Secretory Granule Biogenesis in Sympathoadrenal Cells

IDENTIFICATION OF A GRANULOGENIC DETERMINANT IN THE SECRETORY PROHORMONE CHROMOGRANIN A*

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Chromogranin A (CgA) may be critical for secretory granule biogenesis in sympathoadrenal cells. We found that silencing the expression of CgA reduced the number of secretory granules in normal sympathoadrenal cells (PC12), and we therefore questioned whether a discrete domain of CgA might promote the formation of a regulated secretory pathway in variant sympathoadrenal cells (A35C) devoid of such a phenotype. The secretory granule-forming activity of a series of human CgA domains labeled with a hemagglutinin epitope, green fluorescent protein, or embryonic alkaline phosphatase was assessed in A35C cells by deconvolution and electron microscopy and by secretagogue-stimulated release assays. Expression of CgA in A35C cells induced the formation of vesicular organelles throughout the cytoplasm, whereas two constitutive secretory pathway markers accumulated in the Golgi complex. The lysosome-associated membrane protein LGP110 did not co-localize with CgA, consistent with non-lysosomal targeting of the granin in A35C cells. Thus, CgA-expressing A35C cells showed electron-dense granules ~180–220 nm in diameter, and secretagogue-stimulated exocytosis of CgA from A35C cells suggested that expression of the granin may be sufficient to restore a regulated secretory pathway and thereby rescue the sorting of other secretory proteins. We show that the formation of vesicular structures destined for regulated exocytosis may be mediated by a determinant located within the CgA N-terminal region (CgA-(1–115)), with a necessary contribution of CgA-(40–115), but not the C-terminal region (CgA-(233–439)) of the protein. We propose that CgA promotes the biogenesis of secretory granules by a mechanism involving a granulogenic determinant located within CgA-(40–115) of the mature protein.

The regulated secretory pathway that exists in neurons and neuroendocrine cells is characterized by the concentration and sorting of a pool of secretory proteins into specialized intracellular organelles with a typical electron-dense appearance upon transmission electron microscopy, prompting the morphologic term dense-core granules. These granules may remain in the cell for an extended period of time after their formation, until prompted for exocytotic fusion with the plasma membrane by a secretagogue characteristic for a particular cell type.

The mechanism underlying the initiation and regulation of dense-core secretory granule biogenesis is poorly understood. The formation of secretory granules is believed to be initiated at the trans-Golgi network (TGN) apparatus, where aggregation and condensation of secretory proteins are typically viewed as the initial step prior to granulogenesis. Thus, formation of protein aggregates may occur in the mildly acidic environment that exists in the TGN and in the presence of millimolar concentrations of bivalent cations like Ca²⁺. Aggregation and condensation are the underpinning processes of two basic models of sorting of proteins within the regulated secretory pathway, viz. sorting for entry and sorting by retention (1–3). In the sorting-for-entry model, selective aggregation of the secretory protein may take place in the lumen of the TGN, followed by subsequent binding of a specific structural motif of the protein aggregate to the membrane of the nascent secretory granule or to a sorting receptor therein. In the sorting-by-retention model, sorting takes place in post-TGN immature secretory granules, wherein selective aggregation and condensation occur, leading to preferential retention of regulated secretory proteins while unaggregated (non-retained) proteins are removed from maturing granules by clathrin-coated vesicles through a constitutive-like secretory pathway.

The prohormone chromogranin A (CgA) is a member of the granin family of regulated secretory proteins, which are widely distributed in secretory granules of endocrine, neuroendocrine, and neuronal cells (4). Because of its abundance and its susceptibility to form aggregates in the presence of millimolar Ca²⁺ and in the mildly acidic pH environment that exists in the TGN

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2 The abbreviations used are: TGN, trans-Golgi network; CgA, chromogranin A; CgB, chromogranin B; GFP, green fluorescent protein; EAP, embryonic alkaline phosphatase; HA, hemagglutinin; SgP, signal peptide; EGF, enhanced green fluorescent protein; SEAP, secreted embryonic alkaline phosphatase; PBS, phosphate-buffered saline; siRNA, small interfering RNA; TEM, transmission electron microscopy; FACS, fluorescence-activated cell sorting; GH, human growth hormone; BoNT/C1, botulinum neurotoxin C1 light chain.
and within the immature secretory granule, CgA has long been suspected to contribute to some key aspect of the formation of dense-core secretory granules (5–9). Indeed, recent studies have provided evidence that CgA is required for the formation of dense-core secretory granules and hormone sequestration in sympathoadrenal chromaffin cells. For instance, depletion of CgA in the sympathoadrenal PC12 cell line by antisense or small interfering RNAs reduces the number of dense-core secretory granules (this study and Refs. 10 and 11) and the intracellular levels of other secretory granule proteins (10). Evidence for a granulogenic function of CgA was also documented in vivo, where genetic ablation of the mouse CgA gene (12) or transgenic expression of antisense RNA against CgA in mice (13) impairs the formation of catecholamine storage vesicles in adrenal chromaffin cells (12, 13) and reduces the content of other chromaffin granule constituents such as catecholamines, chromogranin B (CgB), and neuropeptide Y (12). A contribution of CgA to the formation of secretory granules has also been documented across cell lineages. For instance, introduction of CgA into non-neuroendocrine fibroblast cell lines such as CV1, NIH/3T3, and COS-7 cells drives the formation of dense-core vesicles, allowing regulated exocytosis of the granin cargo in response to secretagogue stimulation (10, 11, 14).

We reported previously that information necessary for CgA trafficking within the regulated pathway of chromaffin cells is contained in the N-terminal region of the protein (CgA-(1–115)) of the granin (15, 16). Thus far, little information is available on specific structural determinant(s) in CgA that may be required for the granulogenic function of the protein in sympathoadrenal cells. Thus, we have proposed recently that a V-ATPase-mediated acidicification of the TGN and/or the immature chromaffin granule contributes to the sorting of CgA to the regulated secretory pathway and the biogenesis of dense-core secretory granules by a mechanism that may recruit a sorting/granulogenic determinant located within CgA-(1–115), but not the C-terminal region of the protein (9).

To further refine these studies on the presence of both cis- and trans-determinants for the granulogenic function of CgA, we questioned whether expression of a specific domain of CgA might effectively drive the formation of regulated secretory organelles in a sympathoadrenal cell type devoid of such a phenotype. The sympathoadrenal cell variant A35C lacks dense-core secretory granules or a regulated secretory pathway and does not express several membrane or soluble secretory granule proteins, including synaptophysin, and several members of the granin family such as CgA, CgB, and secretogranin II (17).

In this work, we further probed the granulogenic function of CgA in wild-type sympathoadrenal PC12 cells and studied the intracellular trafficking of a series of human full-length CgA or truncated domains fused to green fluorescent protein (GFP), embryonic alkaline phosphatase (EAP), or an influenza hemagglutinin (HA) epitope after adventitious expression in A35C cells. Our results show that silencing the expression of CgA in normal PC12 cells decreased the number of secretory granules, whereas expression of CgA in the sympathoadrenal cell variant A35C drove the formation of dense-core vesicular organelles containing a releasable cargo of CgA in response to extracellular stimulation of the cells. Our data suggest that expression of CgA in A35C cells restores a functional regulated secretory pathway, allowing regulated trafficking of other secretory proteins (e.g. growth hormone) that would be otherwise constitutively secreted in naive A35C cells. We propose that CgA contains a granulogenic determinant located within its N-terminal (CgA-(40–115)), but not C-terminal (CgA-(233–439)), region.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transient Transfections**—Rat pheochromocytoma PC12 cells were cultured as described previously (16). Rat pheochromocytoma A35C cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum (Gemini Bio-Products), 100 μg/ml streptomycin, and 100 units/ml penicillin (Invitrogen). Supercoiled plasmid DNA for transfection was grown in *Escherichia coli* strain DH5α (Invitrogen) and purified on columns (Qiagen Inc.). Two days before transfection, cells were split onto either poly-L-lysine (Sigma)/collagen (Upstate)-coated 15-mm round glass coverslips (Fisher No. 1) in 12-well Costar plates or uncoated 6- or 12-well Costar plates. Cells were transfected with 1.25 μg (12-well plate), 2 μg (6-well plate), or 10 μg (100-mm plate) of supercoiled plasmid DNA/well using a high efficiency cationic scaffold method (GenePORTER 2, Gelantis) according to the manufacturer’s instructions. Five hours after the beginning of transfection, the culture medium was replaced, and cells were further cultured for 48 h.

**Construction of Expression Vectors**—The expression vectors for human full-length CgA and CgA domain chimeric proteins (including the CgA 18-amino acid signal peptide (SgP), MRSAAVILLLLCLAGQVTA) fused to the enhanced GFP (EGFP) gene (pCMV-CgA-EGFP, pCMV-SgP-EGFP, pCMV-CgA253-EGFP, pCMV-CgA481-EGFP, pCMV-CgA805-EGFP, and pCMV-CgA253-EGFP) or to a truncated domain of the human full-length secreted embryonic alkaline phosphatase (SEAP) gene (pCMV-CgA-EAP, pCMV-CgA253-EAP, and pCMV-CgA481-EAP) were constructed as described previously (9, 15). The pSEAP2-Basic plasmid (Clontech) was used for the eukaryotic expression of full-length SEAP (including the SEAP 17-amino acid signal peptide, MLLLLLLGLRQLSLG). To label proteins at the C terminus with the HA epitope, a double-stranded DNA oligonucleotide incorporating a KpnI and a NotI site and encoding YPYDVPDYA-stop was ligated in-frame into the KpnI and NotI cloning sites of the EGFP gene in pCMV-CgA-EGFP, pCMV-CgA481-EGFP, or pCMV-CgA253-EGFP (15) to generate pCMV-CgA-HA, pCMV-CgA481-HA, or pCMV-CgA253-HA, respectively. The CgA481-HA fragment obtained after digestion of pCMV-CgA481-HA with Xhol and NotI was subcloned into the same sites of the internal ribosome entry site-based bicistronic vector pPRIG (18) to produce pPRIG-CgA481-HA. Plasmid numbering refers to the position of the 3’-end of the CgA fragment subcloned within the original human CgA cDNA (GenBank™ accession number NM_001275) (Fig. 1). All constructs were verified by restriction and nucleotide sequence analyses.

**Chimeric Photoprotein Fluorescence and Immunocytochemistry**—Transfected cells cultured on glass coverslips were fixed for 1 h at room temperature with 2% paraformaldehyde in
phosphate-buffered saline (PBS) (pH 7.4), permeabilized with 0.1% Triton X-100 in PBS (10 min), and exposed to the nucleic acid stain Hoechst 33342 (1 μg/ml; Molecular Probes) for nuclei visualization. Coverslips were subsequently washed with PBS, mounted in buffered Celvol (Celanese), and processed for three-dimensional imaging by deconvolution microscopy. For immunocytochemistry, fixed and permeabilized cells were incubated for 5 min in PBS containing 0.1 M glycine and subsequently exposed for 30 min to PBS containing 5% fetal calf serum to reduce nonspecific antibody labeling. Cells were then incubated for 1 h at room temperature with rabbit anti-human placental alkaline phosphatase polyclonal antibody (1:50; Biomedia), mouse anti-HA monoclonal antibody HA.11 (1:1000; Covance), or rabbit anti-rat LGP110 polyclonal antibody (1:1000 in buffer containing 1% bovine serum albumin in PBS) (19). Cells were then washed and incubated for 30 min with Alexa Fluor 594-conjugated goat anti-mouse IgG or anti-rabbit IgG (F(ab')2 at 1:250; Molecular Probes) together with 1 μg/ml Hoechst 33342. Coverslips were subsequently washed with PBS, mounted in buffered Celvol, and processed for three-dimensional imaging by deconvolution microscopy.

**Three-dimensional Imaging by Deconvolution Microscopy**—Images were captured on a DeltaVision O2 workstation using a ×60 (numerical aperture of 1.4) or ×100 (numerical aperture of 1.4) oil immersion objective. The system included a Photometrics CoolSNAP HQ2 CCD camera mounted on a Nikon inverted fluorescence/differential interference contrast microscope and a mercury arc lamp light source. Fluorescence was detected using a standard DeltaVision 4′,6-diamidino-2-phenylindole/fluorescein isothiocyanate/Texas Red filter set. Pixel intensities were kept in the linear response range of the digital camera. Optical sections along the z axis were acquired with increments of 0.2 μm. The fluorescence data sets were deconvolved and analyzed using DeltaVision softWoRx programs on a Silicon Graphics Octane workstation to generate three-dimensional images of the data sets.

**Silencing of CgA Expression by Small Interfering RNA (siRNA)**—CgA siRNA (5′-CAACAACAACACACGACACUdTdT-3′ (sense) and 5′-AGCUGCGUGUGUGUGUUGdTdT-3′ (antisense)) and scrambled CgA siRNA (CT-CgA siRNA, 5′-GCCACAUACAGGACAAAdTdT-3′ (sense) and 5′-UUUGUGUUGUGUGUGUGGdTdT-3′ (antisense)) were synthesized (Dharmacon) according to a AA(N19)TT pattern (siRNA selection software, Whitehead Institute, Cambridge, MA). Annealed siRNA duplexes were resuspended in RNase-free solution buffered to pH 7.4. One day before transfection...
fection, PC12 cells maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum, 5% fetal bovine serum, and antibiotics were grown on poly-L-lysine-coated 12-well plates. Cells were transfected for 72 h with 4 μg/well siRNA CgA duplex using RNAiFect transfection reagent (Qiagen Inc.) according to the manufacturer’s instructions. Silencing of CgA expression was evaluated by immunoblotting, and the effect of reduced expression of CgA on dense-core secretory granule cellular content was examined by transmission electron microscopy (TEM).

Flow Cytometry—A35C cells grown on 100-mm plates and transiently transfected with the bicistronic expression vector pPRIG (expressing GFP alone) or pPRIG-CgA481-HA (expressing two separate open reading frames: GFP and SgP-CgA-(1–115)-HA) were suspended in Accutase detachment solution (Innovative Cell Technologies) at a concentration of 2 × 10⁶ cells/ml. Cells (7 × 10⁶/sample) were analyzed for GFP fluorescence using a MoFlo cell sorter (DakoCytomation). Fluorescence-activated cell sorting (FACS) resulted in the isolation of either pPRIG- or pPRIG-CgA481-HA-expressing cells, with both populations showing ~72% of cells positive for GFP fluorescence. An aliquot of each cell sample was processed for immunocytochemistry (anti-HA monoclonal antibody) and three-dimensional fluorescence microscopy to confirm the vesicular distribution of SgP-CgA-(1–115)-HA and the concurrent cytosolic expression of GFP. The remaining cells were processed for imaging by TEM.

Electron Microscopy—Cells were fixed overnight at 4 °C in modified Karnovsky’s fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4)), followed by 1% OsO₄ in 0.1 M sodium cacodylate buffer (pH 7.4), and subsequently dehydrated using a graded series of ethanol solutions, followed by propylene oxide and infiltration with modified Karnovsky’s fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4)), followed by 1% OsO₄ in 0.1 M sodium cacodylate buffer (pH 7.4), and subsequently dehydrated using a graded series of ethanol solutions, followed by propylene oxide and infiltration with epoxy resin. After polymerization overnight at 65 °C, thin sections were cut and stained with 4% uranyl acetate in 50% ethanol, followed by bismuth subnitrate. Sections were examined at an accelerating voltage of 60 kV using a Zeiss EM10B electron microscope.

Chemiluminescence Detection of EAP Secretion and Colorimetric Immunooassay of Human Growth Hormone (GH) Secretion—A35C cells transfected with an expression plasmid for a CgA domain-EAP chimera or for GH (pXGH5, Nichols Institute) were grown on 6- or 12-well culture dishes. Cells were washed with calcium secretion buffer (150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, and 10 mM HEPES (pH 7.4)) and subsequently exposed to calcium secretion buffer or barium secretion buffer (150 mM NaCl, 5 mM KCl, 2 mM BaCl₂, and 10 mM HEPES (pH 7.4)) for 15 min. Supernatants were collected, and cell lysates were prepared by quick freeze/thaw in calcium secretion buffer containing 0.1% Triton X-100. EAP enzymatic activity or GH was measured in the culture supernatant and cell lysate. Detection of EAP activity from CgA-EAP chimera-expressing PC12 or A35C cells was achieved with a high sensitivity chemiluminescence assay (Phospha-Light, Applied Biosystems) using a AutoLumat 953 luminometer (EG&G Berthold). GH was measured with a colorimetric enzyme immunoassay (GH enzyme-linked immunosorbent assay, Roche Diagnostics) according to the manufacturer’s instructions using a Spectramax microplate reader. The secretion rate was calculated as a percentage of the total EAP activity or GH present in the cells before stimulation. Total EAP activity or GH is the sum of the amount released plus the amount remaining in the cells.

Secretion Assay of Epitope-tagged CgA—Transfected A35C cells grown on poly-L-lysine-coated 100-mm tissue culture dishes were extensively washed with calcium secretion buffer and sequentially exposed to calcium secretion buffer (15 min, 37 °C, 5% CO₂), followed by incubation in barium secretion buffer (15 min, 37 °C, 5% CO₂). Culture supernatants were collected at the end of each 15-min incubation period, cleared by centrifugation at 4000 × g for 10 min at 4 °C, and concentrated using reverse-phase SepPak C18 silica cartridges (Millipore). The solvent system consisted of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in 100% CH₃CN. Eluates were lyophilized and processed for immunoblotting.

Gel Electrophoresis and Immunoblot Analysis—In some experiments, total cell lysates were prepared by detergent extraction for 20 min at 4 °C in buffer containing 10 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 5 mM EDTA, protease inhibitor mixture (Calbiochem), and 50 mM Tris-HCl (pH 8.0). The lysate were cleared by centrifugation at 23,000 × g for 10 min at 4 °C. Proteins were separated by SDS-PAGE on 10% polyacrylamide gels (NuPAGE, Invitrogen) and transferred onto nitrocellulose sheets (Schleicher & Schuell). Membranes were blocked for 1 h either in buffer containing 5% heat-inactivated fetal calf serum plus 0.05% Tween 20 in PBS or in 5% nonfat dry milk in PBS. Nitrocellulose blots were subsequently incubated for 2 h with mouse anti-HA monoclonal antibody HA.11 (1:1000 in 5% heat-inactivated fetal calf serum plus 0.05% Tween 20 in PBS), rabbit anti-catestatin polyclonal antibody (rat CgA-(363–383); 1:1000 in 5% nonfat dry milk in PBS) (20), rabbit anti-human CgA polyclonal antibody (1:10,000 in 5% nonfat dry milk in PBS) (21), or anti-actin monoclonal antibody I-19 (1:1000 in 5% fetal calf serum; Santa Cruz Biotechnology, Inc.) and washed for 15 min with 0.05% Tween 20 in PBS. Blots were subsequently incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Fcy fragment; 1:7500 in 5% fetal calf serum plus 0.05% Tween 20; BIODESIGN International) or horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000 in 5% nonfat dry milk in PBS; Bio-Rad). Immunoactive bands were visualized by detection of peroxidase activity by chemiluminescence (SuperSignal West Pico, Pierce). In some experiments, protein expression was evaluated by densitometry (NIH Image Version 1.6).

Presentation of Data and Statistics—Values are given as the means ± S.E. of at least triplicate determinations. In the figures, data are representative of a typical experiment repeated twice or more. The number of independent experiments or analyses (n) performed for the results presented in each figure is as follows: Fig. 2, n = 2; Fig. 3, n > 3; Fig. 4, n = 3; Fig. 5, n > 3; Fig. 6, n > 3; Fig. 7, n = 2; Fig. 8, n = 2; Fig. 9, n = 2; Fig. 10, n > 2; Fig. 11, n ≥ 2; and Fig. 12, n ≥ 3. Statistical analysis was performed by Student’s t test or analysis of variance with Dunnett’s or Bonferroni’s post test using the KaleidaGraph statistical software package (Synergy Software). Differences were considered significant when p was < 0.05.
RESULTS

Contribution of CgA to the Biogenesis of Dense-core Granules in Normal Sympathoadrenal PC12 Cells—The effect of reduced expression of CgA on the formation of secretory granules in normal PC12 cells was assessed using a siRNA methodology. A 22-bp siRNA duplex was prepared (CgA siRNA) to target the sequence corresponding to nucleotides 311–333 of the rat CgA mRNA (start codon at position 11001; GenBank accession number AF145445). As shown in Fig. 2, transfection of PC12 cells with CgA siRNA reduced endogenous CgA by 80% when normalized to the control housekeeping protein actin (Fig. 2, A and B). In contrast, the level of expression of CgA was unaffected by a siRNA duplex corresponding to a randomly scrambled sequence of CgA siRNA (CT-CgA siRNA) (Fig. 2, A and B).

Ultrastructural examination of wild-type PC12 cells treated with siRNA transfection reagent showed abundant dense-core granules docked onto or in the vicinity of the plasma membrane (Fig. 2C). A 3-day exposure of PC12 cells to CgA siRNA decreased the number of secretory granules (Fig. 2D); the abundance of dense-core granules, as defined by the number of granules found in an xy section of the mid-cell body, was lowered by 47% (p < 0.001) (Fig. 2E). The overall morphological appearance of the nuclei, mitochondria, and Golgi complex in siRNA-treated cells was indistinguishable from that in control cells, further suggesting the specificity of the change in chromaffin granules.

Vesicular Storage of Human CgA or Regulated Secretory Human CgA Chimeric Proteins in the Sympathoadrenal Cell Variant A35C—We reported previously that CgA fused to GFP (SgP-CgA-GFP) or to an engineered form of human EAP (SgP-CgA-EAP) is trafficked to dense-core chromaffin granules of sympathoadrenal PC12 cells and released by exocytosis (9, 15). The subcellular distribution of SgP-CgA-GFP, SgP-CgA-EAP, or CgA tagged with the short 9-amino acid HA epitope (SgP-CgA-HA) (Fig. 1) was examined by deconvolution microscopy after transient expression of the labeled proteins in A35C cells. Computational three-dimensional reconstruction of the intracellular localization of SgP-CgA-HA, SgP-CgA-GFP, and SgP-CgA-EAP revealed punctate fluorescence signals throughout the cell body (Fig. 3), suggesting storage of these regulated secretory proteins in vesicular structures, even in a cell type lacking regulated secretory organelles (A35C) (17). In contrast, expression of the constitutive secretory pathway markers SgP-GFP (15) and SEAP (9, 22) caused a marked accumulation of fluorescence in the perinuclear region of the cell (Fig. 3), which is consistent with the previously documented Golgi complex accumulation of these constitutively trafficked proteins in PC12 cells (9, 15). The effect of CgA expression on granule formation was also studied at the ultrastructural level by TEM. As documented previously
whether the CgA-positive punctate structures observed in CgA-expressing A35C cells (Figs. 3, 4, and 9) might be the result of the targeting of CgA to lysosomal compartments. Immunostaining of SgP-CgA-GFP-expressing A35C cells for the lysosomal type I integral membrane glycoprotein LGP110 (equivalent to lysosome-associated membrane protein), a ubiquitous marker of late endosomes and lysosomes (19, 26), did not show co-localization with the regulated secretory photoprotein SgP-CgA-GFP (Fig. 6 and inset), consistent with a non-lysosomal storage of the chimera.

Secretagogue-stimulated Secretion of CgA Expressed in A35C Cells—We questioned whether CgA-containing vesicular structures that are formed after expression of CgA in regulated secretory pathway-deficient A35C cells might be competent for regulated exocytosis. Restoration of a regulated secretory pathway by CgA would therefore predict that CgA undergoes regulated secretion. As a prerequisite for this experiment, we assessed the expression of endogenous CgA in A35C cells compared with PC12 cells. As shown in Fig. 7A, immunoreactivity for CgA was not detected in naive untransfected A35C cells, confirming previous observations that this cell line lacks endogenous expression of CgA (17).

Our general strategy was first to establish qualitatively, by immunoblotting, whether CgA (or a CgA subdomain) labeled with HA (SgP-CgA-HA) enters or creates a regulated secretory pathway. Then we tested the extent of such regulated secretion using the more readily quantitated technique of secretion of EAP chimeras (9) following the EAP reporter by quantitative luminometry on post-secretion cell supernatants.

A35C cells were transfected with SgP-CgA-HA, and the release of the granin in the extracellular milieu was assayed by immunoblotting in the presence or absence of the potent sympatoadrenal cell secretagogue BaCl2 (2 mM, 15 min) (9, 15, 27). Ba2+ triggered a large release of CgA immunoreactivity from SgP-CgA-HA-expressing A35C cells, whereas only a low amount of the granin was detected during a similar 15-min incubation period in the absence of stimulation (Fig. 7B).

Further insight into the secretory profile of CgA in A35C cells was obtained using the regulated secretory chimeric protein SgP-CgA-EAP (Fig. 1) (9). We showed previously that CgA fused to the N terminus of a truncated form of SEAP is sorted to the lysosomal type I integral membrane glycoprotein LGP110 (equivalent to lysosome-associated membrane protein), a ubiquitous marker of late endosomes and lysosomes (19, 26), did not show co-localization with the regulated secretory photoprotein SgP-CgA-GFP (Fig. 6 and inset), consistent with a non-lysosomal storage of the chimera.

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chimera through a regulated secretory pathway. In contrast, the release of unfused SEAP (including a 17-amino acid signal peptide, MLLLLLLLLGLRLQLSLG), a typical marker of the constitutive pathway of secretion (9, 22, 28), was stimulated by only ~1.5-fold (Fig. 7C). To ascertain that the release of CgA from transfected cells was the result of exocytic fusion of secretory granules, we examined the effect of transient expression of the botulinum neurotoxin C1 light chain (BoNT/C1) on the secretagogue-evoked release of SgP-CgA-EAP in PC12 and A35C cells. BoNT/C1 cleaves the syntaxin-1 and SNAP-25 (soluble N-ethylmaleimide-sensitive factor attachment protein) components of the SNARE (SNAP receptor) complex of the exocytotic machinery, thereby disrupting the late stage of secretory biogenesis.

**FIGURE 4.** Dense-core granules in A35C cells expressing SgP-CgA-HA compared with wild-type PC12 cells. Control A35C cells (transfection reagent alone; A), SgP-CgA-HA-expressing A35C cells (A and B), or wild-type PC12 cells (C) were processed for TEM. Newly formed dense-core granules are indicated by arrowheads in A. Scale bars = 1 μm. The high magnification electron micrographs in B and C show the presence of electron-dense, membrane-delimited vesicles (arrows). Scale bars = 100 nm.

**FIGURE 5.** Distribution of CgA along and at the tips of neurites in A35C cells. A35C cells were transfected with the expression plasmid encoding full-length CgA labeled with the HA epitope (SgP-CgA-HA). Cells were processed for immunocytochemistry using anti-HA monoclonal and Alexa Fluor 594-conjugated secondary antibodies, and SgP-CgA-HA-positive A35C cells displaying long processes were imaged by three-dimensional deconvolution microscopy as described in the legend of Fig. 3. Substantial accumulation of HA-tagged CgA is seen at the tip of neurites as shown in the enlarged insets in the deconvolved three-dimensional images.

**FIGURE 6.** Intracellular distribution of CgA and the endosomal/lysosomal marker LGP110 in A35C cells. A35C cells were transfected with the expression plasmid encoding SgP-CgA-GFP, processed for immunocytochemistry using anti-LGP110 polyclonal and Alexa Fluor 594-conjugated secondary antibodies, and imaged by three-dimensional deconvolution fluorescence microscopy as described in the legend of Fig. 3. Nuclei were visualized with Hoechst 33342 (blue). Overlap (yellow) in the distribution of SgP-CgA-GFP (green) and LGP110 (red) was very low outside of the perinuclear Golgi region, as shown in the enlarged inset in the merged image.
As expected for a fusion protein targeted to chromaffin granules (9), Ba\(^{2+}\)-evoked release of SgP-CgA-EAP was blunted in PC12 cells cotransfected with BoNT/C1 (p < 0.01) (Fig. 7D). Similarly, we found that Ba\(^{2+}\)-stimulated release of SgP-CgA-EAP from A35C cells was fully inhibited by BoNT/C1 (p < 0.01) (Fig. 7E), which clearly indicates that secretagogue-evoked release of SgP-CgA-EAP in A35C cells is exocytotic. Taken together, these results suggest that introduction of CgA into a cell type lacking regulated secretory organelles (A35C) is sufficient to create a functional regulated secretory pathway.

Expression of CgA in A35C Cells Redirects the Human Regulated Secretory Protein GH from a Constitutive to a Regulated Secretory Pathway—GH may be expressed in sympathoadrenal cells, where it is sorted to the regulated pathway of secretion (30, 31). Consistent with these studies, we found that the release of GH from transfected PC12 cells was increased by Ba\(^{2+}\) (2 mM, 15 min) to a value of 16.2 ± 1.4% over the basal level (p < 0.01) (Fig. 8A), confirming routing of GH to the regulated secretory pathway. Mistargeting of endogenous or exogenously expressed proteins normally trafficked to the regulated secretory pathway has been documented in A35C cells (17). Indeed, we found that Ba\(^{2+}\) stimulation of GH-expressing A35C cells did not significantly increase the net release of GH (p > 0.05) (Fig. 8A and B), indicating that exogenously expressed GH may traffic primarily though a constitutive (basal) pathway of secretion.

Considering that expression of CgA may be sufficient to restore a functional regulated secretory pathway in A35C cells (Fig. 7), we wondered whether CgA might influence the constitutive secretory profile of GH. Ba\(^{2+}\)-evoked GH release from A35C cells was evaluated after cotransfection with pXGH5 together with a control vector (pcDNA3.1) or pCMV-CgA-HA. Consistent with the results shown in Fig. 8A, exposure of GH-expressing A35C cells to BaCl\(_2\) (2 mM, 15 min) did not increase GH release above its basal level (p > 0.05) (Fig. 8B). In sharp contrast, Ba\(^{2+}\)-evoked release of GH from A35C cells cotransfected with CgA was augmented, reaching a value of 7.2 ± 1.5% over the basal level (p < 0.01) (Fig. 8B). Thus, CgA influences trafficking of GH in A35C cells by rerouting this otherwise regulated secretory protein from a constitutive to a regulated secretory pathway.

Identification of a Granulogenic Determinant within the Primary Structure of CgA—We showed previously that information necessary for the regulated trafficking of CgA in PC12 cells is contained within its N-terminal region (CgA-(1–115)), but not in the C-terminal half-region of the protein (9, 15). We therefore examined whether such a determinant for sorting
FIGURE 9. Intracellular distribution and secretagogue-evoked release of the CgA proximal N-terminal domain and C-terminal half-domain expressed in A35C cells. A35C cells were transfected with the expression plasmid encoding the proximal N-terminal domain of CgA (SgP-CgA-(1–115)-HA) or the C-terminal half-domain of CgA (SgP-CgA-(233–439)-HA) tagged with HA (A and B). A, cells expressing HA-tagged deletion mutants of CgA were processed for immunocytochemistry using anti-HA primary and Alexa Fluor 594-conjugated secondary antibodies (red), followed by deconvolution microscopy imaging to generate a three-dimensional/volume view of the subcellular distribution of the recombinant proteins as described in the legend of Fig. 3. Nuclei were visualized with Hoechst 33342. B, shown is the immunochromed detection of Ba\(^{2+}\)-evoked release of SgP-CgA-(1–115)-HA or SgP-CgA-(233–439)-HA. Transfected A35C cells were exposed for 15 min to secretion medium alone or to 2 mM BaCl\(_2\). Culture supernatants were collected, concentrated by reverse-phase chromatography, and processed for immunoblotting using anti-HA antibody.

into the regulated pathway of PC12 cells might also determine the formation of granular structures elicited by CgA in A35C cells.

Deconvolution Fluorescence Microscopy Analysis of the Subcellular Distribution and Immunological Detection of Secretagogue-evoked Release of the CgA Proximal N-terminal Domain and C-terminal Half-domain in A35C Cells—The granule-forming activity of the proximal N-terminal domain of CgA (SgP-CgA-(1–115)-HA) and the C-terminal half-domain of CgA (SgP-CgA-(233–439)-HA) was evaluated in A35C cells by three-dimensional deconvolution microscopy and by immunoblot analysis of the secretagogue-evoked release of the CgA segments. When expressed in A35C cells, SgP-CgA-(1–115)-HA localized to vesicular structures, whereas SgP-CgA-(233–439)-HA accumulated in a perinuclear region of the cell characteristic of the Golgi complex (Fig. 9A). Ba\(^{2+}\) (2 mM, 15 min) stimulated the release of HA immunoreactivity into the extracellular medium of SgP-CgA-(1–115)-HA-expressing A35C cells, whereas no SgP-CgA-(1–115)-HA release was detected in cells exposed to mock (control) secretion medium during a similar 15-min incubation period (Fig. 9B), suggesting that constitutive (basal) release of the protein was minimal in non-stimulated cells. Thus, the secretory profile of SgP-CgA-(1–115)-HA observed in transfected A35C cells is consistent with routing of the protein into a regulated secretory pathway. In contrast, the release of SgP-CgA-(233–439)-HA was not stimulated by Ba\(^{2+}\) as indexed by immunoblotting of the HA-tagged form (Fig. 9B), suggesting that the C-terminal half-domain of CgA did not enter a regulated secretory pathway. Indeed, the release of SgP-CgA-(233–439)-HA seemed actually to diminish slightly after Ba\(^{2+}\) stimulation (Fig. 9B), although this likely reflects the sequential assay protocol (mock stimulation followed by secretagogue) rather than any real decline in release. The contribution of CgA-(1–115) to granule formation was also studied at the ultrastructural level by TEM. Fluorescence flow cytometric sorting of A35C cells transfected with the bicistronic expression vector pPRIG-CgA481-HA (expressing both GFP and SgP-CgA-(1–115)-HA) prior to TEM analysis increased the number of SgP-CgA-(1–115)-HA-expressing A35C cells from 9 to 72%. Efficiency of cell sorting and concurrent expression of GFP with SgP-CgA-(1–115)-HA were first assessed by deconvolution microscopy. As expected, unfused GFP was diffusely distributed in the cell cytosol and nucleus (see Fig. 12A), whereas SgP-CgA-(1–115)-HA localized to vesicular structures (see Fig. 12B). Examination of such transfected A35C cells by TEM revealed the presence of electron-dense granular structures (see Fig. 12B), although with a slightly wider ~110–230-nm range in diameter than the ~180–220-nm range of dense-core granules formed in A35C cells expressing full-length CgA (Fig. 4, B and C). The number of newly formed dense-core vesicles, as defined by the number found in an xy section of the mid-cell body, was 4.76 ± 0.67 (mean ± S.E., n = 17 TEM images). Consistent with the results shown in Fig. 4A, dense-core secretory granules were not detected in control A35C cells expressing unfused GFP alone (see Fig. 12C). Taken together, these results indicate that a determinant located within the N-terminal (CgA-(1–115)), but not C-terminal (CgA-(233–439)) region of the granin may be sufficient to trigger the formation of vesicular structures as well as a regulated secretory pathway in A35C cells.

Subcellular Distribution of CgA Domain-GFP Fusion Proteins in A35C Cells—To further investigate the presence of a discrete domain within the CgA N-terminal region that may contribute to the accumulation of CgA within vesicular structures, we examined the intracellular distributions of a series of full-length or truncated CgA-GFP chimeras by three-dimensional deconvolution microscopy (Fig. 10). When expressed in A35C cells, the signal sequence of CgA (SgP-GFP) (Fig. 1), the short N-terminal hydrophobic disulfide loop-containing domain of CgA (SgP-CgA-(1–39)-GFP) (Fig. 1), or the C-terminal half-region of the granin (SgP-CgA-(233–439)-GFP) (Fig. 1) fused to GFP exhibited a characteristic Golgi complex accumulation (Fig. 10) consistent with the previously reported constitutive trafficking of these photoproteins in PC12 cells (15). In contrast, expression of the longer CgA N-terminal domains (SgP-CgA-(1–115)-GFP and SgP-CgA-(1–224)-GFP) or full-length CgA fused to GFP (Fig. 1) revealed abundant punctate fluorescence signals throughout much of the cell body (Fig. 10), consistent with storage of the chimeras in vesicular organelles.

Secretagogue-evoked Release of CgA Domain-EAP Fusion Proteins in A35C Cells—Full-length CgA and truncated domains of CgA fused to EAP may be used to assess sorting to chromaffin secretory granules and secretagogue-mediated exocytosis in PC12 cells (9). Analysis of the subcellular distribution...
of the CgA N-terminal region (SgP-CgA-(1–115)-EAP) and full-length CgA (SgP-CgA-EAP) in A35C cells revealed a vesicular accumulation of the chimeras throughout the cell body (Fig. 11A). Moreover, exposure of such transfected A35C cells to BaCl2 (2 mM, 15 min) stimulated the release of SgP-CgA-(1–115)-EAP by 1.7-fold (p < 0.01) and the release of SgP-CgA-EAP by 2.8-fold (p < 0.001) over the basal levels (Fig. 11B). In contrast, the short N-terminal domain (CgA-(1–39)) transferred to EAP (SgP-CgA-(1–39)-EAP) exhibited marked accumulation in the Golgi apparatus (Fig. 11A), and the release of this chimera was not augmented in the presence of BaCl2 (Fig. 11B), which points to constitutive trafficking of SgP-CgA-(1–39)-EAP in A35C cells. Thus, the ability of CgA to induce the formation of vesicular structures competent to release a cargo of transiently expressed proteins into the extracellular milieu in response to BaCl2 stimulation may depend on a determinant located within the N-terminal amino acids 40–115 of CgA.

**DISCUSSION**

Little is known about the mechanism by which dense-core secretory granules of endocrine, neuroendocrine, and neuronal cells are formed. Until recently, research into the formation of dense-core secretory granules has been largely aimed at defining cis- or trans-determinants that contribute to the sorting of a cargo of regulated secretory proteins to secretory granules for subsequent release by exocytosis in response to extracellular stimulation. Although no consensus secretory granule-sorting sequence has emerged from these studies, various structural motifs influencing targeting of proteins to secretory granules have been identified. These motifs include hydrophobic disulfide-bonded loop structures found in a variety of regulated secretory proteins such as pro-opiomelanocortin, CgA, and CgB (15, 28, 32, 33); amphipathic α-helical domains that mediate interaction with membranes (15, 34, 35); and Ca2+-binding domains that promote protein aggregation prior to sorting (36, 37). Several studies on the secretory prohormone CgA have suggested a direct correlation between the sorting process and the formation of secretory granules (9–13). Indeed, CgA may play a critical role in the biogenesis of dense-core secretory granules of neuroendocrine cells, and this aspect of CgA func-
tion has been documented in chromaffin cells of the adrenal medulla both in living cells (this study and Refs. 9–11) and in vivo (12, 13). Although evidence in support of a central role of CgA in the formation of dense-core granules is thus compelling, there is not yet a general consensus on such a granulogenic function in the wake of a novel CgA-null mouse strain that shows an essentially normal phenotype (38); in particular, ultrastructural analyses of that strain’s pituitary and adrenal medulla did not suggest changes in the structure, size, or abundance of dense-core secretory granules. On the other hand, expression of several granins, including CgB and secretogranin II, is substantially increased in the adrenal tissues of these CgA knock-out mice, suggesting that other granins might compensate for CgA deficiency and account for the seemingly normal ultrastructural phenotype of these mutant mice (38). Intriguingly, this report also documented substantial up-regulation of urinary catecholamine excretion in knock-out animals, perhaps as a consequence of perturbed vesicular storage of catecholamine in the absence of CgA that would be incompletely rescued by other granin members.

In this study, we aimed to understand whether a determinant for the biogenesis of dense-core chromaffin granules might localize within a specific region of CgA. Using a series of human full-length CgA or truncated domains fused to reporters (GFP, EAP, or the epitope tag HA), we investigated the effect of CgA expression on the formation of regulated secretory granules in the sympathoadrenal PC12 cell variant A35C, which lacks dense-core granules and a functional regulated secretory pathway.

CgA and the Formation of Dense-core Granules in Normal Sympathoadrenal PC12 Cells—As a prerequisite to experiments aimed at defining a putative secretory granule-forming function of CgA in a mutant sympathoadrenal cell devoid of secretory organelles, we first ascertained the role of CgA in regulating catecholamine storage granule biogenesis in normal sympathoadrenal PC12 cells. siRNA targeting rat CgA mRNA had a significant inhibitory effect on the formation of dense-core granules in PC12 cells (Fig. 2); a 50% decline in the number of these granules was achieved upon 80% reduction of endogenous CgA expression. Such a decrease in chromaffin granule abundance is remarkably consistent with a previous study showing partially reduced granulogenesis in PC12 cells in response to siRNA-mediated inhibition of CgA expression (11). Consistent with that study, we also observed that the reduction in granule number (50%) was not proportional to the reduced level of CgA expression (80%), which suggests a possible granulogenic function of other granule cargo proteins, perhaps CgB as suggested by other investigators (11, 14). Whether CgA might be the sole contributor to chromaffin granule formation remains unsettled. Nevertheless, the contribution of CgA appears to be critical to the mechanisms underlying the formation of functional secretory granules in sympathoadrenal cells in vivo. Indeed, a reduction in or the absence of CgA expression not only perturbs the storage and release of several dense-core granule constituents, including catecholamines, but also leads to the formation of dense-core granules with aberrant morphology in CgA knock-out or CgA-deficient mice (12, 13).

CgA and the Formation of Dense-core Secretory Organelles in Mutant Sympathoadrenal A35C Cells—Previous studies have identified several clonal variants of PC12 cells, including A35C cells, defective in the expression of many components of secretory organelles, resulting in the absence of dense-core granules and incompetence for regulated secretion of peptide and amine hormones (17, 39, 40). Although our understanding of the mechanisms sustaining this phenotypic defect is incomplete, such incompetence for neurosecretion might be the consequence of an inhibitory effect that represses neuroendocrine-specific gene expression at the transcriptional level (17, 40). In this study, we have shown that introduction of human full-length CgA into A35C cells induced the formation of vesicular organelles competent for regulated exocytosis.

Expression of CgA labeled with HA or as a fluorescent (GFP) or chemiluminescent (EAP) fusion protein formed cytoplasmic vesicular structures distributed throughout much of the A35C cell bodies (Figs. 3, 6, and 10). These vesicular structures were not formed by the secretory proteins SEAP and SgP-GFP, the trafficking of which to the constitutive secretory pathway has been demonstrated previously (9, 15, 22, 28). Ultrastructural examination of these CgA-induced organelles by electron microscopy revealed membrane-bounded structures with a characteristic electron-dense appearance. However, the diameter (180–220 nm) of these newly formed vesicles was slightly larger than the size (100–130 nm) of catecholamine storage granules found in our clone of wild-type PC12 cells (Fig. 4), but nevertheless well within the broad diameter range (~30–330 nm) reported for chromaffin granules in rodents (12, 13, 41, 42).

Extension and remodeling of neurites that typically accompany neuronal differentiation have been reported in PC12 cell variants lacking the neurosecretory machinery (43). Indeed, we found that a subset of A35C cells may spontaneously display long neurite extensions (Fig. 5). In such cells, CgA-containing vesicular organelles distributed along the processes, and substantial accumulation of fluorescence was seen at the tips of the neurite extensions (Fig. 5). Such a distribution is remarkably similar to the trafficking behavior of secretory granules during neurite extension of wild-type or neuronally differentiated PC12 cells (23, 24) and further suggests that the vesicular organelles formed upon CgA expression may qualify as functional secretory organelles. Previous studies in non-neuroendocrine or secretory organelle-deficient neuroendocrine cells have argued that adventitious expression of CgA merely induces the formation of secretory lysosomes rather than genuine secretory granules (25). In contrast, our current findings show that CgA accumulated in vesicular structures devoid of the late endosomal/lysosomal marker LGP110 (Fig. 6) and that secretagogue stimulation of CgA-expressing A35C cells evoked exocytotic release of the granin independently of the label (HA or EAP) used to report secretion (Figs. 7 and 11). The ability of CgA to form functional secretory granules was further supported by its influence on human GH trafficking in A35C cells (Fig. 8). GH may be considered as the epitome of a secretory protein (37, 44); when expressed in neuroendocrine cells that do not normally produce it (e.g. PC12 cells) (Fig. 8), GH is typically sorted to dense-core secretory granules, and its release by exocytosis responds to secretagogue stimulation (Fig. 8) (30, 31,
truncated domains labeled with HA (or fused to GFP or EAP) in A35C cells, and electron-dense vesicle formation indicated that a determinant contained within CgA-(1–115) (but not the C-terminal region, CgA-(233–439)) is required for the formation of granules and the restoration of a regulated secretory pathway in A35C cells (Figs. 9–12). Additional analyses of the granulogenic activity of CgA-(1–115) revealed that the CgA hydrophobic disulfide-bonded loop structure (CgA-(17–38)) did not provide sufficient information for the formation of vesicular organelles in A35C cells, as the chimeric proteins SgP-CgA-(1–39)-GFP and SgP-CgA-(1–39)-EAP showed an intracellular distribution (Figs. 10 and 11A) or secretagogue-stimulated secretory profile (Fig. 11B) characteristic of a protein destined to the constitutive secretory pathway. This result is consistent with previous data showing that the disulfide-bonded loop motif is not sufficient for routing CgA into the regulated pathway of PC12 cells (15) and that CgA lacking the 17–38 loop domain is properly targeted to secretory granules of pituitary AtT-20 cells (46). On the other hand, we found that the CgA region spanning amino acids 40–115 may provide additional (indeed, necessary) granulogenic information that allows the recovery of a regulated secretory pathway phenotype (Figs. 9 and 11).

What mechanism might underlie the granulogenic function of CgA? Although the biophysical process underpinning the biogenesis of secretory granules is not well understood, the formation of protein aggregates typically represents the initial step leading to granulogenesis (1, 2, 37, 44, 47). For CgA, we proposed previously that perturbation of a pH/Ca2+-dependent multimerization mechanism of CgA that mobilizes a trafficking determinant within the N-terminal (CgA-(1–115)), but not C-terminal (CgA-(233–439)), region of the protein may perturb the formation of dense-core secretory granules in wild-type PC12 cells (9, 15), perhaps by modulating the ability of CgA to form aggregates. However, aggregation of secretory proteins alone is unlikely to fully account for the process by which secretory granules are formed in A35C cells. For instance, GH readily aggregates in the TGN of neuroendocrine cells (44, 48), but its expression in A35C cells did not produce a regulated secretory pathway (Fig. 8). Also, pH/Ca2+-dependent multimerization/aggregation properties have been documented for the CgA C-terminal region (5, 49), but the expression of the CgA C-ter-

44). Despite GH being a regulated secretory protein that forms aggregates in a slightly acidic environment and in the presence of millimolar amounts of bivalent cations (37, 44), expression of GH in A35C cells was unable to create a regulated secretory pathway (Fig. 8A). In contrast, introduction of CgA together with GH into A35C cells rerouted GH from a constitutive to a regulated secretory pathway (Fig. 8B), which strongly suggests that the vesicular structures formed by CgA behave as functional secretory granules capable of exporting a cargo of secretory proteins to the cell surface for release. This result is consistent with previous reports that expression of CgA recovered dense-core granule formation and the regulated secretion of pro-opiomelanocortin in the variant pituitary cell line 6T3 (10, 45).

A Granulogenic Determinant in the CgA N-terminal Region—We showed previously that the CgA N-terminal region (CgA-(1–115)) contains information required for sorting to dense-core granules of wild-type PC12 cells (9, 15), with CgA-(77–115) acting as a necessary although not sufficient sorting determinant. Here, we questioned whether a segment of CgA implicated in sorting might also function as a granulogenic determinant for the formation of secretory granules in sympathoadrenal cells. Analyses of the subcellular distribution, release profiles of
minal half-domain (CgA-(233–439)) in A35C cells did not result in granule formation or the appearance of a regulated pathway of secretion (Figs. 9 and 10).

Possible mechanisms for the granulogenic function of CgA may include association of a cis-determinant (e.g. CgA-(40–115)) within the aggregate of CgA with the membrane, or a particular lipid therein (47, 50, 51), or with partner proteins (46) to provide a driving force for the budding of secretory granules from the TGN. Our previous studies suggested that a predicted amphipathic α-helix (His79–Leu99) in the structure of the N-terminal region of CgA could be important for the sorting of CgA to secretory granules of normal PC12 cells (15), perhaps by mediating association of the protein with the membrane of the nascent granule. Interestingly, the vesicular sorting of CgA in neuroendocrine AtT-20 cells is dependent on the binding of a domain of CgA spanning amino acids 48–111 to the lipid raft-associated protein secretogranin III (46, 52). Finally, it has been proposed recently that CgA, or a derived fragment, may regulate the formation of dense-core granules in pituitary 

docrine cells. We have shown that expression of CgA in normal sympa-

tory pathway and promotes the formation of vesicular 

ule abundance, whereas expression of CgA in mutant sympa-
thetaedral cells (A35C) restores a functional regulated secretory 
pathway and promotes the formation of vesicular organelles that exhibit many features of dense-core secretory granules. Our data show for the first time that the granulogenic determinant within CgA may be located within its N-terminal region (CgA-(40–115)).

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