Identification of a novel sorting determinant for the regulated pathway in the secretory protein chromogranin A

Laurent Taupenot[1,3,*], Kimberly L. Harper[1,3], Nitish R. Mahapatra[1,3], Robert J. Parmer[1,3], Sushil K. Mahata[1,3] and Daniel T. O'Connor[1,2,3]

[1]Department of Medicine and [2]Center for Molecular Genetics, University of California at San Diego, La Jolla, CA 92093, USA
[3]Veterans Affairs San Diego Healthcare System, San Diego, CA 92161, USA
*Author for correspondence (e-mail: ltaupenot@ucsd.edu)

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Summary

Chromogranin A (CgA) is the index member of the chromogranin/secretogranin (or 'granin') family of regulated secretory proteins that are ubiquitously distributed in amine- and peptide-containing secretory granules of endocrine, neuroendocrine and neuronal cells. Because of their abundance and such widespread occurrence, granins have often been used as prototype proteins to elucidate mechanisms of protein targeting into dense-core secretory granules. In this study, we used a series of full-length, point mutant or truncated CgA-green fluorescent protein (GFP) chimeras to explore routing of CgA in neuroendocrine PC12 cells. Using sucrose gradient fractionation and 3D deconvolution microscopy to determine the subcellular localization of the GFP chimeras, as well as secretagogue-stimulated release, the present study establishes that a CgA-GFP fusion protein expressed in neuroendocrine PC12 cells is trafficked to the dense core secretory granule and thereby sorted to the regulated pathway for exocytosis. We show that information necessary for such trafficking is contained within the N-terminal but not the C-terminal region of CgA. We find that CgA's conserved N-terminal hydrophobic Cys_{17}-Cys_{38} loop structure may not be sufficient for sorting of CgA into dense-core secretory granules, nor is its stabilization by a disulfide bond necessary for such sorting. Moreover, our data reveal for the first time that the $CgA_{77\text{-}115}$ domain of the mature protein may be necessary (though perhaps not sufficient) for trafficking CgA into the regulated pathway of secretion.

Key words: Chromogranin A, Traffic, Sorting, Secretion, Chromaffin, Catecholamine

Introduction

Neurons and neuroendocrine cells contain membrane-delimited pools of peptide or amine hormones or neurotransmitters, typically with a characteristic electron-dense appearance on transmission electron microscopy, prompting the morphologic term 'granules' for such vesicles. These vesicles remain in the cell for extended periods of time, only undergoing exocytotic release after secretory stimulation characteristic for a particular cell type. This neuroendocrine version of secretion is often referred to as the 'regulated' secretory pathway, in contrast to 'constitutive' secretion, wherein the secretory rate is a strict function of the biosynthetic rate of the secreted substance, and secretory cargo leaves the cell promptly after transit through the trans-Golgi network (TGN) even in the absence of stimulation (Halban and Irminger, 1994; Kelly, 1985). These secretory pathways may co-exist with a 'constitutive-like' secretory pathway reflecting protein sorting that occurs in immature secretory granules yielding to a constitutive-like secretion of non-retained proteins (Arvan and Castle, 1998).

Chromogranin A (CgA) is the index member of the chromogranin/secretogranin (or 'granin') family of regulated secretory proteins initially described in the core of the adrenal medullary chromaffin granules, but subsequently identified in secretory granules throughout the neuroendocrine system and in a variety of neurons, both central and peripheral. Dual extracellular and intracellular functions have been ascribed to CgA. Extracellular roles include generation of bioactive peptides such as the nicotinic cholinergic antagonist catestatin (Mahata et al., 1997; Taupenot et al., 2000), or pancreastatin which impairs glucose-stimulated insulin release from pancreatic islet β-cells (Sanchez-Margalet et al., 1996). Intracellularly, CgA is now believed to play a pivotal role in the initiation and regulation of dense-core secretory granule biogenesis and hormone sequestration in neuroendocrine cells (Iacangelo and Eiden, 1995; Kim et al., 2001).

The mechanism by which secretory proteins such as CgA are sorted at the TGN to the regulated pathway is a topic of debate and active investigation. Several models of recruitment within this secretory pathway have been proposed. These mechanisms include pH- and Ca^{2+}-dependent aggregation, signal/receptor-mediated targeting of pro-hormones into the regulated pathway, or direct binding to particular 'lipid raft' domains on the inner face of the granule membrane (Loh et al., 1997; Thiele et al., 1997; Tooze et al., 2001).

Selective aggregation of regulated secretory proteins at the level of the TGN, a process also known as 'sorting by retention', occurs under conditions of high Ca^{2+}/acidic pH,

which would be a means of segregation away from constitutively secreted proteins and prevent the regulated secretory proteins to escape from maturing granules in the process of constitutive-like secretory pathway (Arvan and Castle, 1998; Chanat and Huttner, 1991; Gerdes et al., 1989; Gorr et al., 1989). For CgA and other granins such as chromogranin B (CgB) and secretogranin II (SgII), this aggregation depends on millimolar Ca^{2+} concentrations and on a mildly acidic pH, conditions that are fulfilled in the lumen of the TGN (Chanat and Huttner, 1991; Gorr et al., 1989).

The receptor-mediated transport model, also referred to as 'sorting for entry', proposes that a specific sorting signal in the secretory protein binds to a receptor, perhaps located in the TGN, facilitating appropriate trafficking of the protein to the regulated pathway of secretion (Arvan and Castle, 1998; Cool et al., 1997; Loh et al., 1997). The identification of such sorting signal domains in regulated secretory proteins such as CgB (Chanat et al., 1993) or proopiomelanocortin prohormone [POMC (Cool et al., 1997)] is consistent with the signal-receptor-mediated targeting hypothesis. Near its N-terminus, CgB has a conserved intramolecular disulfide loop domain bounded by cysteine residues and containing a preponderance of aliphatic hydrophobic residues. A series of investigations suggested that this loop structure (when stabilized by the disulfide bridge) is both necessary and sufficient for the sorting of CgB from the TGN to secretory granules of PC12 cells (Chanat et al., 1994; Chanat et al., 1993; Glombik et al., 1999; Kromer et al., 1998). Since CgB and CgA share this disulfide loop structure, it has been assumed that this structure would also be a sorting determinant for CgA. However, evidence of a potential role of the CgA loop structure and/or the integrity of its disulfide bridge in sorting from the TGN remains limited, and pH- and Ca^{2+}-dependent aggregation of CgB in vitro are independent of the presence of the hydrophobic disulfide-stabilized loop (Chanat et al., 1994; Glombik et al., 1999).

We previously showed that the N-terminal half region of CgA expressed in PC12 cells as an in-frame fusion with a protein not ordinarily secreted (bacterial chloramphenicol acetyltransferase, or CAT) is trafficked into the regulated secretory pathway, and co-secreted along with catecholamines after exocytotic trigger stimuli (Parmer et al., 1993). In the present study, we aimed to investigate whether a novel CgA-GFP (green fluorescent protein) fusion protein is targeted in situ to catecholamine storage vesicles of CgA in sympathoadrenal PC12 cells, providing a novel way to explore whether discrete domains in the CgA primary structure are crucial for trafficking of the protein to the regulated pathway of secretion. To identify potential determinants for sorting of CgA, we prepared a series of mutants of human CgA fused to the N-terminal end of GFP.

We now report that a CgA-GFP fusion protein expressed in neuroendocrine PC12 cells is trafficked to the dense core secretory granule and thereby sorted to the regulated pathway for exocytosis. We show that information necessary for such trafficking is contained within the N-terminal but not the C-terminal half region of CgA. We find that CgA's N-terminal hydrophobic loop structure may not be sufficient, and its stabilization by a disulfide bond not necessary, for sorting of CgA into dense-core secretory granules. A domain spanning residues 77 to 115 of the CgA mature protein seems to be a necessary (though perhaps not sufficient) sorting determinant for the regulated pathway.

Materials and Methods

Cell culture and transient transfections

PC12 rat pheochromocytoma cells (obtained from David Schubert, Salk Institute, La Jolla, CA) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum (Gemini Bioproducts), streptomycin (100 μg/ml) and penicillin (100 U/ml) (Life Technologies). Supercoiled plasmid DNA for transfection was grown in Escherichia coli strain DH-5α (Life Technologies), and purified on columns (Qiagen). Two days before transfection, PC12 cells were split onto either poly-L-lysine coated 18-mm round glass no.1 coverslips (Fisher Scientific) in 12-well Costar plates or onto poly-L-lysine coated 100 mm tissue culture Falcon dishes. Cells were transfected with 1.8 μg (12-well plate) or 10 μg (100 mm dish) of supercoiled plasmid DNA per well, using a high efficiency cationic scaffold method (GenePorter II, Gene Therapy Systems) according to the manufacturer's instructions. Five hours after the beginning of the transfection, culture medium was replaced and cells were further cultured for 48-72 hours. In some experiments, PC12 cells were treated with NGF (2.5S, 100 ng/ml; Life Technologies) 48 hours prior transfection and further differentiated by NGF for an additional 72 hours.

Cloning and mutagenesis

Full length human CgA (SgP-CgA, including the 18 amino acid signal peptide [MRSAAVLALLLCAGQVTA], or SgP) and several human CgA domains (SgP-CgA_{1-16}, SgP-CgA_{1-37}, SgP-CgA_{1-39}, SgP-CgA_{1-77}, SgP-CgA_{1-115}, SgP-CgA_{1-153}, SgP-CgA_{1-168}, SgP-CgA_{1-209}, SgP-CgA_{1-224}) were obtained by PCR using specific oligonucleotide primers incorporating an *Xho*I restriction site followed by a Kozak translation initiation consensus sequence (GCCACC-ATG) at the 5′ end, and a *Kpn*I restriction endonuclease site at the 3′ end of each domain. pCMV-hCgA served as a template [gift from Lee Helman (Helman et al., 1988)]. The amplified fragments were purified, digested with *Xho*I and *Kpn*I and subcloned in frame into the same sites of the mammalian expression vectors for the enhanced fluorescence variant of wild-type GFP (enhanced GFP, EGFP), pEGFP-N1 or pEGFP-N2 (Clontech), under the control of the cytomegalovirus promoter to produce plasmids pCMV-CgA184-EGFP, pCMV-CgA247-EGFP, pCMV-CgA253-EGFP, pCMV-CgA367-EGFP, pCMV-CgA481-EGFP, pCMV-CgA595-EGFP, pCMV-CgA640-EGFP, pCMV-CgA763-EGFP, pCMV-CgA805-EGFP, pCMV-CgA-EGFP, respectively.

SgP-GFP was obtained by PCR using specific oligonucleotide primers incorporating the18 amino acid signal peptide of human CgA as well as *Xho*I and *Not*I restriction endonuclease sites. The amplified fragment was purified, digested with *Xho*I and *Not*I and subcloned in frame into the same sites of the vector pEGFP-N1 to produce pCMV-SgP-EGFP. The N-terminal deletion mutant of human CgA (SgP-$CgA_{233-439}$) was obtained by site-directed mutagenesis (QuickChange, Stratagene) performed according to the supplier guidelines. Briefly, specific mutagenic oligonucleotide primers were used to sequentially add *Hin*dIII restriction endonuclease sites at positions 137-142 and 830-835 of the human CgA cDNA. The amplified plasmid was digested by *Hin*dIII and ligated to produce the plasmid pCMV-ΔNCgA-EGFP. Disruption of the disulfide loop of human CgA (SgP-CgAΔC_{17}E-GFP) was achieved by site-directed mutagenesis (QuickChange, Stratagene); the amino acid residue 17 in SgP-CgA-GFP was mutated from a cysteine to a glutamic acid to produce the plasmid pCMV-CgAΔC/E-EGFP.

Creation of the fusion protein SgP-GFP-CgA_{77-115} was achieved by PCR and by site-directed mutagenesis. The EGFP gene termination

signal (TAA) located at position 1393-1395 of plasmid pCMV-SgP-EGFP was mutated (to codon GCA) by site-directed mutagenesis (QuickChange, Stratagene) to produce pCMV-SgP-EGFP(stopmut). Domain CgA365-481 of human CgA cDNA was obtained by PCR using specific oligonucleotide primers incorporating a *Not*I restriction site at the 5′ end, and a *Not*I site preceeded by a termination signal (TAA) at the 3′ end. pCMV-CgA-EGFP served as a template. The amplified fragment was purified, digested with *Not*I and subcloned in-frame into the *Not*I site of the vector pCMV-SgP-EGFP(stopmut), to produce pCMV-SgP-EGFP-CgA365-481.

All of the constructs were verified by restriction analysis and nucleotide sequence analysis. In addition, the expression of each chimera was confirmed by immunoblotting of total cell lysate wherein GFP chimera-transfected PC12 cells were lysed in a buffer containing: 50 mM Tris-HCl (pH 7.4), 10 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 1 mM DTT, protease inhibitor cocktail (Protease inhibitor cocktail Set I, Calbiochem), and centrifuged at 20,000 ***g*** for 30 minutes at 4°C. Protein lysate of each samples was then processed for immunoblotting using with a purified polyclonal rabbit anti-GFP (Living Colors A.v. peptide antibody, Clontech).

3D-imaging by deconvolution microscopy

Images were captured on a DeltaVision deconvolution microscopy system (Applied Precision) operated by SoftWoRx software (Applied Precision) on a Silicon Graphics O_2 workstation using with 60× (NA 1.4) or 100× (NA 1.4) oil immersion objectives. The system included a Photometrics CCD camera mounted on a Nikon inverted fluorescence/dic microscope, and a mercury arc lamp light source. Pixel intensities were kept in the linear response range of the digital camera. 40-50 optical sections along the Z axis were acquired with increments of 0.2 μm. The fluorescent data sets were deconvoluted and analyzed by Delta Vision SoftWoRx programs on a Silicon Graphics Octane workstation (Applied Precision) to generate optical sections or 3D images of the data sets. In some experiments, the extent of colocalization of distinct fluorescent signals was assessed in 3D using Nearcount Image Analyzer software (developed by David Nadeau, UCSD/San Diego Supercomputer Center). Adobe Photoshop 5.5 software was used for additional processing of the images. The following excitation and emission wavelengths were used for imaging: GFP, λ_{ex} 490±10/λ_{em} 528±238 nm; cyan fluorescent protein (CFP, for Golgi visualization; see below), λ_{ex} 436±10/λ_{em} 465±30 nm; Alexa Fluor 594 conjugate (for antibody staining), λ_{ex} 555±28 /λ_{ex} 580±20 nm; and Hoechst 33342 (nuclear DNA stain), λ_{ex} 350/λ_{em} 461 nm.

Chimeric photoprotein fluorescence and immunocytochemistry

Chimeric photoproteins-transfected PC12 cells cultured on poly-L-lysine coated glass coverslips were fixed for 1 hour at room temperature with 2% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, permeabilized with 0.1% Triton X-100 in PBS (10 minutes), and exposed to 1 μg/ml of nucleic acid stain Hoechst 33342 (Molecular Probes) for nuclei visualization. Coverslips were subsequently washed with PBS, mounted in buffered Gelvatol and processed for 3D imaging by deconvolution microscopy. For immunocytochemistry, fixed PC12 cells were incubated for 5 minutes in PBS-glycine (0.1 M) buffer and subsequently exposed for 30 minutes to PBS containing 5% fetal calf serum to reduce non specific antibody labeling. Cell were then incubated for 1 hours at room temperature with a mouse monoclonal anti-rat dopamine β-hydroxylase (1:100: PharMingen) in buffer containing 1% bovine serum albumin in PBS. Cells were then washed and incubated for 30 minutes with a Alexa-Fluor-594-conjugated (red) goat anti-mouse IgG, F(ab′)$_2$ (1:250; Molecular Probes) together with 1 μg/ml of nucleic acid stain Hoechst 33342 (Molecular Probes). Coverslips were subsequently washed with PBS, mounted in buffered Gelvatol and processed for 3D imaging by deconvolution microscopy.

Sucrose gradient fractionation

Transiently transfected PC12 cells were labeled for 2 hours with 1 μCi/ml [^{3}H]-L-norepinephrine (71.7 Ci/mmol, DuPont-NEN) in PC12 medium, washed with buffer (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES buffer, pH 7.4). Cells were subsequently harvested by scraping into ice-cold PBS containing protease inhibitors cocktail (Protease inhibitor cocktail set I, Calbiochem), and resuspended in 1 ml ice-cold 0.32 M sucrose, EDTA 1.5 mM, Tris 10 mM pH 7.4, supplemented with protease inhibitor cocktail. Cells were passed 16 times through a 30-gauge needle, and the lysate was centrifuged (800 ***g*** for 10 minutes at 4°C) to pellet unbroken cells and nuclei. The supernatant was layered over a continuous sucrose density gradient (0.6-2.2 M), and centrifuged at 100,000 ***g*** for 90 minutes at 4°C. Fractions were collected and assayed for [^{3}H]-L-norepinephrine by scintillation counting, sucrose concentration by refractometry, and detection of GFP fluorescence using a cuvette-based fluorometer (TD700, Turner Designs).

Secretion assay

For the stimulated release assay, transiently transfected PC12 cells grown on poly-L-lysine-coated 100 mm tissue culture dishes were extensively washed with secretion medium (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES pH 7.4), and subsequently exposed to 2 mM $BaCl_2$ in Ca^{2+}-free secretion medium for 15 minutes. For the constitutive release assay, transiently transfected PC12 cells grown on poly-L-lysine-coated 100 mm tissue culture dishes were extensively washed with Locke's buffer (140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.01 mM EDTA, 10 mM glucose, 0.56 mM ascorbic acid, 15 mM HEPES pH 7.4), and further incubated in Locke's medium for 3 hours at 37°C in 5% CO_2.

Extracellular media were collected, cleared by centrifugation (10 minutes, 4000 ***g***, 4°C), and concentrated using reverse phase Sep-Pak C-18 silica cartridges (Waters Millipore). The solvent system consisted of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in 100% CH_3CN. Eluates were lyophilized and immunoblotted using a purified polyclonal rabbit anti-GFP (Living Colors A.v. peptide antibody, Clontech).

Gel electrophoresis and immunoblotting analysis

Proteins were separated by SDS-PAGE on 10% polyacrylamide gels and transferred onto nitrocellulose sheets (Schleicher and Schuell). Membranes were blocked in buffer containing 5% nonfat dry milk in PBS for 1 hours, incubated for 2 hours with a purified polyclonal rabbit anti-GFP (1:100, Living Colors A.v. peptide antibody, Clontech), and washed for 15 minutes with 0.05% Tween 20 in PBS. Blots were subsequently incubated for 1 hours with an anti-rabbit horseradish peroxidase conjugate secondary antibody (1:3500; BioRad) in blocking buffer. Immunoreactive bands were detected by chemiluminescence (Supersignal West Pico, Pierce).

Results

Subcellular localization of a human CgA-GFP chimeric photoprotein: the CgA-GFP chimera enters chromaffin granules of the regulated secretory pathway

3D deconvolution microscopy

We first designed expression plasmids encoding the enhanced fluorescence variant of wild-type GFP (enhanced GFP, EGFP) fused at GFP's N-terminus to the C-terminus of full-length human CgA (pCMV-CgA-EGFP; Fig. 3) or the 18-amino acid signal peptide of human CgA (pCMV-SgP-GFP). EGFP (mutations Ser_{cc} to Thr_{65} and Phe_{64} to Leu_{64}) has a single, red-shifted excitation peak and fluoresces about 35

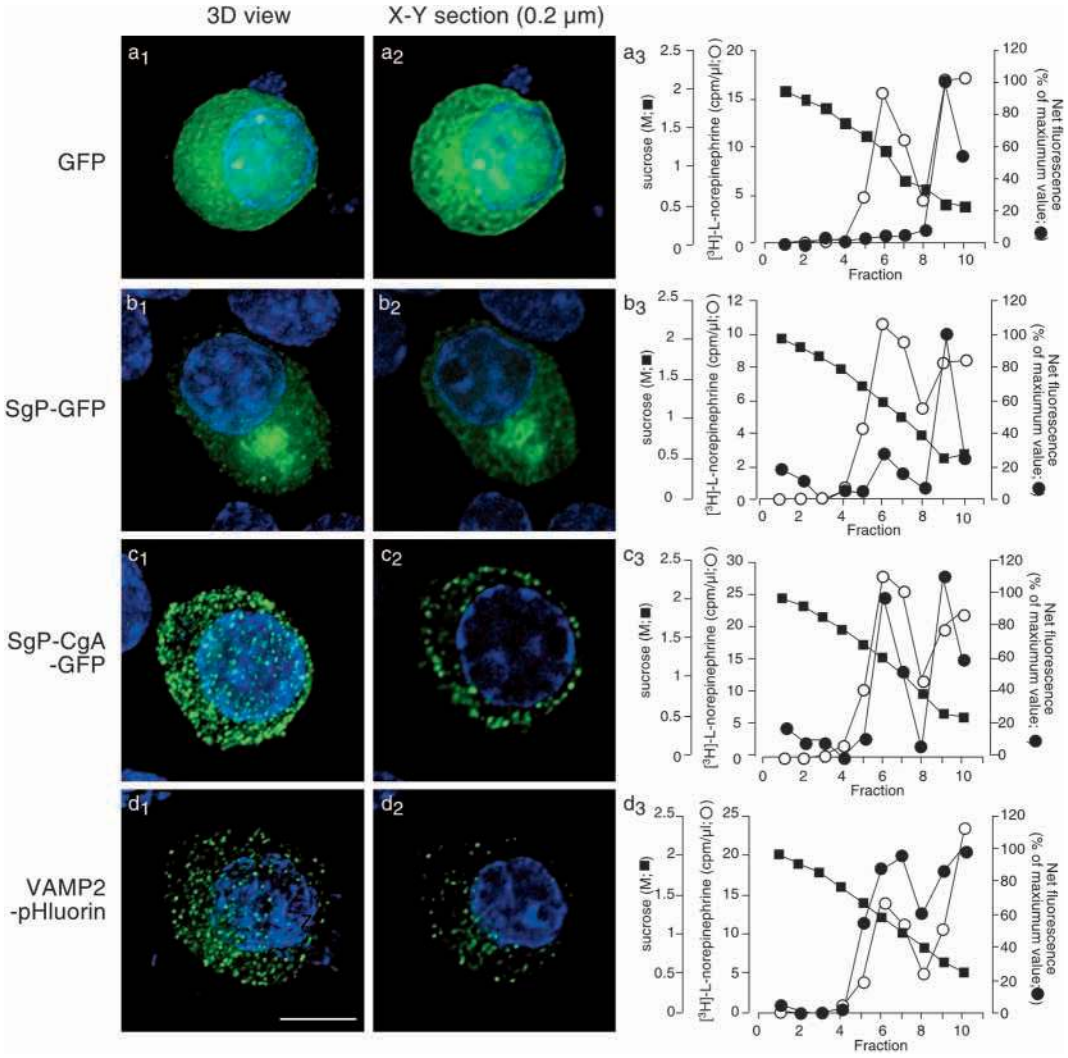


Fig. 1. Subcellular distribution of chimeric photoproteins in PC12 cells. 48 hours after transfection with expression plasmids encoding GFP, SgP-GFP, SgP-CgA-GFP or VAMP2-pHluorin, aldehyde-fixed cells were examined by deconvolution microscopy. Optical sections along the Z axis were acquired with increments of 0.2 µm using 60× or 100× oil immersion objectives (1.4 NA). GFP was excited at λ_{ex} 490±10 nm and imaged at λ_{em} 528±238 nm. Nuclei were visualized with Hoechst 33342 (λ_{ex} 350 nm/λ_{em} 461 nm). Data were processed to generate 3D/volume (a1, b1, c1, d1) or section (0.2 µm; a2, b2, c2, d2) views. Bars, 5 µm. Postnuclear supernatants prepared from [^{3}H]-L-norepinephrine-labeled cells transiently expressing GFP (a3), SgP-GFP (b3), SgP-CgA-GFP (c3) or VAMP2-pHluorin (d3), were centrifuged to equilibrium on sucrose density gradients (0.6-2.2 M). Fractions were collected from the bottom of the gradient and assayed for [^{3}H]-L-norepinephrine, sucrose concentration and detection of GFP fluorescence.

times more intensely than wild type GFP when excited at 488 nm.

The distribution of expressed GFP fusion proteins was assessed by deconvolution microscopy in paraformaldehyde-fixed PC12 cells (Fig. 1). Unfused GFP was diffusely distributed in the cell cytosol (Fig. 1a$_1$) and the nucleus (Fig. 1a$_2$). The extensive fluorescence signal in the nucleus is likely due to the low molecular weight (~27 kDa) of GFP that allows the protein to pass through nuclear pores by diffusion. Addition of the 18-amino acid signal peptide of human CgA to the N-terminus of GFP caused a marked accumulation of fluorescence in the perinuclear region of the cell typically occupied by the Golgi complex (Fig. 1b$_1$). As expected, a 0.2 µm optical sections acquired in the middle region of the cells indicated no detectable SgP-GFP fluorescence in the nucleus. This finding is consistent with the notion that the signal peptide within the chimera directs the hybrid protein to the endoplasmic reticulum's lumen (Fig. 1b$_2$).

In contrast, 3D analysis of SgP-CgA-GFP distribution revealed a non-uniform punctate distribution of the fluorescence signal throughout much of the cytoplasm (Fig. 1c$_1$) but was fully excluded from the nucleus (Fig. 1c$_2$). Moreover, 0.2 µm optical sections acquired in the middle region of SgP-CgA-GFP-expressing PC12 cells, revealed discrete, abundant peripheral/subplasmalemal punctate fluorescence signals, indicating storage of the recombinant protein in dense-core secretory granules, perhaps docked at the plasma membrane. A similar fluorescence pattern was seen in cells expressing VAMP2-pHluorin (Miesenböck et al., 1998), a pH-sensitive mutant of GFP fused in-frame with

VAMP2/synaptobrevin (Fig. 1d_1,d_2), a trans-membrane protein of small synaptic and large dense core vesicles (Miesenböck et al., 1998; Winkler, 1997). Importantly, identical subcellular distribution patterns for these GFP chimeras were also observed in living PC12 cells, indicating that fixation of cells with paraformaldehyde did not produce artifacts in the distribution of these fusion proteins (data not shown).

Subcellular fractionation

To further explore the subcellular localization of these GFP fusion proteins, we performed equilibrium sucrose density gradient fractionations on post-nuclear supernatants prepared from [^{3}H]-L-norepinephrine-loaded PC12 cells expressing GFP alone, SgP-GFP, SgP-CgA-GFP, or VAMP2-pHluorin. Unfused GFP fluorescence was found only in subcellular fractions corresponding to the cytosol (~0.5-0.7 M sucrose; Fig. 1a_3).

Fractionation of cells expressing SgP-GFP revealed that fluorescence intensity was low in the region containing chromaffin granules (Fig. 1b_3), as expected for a protein that may be targeted only to the constitutive pathway of secretion. In contrast, [^{3}H]-L-norepinephrine co-localized with either SgP-CgA-GFP or VAMP2-pHluorin in the same subcellular fractions found at ~1.2-1.4 M sucrose (Fig. 1c_3,d_3). The ~1.2-1.4 M sucrose peak was consistent with the buoyant density previously reported for chromaffin granules isolated from PC12 cells (Parmer et al., 1997; Parmer et al., 1993). In addition, some [^{3}H]-L-norepinephrine and GFP fluorescence were found at the top of the gradient (~0.5-0.7 M sucrose; Fig. 1c_3,d_3). This additional peak at the top of the gradient is consistent with release of granular components from vesicles lysed during the homogenization step (i.e. before application of the sample to the gradient) (Parmer et al., 1997; Parmer et al., 1993).

Nerve growth factor (NGF) differentiation to neurites

Accumulation of secretory granules in growth cones has been reported in NGF-treated PC12 cells (Kaether and Gerdes, 1995; Lang et al., 1997; Lochner et al., 1998). When SgP-CgA-GFP-expressing PC12 cells were differentiated with NGF, an enhancement of fluorescence in neurite endings was detected, further suggesting that SgP-CgA-GFP is routed to chromaffin secretory granules (data not shown).

Immunological detection and secretagogue-stimulated secretion

The intracellular localization of SgP-CgA-GFP was also investigated by immunoblotting the chimeric protein in subcellular fractions obtained from equilibrium sucrose density gradient fractionations of SgP-CgA-GFP-transfected PC12 cells, as well as immunodetection of chimera release from SgP-CgA-GFP-transfected PC12 cells exposed to Ba^{2+}, a cation permeant through membrane Ca^{2+} channels which triggers catecholamine release by exocytosis (Heldman et al., 1989).

As shown in Fig. 2, immunoblotting of GFP immunoreactivity in sucrose gradient fractions from SgP-CgA-GFP-expressing PC12 cells localized SgP-CgA-GFP and norepinephrine to the same subcellular fractions, with densities characteristic of chromaffin granules (Fig. 2A,B). SgP-CgA-GFP immunoreactivity was detected in catecholamine-containing fractions 11-15 (Fig. 2B), revealing a product band with SDS-PAGE mobility of ~105 kDa, consistent with the mass predicted for the full-length human CgA-GFP chimera (~75 kDa for human CgA plus 27 kDa for GFP). Human and other species' CgA displays abnormal SDS-PAGE mobility, and its apparent molecular mass value (70-75 kDa) is substantially higher than its predicted molecular mass (~50

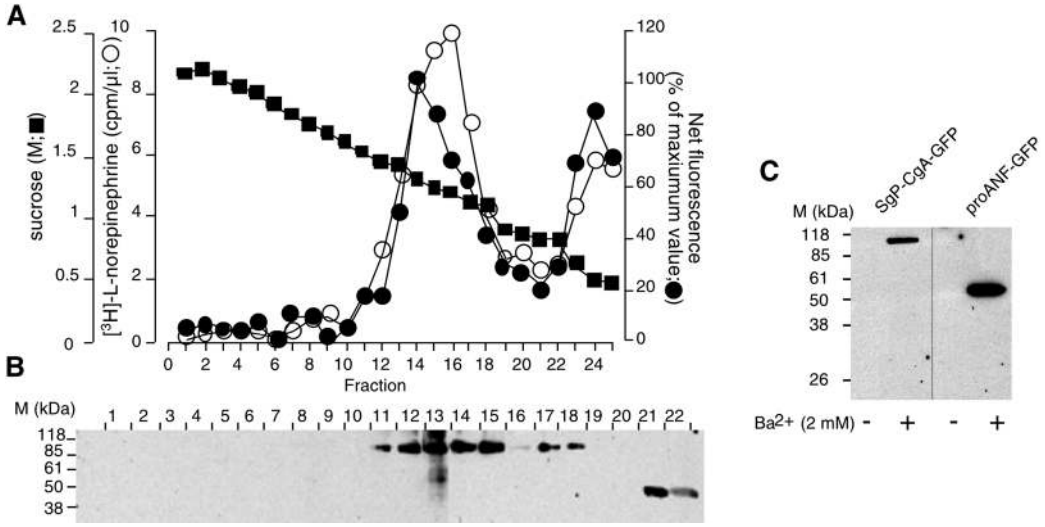


Fig. 2. Biochemical detection of SgP-CgA-GFP in sucrose density gradient fractions and in the secreted medium from PC12 cells. (A) Subcellular localization of SgP-CgA-GFP in PC12 cells. Postnuclear supernatants prepared from [^{3}H]-L-norepinephrine-labeled PC12 cells transiently expressing SgP-CgA-GFP were centrifuged to equilibrium on sucrose density gradients. Fractions were assayed for sucrose concentration, GFP fluorescence and scintillation counting. (B) Immunochemical detection of the SgP-CgA-GFP chimera in sucrose density gradient fractions. Gradient fractions were concentrated using SepPak C-18 cartridges and processed for immunoblot using an anti-GFP antibody. (C) Immunochemical detection of stimulated release of SgP-CgA-GFP or proANF-GFP. Regulated secretion from SgP-CgA-GFP- or proANF-GFP-expressing PC12 cells was triggered by 2 mM $BaCl_2$. Extracellular media were concentrated using C-18 SepPak cartridges and processed for immunoblot using an anti-GFP antibody. Immunoreactivity was visualized by chemiluminescence.

kDa) (Helman et al., 1988; Taupenot et al., 1995), a result of CgA's highly anionic pI. GFP-immunoreactive fragments of lower molecular masses were also detected in fractions with sucrose densities ~0.5-0.7 M (Fig. 2B); such fragments likely correspond to proteolytic degradation of SgP-CgA-GFP during release of granular components from vesicles lysed by homogenization.

Correct targeting of SgP-CgA-GFP to chromaffin secretory granules predicts that the chimera would undergo regulated exocytosis. We therefore tested the effect of the potent secretagogue Ba^{2+}, a divalent cation whose entry through membrane Ca^{2+} channels that triggers catecholamine release by exocytosis from chromaffin cells (Heldman et al., 1989). As shown in Fig. 2C, 15 minutes exposure to 2 mM $BaCl_2$ triggered the release of GFP-tagged CgA into the extracellular medium, consistent with exocytotic release of the chimera from catecholamine storage vesicles. Furthermore, chimera release paralleled GFP-tagged neuropeptide pre-pro-atrial natriuretic factor (proANF-GFP) secretion, another chimera reported to be efficiently sorted to the regulated pathway of secretion in chromaffin cells (Han et al., 1999). No chimera release was detected in SgP-CgA-GFP- or proANF-GFP-transfected cells exposed to mock (control) secretion medium, suggesting that constitutive release of the chimeras was minimal in non-stimulated cells (Fig. 2C).

Identification of a routing determinant for secretory granules of the regulated pathway in the N-terminal half of chromogranin A

Earlier studies from our group found a dominant targeting signal for the regulated pathway within the N-terminal half domain of CgA (Parmer et al., 1993). To understand the necessary (required) region within the N-terminal domain of CgA with information for targeting of the protein to dense-core secretory granules, we designed a series of CgA fragment-GFP fusion proteins (Fig. 3). Cellular expression of each chimera was first confirmed by immunoblotting lysates from transfected PC12 cells, using an antibody to GFP. As shown in Fig. 4, immunoblotting revealed CgA fragment/GFP fusion proteins of apparent molecular mass values for the major bands consistent with either their calculated molecular masses (for shorter fragments of CgA), or with a predicted, higher mass values reflecting the abnormal SDS-PAGE mobility of larger CgA domains.

3D visualization of the subcellular distribution of CgA fragment-GFP chimeric photoproteins in PC12 cells

We then analyzed the subcellular distributions of CgA fragment/GFP chimeric photoproteins in PC12 cells by deconvolution microscopy followed by computational 3D reconstruction (Fig. 5). When expressed in PC12 cells, the shorter hybrid proteins SgP-CgA_{1-16}-GFP and SgP-CgA_{1-37}-GFP (missing the disulfide bond) (Fig. 5) exhibited a subcellular distribution pattern markedly different from SgP-CgA-GFP, but similar to the one observed in cells expressing SgP-GFP (Fig. 1). Indeed, the fluorescence of these shorter chimeras accumulated in a region of the cell bodies usually occupied by the Golgi complex. A perinuclear build-up of fluorescence was also observed in cells expressing the C-terminal half of CgA fused to GFP (SgP-$CgA_{233-439}$-GFP, Fig. 5). This finding further supports the proposal that a targeting signal for the sorting of CgA from the TGN to secretory granules is contained within its N-terminal domain (Parmer et al., 1993).

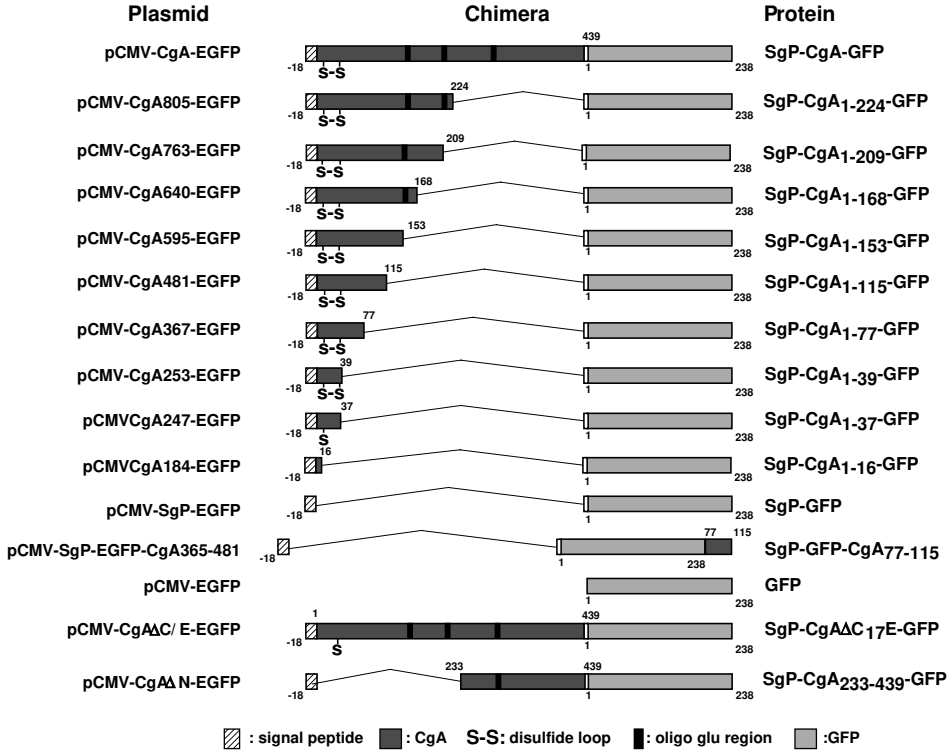


Fig. 3. Schematic representation of chimeric photoproteins (CgA fragment-GFP fusion proteins) designed to study CgA trafficking to the dense core secretory granules of PC12 cells. Construction of plasmids encoding GFP fusion proteins was performed as described in Materials and Methods. Plasmid numbering refers to the position of the 3′ end of the CgA fragment subcloned, within the original human CgA cDNA (GenBank NM_001275). For example, in pCMV-CgA805-EGFP, the 3′ end of the subcloned CgA fragment (at amino acid +224) is located at 805 bp in the original cDNA. Amino acid residue positions are indicated by numbers alongside the chimera. Proteins are drawn proportional to scale.

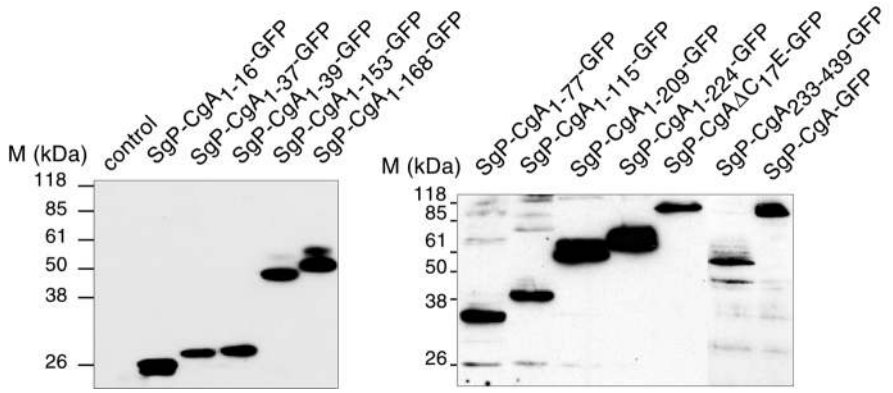


Fig. 4. Immunochemical detection of the expression of GFP fusion proteins in PC12 cells. Total PC12 cell lysates were prepared 72 hours after transfection with expression plasmids encoding the GFP fusion proteins indicated. Expression of GFP fusion proteins was evaluated by SDS-PAGE followed by immunoblotting using an anti-GFP antibody. Immunoreactivity was visualized by chemiluminescence. Estimation of size (M_m, calculated molecular mass, based upon primary structure of the chimera; M_a, apparent molecular mass by SDS-PAGE): SgP-CgA_{1-16}-GFP (28.6 kDa; 27 kDa); SgP-CgA_{1-37}-GFP (30.9 kDa; 29.2 kDa); SgP-CgA_{1-39}-GFP (31.2 kDa; 29.5 kDa); SgP-CgA_{1-77}-GFP (35.6 kDa; 35 kDa); SgP-CgA_{1-115}-GFP (39.9 kDa; 40 kDa); SgP-CgA_{1-153}-GFP (43.8 kDa; 48.5 kDa); SgP-CgA_{1-168}-GFP (45.6 kDa; 52.5 kDa); SgP-CgA_{1-209}-GFP (49.9 kDa; 62 kDa); SgP-CgA_{1-224}-GFP (51.6 kDa; 71 kDa); SgP-CgA-GFP (75.8 kDa; 105 kDa); SgP-CgAΔC_{17}E-GFP (75.8 kDa; 105 kDa); SgP-$CgA_{233-439}$-GFP (50.3 kDa; 56 kDa).

Chimeric SgP-CgA_{1-39}-GFP and SgP-CgA_{1-77}-GFP, which both contain an unaltered disulfide-bonded hydrophobic loop structure (at Cys_{17}-Cys_{38}), exhibited a subcellular distribution pattern similar to SgP-GFP, SgP-CgA_{1-16}-GFP or SgP-CgA_{1-37}-GFP, rather than SgP-CgA-GFP (Fig. 5), suggesting that CgA's disulfide loop may not contribute to (i.e., is not sufficient for) CgA targeting to chromaffin secretory granules. Indeed, when the disulfide-bonded loop of CgA was disrupted by point-mutation of Cys_{17} (SgP-CgAΔC_{17}E-GFP), the subcellular distribution pattern of SgP-CgAΔC_{17}E-GFP remained similar, if not identical, to the pattern detected in cells expressing the full-length CgA-GFP chimera with an intact disulfide bridge (SgP-CgA-GFP, Fig. 5). Interestingly, a gradual appearance of a more prominent punctate/granular pattern could be detected as the length of the fragment of CgA fused to GFP increased from +16 to +77 amino acid residues (SgP-CgA_{1-16}-GFP, SgP-CgA_{1-37}-GFP SgP-CgA_{1-39}-GFP, SgP-CgA_{1-77}-GFP, Fig. 5). This observation suggests that a trafficking information present within the first 77 amino acid residues region of the mature CgA protein allows the chimera to begin to traffic more efficiently out of the TGN and, to some extent, may initiate its packaging into vesicular structures.

In sharp contrast, no juxtanuclear cluster of fluorescence could be detected in PC12 cells transiently expressing SgP-CgA_{1-115}-GFP, SgP-CgA_{1-153}-GFP, SgP-CgA_{1-168}-GFP, SgP-CgA_{1-209}-GFP or SgP-CgA_{1-224}-GFP fusion proteins (Fig. 5). In these cells, the subcellular distribution patterns of the CgA fragment-GFP chimeric photoproteins were homogenous, highly punctate, and largely identical to the distribution pattern of native human CgA fused to GFP (SgP-CgA-GFP; Figs 1, 5). Taken together, these deconvolution microscopic observations provide several clues to trafficking determinants in the N-terminus of CgA. First, the disulfide-bonded loop structure (Cys_{17}-Cys_{38}) may not be sufficient, and the disulfide bridge not required, for correct targeting of CgA to chromaffin secretory granules. However, the CgA region spanning amino acids 77 to 115 may provide crucial (indeed, necessary) trafficking information that prevents CgA domain-GFP hybrids from clustering in the perinuclear/Golgi area of PC12 cell bodies, thus efficiently driving the chimeras from the TGN to vesicular structures corresponding to chromaffin dense-core secretory granules.

The Golgi apparatus: 3D quantification of co-localization between CgA domain-GFP fusion proteins and β1,4-galactosyltransferase cyan fluorescent protein (GALT-CFP) chimera, a Golgi apparatus marker

The initial observation that SgP-CgA_{1-16}-GFP, SgP-CgA_{1-37}-GFP, SgP-CgA_{1-39}-GFP or SgP-CgA_{1-77}-GFP chimeras accumulated largely in PC12 cells juxtanuclear structures reminiscent of the Golgi compartment, prompted us to investigate a putative colocalization of these CgA domain-GFP fusion proteins with a β1,4-galactosyltransferase cyan fluorescent protein (GALT-CFP) chimera, a selective marker for the Golgi complex.

The subcellular distributions of the GFP and CFP chimeras was analyzed by deconvolution microscopy, and the extent of the colocalization between GFP chimeras and GALT-CFP was subsequently quantified in 3D using Nearcount Image Analysis software (Fig. 6). Sets of 0.2 μm X-Y image stacks were analyzed for two colocalization parameters, 'overlap' and 'nearness'. 'Overlap' was defined as the total number of blue (CFP) pixels within the cell region that were above a threshold value and had an above-threshold green (GFP) pixel at the same location (that is, at the same pixel position as GFP).

To take into account the wavelength-dependent X-Y plane shift of the different (green GFP versus blue CFP) images during multicolor imaging (Lochner et al., 1998; Scalettar et al., 1996), we also considered a 'nearness' colocalization parameter. 'Nearness' was defined as the total number of blue (CFP) pixels within the cell region that were above a threshold value and had an above-threshold green (GFP) pixel within a 3D 2×2×2 pixel window centered on a green pixel (that is, within ±2 pixels of a green pixel in any XYZ volume).

Fig. 6 shows the distribution patterns of SgP-GFP, SgP-CgA_{1-16}-GFP, SgP-CgA_{1-37}-GFP SgP-CgA_{1-39}-GFP, SgP-CgA_{1-77}-GFP, SgP-CgA_{1-168}-GFP, SgP-CgA-GFP or SgP-CgAΔC_{17}E-GFP together with GALT-CFP. As expected, the GALT-CFP distribution pattern was clustered in a perinuclear region characteristic of the Golgi complex. As noted upon visual inspection of the 3D/volume views and of the 0.2 μm X-Y optical sections selected from a middle region of the cells, colocalization between GFP-tagged SgP, SgP-CgA_{1-16}, SgP-CgA_{1-77} and GALT-CFP was high, with overlap values ranging

from 76.2 to 77.2%, and nearness values ranging from from 81.6 to 83.6% (Fig. 6). These findings demonstrate that GFP chimeras containing a CgA domain extending from –18 to not beyond +77 amino acid residues accumulate in a subcellular region of PC12 cells that corresponds to the Golgi complex.

In contrast, the extent of colocalization between GALT-CFP and SgP-CgA_{1-168}-GFP, SgP-CgA-GFP or SgP-$CgA\Delta C_{17}E$-GFP was markedly reduced, with average overlap values ranging from 38.8 to 43.7%, and average nearness values ranging from 42.7 to 44.7% (Fig. 6). This reduced colocalization indicates that the CgA domain ranging from 77 to 168 amino acid residues provides an important trafficking signal that promotes exit of CgA-GFP from the TGN, but that an intact disulfide bond between Cys_{17}-Cys_{38} is not required for such trans-Golgi exit.

Secretory granules: 3D quantification of the colocalization of CgA domain-GFP fusion proteins and dopamine β-hydroxylase (DβH), a dense core secretory granule marker

To further investigate the transport of CgA fragment/GFP fusion proteins into the regulated secretory pathway, we assessed the subcellular colocalization of the chimeras with dopamine β-hydroxylase (DβH), an enzyme found in both membrane and the soluble core of catecholamine secretory granules (Kirshner and Goodhall, 1957). Detection of DβH was achieved by immunocytochemistry of aldehyde-fixed PC12 cells expressing CgA fragment/GFP chimeras using a monoclonal antibody against rat DβH. The subcellular distribution of GFP hybrids and DβH was visualized by deconvolution microscopy, and the degree of the colocalization between GFP chimera fluorescence and immunoreactivity for DβH was subsequently quantified in 3D using Nearcount Image Analysis software (Fig. 7).

Fig. 7 shows high magnification volume views of the distribution patterns of SgP-GFP, SgP-CgA_{1-77}-GFP, SgP-CgA_{1-115}-GFP, SgP-CgA_{1-224}-GFP, SgP-CgA-GFP, SgP-$CgA\Delta C_{17}E$-GFP, SgP-$CgA_{233-439}$-GFP or proANF-GFP, together with DβH immunostaining.

To take into account the wavelength-dependent X-Y plane shift of the different [green GFP versus red DβH (Alexa Fluor 594)] images during multicolor imaging (Lochner et al., 1998; Scalettar et al., 1996), as well the possibility of incomplete

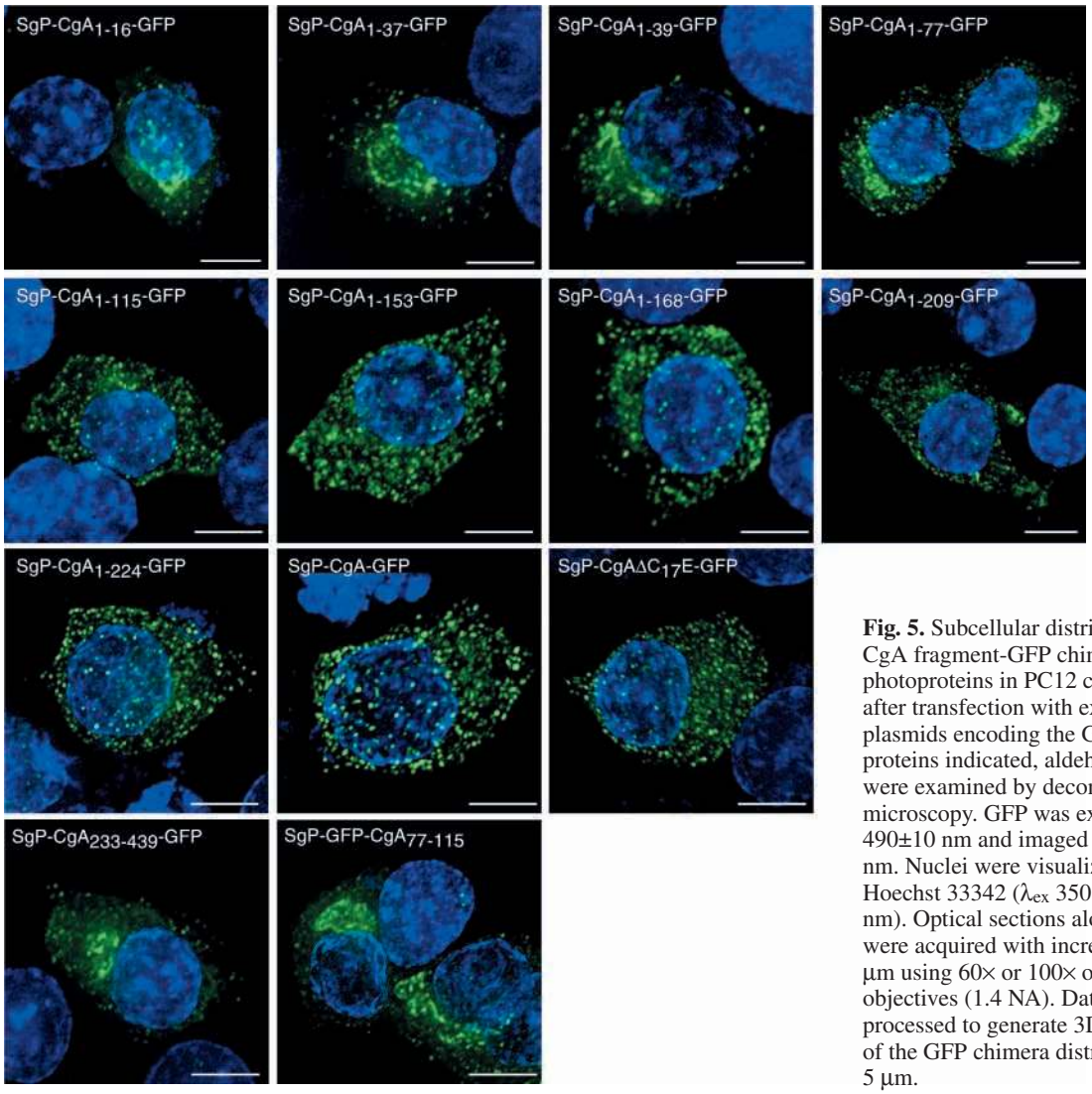
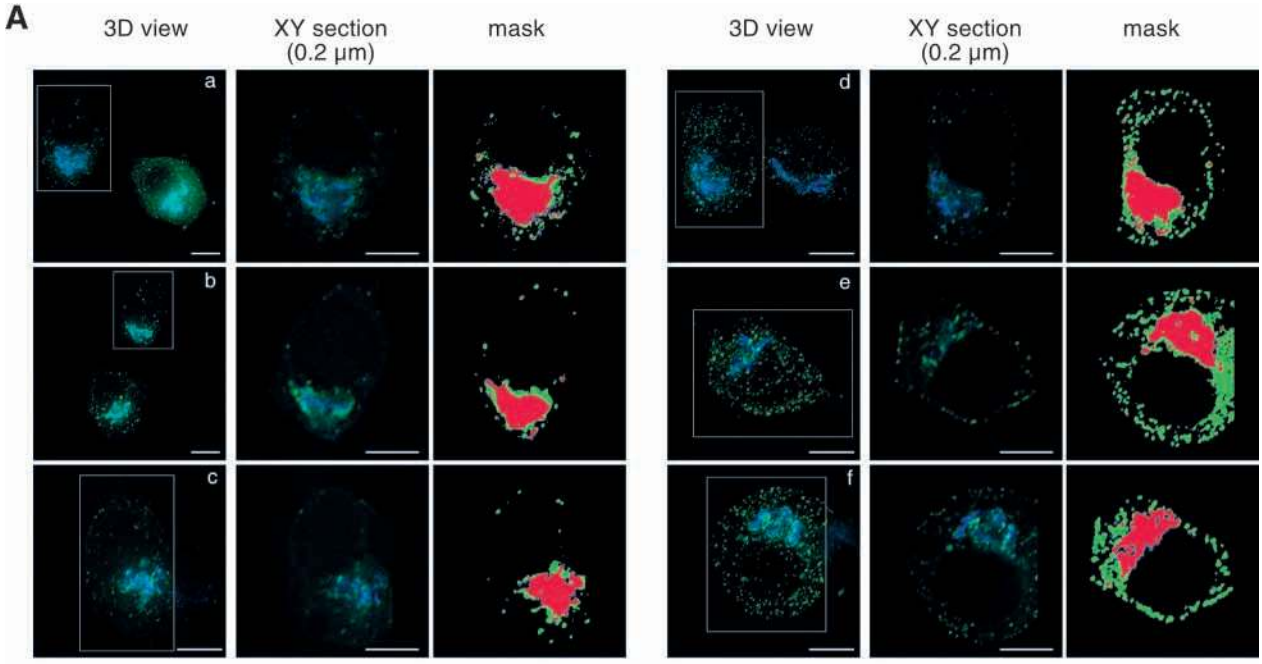


Fig. 5. Subcellular distribution of the CgA fragment-GFP chimeric photoproteins in PC12 cells. 48 hours after transfection with expression plasmids encoding the GFP fusion proteins indicated, aldehyde-fixed cells were examined by deconvolution microscopy. GFP was excited at λ_{ex} 490±10 nm and imaged at λ_{em} 528±238 nm. Nuclei were visualized with (blue) Hoechst 33342 (λ_{ex} 350 nm/λ_{em} 461 nm). Optical sections along the Z axis were acquired with increments of 0.2 μm using 60× or 100× oil immersion objectives (1.4 NA). Data were processed to generate 3D/volume views of the GFP chimera distributions. Bars, 5 μm.

penetration of the secretory granule by the anti-DβH antibody, we once again considered the 'nearness' colocalization parameter. 'Nearness' was defined as the total number of red (Alexa Fluor 594; DβH) pixels within the cell region that were above a threshold value and had an above-threshold green (GFP) pixel within a 3D 2×2×2 pixel window centered on a green pixel (that is, within ±2 pixels of a green pixel in any XYZ volume). Of further note, this 'nearness' parameter also allows accommodation of the physical span of a chromaffin granule, which is ~9-15 pixels in these images (as determined for either CgA-GFP or proANF-GFP; Fig. 7). Thus, 'nearness' may be a superior index of co-inhabitation of the same secretory granule by two molecules.

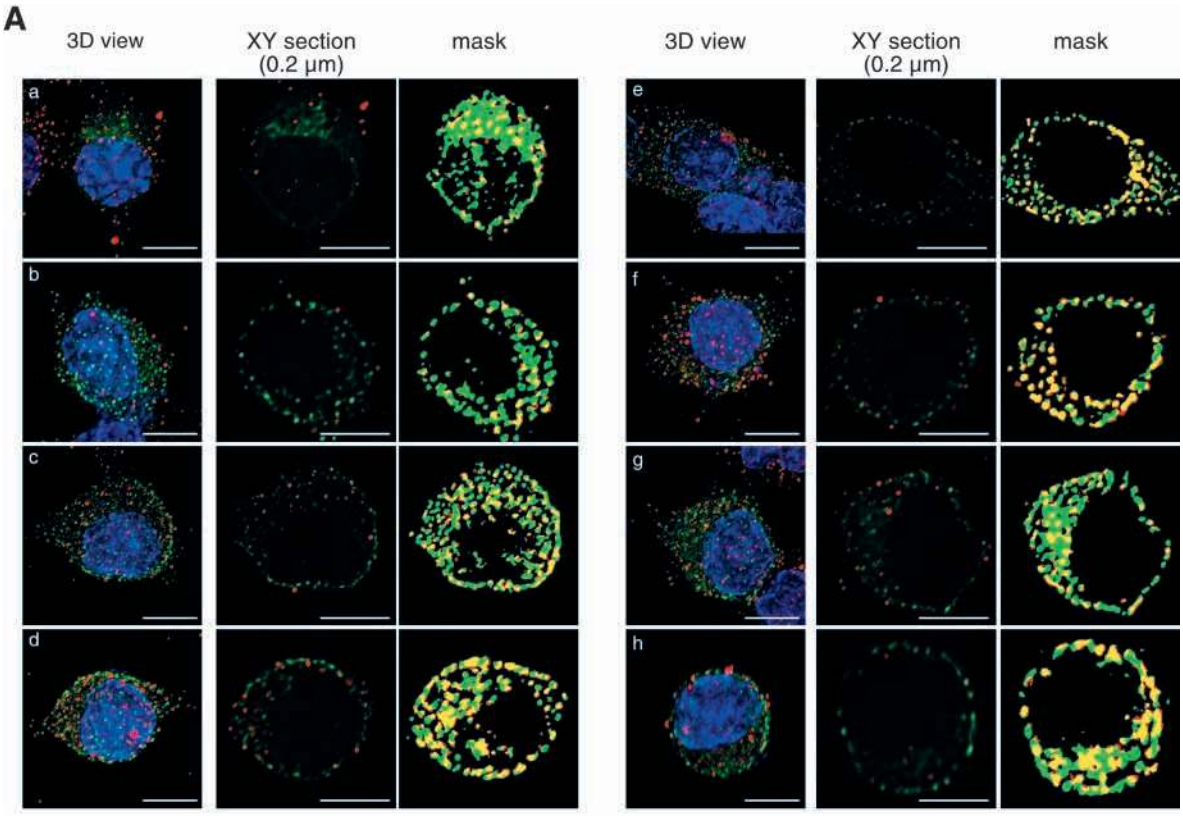
The extent of colocalization of SgP-GFP, SgP-CgA$_{1\text{-}77}$-GFP, or SgP-CgA$_{233\text{-}439}$-GFP with DβH was low, with average overlap values ranging from 14 to 21%, and average nearness values ranging from 16.4 to 28.3% (Fig. 7). In contrast, the extent of colocalization between DβH and GFP was substantially higher in cells expressing SgP-CgA$_{1\text{-}115}$-GFP, SgP-CgA$_{1\text{-}224}$-GFP, SgP-CgA-GFP, or SgP-CgAΔC$_{17}$E-GFP,



B

GFP chimera	Overlap (%)	Nearness (%)	Optical Z sections
SgP-GFP	76.0	83.6	n=25
SgP-CgA$_{1\text{-}39}$-GFP	77.2	81.6	n=25
SgP-CgA$_{1\text{-}77}$-GFP	76.2	82.7	n=16
SgP-CgA$_{1\text{-}168}$-GFP	43.7	43.0	n=36
SgP-CgA-GFP	38.8	42.7	n=35
SgP-CgAΔC$_{17}$E-GFP	44.2	44.7	n=24

Fig. 6. The Golgi apparatus. Analysis for colocalization between CgA fragment-GFP chimeric photoproteins and β1,4-galactosyltransferase cyan fluorescent protein (GALT-CFP) chimera, a Golgi apparatus marker. (A) 48 hours after transfection with expression plasmids encoding SgP-GFP (a), CgA$_{1\text{-}77}$-GFP (b), CgA$_{1\text{-}115}$-GFP (c), CgA$_{1\text{-}224}$-GFP (d), SgP-CgA-GFP (e), CgAΔC$_{17}$E-GFP (f) fusion proteins, together with the expression plasmids encoding for the targeting sequence of β1,4-galactosyltransferase fused to CFP (GALT-CFP), aldehyde-fixed cells were examined by deconvolution microscopy. GFP was excited at λ_{ex} 490±10 nm and imaged at λ_{em} 528±38 nm; CFP was excited at λ_{ex} 436±10 nm and imaged at λ_{em} 465±30 nm. Optical sections along the Z axis were acquired with increments of 0.2 μm using a 100× oil immersion objective (1.4 NA). Data were processed to generate combined 3D/volume views of the GFP and CFP chimera distribution. Representative 0.2 μm XY optical sections acquired in the middle region of the cells, and corresponding three-color mask images generated by Nearcount Image Analyzer are shown. The extent of cyan (CFP) and green (GFP) fluorescent signal 3D colocalization in cells within the boxed areas (crop region) was assessed using Nearcount Image Analyzer software. (B) Nearcount image quantification of GFP (green) chimeras and CFP-Golgi (blue) 3D colocalization. 'Overlap' is defined is defined as the total number of blue (CFP) pixels within the crop region that are above a threshold value and have an above-threshold green (GFP) pixel at the same location (same pixel). 'Nearness' is defined as the total number of blue (CFP) pixels within the crop region that are above a threshhold value and have an above-threshhold green (GFP) pixel within a 3D 2×2×2 pixel window centered on a green (GFP) pixel. Areas of overlap or nearness are displayed in red ('mask'). Bars, 5 μm.



B

GFP chimera	Overlap (%)	Nearness (%)	Optical Z sections
SgP-GFP	14.0	16.4	n=28
SgP-CgA$_{1-77}$-GFP	14.1	19.5	n=44
SgP-CgA$_{1-115}$-GFP	34.4	48.9	n=29
SgP-CgA$_{1-224}$-GFP	59.0	77.7	n=23
SgP-CgA-GFP	46.5	65.7	n=28
SgP-CgAΔC$_{17}$E-GFP	54.5	71.8	n=32
SgP-CgA$_{233-439}$-GFP	21.0	28.3	n=34
ProANF-GFP	40.1	52.7	n=32

Fig. 7. Secretory granules. Analysis for colocalization between CgA fragment-GFP chimeric photoproteins and dopamine β-hydroxylase (DβH), a dense-core secretory (chromaffin) granule marker. (A) 48 hours after transfection with expression plasmids encoding SgP-GFP (a), SgP-CgA$_{1-77}$-GFP (b), SgP-CgA$_{1-115}$-GFP (c), SgP-CgA$_{1-224}$-GFP (d), SgP-CgA-GFP (e), SgP-CgAΔC$_{17}$E-GFP (f), SgP-CgA$_{233-439}$-GFP (g) or proANF-GFP (h), cells were aldehyde-fixed and processed for immunocytochemistry. Cells were incubated with a mouse anti-rat DβH followed by Alexa-Fluor-594 (red)-conjugated goat anti-mouse. GFP was excited at λ_{ex} 490±10 nm and imaged at λ_{em} 528±38 nm; the red Alexa Fluor 594 conjugate was excited at λ_{ex} 555±28 nm and imaged at λ_{em} 580±20 nm. Nuclei were visualized with Hoechst 33342 (λ_{ex} 350 nm/λ_{em} 461 nm). Optical sections along the Z axis were acquired with increments of 0.2 μm with a DeltaVision imaging system using a 100× oil immersion objective. Volume views (left panel) of the GFP chimeras and Alexa-Fluor-594-conjugated antibody distributions are shown. The extent of intracellular 3D colocalization of red (Alexa 594) and green (GFP) fluorescent signals was assessed using Nearcount Image Analyzer software. Representative 0.2 μm XY optical sections acquired in the middle region of the cells, and corresponding three-color mask images generated by Nearcount Image Analyzer are shown. (B) Nearcount image quantification of GFP (green) chimeras and DβH (red) 3D colocalization. The area of analysis (crop region) was defined as a rectangle that included the whole cell. 'Overlap' is defined as the total number of red (Alexa 594) pixels within the crop region that are above a threshold value and have an above-threshold green (GFP) pixel at the same location (same pixel). 'Nearness' is defined as the total number of red (Alexa 594) pixels within the crop region that are above a threshold value and have an above-threshold green (GFP) pixel within a 3D 2×2×2 pixels window centered on a green (GFP) pixel (i.e. ±2 pixels). At the total magnification used here, a chromaffin granule spans ~9-15 pixels (as determined by CgA-GFP or proANF-GFP). Areas of overlap or nearness are displayed in yellow ('mask'). Bar, 5 μm.

as evidenced by average overlap values ranging from 34.4 to 59%, and average nearness values ranging from 48.9 to 77.7% (Fig. 7). Colocalization with DβH was also determined for proANF-GFP, a well-described fluorescent marker of neuroendocrine secretory granules (Burke et al., 1997; Han et al., 1999) (Fig. 7). Nearcount analysis indicated that proANF-GFP colocalized with DβH with parameters of the same magnitude as those measured for SgP-CgA-GFP (40.1% overlap, 52.7% nearness; Fig. 7).

Secretagogue-mediated release of CgA domain-/GFP chimeric photoproteins

We investigated secretagogue-mediated release of the GFP chimeras from the regulated secretory pathway. Sorting was assayed by immunoblotting secretion media collected from PC12 cells, in the presence or the absence of $BaCl_2$ (2 mM, 15 minutes). As displayed in Fig. 8, regulated secretion of the GFP-tagged chimeras SgP-$CgA_{1\text{-}115}$, SgP-$CgA_{1\text{-}168}$, SgP-$CgA_{1\text{-}209}$, SgP-$CgA_{1\text{-}224}$, or a GFP-tagged CgA devoid of its disulfide bridge ($CgA\Delta C_{17}E$-GFP) was detected following Ba^{2+} stimulation and paralleled proANF-GFP release.

In contrast, Ba^{2+} stimulation did not release GFP immunoreactivity from PC12 cells expressing GFP chimeric proteins from either the more proximal CgA N-terminal domains SgP-$CgA_{1\text{-}39}$ and SgP-$CgA_{1\text{-}77}$, or the C-terminal half fragment $CgA_{233\text{-}439}$.

In the absence of stimulation, null or trace amounts of GFP immunoreactivity were detected in the extracellular media after transfection/expression of any of these chimeras, suggesting that, within this 15-minute stimulation period, basal secretion of each of these chimeras was limited. Can the N-terminal chimeras exit the Golgi apparatus into the secretory pathway in general? Importantly, we found that SgP-GFP and SgP-$CgA_{1\text{-}39}$ were secreted into the basal media following 3 hours of incubation in non-stimulating conditions (data not shown). This result suggests that the lack of regulated release of the chimeras bearing only the $CgA_{1\text{-}77}$ domain cannot be attributed to 'trapping' of such chimeras in the Golgi complex, since they are able to exit the trans-Golgi network and thereby enter the constitutive (basal) pathway of secretion

The results reported here seem to locate a novel sorting determinant in CgA's N-terminal region which is: (i) not restricted to the Cys_{17}-Cys_{38} loop domain, (ii) independent of the presence of a disulfide bridge, and (iii) critically dependent upon sequences between amino acid residues 77 through 115 in mature CgA.

The $CgA_{77\text{-}115}$ domain: 3D visualization of the subcellular distribution of a SgP-GFP-$CgA_{77\text{-}115}$ chimera in PC12 cells

Finally, we aimed to determine whether the $CgA_{77\text{-}115}$ domain, which seems to be necessary for correct trafficking into the regulated pathway of secretion (Figs 7, 8), also contains sufficient sorting information for such entry. We therefore tested whether transfer of this domain to the constitutively secreted SgP-GFP chimera (SgP-GFP-$CgA_{77\text{-}115}$; Fig. 3) can reroute the hydrid protein to secretory granules. Subcellular localization of SgP-GFP-$CgA_{77\text{-}115}$ was assessed by deconvolution microscopy followed by computational 3D

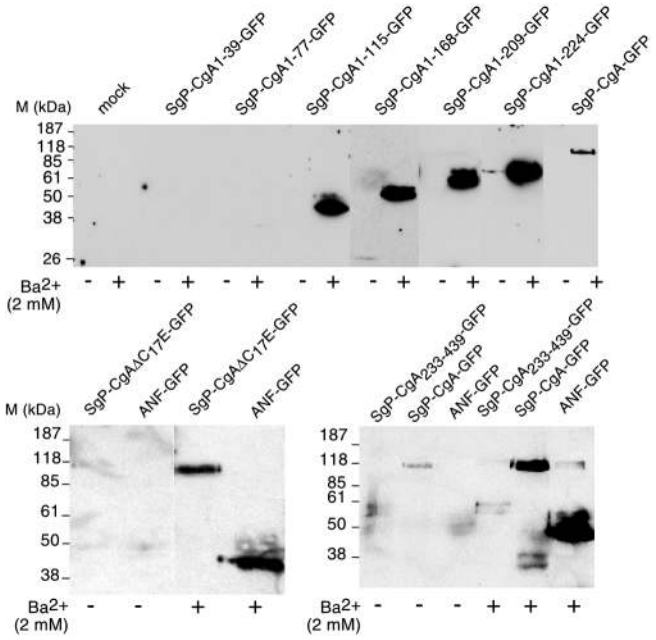


Fig. 8. Immunochemical analysis of CgA domain-GFP fusion protein release upon stimulation of regulated secretion. PC12 cells were transiently transfected with expression plasmids encoding the GFP fusion proteins indicated. 48 hours after transfection, regulated secretion was triggered by 2 mM $BaCl_2$ for 15 minutes. Extracellular media were collected and concentrated using C-18 SepPak cartridges, and processed for immunoblot using an anti-GFP antibody. Immunoreactivity was visualized by chemiluminescence.

reconstruction (Fig. 5). When expressed in PC12 cells, SgP-GFP-$CgA_{77\text{-}115}$ chimera exhibited a subcellular distribution identical to the ones observed in cells expressing the constitutively trafficked SgP-GFP, SgP-$CgA_{1\text{-}16}$, SgP-$CgA_{1\text{-}16}$, SgP-$CgA_{1\text{-}37}$, SgP-$CgA_{1\text{-}39}$ or SgP-$CgA_{1\text{-}77}$ chimeras (Figs 1, 5, 6; substantial peri-nuclear Golgi accumulation), suggesting that the domain Lys_{77}-Arg_{115} of CgA, although necessary for appropriate trafficking (Figs 7 8), may not be a sufficient sorting determinant towards the regulated pathway.

Discussion

Chromogranin A trafficking

Chromogranin A is the index member of the 'granin' family of regulated secretory proteins which are ubiquitously distributed in amine- and peptide-containing secretory granules of endocrine, neuroendocrine and neuronal cells. Because of their abundance and such widespread occurrence, granins have often been used as prototype proteins to elucidate mechanisms of protein targeting into dense-core secretory granules. However, while several studies address CgB sorting into catecholamine storage vesicles, much remains to be learned about CgA trafficking into the regulated pathway of secretion. GFP-tagged molecules in secretory granules have frequently been used as a tool to investigate the dynamics of secretory granule movement (Burke et al., 1997; Han et al., 1999; Hirschberg et al., 1998; Lang et al., 1997; Lochner et al., 1998; Miesenböck et al., 1998) rather than the process of secretory protein routing within the constitutive or regulated secretory pathways. In this

work we used a series of full-length, point mutant or truncated CgA-GFP recombinant proteins to explore routing of CgA in PC12 cells. Using a combination of subcellular fractionation and 3D microscopy techniques to determine the subcellular localization the GFP chimeras, as well as secretagogue-stimulated secretion, the present study establishes that human CgA flanked by a signal sequence is able to convert GFP from a cytosolic protein to a soluble component of catecholamine secretory vesicles. Moreover, our studies reveal for the first time the necessary contribution of the amino acid residues 77-115 of the mature protein in trafficking CgA into the regulated pathway of secretion in neuroendocrine PC12 cells.

A prerequisite for the significance of this study was to establish that a human CgA-GFP chimeric photoprotein could correctly localize to dense-core catecholamine secretory granules. 3D deconvolution microscopy of PC12 cells expressing SgP-CgA-GFP revealed a bright, subplasmalemmal, punctate/vesicular fluorescence (Figs 1, 5, 6, 7) which concentrated in neurite termini in NGF-differentiated cells, indicating storage of GFP-tagged CgA within chromaffin granules. Sucrose gradient studies colocalized SgP-CgA-GFP and catecholamines to the same subcellular fraction at 1.2-1.4 M sucrose (Figs 1, 2); the 1.2-1.4 M sucrose peak is consistent with the buoyant density reported previously for chromaffin granules from PC12 cells (Parmer et al., 1997; Parmer et al., 1993; Roda et al., 1980; Schubert and Klier, 1977). Sucrose gradient fractionation results may be limited by the potential for co-purification of heterogeneous organelles to the same fraction (Roda et al., 1980; Schubert and Klier, 1977); hence, we localized the chimeras in other ways, both optical and pharmacological.

Further insights into the subcellular location of SgP-CgA-GFP were therefore acquired using 3D deconvolution microscopy of the transport of CgA-GFP hybrid through the Golgi complex to dense-core granules. SgP-CgA-GFP colocalization with Golgi apparatus marker β1,4-galactosyltransferase was low (38.8-42.7%), suggesting that the chimera was normally routed through cisternal structures of the Golgi but eventually exiting the trans side of the apparatus to reach dense-core granules. Indeed, we found that 65% of SgP-CgA-GFP fluorescence colocalized with DβH, an enzymatic marker of catecholamine secretory granules (Fig. 7), further demonstrating that SgP-CgA-GFP is effectively sorted out of the TGN into dense-core granules.

Colocalization between the SgP-CgA-GFP chimera and DβH was not absolute (Fig. 7); the extensive although incomplete colocalization may reflect the limited accessibility of antibodies to DβH in aldehyde-permeabilized cells, accounting for secretory granules positive for GFP but immunonegative for DβH. Conversely, secretory granules already synthesized before chimera transfection would be devoid of GFP fluorescence but display immunopositivity for DβH. Incomplete colocalization with DβH was also observed for proANF-GFP (Fig. 7), previously documented as a resident protein of dense-core granules of PC12 cells [This report; (Burke et al., 1997; Han et al., 1999). Indeed, colocalization parameters for proANF-GFP were of the same magnitude (40.1-52.7%) as those measured for SgP-CgA-GFP. Such extensive though incomplete colocalization is consistent with values reported in studies localizing other GFP fusion proteins, such as CgB-GFP (Kaether and Gerdes, 1995; Lang et al., 1997) or tPA(tissue plasminogen activator)-GFP (Lochner et al., 1998), with markers of the regulated pathway of secretion.

Sorting signals within CgA

We next aimed to establish that the fluorescence of a GFP-tagged CgA can be used to identify potential sorting signals for CgA in neuroendocrine cells. To accomplish this goal, we designed a series of GFP chimeras that comprised N-terminal and C-terminal truncated CgA domains as well as a mutant of full-length human CgA devoid of a disulfide bond (Fig. 3). The C-terminal domain of CgA has been reported to exhibit Ca^{2+}/pH-dependent homodimerization/homotetramerization (Yoo and Lewis, 1993a) which may initiate aggregation-mediated sorting of CgA to secretory granules (Chanat and Huttner, 1991; Gerdes et al., 1989; Gorr et al., 1989). In support of this proposal, Cowley et al., recently showed that a C-terminal multimerization domain directs CgA sorting to the regulated secretory pathway of endocrine GH4C1 (pituitary somatotrope) cells (Cowley et al., 2000). Our 3D deconvolution data (Figs 5, 7) and barium chloride-evoked secretion data (Fig. 8) obtained for SgP-$CgA_{233-439}$-GFP and SgP-CgA_{1-224}-GFP, clearly establish that PC12 cells require an N-terminal domain for sorting of CgA to secretory granules and that aggregation properties described for the C-terminal region (Chanat and Huttner, 1991; Gerdes et al., 1989; Gorr et al., 1989) do not contribute to such sorting. These results are consistent with earlier studies from our group (Parmer et al., 1993) and with a recent proposal that CgA may contain distinct, cell-specific sorting domains (Cowley et al., 2000).

The disulfide-bonded hydrophobic loop domain

Disulfide-bonded loop structures found in several regulated secretory proteins such as POMC or CgB have been proposed to play a role in the sorting process. For example, CgB contains near its N-terminus a stretch of hydrophobic amino acid residues, flanked by two cysteines, which is substantially conserved even between CgA and CgB of various species. Reductive cleavage of CgB's loop structure (Chanat et al., 1993; Gorr et al., 1999) indicated the necessity of the disulfide bond for sorting in PC12 cells. Studies on a loopless mutant of CgB and on chimeric proteins of the loop domain of CgB fused to the constitutively secreted reporter protein α_1-antitrypsin (Glombik et al., 1999; Kromer et al., 1998) suggest a necessary and indeed sufficient role for the hydrophobic loop domain in sorting of CgB to the regulated pathway of secretion. In contrast with these previous studies, we show in this report that disruption of CgA's Cys_{17}-Cys_{38} disulfide bridge by point mutagenesis (SgP-$CgA\Delta C_{17}E$-GFP) did not affect its colocalization with the dense-core secretory granule marker DβH (Fig. 7) or its regulated secretion (Fig. 8). Hence stabilization of CgA's N-terminal hydrophobic Cys_{17}-Cys_{38} domain by a disulfide bridge may not be necessary for sorting in PC12 cells. Moreover, the inability of the loop-containing chimeras SgP-CgA_{1-39}-GFP and SgP-CgA_{1-77}-GFP to steer GFP to the regulated pathway of secretion further indicates that neither the disulfide bond nor the first 77 amino acid residues of CgA (spanning the Cys_{17}-Cys_{38} loop), hold sufficient information for sorting of CgA to neuroendocrine PC12 granules.

Conventional transfection experiments to screen for granin sorting domains might be difficult to interpret because of the propensity of the exogenous and endogenous granins to form intermolecular aggregates (Kromer et al., 1998; Tooze et al., 2001). Indeed, CgA may contain two distinct oligomerization domains, one at the disulfide-bonded loop $CgA_{17\text{-}38}$ (Thiele and Huttner, 1998), and the other towards the C-terminus (Yoo and Lewis, 1993a), that might contribute to exogenous-endogenous granin interactions. However, the current results argue against an endogenous granin loop-mediated or C-terminal domain-mediated 'rescue' sorting of loop-free exogenous CgA (SgP-CgAΔC_{17}E-GFP), since neither the C-terminal half of CgA (SgP-$CgA_{233\text{-}439}$-GFP), the N-terminal hydrophobic disulfide loop domains of CgA (SgP-$CgA_{1\text{-}39}$-GFP and SgP-$CgA_{1\text{-}77}$-GFP), nor the N-terminal domain with disulfide bond disruption (lacking Cys_{38}; SgP-$CgA_{1\text{-}37}$-GFP) were trafficked to dense-core granules or were exocytosed in a regulated manner (Figs 7, 8).

Another necessary sorting domain: $CgA_{77\text{-}115}$

Our studies point to an important contribution of the amino acid residues 77-115 in promoting the sorting process of CgA. Indeed, the addition of 39 amino acid residues to the truncated SgP-$CgA_{1\text{-}77}$-GFP fusion protein, to form SgP-$CgA_{1\text{-}115}$-GFP, markedly increased its transport through and out of the Golgi complex (Fig. 6), its degree of colocalization with DβH (Fig. 7), and its secretagogue-regulated release (Fig. 8). One interpretation of these results is that the $CgA_{77\text{-}115}$ domain possesses an independent sorting signal, necessary [though not sufficient (SgP-GFP-$CgA_{1\text{-}115}$; Fig. 5)] for regulated pathway entry of CgA.

On the basis of the amino acid sequences and secondary structures of 15 proteins sorted to regulated secretory granules, Kizer and Tropsha proposed a degenerate secondary structure sorting motif consisting of two or more leucines occupying one side of a highly amphipathic α-helix with a serine or threonine positioned N-terminal to the leucines (Kizer and Tropsha, 1991). We found that the domain His_{79}-Leu_{90} of human CgA (H_{79}SGFEDELSEVL_{90}) conformed exactly (12/12 match) to three turns of a putative amphipathic α-helical motif, wherein hydrophobic residues occur every at fourth residue in the primary structure, using the alternating search motif XXX(FILMVWY)XXX(FILMVWY)XXX(FILMVWY), where (FILMVWY) represents the single occurrence of any hydrophobic residue at that position, and XXX indicates 3 intervening amino acids. Perhaps such a discrete segment of CgA could be recognized as a sorting determinant to the regulated pathway, by virtue of its putative membrane interaction. It also conceivable that the segment Lys_{77}-Arg_{115} of CgA might provide a scaffold for the correct conformational expression of a sorting domain, perhaps the Cys_{17}-Cys_{38} hydrophobic domain of CgA, which exhibits some of the properties expected of a sorting signal for regulated secretory proteins (Gorr and Darling, 1995); in that case, two CgA domains separated in primary structure might cooperate in 3 dimensions to direct sorting.

How the sorting element(s) located within the $CgA_{1\text{-}115}$ domain function in a cellular context is not clear from our studies. As outlined in the introduction, several mechanisms might contribute in a separate or concerted manner to the sorting of secretory proteins from the TGN into the regulated pathway of secretion. Such mechanisms include formation of insoluble aggregates of the regulated secretory proteins,as well as direct or indirect binding of the regulated secretory proteins to the nascent granule membrane or to a sorting receptor therein (Arvan and Castle, 1998; Chanat and Huttner, 1991; Cool et al., 1997; Loh et al., 1997; Thiele et al., 1997; Tooze et al., 2001; Yoo, 1993b). For example, the N-terminal sorting domain of POMC seems to interact with carboxypeptidase E (CPE) which has been postulated to play a role as a sorting receptor for several regulated secretory proteins (Loh et al., 1997); however CPE, does not act as a sorting receptor for the granins (Natori et al., 1998; Normant and Loh, 1998). An as another alternative, the near N-terminal region of CgA devoid of flanking cysteines (i.e., CgA residues 18-37) can bind to and modulate the activity of the inositol (1,4,5)-trisphosphate receptor, which might behave as a sorting receptor for CgA to secretory granules (Yoo, 1993b; Yoo and Kang, 1997; Yoo et al., 2000).

Since this report supports the idea that the hydrophobic loop structure of CgA is not a sufficient determinant for sorting, it is appealing to propose that the $CgA_{77\text{-}115}$ region contains an additional sorting determinant which may provide an optimal conformational framework for binding of CgA's loop domain to a granule membrane sorting receptor, such as, perhaps, the intravesicular domain of the inositol (1,4,5)-trisphosphate receptor. Additional mutagenesis and deletion experiments might determine whether transfer of this specific domain of CgA to constitutively secreted proteins re-routes them to the chromaffin granule. Several clusters of glutamic amino acid residues occur in the primary structure of CgA (Fig. 3) and might have the potential to promote selective pH- and Ca^{2+}-dependent aggregation of CgA in the TGN, a process involved in targeting the protein to the regulated pathway of secretion. Evaluation of the putative effect of such acidic domains on the sorting efficiency of chimeras spanning $CgA_{1\text{-}153}$, $CgA_{1\text{-}168}$, $CgA_{1\text{-}209}$ or $CgA_{1\text{-}224}$ (Fig. 3) might be of interest.

In summary, these studies demonstrate that the N-terminal half of human CgA contains information both necessary and sufficient for diverting a heterologous, ordinarily non-secreted cytoplasmic protein into the regulated pathway of secretion in neuroendocrine PC12 cells. In addition, our studies indicate that stabilization of CgA's hydrophobic loop by a disulfide bridge is not a requirement for this event. Finally, this report reveals that the N-terminal hydrophobic loop domain may not be sufficient for sorting of CgA to chromaffin granules; such trafficking seems to require the contribution of an additional, novel and necessary (though perhaps not entirely sufficient) determinant located between residues 77-115 in the mature protein.

Since neither the disulfide loop domain ($CgA_{17\text{-}38}$; Figs 6, 7, 8) nor the $CgA_{77\text{-}115}$ domain (Fig. 5) seem to be sufficient for routing CgA into the regulated pathway, how might such trafficking be accomplished? Our results suggest that multiple domains in the N-terminal half of CgA might cooperate in ways that are not easily explained by short stretches of primary structure; perhaps such sub-domains must cooperate within larger, as-yet-uncharacterized regions of tertiary structure to achieve the necessary signal for regulated pathway entry. Such an understanding awaits further development of 3D structural information on CgA.

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