Post-nuclear supernatants from PC12 cells transfected with VGF-(1–65):GFP:VGF-(452–617) or VGF-(1–65):GFP:VGF-(452–544) were fractionated by equilibrium density centrifugation, and LDCV-containing fractions 5–10 were pooled and centrifuged as described under “Experimental Procedures.” Input vesicles were evenly divided (5th and 6th lanes), saponin-permeabilized, and incubated under non-aggregative (pH 7.4, KCl, EDTA) or aggregative (10 mM Ca\(^{2+}\), pH 6.4, CaCl\(_2\)) conditions. Supernatant (S) and pellet (P) fractions were obtained by centrifugation and were analyzed by Western blotting with anti-GFP (fusion protein) and anti-VGF (native VGF).

**FIGURE 9. Deletion of VGF-(545–617) decreases Ca\(^{2+}\)/pH-dependent aggregation.** Post-nuclear supernatants from PC12 cells transfected with VGF-(1–65):GFP:VGF-(452–617) or VGF-(1–65):GFP:VGF-(452–544) were fractionated by equilibrium density centrifugation, and LDCV-containing fractions 5–10 were pooled and centrifuged as described under “Experimental Procedures.” Input vesicles were evenly divided (5th and 6th lanes), saponin-permeabilized, and incubated under non-aggregative (pH 7.4, KCl, EDTA) or aggregative (10 mM Ca\(^{2+}\), pH 6.4, CaCl\(_2\)) conditions. Supernatant (S) and pellet (P) fractions were obtained by centrifugation and were analyzed by Western blotting with anti-GFP (fusion protein) and anti-VGF (native VGF).

**TABLE THREE**

| Aggregation Test | VGF-(1–65):GFP:VGF-(452–617) anti-VGF | VGF-(1–65):GFP:VGF-(452–617) anti-GFP | VGF-(1–65):GFP:VGF-(452–544) anti-VGF | VGF-(1–65):GFP:VGF-(452–544) anti-GFP |
|-----------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
| pH 7.4, KCl EDTA supernatant | 77.8 ± 5.5 | 38.1 ± 0.5 | 82.0 ± 1.0 | 89.8 ± 5.9 |
| pH 7.4, KCl EDTA pellet | 22.2 ± 5.5 | 61.9 ± 0.5 | 18.0 ± 1.0 | 10.2 ± 5.9 |
| pH 6.4, CaCl\(_2\) supernatant | 26.2 ± 12.3 | 17.9 ± 0.05 | 25.0 ± 9.1 | 30.0 ± 7.0 |
| pH 6.4, CaCl\(_2\) pellet | 73.8 ± 12.3 | 82.1 ± 0.05 | 75.0 ± 9.1 | 70.0 ± 7.0 |

VGF Sorting and Regulated Secretion

**TABLE THREE**

Quantification of VGF and VGF:GFP fusion protein aggregation in LDCV vesicle fractions under non-aggregating and aggregating conditions

Aggregation assays were carried out as described in the legend to Fig. 9 and under “Experimental Procedures.” Western blots were developed using ECL, and autoradiograms were scanned and quantified by densitometry. Permeabilized vesicles were evenly divided into two fractions and were incubated either in non-aggregating (pH 7.4) or aggregating (pH 6.4, Ca\(^{2+}\)) conditions. Supernatant and pellet were obtained by centrifugation and analyzed by Western blotting with anti-GFP (fusion protein) and anti-VGF (native VGF) (data not shown). As is shown in Fig. 9, the VGF-(1–65):GFP:VGF-(454–617) fusion protein was found almost exclusively in the supernatant under non-aggregating conditions, whereas ~65% was found in the membrane pellet under aggregating conditions (Fig. 9 and TABLE THREE). Therefore, removal of the C-terminal domain (amino acids 545–617) decreases Ca\(^{2+}\)/pH-dependent aggregation. As is shown in Fig. 11, VGF-(1–65):GFP:VGF-(452–544) were fractionated by equilibrium density centrifugation, both secretory vesicle membrane pellet and supernatant were subjected to gel electrophoresis and Western blotting using antisera directed against VGF or GFP. As is shown in Fig. 9, the VGF-(1–65):GFP:VGF-(454–617) fusion protein was found almost exclusively in the pellet under aggregating conditions and primarily in the pellet in non-aggregating buffer. The VGF-(1–65):GFP:VGF-(454–617) fusion protein is therefore less soluble than native VGF under aggregating or non-aggregating conditions. By comparison, the truncated fusion protein VGF-(1–65):GFP:VGF-(454–544) was detected exclusively in the supernatant under non-aggregating conditions, whereas ~65% was found in the membrane pellet under aggregating conditions (Fig. 9 and TABLE THREE). Therefore, removal of the C-terminal domain (amino acids 545–617) decreases Ca\(^{2+}\)/pH-dependent aggregation. Post-nuclear supernatants from PC12 cells transfected with VGF-(1–65):GFP:VGF-(452–617) or VGF-(1–65):GFP:VGF-(452–544) were fractionated by equilibrium density centrifugation, and LDCV-containing fractions 5–10 were pooled and centrifuged as described under “Experimental Procedures.” Input vesicles were evenly divided (5th and 6th lanes), saponin-permeabilized, and incubated under non-aggregative (pH 7.4, KCl, EDTA) or aggregative (10 mM Ca\(^{2+}\), pH 6.4, CaCl\(_2\)) conditions. Supernatant (S) and pellet (P) fractions were obtained by centrifugation and were analyzed by Western blotting with anti-GFP (fusion protein) and anti-VGF (native VGF).
that were found to be necessary and sufficient for regulated secretion, and these results are summarized in Fig. 12 (panel A) where the positions of potential PC cleavage sites are also noted. The 65-amino acid N-terminal VGF domain contains a single PC cleavage site that is utilized in the central nervous system (45), whereas the C-terminal domain contains three dibasic sites and a monobasic RPR motif, at least two of which are cleaved by PC1 and PC2 in vitro, whereas the remainder are processed by enzymes that have not yet been identified (42). In addition, secondary structure predictions indicated that the 73-amino acid C-terminal VGF domain contains two α-helices that could represent interacting loops for sorting, whereas the N-terminal domain contains a single α-helix within the signal peptide.

Additional deletion analysis was therefore carried out, and VGF:GFP fusion proteins were expressed transiently in PC12 cells. Deletion of the N-terminal 61RAAR64 cleavage site did not block regulated release of the VGF-(1–53):GFP:VGF-(454–617) and VGF-(1–23):GFP:VGF-(454–617) fusion proteins from PC12 cells (Fig. 12, panel B). Deletion analysis of the C-terminal 72 amino acid tail indicated that regulated release was retained in fusion proteins that contained the 564RRR566 PC cleavage site within the short predicted α-helical loop 1 (see Fig. 12, panels A and C). Deletion of this RRR site, associated either with complete deletion or partial disruption of the 10-amino acid α-helical loop 1, resulted in release via the constitutive pathway (Fig. 12, panel C).

Selective Mutation of the Arg566 Cleavage Site Blocks Regulated Release of VGF-(1–65):GFP:VGF-(545–617) Whereas Proline Insertion into the α-Helix Containing the PC Cleavage Site Does Not—To determine whether both the VGF α-helical loop and convertase cleavage site were required for regulated secretion, we mutated the 564RRR566 cleavage site to 564AAA566 or 564AAR566 in the parental construct VGF-(1–65):GFP:VGF-(545–617), and in another mutant, we inserted flanking proline residues around the 564RRR566 cleavage site (P564RRR566P), had no effect on regulated release.

PCs Play a Role in the Regulated Secretion of VGF—To assess indirectly the importance of PCs and their cleavage sites to sorting, we treated PC12 cells with the membrane-permeable PC inhibitor decapeptide dec-FIGURE 10. Western analysis for VGF and VGF-(1–65):GFP:VGF-(452–617) in detergent-resistant raft fractions. Post-nuclear supernatants from PC12 cells and PC12 cells transfected with VGF-(1–65):GFP:VGF-(452–617) were extracted in 1% Triton X-100 in 50 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 7.4, for 30 min on ice. Cell extracts were centrifuged through 0.15:1.1:1.2M sucrose step gradients; gradients were fractionated from the top, and each fraction was trichloroacetic acid-precipitated and subjected to SDS-PAGE and Western analysis with antisera directed against VGF, GFP, flotillin-1, or the transferrin receptor. Analysis for VGF is shown in panel A and VGF-(1–65):GFP:VGF-(452–617) in panel B.

FIGURE 11. The C-terminal VGF-(545–617) domain is sufficient with VGF-(1–65) N-terminal domain to target GFP to the regulated release pathway. Panel A, equilibrium density gradient fractionation of post-nuclear supernatants from mixed stable transfectants was carried out as described in Fig. 5. Western analysis was carried out to localize endogenous VGF, transfected VGF-(1–65):GFP:VGF-(545–617), synaptophysin, and lactate dehydrogenase. Panel B, basal and KCl-stimulated release from mixed stable PC12 cell transfectants were measured by Western analysis using anti-VGF (endogenous VGF, upper panel) and anti-GFP (transfected fusion protein, lower panel) as described in Fig. 6. Panel C, sucrose gradient fractions containing LDCVs were saponin-permeabilized, subjected to aggregating (agg) or non-aggregating (non-agg) conditions, and analyzed as described in Fig. 9 for native VGF (upper panel) and transfected VGF-(1–65):GFP:VGF-(545–617) (lower panel).
FIGURE 12. An N-terminal signal peptide-containing domain with the VGF C-terminal α-helical loop and embedded PC cleavage site mediates sorting into the regulated release pathway in PC12 cells. Secondary structural predictions and locations of PC cleavage sites within the VGF N- and C-terminal domains are shown in panel A. For sequence analysis and structure prediction, we employed a suite of programs described under the “Experimental Procedures.” Panels B and C, plasmid constructs were transiently transfected into PC12 cells; domains included in these fusion proteins and their relationship to the secondary structure and paired basic residues are indicated in panel A. Panel B, sequences containing deletions within the N-terminal domain that remove the RAAR site were fused to GFP and the VGF-(454–617) C-terminal domain. Panel C, sequences containing deletions within the C-terminal 72-amino acid domain were fused to GFP and the VGF-(1–65) N-terminal domain. Basal and KCl-stimulated (stim) release were measured by Western analysis using anti-GFP (upper panel) to detect the transfected fusion protein and anti-VGF (lower panel) to detect endogenous VGF. aa, amino acids.

DISCUSSION

Our data define a C-terminal domain, containing a critical 564RRR566 cleavage site, that is both necessary and sufficient, together with an N-terminal signal peptide-containing domain, to target the soluble neuronal and endocrine polypeptide VGF into the regulated secretory pathway in PC12 and INS-1 cells. Targeting of soluble proteins destined for release from the regulated secretory pathway has been hypothesized to depend on sorting signals that control entry into the regulated secretory vesicle and/or protein domains that control aggregation and retention in the mature granule (2–5). To determine whether one or more active sorting mechanisms are required, putative targeting domains have been epitope-tagged or fused to cytoplasmic proteins such as GFP, and after transfection into various endocrine and neuroendocrine cells, the secreted proteins have been localized. With the exception of two studies that examined targeting of proinsulin (47) and NPY (19) to the regulated secretory pathway, where a signal sequence alone was sufficient to sort GFP to the regulated pathway, targeting has generally been shown to rely on either a helical loop or on critical paired amino acid residues that are sites of PC cleavage. For example, prosomatostatin (48) and vasoressin (34) sorting are dependent on N-terminal α-helical domains, and POMC targeting requires an N-terminal amphipathic loop that has been demonstrated to interact with CPE, the proposed sorting receptor for POMC (43). Although this DLEL-containing loop, constrained by cysteine residues, is also found in proenkephalin and proinsulin where it has been suggested to function as a sorting motif, neither chromogranin A (49) nor VGF, for example, utilize CPE as a sorting signal receptor. This suggests that additional sorting receptors remain to be identified, and chromogranin A, although it contains a POMC-like sorting signal, has been shown to interact with the inositol 1,4,5-triphosphate receptor (50–52).

We found that several N-terminal VGF fragments each containing the signal peptide, or the Ig leader, were insufficient for LDCV sorting or regulated release from PC12 and INS-1 cells. Previously, the signal peptide of NPY (NPY-(1–28)) alone was found to be sufficient to target GFP into PC12 secretory granules, and regulated release of this fusion
FIGURE 13. Mutation of either the \textsuperscript{564}RRR\textsuperscript{566} convertase cleavage site or the adjacent \textsuperscript{569}HFHH\textsuperscript{572} motif in VGF-(1–65):GFP/VGF-(545–617) blocks regulated release, whereas insertion of flanking proline residues P \textsuperscript{564}RRR\textsuperscript{566}P into VGF-(1–65):GFP/VGF-(545–617) does not. As shown in panel A, mutations in the C-terminal domain were made to assess independent contributions to VGF sorting of the \textsuperscript{564}RRR\textsuperscript{566} PC cleavage site (\textsuperscript{564}AAR\textsuperscript{566} and \textsuperscript{564}AAA\textsuperscript{566}), the predicted \(\alpha\)-helical domain (\(\textsuperscript{564}RRR\textsuperscript{566}\)), and the positively charged \textsuperscript{569}HFHH\textsuperscript{572} motif (\textsuperscript{569}AF\textsuperscript{572}). Panel B, PC12 cells were transiently transfected, and basal and KCl-stimulated release of mutant VGF-(1–65):GFP/VGF-(545–617) fusion proteins, and endogenous VGF, were analyzed by Western blotting as described in the legend to Fig. 12. Panel C, 3-h basal- and 3-h KCl-stimulated release of VGF were quantified in untreated PC12 cells, and those treated with the general PC inhibitor dec-RVKR-CMK (100 \(\mu\)M) for 48 (upper panel) or 96 h (lower panel). The two arrows indicate the VGF doublet (90 and 80 kDa) and the asterisk denotes one of the major processed forms; levels of the 80-kDa VGF and processed VGF are higher in media and cell extracts (not shown) from untreated PC12 cells in comparison to dec-RVKR-CMK-treated cells.

VGF and members of the chromogranin/secretogranin family are acidic proline-rich neuronal and neuroendocrine polypeptides that, although not structurally similar, share a number of features, including LDCV localization, regulated release, and multiple paired basic amino acid sequences that are cleaved to generate peptides. Sorting of chromogranin A and chromogranin B also appears to require N- and C-terminal domains depending on the cell type. The N-terminal disulfide bond is necessary for sorting of chromogranins A and B in neuroendocrine PC12 cells but not endocrine GH4C1 cells (26, 37, 55–57), whereas the 90-amino acid C-terminal domain of chromogranin A is required for sorting and aggregation in GH4C1 but not PC12 cells (37). The predicted peptide sequence of VGF (7), however, contains only a single cysteine residue in the signal peptide, so an N-terminal disulfide-stabilized loop cannot be a requirement for VGF sorting.

Models for regulated secretion generally include sorting of proteins into secretory granules by receptor-mediated transport or by association with lipids within rafts or via aggregation induced by low pH and calcium. Currently, little is known about the state of condensation or stabilization loop cannot be a requirement for VGF sorting.

VGF and members of the chromogranin/secretogranin family are acidic proline-rich neuronal and neuroendocrine polypeptides that, although not structurally similar, share a number of features, including LDCV localization, regulated release, and multiple paired basic amino acid sequences that are cleaved to generate peptides. Sorting of chromogranin A and chromogranin B also appears to require N- and C-terminal domains depending on the cell type. The N-terminal disulfide bond is necessary for sorting of chromogranins A and B in neuroendocrine PC12 cells but not endocrine GH4C1 cells (26, 37, 55–57), whereas the 90-amino acid C-terminal domain of chromogranin A is required for sorting and aggregation in GH4C1 but not PC12 cells (37). The predicted peptide sequence of VGF (7), however, contains only a single cysteine residue in the signal peptide, so an N-terminal disulfide-stabilized loop cannot be a requirement for VGF sorting.

Models for regulated secretion generally include sorting of proteins into secretory granules by receptor-mediated transport or by association with lipids within rafts or via aggregation induced by low pH and calcium. Currently, little is known about the state of condensation or aggregation of VGF in LDCVs, although both our studies and those of Gentile et al. (58) indicate that VGF cofractionates with insoluble secretory granule membranes under conditions of low pH and high calcium. Of note, aggregation is neither necessary nor sufficient for release of chromogranin B from the regulated secretory pathway in PC12 cells (4). Similarly, our data indicate that the C-terminal VGF deletions that block regulated release do not decrease the formation of fusion protein aggregates relative to endogenous VGF, suggesting that aggregation by
itself is not sufficient for targeting VGF or VGF:GFP fusion proteins into the regulated pathway. Deletions that removed the VGF C-terminal domain (e.g. VGF-(1–65):GFP:VGF-(454–544)) also substantially decreased but did not entirely eliminate fusion protein identified in LDCV vesicle fractions (see Fig. 5) or in VGF-containing LDCV-like particles by confocal microscopy (see Fig. 4 and TABLE ONE), suggesting that the C-terminal VGF domain (amino acids 545–617) may be required for efficient LDCV sorting, retention, or both.

Endogenous VGF is expressed in both PC12 and INS-1 cells, but several pieces of data suggest that it is unlikely to contribute sorting information through binding or aggregation with truncated VGF:GFP fusion proteins. First, we noted in our confocal studies that endogenous VGF and VGF:GFP fusion proteins (e.g. VGF-(1–65):GFP:VGF-(545–617)) only partially colocalize in LDCV-like particles. Although one explanation for this may be that these VGF:GFP proteins are not targeted exclusively to LDCVs, previous EM studies indicated that endogenous VGF was not found in all PC12 cell LDCVs but rather only in a subset (8), and gradient fractionation suggests that vesicles containing VGF and/or VGF-(1–65):GFP:VGF-(545–617) are of similar density. Finally by yeast two-hybrid screening, we have noted no interactions between VGF or the VGF C-terminal domain and any other VGF-encoding plasmids.7 More rigorous testing of a possible requirement for endogenous VGF in the regulated secretion of VGF:GFP fusion proteins should be possible through the analysis of VGF knock-out mice that express transgenic VGF-(1–65):GFP:VGF-(545–617) in neuronal and/or endocrine cells.

In general, sorting strategies appear to be cell type-specific and to vary depending on the secreted polypeptide examined (2–5). In fact, a recent report described the transfection of VGF-encoding constructs into Fischer rat thyroid cells, a poorly differentiated, polarized epithelial cell line that does not express VGF, and these studies noted that VGF and all deletion mutants examined were similarly sorted into dense core granules and were released via an apical secretory pathway (58). Together with our data, this suggests that the recognition of sorting domains that target VGF into the regulated pathway are cell type-specific. It is also interesting that similar VGF fragments in both studies were found to aggregate almost identically in permeabilized secretory granules under conditions of low pH and high calcium, suggesting that aggregation may be sufficient to properly target the contents of a vesicle destined for a polarized domain (e.g. apical versus basolateral membrane in epithelial cells) but may not be sufficient to distinguish regulated from constitutive pathways in a neuronal or endocrine cell. Although VGF is associated with LDCV membrane pellets, we did not detect VGF, nor significant amounts of VGF-(1–65):GFP:VGF-(454–617), in lipid rafts. Thus neither VGF nor chromogranin A (39) is localized in a detergent-resistant membrane fraction. It does remain possible that such an association occurs but that it is transient, occurring in immature secretory vesicles, or is of low affinity. Alternatively, VGF may associate with another protein (e.g. a sorting receptor) found in rafts, and this interaction could be transient or detergent-sensitive.

A C-terminal α-helical domain in PC1 was shown previously to be critical for targeting PC1 fusion proteins to the regulated secretory pathway (59). We used a number of independent algorithms to confidently and reproducibly predict the secondary structure of the VGF N- and C-terminal domains, which suggested by consensus that the C-terminal region contained two α-helical loops (Fig. 12). Within the VGF C terminus, several PC cleavage sites that may be candidate sorting signals were noted, similar in position to the C-terminal 617RR618 of PC1 that is involved in sorting to the regulated pathway (44); our data indicate that the 564RR566 in the VGF C terminus is critical for regulated release, whereas the α-helical loop is not.

Dibasic sequences have been suggested previously to play an active role in sorting proteins into the regulated secretory pathway; are essential for targeting PC1 (44), pro-NPY (53), NPY (53), prorenin (60), and pronepotensin (61); and are relatively commonly found in neuropeptides, PCs, members of the chromogranin-secretogranin family, the PC2-targeting protein 7B2, and hormones that are all stored in and released from secretory vesicles. In addition, dibasic sequences from pro-neurotensin can partially re-direct the constitutively secreted protein β-lactamase into the regulated pathway (62). The precise mechanism by which these positively charged residues mediate secretion is unclear, although they have been suggested to interact with the acidic polar heads of phospholipids (63, 64), possibly in lipid raft microdomains, or with enzymes that cleave or modify dibasic sites, including PCs and CPE, that could therefore act as sorting receptors (43). However, neither PC1, PC2, nor CPE seems to function as a general sorting receptor for all secreted hormones and neuropeptides; down-regulation of CPE in vitro or in vivo blocks regulated secretion of select hormones and growth factors (43, 65–68), whereas distribution studies have identified cells that do not express either PC1 or PC2 yet secrete polypeptides via the regulated pathway (62).

Consistent with a possible role for convertases in VGF secretion, treatment of PC12 cells with the PC enzyme inhibitor dec-RVKR-CMK partially blocked regulated release of VGF at similar concentrations (~100 μM) to those demonstrated previously to block constitutive secretion of soluble amyloid precursor protein α (21). In addition to a partial block of regulated VGF release at 48 h and a complete block at 96 h, and no significant change in basa VGF release, we also noted decreased VGF processing in cell extracts and media from dec-RVKR-CMK-treated PC12 cultures, an indication that convertase activity was broadly inhibited at these dec-RVKR-CMK concentrations. Although this does not prove that one or more proconvertases sort VGF in PC12 cells, it does suggest that both proconvertase catalytic activity and the RRR site are critical for regulated release. Which PCs might be involved in VGF processing and regulated secretion from PC12 cells? PC12 cells grown in the absence of nerve growth factor do not synthesize PC1 or PC2, contain low levels of PC5 (69, 70), and express furin, which is involved in prosomatostatin processing (71). PACE4 and PC7 are broadly expressed PCs (72–74); the latter is found in PC12 and insulinoma cells (75). In addition to VGF, PC12 cells process pro-SAAS and secrete pro-SAAS-derived peptides SAAS, PEN, and bigLEN (76), and pronepotensin (62), via the regulated pathway.

Because of the possible involvement of PCs in VGF sorting and the previous demonstration that a positively charged histidine residue, 6 amino acids N-terminal to the dibasic cleavage site (p6 position), is required for furin-mediated α4 integrin processing (46), coupled with the suggestion that histidines other than those at the P6 position might regulate PC processing (46), we investigated whether a cluster of histidine residues immediately C-terminal to 564RR566 of VGF is required for regulated release. Specific histidine residues that become positively charged at low pH apparently can restrict PC cleavage to the acidic environment of the secretory pathway (77); whether histidine residues might regulate a putative sorting interaction involving PCs, however, is unknown. We noted a partial blockade of regulated VGF:GFP secretion in the HFHH to AFAA mutation, similar to the partial effect that the His to Ala mutation at the P6 position has on α4 integrin cleavage by pro-hormone convertase 5A (46). Taken together, these data suggest that the 564RR566 PC cleavage site, an adjacent HFHH domain, and PC

---

7 T. Ito, J. Wu, and S. Salton, unpublished data.
catalytic activity each contribute to VGF sorting and its regulated release. Our data, demonstrating the importance of VGF sorting of prohormone convertase catalytic activity and a C-terminal RRR PC cleavage site, and previous studies indicating that dibasic sites are essential for targeting a number of polypeptides to the regulated secretory pathway, suggest that the molecular mechanisms that control regulated secretion in neuronal and endocrine cells are likely to involve interaction with specific dibasic sorting signals, perhaps utilizing members of the prohormone convertase family as sorting receptors.

REFERENCES

1. Bredt, D. S. (1998) Cell 94, 691–694
2. Arvan, P., and Castle, D. (1998) Biochem. J. 332, 593–610
3. Dannies, P. S. (1999) Endocr. Rev. 20, 3–21
4. Gorr, S. U., Jain, R. K., Kuehn, U., Joyce, P. B., and Cowley, D. J. (2001) Mol. Cell. Endocrinol. 172, 1–6
5. Tooze, S. A., Martens, G. J., and Huttner, W. B. (2001) Trends Cell Biol. 11, 116–122
6. Levy, A., Eldridge, I. D., and Paterson, B. M. (1985) Science 229, 393–395
7. Salton, S. R. (1991) J. Neurosci. 11, 9315–9324
8. Hahn, S., Mizuno, T. M., Wu, T. J., Wisor, J. P., Priest, C. A., Kozak, C. A., Boozer, C. N., Lee, C., El Meskini, R., Jin, L., Marx, R., Bruzzaniti, A., Lee, J., Emeson, R., and Mains, R. (2001) Exp. Cell Res. 269, 6140–6150
9. Feliciangeli, S., Kitabgi, P., and Bidard, J. N. (2001) J. Biol. Chem. 276, 6041–6049
10. Bundgaard, J., Rørberg, H., and Rehfeld, J. F. (2004) J. Biol. Chem. 279, 5484–5493
11. Chretien, M., Day, R., Marcinkiewicz, M., and Day, R. (1996) Biochem. Biophys. Res. Commun. 233, 680–686
12. VGF Sorting and Regulated Secretion in Neuronal and Endocrine Cells are Likely to Involve Interaction with Specific Dibasic Sorting Signals, Perhaps Utilizing Members of the Prohormone Convertase Family as Sorting Receptors.