Secretory Protein Traffic
Chromogranin A Contains a Dominant Targeting Signal for the Regulated Pathway

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Abstract
Secretory proteins are targeted into either constitutive (secreted upon synthesis) or regulated (stored in vesicles and released in response to a secretagogue) pathways. To investigate mechanisms of protein targeting into catecholamine storage vesicles (CSV), we stably expressed human chromogranin A (CgA), the major soluble protein in human CSV, in the rat pheochromocytoma PC-12 cell line. Chromaffin cell secretagogues (0.1 mM nicotinic cholinergic agonist, 55 mM K+, or 2 mM Ba++) caused cosecretion of human CgA and catecholamines from human CgA-expressing cells. Sucrose gradients colocalized human CgA and catecholamines to subcellular particles of the same buoyant density. Chimeric proteins, in which human CgA (either full-length [457 amino acids] or truncated [amino-terminal 226 amino acids]) was fused in-frame to the ordinarily nonsecreted protein chloramphenicol acetyltransferase (CAT), were expressed transiently in PC-12 cells. Both constructs directed CAT activity into regulated secretory vesicles, as judged by secretagogue-stimulated release. These data demonstrate that human CgA expressed in PC-12 cells is targeted to regulate secretory vesicles. In addition, human CgA can divert an ordinarily non-secreted protein into the regulated secretory pathway, consistent with the operation of a dominant targeting signal for the regulated pathway within the peptide sequence of CgA. (J. Clin. Invest. 1993. 92:1042–1054.) Key words: catecholamine • chromaffin cells • chromogranin A • PC-12 • pheochromocytoma • regulated secretion

Introduction
Secretory proteins are targeted into either constitutive or regulated pathways (1, 2). Proteins entering the regulated pathway are concentrated and stored in vesicles, and subsequently released upon stimulation by a secretagogue. In the constitutive pathway, newly synthesized protein is not stored but leaves the Golgi complex in short-lived membrane vesicles that fuse immediately with the plasma membrane even in the absence of any extracellular signal.

Catecholamine storage vesicles are specialized subcellular organelles within chromaffin cells of the adrenal medulla and in sympathetic neurons (3). In addition to catecholamines, these organelles contain ATP, Ca++, peptides, and proteins (4, 5), all of which are released in response to secretagogues by the process of exocytosis (6). These vesicles are thus an example of regulated secretory vesicles. The mechanisms of assembly of catecholamine storage vesicles, and, in particular, how proteins are targeted to such vesicles are poorly understood.

Chromogranin A (CgA) is the major soluble protein in the core of catecholamine storage vesicles (7) and is found as well in dense core secretory vesicles throughout the neuroendocrine system (8–11). In addition, recent evidence suggests that CgA and other members of the chromogranin/secretogranin (“granin”) family of proteins may have a central role in the packaging of other proteins into the regulated secretory pathway (12). Thus, CgA may be a model protein for studies of protein targeting into the regulated pathway of secretion. Also, CgA is the source of biologically active peptides, such as paracentatin (13), which suppresses islet beta cell secretion, and chromostatin, which suppresses secretagogue-stimulated catecholamine release (14).

To investigate mechanisms of protein targeting into catecholamine storage vesicles, we expressed human chromogranin A in the rat PC-12 cell line, a catecholaminergic rat pheochromocytoma cell line (15), which exhibits both regulated and constitutive pathways for secretion and sorts secreted proteins into one or the other of these pathways (16, 17). In the present report, we demonstrate the expression of human CgA in rat pheochromocytoma cells, the targeting of expressed human CgA to dense core catecholamine storage vesicles, and the ability of human CgA to divert a heterologous, ordinarily non-secreted protein (chloramphenicol acetyltransferase [CAT]) into the regulated secretory pathway. In addition, we demonstrate that information sufficient for regulated vesicular targeting is contained within the amino-terminal half of the human CgA peptide sequence.

Methods

Construction of the expression vector, pRVS-hCgA
The mammalian expression vector, pRVS-hCgA, containing the full-length human CgA cDNA under the control of the Rous sarcoma virus long-terminal repeat (R-LSV-LTR) was constructed as shown in Fig. 1. pGM-hCgA (from Dr. Lee Helman, National Cancer Institute) was the source of the human CgA cDNA (18). pRVS-L (from Dr. Suresh Subramani, University of California, San Diego) (19) was the source of the RSV-LTR. After conversion of the Smal site to an Xhol site in pRVS-L with synthetic linkers and subsequent digestion of resultant vector pRVS-L, with HindIII and Xhol to remove the firefly luciferase gene, the human CgA cDNA (liberated from pGem-hCgA by digestion with HindIII and SalI) was spliced into this HindIII-Xhol site. (Cohe-

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Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; CgA, chromogranin A; RSV-LTR, Rous sarcoma virus-long terminal repeat.
sive ends from Xhol and SalI digestions are compatible; subsequent ligation results in loss of SalI and Xhol recognition sites and generation of a TaqI site.) The resultant construct contains the human CgA cDNA under the control of RSV-LTR, an SV40 small t splice signal and polyadenylation signal (A<sub>n</sub>) for efficient message processing, and a prokaryotic segment from pBR-322 including the origin of replication (ori) and β-lactamase gene (amp<sup>B</sup>) for growth in *Escherichia coli*. Construction was verified by diagnostic restriction digests and DNA sequencing.

### Cell culture

Rat pheochromocytoma PC-12 cells (obtained from Dr. Bruce Howard of University of California, Los Angeles) were grown at 37°C, 6% CO<sub>2</sub>, in 10-cm plates in DME medium supplemented with 5% FCS, 10% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

### Stable transfection of rat pheochromocytoma PC-12 cells

Using the calcium phosphate method (20), PC-12 cells (at 20–30% confluence) were cotransfected with 10 μg pRSV-hCgA plus 2 μg of the plasmid pSV2-neo (21), which encodes bacterial phosphotransferase and confers neomycin resistance. Control cells were transfected with pSV2-neo alone. Transfected cells were selected with the antibiotic Geneticin (G418; Gibco BRL Life Technologies, Inc., Gaithersburg, MD) at 0.5 mg/ml (active). Individual stable clones were recovered, transferred to separate wells of 24-well plates for subculture, and grown to mass culture in 10-cm plates. Homogenates of stably transfected cells were obtained by resuspension of harvested cell pellets in 50 mM ammonium acetate, pH 6.5, 0.1% Triton X-100, followed by two freeze-thaw cycles to promote cell disruption. Homogenates were screened for the production of human CgA by RIA specific for human CgA (22).

### SDS-PAGE and immunoblotting

Prestained molecular weight size standards, 15 μg of protein from human pheochromocytoma chromaffin vesicle lysate, and 50 μg of protein from cell lysates of PC-12 cells transfected with either pSV2-neo alone or pSV2-neo and pRSV-hCgA, were electrophoresed through 10% SDS-PAGE slab gels, transferred to nitrocellulose, and stained for immunoreactive human CgA with rabbit anti-human CgA antisera (22) followed by visualization with peroxidase coupled goat anti-rabbit IgG plus the chromogenic substrate 4-chloro-1-naphthol.

### Electron microscopy of PC-12 cells

Stably transfected or wild-type PC-12 cells were grown in Permanox (Lux, Tucson, AZ) culture dishes, washed three times in PBS (pH 7.4)
Figure 2. (A) Construction of the expression vectors pRSV-hCgA457-CAT and pRSV-hCgA226-CAT. The expression vector pRSV-hCgA457-CAT encoding the full-length human CgA fused in-frame to CAT was constructed as follows. Beginning with the full-length 1.8 kb human CgA cDNA as template (upper box), a 1.4-kb insert was synthesized with PCR methods (28), using synthesized primers which engineered unique Hind III sites (5'-AAATAAGCTTCCGCCACACCGCCA-3' as 5' primer and 5'-AAATAAGGCTTCCCCCGCGTAGTGGCTG-3' as 3' primer) into the 5' and 3' termini of the final 1.4-kb product. The PCR product contained the full open reading frame of human CgA but was altered by the 3' primer such that the stop codon of the initial human CgA cDNA was substituted with the codon AGC, encoding a serine residue and allowing translation to continue through this point; after Hind III digestion and purification, the PCR product was ligated into the unique Hind III site.
to remove growth media, and fixed at room temperature in modified Karnovsky’s solution for 30 min. After three buffer washes with 0.2 M sodium cacodylate (pH 7.4), the cells were postfixed in buffered 1% osmium tetroxide for 30 min. After three distilled water rinses, the cells were dehydrated in a graded ethanol series (3 min each) followed by a 10-min wash series in (90%, 95%, 97%) hydroxypropyl methacrylate. After dehydration, the cells were embedded in a thin layer (approximately one-fourth the depth of the dish) of Epon 812. After polymerization at 65°C oven, the hardened plastic containing the cells was removed from the dish and floated for 15 s in a near boiling solution of 1% toluidine blue in 1% sodium borate. Cells were cut out from the embedded plate of plastic and glued to blank blocks of Epon 812. Thin sections were cut and mounted on unsupported 200-mesh grids. Sections were stained in saturated ethanol-uranyl acetate and bismuth subnitrate before viewing in a model EM-10B electron microscope (Carl Zeiss, Inc., Thornwood, NY).

Sucrose density gradient studies
After G-418 selection, PC-12 cells stably transfected with pRSV-hCgA plus pSV2-neo or with pSV2-neo alone were labeled for 2 h with [3H]norepinephrine (Amerham Corp., Arlington Heights, IL) at 1 μCi/ml in PC-12 medium, washed twice with buffer (150 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM ascorbic acid, 10 mM Hepes, pH 7), homogenized in 0.3 M sucrose, 10 mM Hepes, pH 7, layered over a continuous sucrose density gradient (0.3–2.5 M), and centrifuged at 100,000 g for 60 min at 2°C as described (23–26). Fractions were collected and assayed for [3H]norepinephrine by liquid scintillation counting, human CgA by RIA, and sucrose concentration by refractometry.

Secretagogue-mediated release of human CgA and norepinephrine
PC-12 cells stably transfected with pRSV-hCgA plus pSV2-neo or with pSV2-neo alone were plated on poly-d-lysine–coated polystyrene dishes (Falcon, Labware, Oxnard, CA), labeled for 2 h with [3H]norepinephrine at 1 μCi/ml in PC-12 medium, washed twice with release buffer (150 mM NaCl, 5 mM KCl, 2 mM CaCl2, 10 mM Hepes, pH 7), and incubated at room temperature (air-conditioned lab with near constant temperature readings [22.5±0.1°C, n=7]) for 30 min in release buffer with or without the following secretagogues: the nicotinic agonist carbacholam chloride (carbachol; Sigma Chemical Co., St. Louis, MO) at 0.1 mM; potassium chloride at 55 mM; or barium chloride at 2 mM. (Release buffer for experiments involving KCl as secretagogue included NaCl at 100 mM and release buffer was devoid of CaCl2 when BaCl2 was used as secretagogue.) After aspirating the release buffer, cells were harvested and lysed in 150 mM NaCl, 5 mM KCl, 10 mM Hepes, pH 7, and 0.1% Triton X-100. Release buffer and cell lysates were assayed for human CgA by RIA and [3H]norepinephrine by liquid scintillation counting, and results were expressed as percent secreted: amount in release buffer/total (amount in release buffer + amount in cell lysate). In addition, release of norepinephrine and human CgA from transfected PC-12 cells was determined after exposure for 30 min to the weak bases chloroquine at 1 mM or ammonium chloride at 10 mM.

Human CgA radioimmunoassay
Human CgA was assayed as previously described by species specific RIA, which recognizes human but not rat (including endogenous PC-12) epitopes (22).

Protein concentrations on lysates of transfected cells and on lysates of chromaffin vesicles prepared from human pheochromocytoma tumors (5) were determined by the Coomassie Blue dye binding technique (27).

Human CgA/CAT fusion studies
Construction of the expression vectors pRSV-hCgA4-CAT and pRSV-hCgA5-CAT.

An expression vector (pRSV-hCgA4-CAT) encoding a chimeric protein consisting of full-length human CgA fused in-frame to the bacterial enzyme CAT was constructed as diagrammed in Fig. 2. Beginning with the full-length human CgA cDNA as template, a 1.4-kb insert was synthesized with standard PCR methods (28), using Vent DNA polymerase (New England Biolabs, Beverly, MA), a thermostable high-fidelity DNA polymerase with 3′-5′ proofreading exonuclease activity (29), 50 mM dNTPs in PCR buffer (10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl, pH 8.0, 5 mM MgSO4, 0.1% Triton X-100) and synthetic primers (at 2 pmol each) which engineered unique Hind III sites (5′-AAATAAGCTTTGCGCCCCACCGCGCA-3′ as 5′ primer and 5′-AAATAGCTTCCCGCGCATGTCCTG3′ as 3′ primer) into the 5′ and 3′ termini of the final 1.4-kb product, using 35 cycles of 94°C for 1 min, 42°C for 1 min, and 65°C for 1.5 min, in an EZ Cycler thermostorer (Ercocomp., Inc., San Diego, CA). The product contained the full open reading frame of human CgA but was altered by the 3′ primer such that the stop codon of the initial human CgA cDNA was substituted with the codon AGC, encoding a serine residue and allowing translation to continue through this point; the PCR product was digested at 37°C for 18 h with Hind III, purified on a 1% agarose gel, and ligated into the unique Hind III site of the expression vector pRSV-CAT (30). The resultant construct contains the RSV-LTR promoter driving full-length human CgA cDNA (encoding 457 amino acid residues) fused in-frame to the full-length CAT-encoding region. Also, since the Hind III cloning site in pRSV-CAT is 36 bp upstream of the ATG initiation codon for CAT, expression of the final fusion product results in the insertion of an additional 12 amino acid residues (SLARFSGAKEAKM) between full-length human CgA and CAT.

An additional expression vector (pRSV-hCgA5-CAT) containing a mutated human CgA cDNA encoding the amino-terminal 226 amino acid residues of human CgA (including the 18-residue signal peptide plus the amino-terminal 208 residues of mature CgA after signal peptide cleavage) fused in-frame to CAT was also constructed using the above PCR methods with 5′-AAATAAGCTTGGCGCCACACC-GCCA-3′ as 5′ primer and 5′-AAATAGCTTGCGCCACCTGGG3′ as the 3′ primer and full-length human CgA cDNA as template. The resultant 0.7-kb product was digested with Hind III and inserted into the Hind III cloning site of pRSV-CAT as above. The final construct contains the coding region for the amino-terminal 226 amino acid residues of human CgA fused in-frame to the coding region for of the expression vector pRSV-CAT (30). The vector pRSV-hCgA5-CAT containing a mutated human CgA cDNA encoding the amino-terminal 226 amino acid residues of human CgA (including the 18-residue signal peptide plus the amino-terminal 208 residues of mature CgA after signal peptide cleavage) fused in frame to CAT was constructed using PCR methods with the primers, 5′-AAATAAGCTTGGCGCCACACC-GCCA-3′ as 5′ primer and 5′-AAATAGCTTGCGCCACCTGGG3′ as the 3′ primer and full-length human CgA cDNA as template (lower box). The resultant 0.7-kb product was digested with Hind III and inserted into the Hind III cloning site of pRSV-CAT as above. (Hinged arrows represent synthesized primers for PCR; H3 indicates Hind III sites.) (B) Structure of CgA/CAT fusion proteins. Predicted expression products of pRSV-hCgA4-CAT (hCgA4-CAT) and pRSV-hCgA5-CAT (hCgA5-CAT). The human CgA and CAT sequences are designated by the open and hatched bars, respectively. Numerals above each bar refer to the amino acid positions of the human CgA sequence (before signal peptide cleavage). Numerals below each bar refer to the amino acid positions of CAT. The stippled area represents a 12-amino acid insertion (SLARFSGAKEAKM) caused by expression of the 36-bp DNA sequence between the HindIII cloning site and the ATG initiation codon for CAT in the parental pRSV-CAT vector. The first 18 amino acid residues of hCgA constitute the hydrophilic signal peptide sequence responsible for targeting the nascent polypeptide to the membrane of the rough endoplasmic reticulum and thus allowing for entry into the secretory pathway.
CAT, under the control of the RSV-LTR. Expression of the final fusion product of this vector results in the insertion (between the 226 amino-terminal amino acid residues of human CgA and CAT) of the same additional 12 amino acid residues (SLARFSGAEEKM) described above for pRSV-hCgA457-CAT. Both of these expression vectors (pRSV-hCgA457-CAT and pRSV-hCgA226-CAT) were analyzed by restriction endonuclease mapping to confirm orientations, and fragment junctions were confirmed by DNA sequencing in both cases.

Transient transfection of PC-12 cells. PC-12 cells grown to 50% confluence in 6-cm plates were transfected using the lipofection procedure (31, 32) with 20 µg of plasmid DNA (either pRSV-hCgA457-CAT or pRSV-hCgA226-CAT, pRSV-CAT, or pBluescript [Stratagene, Inc., La Jolla, CA] as a mock transfection control) and 20 µg of lipofectant (Gibco BRL Life Technologies, Inc.). After 48–60 h, transfected cells were analyzed by metabolic labeling and immunoprecipitation to confirm translation products, or by secretagogue-stimulation to investigate corelease of [3H]norepinephrine and CAT activity as described below.

Metabolic labeling and immunoprecipitation. Transiently transfected PC-12 cells were starved for 1 h in methionine-free medium, then labeled with 50 µCi/plate [35S]methionine (ICN Biomedicals, Inc., Costa Mesa, CA) for 4 h at 37°C. Cells were washed with PBS and lysed in 0.1 M NaCl, 0.1 M Tris, pH 8.0, 10 mM EDTA, 1% Triton X-100, 1 mM PMSF (Sigma Chemical Co.), and 50 KIU/ml aprotinin (Calbiochem Corp., La Jolla, CA). Cell debris was removed by centrifugation and 75 µl of each of the cell supernatants were incubated with 2 µl of preimmune serum for 1 h at 4°C, followed by 10 µl 50% protein A Sepharose (Pharmacia-LKB Biotechnology, Inc., Piscataway, NJ) for 30 min at 4°C on a shaker platform, to reduce nonspecific binding. After centrifugation, the supernatants were incubated for 18 h at 4°C with either 2 µl of polyclonal anti-CAT antibody (Five Prime to Three Prime, Inc., Boulder, CO) or 10 µl polyclonal rabbit anti-human CgA antiserum (22). After incubation with 20 µl of 50% protein A Sepharose for 2 h at 4°C and centrifugation, the pellets were washed, boiled for 5 min, and electrophoresed through 8% SDS-polyacrylamide gels. The gels were placed in the enhancing solution (EM Corp., Chestnut Hill, MA) for 45 min, then placed in 5% cold glycerol for 45 minutes, dried, and exposed at −80°C to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY).

Secretagogue stimulation of transiently transfected cells. 48–60 h after transfection with the above constructs, PC-12 cells, grown in poly-d-lysine-coated polystyrene dishes (Falcon Labware), were labeled for 2 h with [3H]norepinephrine at 1 µCi/ml in PC-12 medium, washed twice with release buffer (150 mM NaCl, 5 mM KCl, 10 mM Heps, pH 7), and incubated at room temperature for 30 min in 1 ml of release buffer with or without the secretagogue barium chloride at 2 mM. After aspirating the release buffer, cells were harvested in 1 ml of 150 mM NaCl, 5 mM KCl, 10 mM Heps, pH 7, and lysed with three freeze-thaw cycles. Release buffer and cell lysates were analyzed for [3H]-norepinephrine by liquid scintillation counting and for CAT activity by the method of organic extraction with ethyl acetate (33). Briefly, the CAT activity assay mixture contained (in a final volume of 250 µl) 177 µl of 50 mM Tris-HCl, pH 7.5, 70 µl of release buffer or cell lysate (heat inactivated at 65°C for 10 min), 1 µl (0.05 µCi) of [4C]acetyl coenzyme A (55 mCi/mmol; Amersharm Corp.), 1 µl of 37 mM/mL chloramphenicol (United States Biochemical Corp., Cleveland, OH), and 1 µl of 25 mM acetyl coenzyme A (Sigma Chemical Co.). After incubation for 120 min at 37°C, the assay mixture was extracted twice with 250 µl of ethyl acetate and the organic phase was assayed for [4C]-chloramphenicol by liquid scintillation counting. Results are expressed as percent secreted (of either [3H]norepinephrine or CAT activity): amount in release buffer/total (amount in release buffer + amount in cell lysate).

In additional control experiments, release of [3H]norepinephrine and CAT activity was assessed in cells transiently transfected with an expression vector, pRSV-v-sisCAT, in which the signal peptide from the secretory protein, v-sis (a signal sequence which has been extensively characterized in transfected mammalian cells (34–36)), was fused in-frame to CAT. pRSV-v-sisCAT was constructed as follows:

PC-12 cells transfected with pSV2-neo and pRSV-hCgA (lane 4), but not in PC-12 cells transfected with pSV2-neo alone (lane 3).

Statistics
Results of secretion experiments are expressed as mean±SEM. Results were analyzed by ANOVA followed by Student-Newman-Keuls post-hoc tests for multiple comparisons (37).

Results
Expression of human CgA in PC-12 cells
Human CgA immunoreactivity was detected in cell homogenates from five of six recovered clones of PC-12 cells co-transfected with pRSV-hCgA and pSV2-neo and in none of six recovered clones of PC-12 cells transfected with pSV2-neo alone as a control.

Fig. 3 shows Western blot analysis demonstrating expression of human CgA in stably transfected rat pheochromocytoma PC-12 cells (clone 2C). Human CgA is observed as an M, 75-kD band crossreacting with a species-specific anti-human CgA antibody in cells cotransfected with pRSV-hCgA and pSV2-neo, but not in control cells transfected with pSV2-neo alone (clone 4A).

Electron microscopy
Transmission electron micrographs of PC-12 cells cotransfected with pRSV-hCgA and pSV2-neo are shown in Fig. 4, demonstrating normal-appearing dense core secretory vesicles in a typical distribution at the cell periphery. Cell appearance,
number, and distribution of dense core secretory vesicles did not differ from cells transfected with pSV2-neo alone or from wild-type PC-12 cells (data not shown).

Subcellular localization studies

Sucrose gradient studies. To investigate the subcellular localization of the expressed human CgA in PC-12 cells transfected with pRSV-hCgA and pSV2-neo, cells were subjected to sucrose density gradient fractionation, the results of which are shown in Fig. 5. In cells transfected with pRSV-hCgA and

Figure 4. Transmission electron micrographs of PC-12 cells cotransfected with pRSV-hCgA and pSV2-neo, demonstrating normally appearing dense core secretory vesicles (catecholamine storage vesicles) in a typical distribution at the cell periphery. Cell appearance, number, and distribution of dense core secretory vesicles did not differ from cells transfected with pSV2-neo alone or from wild-type PC-12 cells. Magnification is 15,840 diameters for upper panel and 79,200 diameters for lower panel.

Figure 5. Sucrose gradient studies. PC-12 cells transfected with pRSV-hCgA and pSV2-neo (Clone 2C, upper panel) were labeled with [3H]norepinephrine, homogenized in 0.3 M sucrose, 10 mM Hepes, pH 7, layered over a continuous sucrose gradient (0.3–2.5 M), and centrifuged at 100,000 g for 60 min at 2°C. Fractions were collected, lysed by addition of Triton X-100 to a final concentration of 0.1%, and assayed for [3H]norepinephrine by liquid scintillation counting, human CgA by RIA, and sucrose concentration by refractometry. Results showed colocalization of transfected CgA and norepinephrine to the same subcellular fraction at a buoyant density of 1.3–1.4 M sucrose. Control PC-12 cells transfected with pSV2-neo alone (Clone 4A, lower panel) also showed a norepinephrine peak at 1.3–1.4 M sucrose but no detectable CgA.
pSV2-neo, there was colocalization of transfected human CgA and norepinephrine to the same subcellular fraction at a buoyant density of 1.3–1.4 M sucrose. Control PC-12 cells transfected with pSV2-neo alone also showed a norepinephrine peak at 1.3–1.4 M sucrose but no detectable human CgA.

Secretagogue-mediated corelease of human CgA and catecholamine. Subcellular localization of the expressed human CgA was also investigated by experiments in which release of both human CgA and [3H]norepinephrine was determined after transfected cells were exposed to a variety of secretagogues (Fig. 6). Exposure of PC-12 cells stably transfected with pRSV-hCgA plus pSV2-neo to the nicotinic agonist carbamylcholine at 0.1 mM, to 55 mM KCl, or to 2 mM BaCl2, resulted in significant release over baseline (no secretagogue) of both [3H]norepinephrine (P < 0.001 for all three agents) and human CgA (P < 0.001 for all three agents). Secretion of human CgA paralleled norepinephrine secretion. Control experiments on PC-12 cells transfected with pSV2-neo alone showed secretion of [3H]norepinephrine in response to secretagogue but no detectable human CgA in either basal or stimulated states.

Effect of alkalinizing agents. PC-12 cells stably expressing human CgA were also exposed to the weak bases (lysosomotropic amines) chloroquine (1 mM) or ammonium chloride (10 mM), which act as alkalinizing agents to disrupt intravesicular acidification (38). Fig. 7 demonstrates that exposure of the transfected cells to either of these agents was associated with significant (P < 0.001) release of [3H]norepinephrine compared with basal values, but no change in release of human CgA.

Human CgA/CAT fusion studies
To determine if human CgA was capable of directing a heterologous, ordinarily nonsecreted protein into the catecholamine storage vesicle, we made plasmid constructs in which human CgA was fused in-frame to the bacterial enzyme chloramphenicol acetyltransferase, and expressed the chimeric proteins in PC-12 cells.

Fig. 8 shows SDS-PAGE analysis of the expression products of the plasmid vectors pRSV-hCgA447-CAT and pRSV-hCgA226-CAT after transient transfection and metabolic labeling in PC-12 cells. Major protein bands were immunoprecipitated in a nearly identical pattern with both anti-human CgA (Fig. 8, left panel) and anti-CAT antibodies (Fig. 8, right panel). Expression of pRSV-hCgA447-CAT resulted in a major protein band with a mobility on SDS-PAGE of ~100–110 kD, consistent with that predicted for the full-length human CgA/CAT chimera (~75 kD for human CgA [see Fig. 3] plus 26.6 kD for CAT [39]). Human CgA has a highly anomalous electrophoretic mobility in SDS polyacrylamide gels. Whereas the predicted molecular mass of CgA is ~50 kD, the apparent molecular mass by SDS-PAGE is 70–75 kD [18, 40–44; and Fig. 3]. Expression of pRSV-hCgA226-CAT resulted in a major band of ~60–65 kD, consistent with the approximate mobility predicted for fusion of 226 amino acid residues of human CgA to CAT (226/457 or 49% of 75 kD = 37 kD for the CgA fragment; 37 kD for this CgA fragment plus 26.6 kD for CAT = 64 kD for the hCgA226-CAT fusion product).

Cells transfected with the CgA/CAT fusion constructs displayed CAT enzyme activity. We investigated the subcellular distribution of the transient expression products by evaluating
in 0.1 M NaCl, 0.1 M Tris, pH 8.0, 10 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 50 KIU/ml aprotinin. Cell lysates were immunoprecipitated with either polyclonal rabbit anti-human CgA antiserum (left panel) or polyclonal anti-CAT antibody (right panel), and electrophoresed through 8% SDS-polyacrylamide gels. After fluorographic enhancement, the gels were dried and exposed at −80°C to Kodak X-OMAT AR film for 5 days. Molecular mass standards are indicated. Duplicate lanes represent cell lysates from PC-12 cell plates transfected in duplicate. Expression product of pRSV-hCgA457-CAT migrates at ≈ 100−110 kDa; expression product of pRSV-hCgA226-CAT migrates at ≈ 60−65 kDa.

secretagogue-mediated corelease of CAT activity and norepinephrine as an index of targeting into the regulated secretory pathway. Fig. 9 shows results of secretion studies performed in PC-12 cells 48−60 h after transient transfection with pRSV-CAT, pRSV-hCgA457-CAT, and pRSV-hCgA226-CAT. Release of [3H]norepinephrine (Fig. 9, lower panel) upon exposure to barium was significant (P < 0.001, compared with basal values) from cells transfected with all three plasmids and the values did not differ among the three groups. pBluescript-[mock]-transfected cells also released [3H]norepinephrine upon exposure to barium to a similar extent [5.3±1.0% to 60.0±2.0%, P < 0.001]. Barium released CAT activity (Fig. 9, upper panel) from cells transfected with pRSV-hCgA457-CAT and pRSV-hCgA226-CAT (P < 0.001, compared with the corresponding basal value), but not from cells transfected with pRSV-CAT.

In additional control experiments, we assessed the secretion characteristics of PC-12 cells transfected with pRSV-v-sis65-CAT, encoding a chimeric protein consisting of the v-sis signal peptide fused in-frame to CAT. Release of [3H]norepinephrine in response to 2 mM barium (from 4.7±0.12% to 67.6±0.6%, n = 8, P < 0.001) was comparable to cells transfected with the above CgA/CAT fusion constructs. Release of CAT activity from these cells did not change in response to barium (from 8.9±0.20% to 7.4±0.5%, n = 8, P not significant).

Discussion

In studying protein targeting into catecholamine storage vesicles, we focused on CgA for several reasons: (a) CgA is the major soluble protein in the core of the catecholamine storage vesicles (7). (b) CgA itself may be important in the targeting of other secretory proteins to the regulated pathway. Recent evidence suggests that proteins of the chromogranin/secretogranin (“granin”) family may act as linker or adapter molecules involved in the aggregation and packaging of peptide hormones and neuropeptides, facilitating their entry into the regulated secretory pathway (12). (c) CgA may act within the chromaffin granule by binding other nonprotein vesicular constituents including Ca**+ (9, 45−47), ATP (48), and the catecholamines themselves (47), thereby stabilizing the intragranular complex to allow high concentrations of these compounds. (d) CgA may play a role in regulation of exocytotic release from the catecholamine storage vesicle. Recent studies have demonstrated that CgA is the parent molecule for the peptide “chromostatin,” a potent inhibitor of catecholamine release from chromaffin cells (14), thereby providing an autocrine/paracrine neuromodulatory role. (e) As suggested by nuclear magnetic resonance (49) and circular dichroism (50) studies, CgA exists in solution predominantly in a random coil, extended chain conformation. Thus, one might predict that the virtually structureless nature of CgA would render this protein less susceptible to tertiary structure disruptions after mutation and gene fusions designed to identify specific targeting domains. This may be an important consideration since some proteins’ secretory transport beyond the endoplasmic reticulum can be severely compromised by tertiary structure disruption (51−53). (f) Finally, CgA is widely distributed in dense core secretory vesicles throughout the neuroendocrine system (8−11). Thus, identifying mechanisms of targeting CgA to catecholamine storage vesicles might have broad implications for understanding secretory protein trafficking in general.

We transfected human CgA into rat chromaffin cells, a strategy that takes advantage of the interspecies divergence in amino acid sequence and epitopes of CgA (44) but still allows investigation of a resident chromaffin cell secretory protein in chromaffin cells. PC-12 cells transfected with the vector pRSV-hCgA, but not mock-transfected PC-12 cells, stably expressed chromogranin A targeting
Subcellular localization of the expressed human CgA was evaluated by both sucrose density gradient studies and secretagogue-mediated release studies.

Sucrose gradient studies colocalized expressed human CgA and catecholamine to the same subcellular fraction at 1.3–1.4 M sucrose. Interpretation of sucrose gradient studies may be limited by copurification of heterogeneous organelles to the same fraction (24–26). Nonetheless, the peak at 1.3–1.4 M sucrose is consistent with the buoyant density previously demonstrated for chromaffin granules isolated from PC-12 cells (1.2–1.4 M sucrose) (24, 25).

We used several well-established chromaffin-cell exocytotic secretagogues to assess the subcellular location of transfected human CgA. Since only proteins correctly targeted to granules are responsive to secretagogue, the extent of secretagogue-induced release relative to basal secretion is a measure of the efficiency at which the protein is targeted to the regulated pathway (54). Stimulation of human CgA-expressing PC-12 cells with the nicotinic agonist carbachol, potassium chloride, or barium chloride all resulted in cosecretion of human CgA and catecholamines (Figure 6).

In these secretion studies (Figure 6), we found a consistent disparity between the percentage of norepinephrine and the percentage of CgA released in response to secretagogue, with greater values for norepinephrine release in all cases. We previously demonstrated time-dependent differences between catecholamine release and CgA release from chromaffin cells (55). At first glance, this may suggest that CgA and norepinephrine are segregated into separate releasable pools. However, Pimplikar and Huttner (56) recently showed that the chromogranins bind to chromaffin granule membranes in a saturable and specific manner, so that some of the CgA initially secreted upon stimulation into the release medium may be rapidly bound to the chromaffin cell membrane and therefore not measured in the secreted fraction. Previous studies by Settlement et al. (57) also demonstrated that a fraction of CgA exists in a membrane-associated form. In addition, release of CgA after synthesis requires trafficking through the endoplasmic reticulum and Golgi stacks before final localization in the dense core secretory vesicles. Thus, a percentage of the measured total cellular CgA may still be in route to the secretory vesicle and, therefore, unavailable for exocytotic release. In contrast, since the norepinephrine is supplied to the cells as an exogenous label and does not require synthesis or routing through the endoplasmic reticulum or Golgi, a greater percentage of cell norepinephrine may be immediately available for release in response to secretagogue stimulation. Still another explanation is the existence (in addition to the regulated vesicles containing both norepinephrine and CgA) of a population of recycled vesicles (58) which, having already released their CgA, contain only (reloaded) norepinephrine. Nonetheless, secretion studies with a variety of secretagogues (Fig. 6) showed parallel secretion of norepinephrine and CgA such that each increment in norepinephrine release was associated with a corresponding increase in CgA release, consistent with colocalization of norepinephrine and CgA to the same subcellular pool. Colocalization of norepinephrine and transfected CgA to the same subcellular fraction was also supported by sucrose density gradients (Fig. 5).

Additional characterization of catecholamine and human CgA targeting to the regulated secretory vesicles was provided by experiments in which transfected PC-12 cells were treated with the weak bases chloroquine and ammonium chloride.
(Fig. 7). Catecholamine storage vesicles and other neurosecretory granules are characterized by the presence of a proton pumping ATPase which maintains an acidic intravesicular pH (pH = 5) essential for concentration of catecholamines (59). Disruption of the proton gradient by weak bases such as chloroquine and ammonium chloride depletes cells of catecholamines by nonexocytotic catecholamine release, without affecting the intravesicular composition of proteins synthesized and sorted before addition of alkalizing agent (38). If expressed human CgA is correctly targeted to the regulated secretory vesicle, then subsequent short-term exposure of PC-12 cells to weak bases would be expected to result in dissociation of release of catecholamines from that of CgA. Both chloroquine and ammonium chloride acutely released norepinephrine but not human CgA from transfected PC-12 cells (Fig. 7), consistent with such a dissociation, and hence retention of previously sorted CgA in catecholamine storage vesicles in the face of this nonexocytotic stimulus. Longer exposure to these compounds may divert newly synthesized regulated proteins away from dense core secretory vesicles (60).

We also tested whether human CgA could divert an ordinarily nonsecreted heterologous protein to the catecholamine storage vesicle. To accomplish this aim, we fused human CgA in-frame to the bacterial CAT gene. Under normal circumstances, the CAT enzyme is not found in eukaryotic cells. When expression vectors encoding CAT are transfected into mammalian cells, the expressed protein is cytoplasmic, distributed diffusely throughout the cytosol (30, 61–63). In our experiments, expressed CgA/CAT fusion proteins were of the appropriate predicted mobility on SDS-PAGE and were immunoprecipitated with both anti-human CgA and anti-CAT antibodies (Fig. 8), confirming their chimeric structure. We then evaluated the subcellular distribution of these products by assessing corelease of norepinephrine and CAT activity as an index of the secretagogue-dependent releasable pool, i.e., the regulated secretory pathway. In cells transfected with pRSV-CAT, exposure to barium did not release CAT activity (Fig. 9), consistent with a cytosolic distribution of expressed CAT (30, 61–63). On the other hand, when CgA was fused to CAT, a four- to fivefold increment in CAT activity was detected in the release medium after barium (Fig. 9), consistent with targeting of the fusion protein into the regulated secretory pathway. Moreover, fusion of the amino-terminal 226 amino acid residues of human CgA to CAT also caused a four- to fivefold increment in CAT release after barium. Thus, CgA was capable of diverting CAT into the regulated secretory pathway, and sufficient information for this event is located in CgA’s amino-terminal 226 amino acid residues. Of note, the ratio of barium-stimulated to basal release percentages for the chimeric proteins was 4.6 for hCgA457-CAT and 4.9 for hCgA226-CAT (Fig. 9, upper panel), compared with a ratio of 16.9 for human CgA alone (Fig. 6). This suggests that the overall sorting efficiency of the chimeric proteins may have been less than that of human CgA alone.

Although not statistically significant by ANOVA, values for 30-min basal (unstimulated) secretion of CAT activity were slightly greater for cells transiently transfected with pRSV-hCgA457-CAT (7.3±1.1%) or pRSV-hCgA226-CAT (5.9±0.5%) compared to cells transfected with pRSV-CAT (2.2±0.2%). Previous studies have suggested that a small percentage of newly synthesized CgA (up to 10% over a 4-h period) is secreted by the constitutive pathway (64). Since the CgA/CAT fusion proteins had slightly higher basal rates of secretion than the cytoplasmically localized wild-type CAT, a portion of the fusion proteins may have entered the constitutive pathway, or had an increased spontaneous release rate from the regulated pathway (65). Fusion of the v-sis signal peptide sequence to CAT also resulted in slightly greater basal release rates of CAT activity (8.9±0.2%), consistent with entry of the v-sis/CAT chimera into the constitutive pathway, but not into the regu-

**Figure 10.** Putative structural domains in human CgA. The amino acid residue number indicated represents the position in the mature protein after signal peptide cleavage. The chromostatin region represents a 20-amino acid peptide sequence homologous to bovine chromostatin (14). Peptide sequence information is from Konecki et al. (40) and Helman et al. (18) as analyzed previously by Farmer et al. (44).
lated pathway, since exposure to barium did not result in further increment of CAT activity in release medium from prSV-v-sisCAT-transfected cells.

Results of our CgA/CAT chimera studies are consistent with previous findings on chimeric polypeptides in which fusion of a regulated secretory protein to a heterologous protein resulted in diversion of the latter into the regulated pathway in other secretory cell types (66-69). Using another approach, Rosa et al. (70) introduced (by mRNA injection) antibodies to chromogranin B (secretogranin I) into PC-12 cells and demonstrated that these antibodies were bound to endogenous chromogranin B and diverted into the regulated pathway. Taken together, these results are consistent with the hypothesis that the targeting of proteins into secretory granules is an active, dominant, signal-mediated process. The trans-Golgi network plays an important role in the process of sorting proteins into the regulated secretory pathway (71-74). Two possible mechanisms have been proposed for targeting into the regulated pathway and for sorting regulated secretory proteins away from constitutive proteins at the trans-Golgi cisternae: (a) receptor-mediated protein targeting and (b) selective protein aggregation and condensation. In the first mechanism, correct targeting to the regulated pathway results from specific binding to carrier proteins or receptors in the Golgi membrane (75) which recognize sorting signals on targeted proteins. Alternatively, in the second mechanism, sorting of regulated secretory products from other soluble (constitutive) proteins in the pathway results from formation of membrane aggregates triggered by conditions in the trans-Golgi region (acidic pH and millimolar concentrations of calcium ions) (12, 76). Chanat and Huttner (76) demonstrated that selective aggregation of proteins of the chromogranin/secretogranin (“granin”) family could be induced by a low pH and high calcium milieu corresponding to that in the trans-Golgi network lumen. Indeed, this propensity for aggregation was remarkably selective for the granins, suggesting that the granin family may play a crucial role in packaging other polypeptides into the regulated pathway by molecular “hitchhiking” (77) or binding to the aggregated granin complex (12). Since posttranslational modification of the chromogranins was not necessary to promote their aggregative properties, the structural features important for aggregation seem to reside in the polypeptide backbone of these proteins (76). Thus, either mechanism (receptor-dependent targeting or selective aggregation), could be mediated or facilitated by a specific region or regions within the primary structure of CgA. Moreover, our fusion studies suggest that sufficient information for targeting is provided by the amino-terminal portion of CgA.

Previous studies have revealed specific targeting domains for proteins destined for a variety of subcellular locations, including the endoplasmic reticulum (78), Golgi complex (79), lysosomes (80), mitochondria (81), peroxisomes (61, 62), and nucleus (82). However, studies to identify targeting signals for sorting to the regulated pathway of secretion have been inconsistent. For example, whereas the pro segment of proinsulin may contain important information for targeting to the regulated pathway (68, 69, 83), mutagenesis studies have thus far failed to reveal discrete, regulated vesicular targeting sequences for a variety of regulated secretory proteins including proinsulin (54), prorenin (84, 85), and parathyroid hormone (86).

What specific domains, particularly within the amino-terminus of CgA, might function in targeting CgA into dense core secretory granules? Structural features contained in the amino-terminal 226 amino acid residues of CgA (that portion of CgA encoded by prSV-hCgA172-CAT) include the 18-residue amino-terminal signal peptide (residues -18 to -1), two cysteine residues (residues +17 and +38), a consensus sequence for N-linked glycosylation (NQS, residues +92 to +94), a 20 amino acid chromostatine sequence (residues +124 to +143) (14), and an oligoglutamic acid cluster (residues +154 to +161) (Fig. 10). Of note, CgA is an extremely hydrophilic protein with the exception of two hydrophobic regions that are well conserved across species. In addition to the 18-residue signal peptide (which is cleaved during cotranslational movement of the protein across the endoplasmic reticulum), a short hydrophobic area occurs near the amino terminus in the region bounded by the two cysteine residues at positions +17 and +38 in the mature protein after signal peptide cleavage (44). These cysteine residues form an intramolecular disulfide loop (87), and the conserved hydrophobic character of this region, given the otherwise quite hydrophilic quality of CgA, has suggested that this domain may interact with Golgi membranes during targeting of CgA to secretory vesicles (44). Recent analysis of the CgA gene by our group showed that CgA’s eight exons correspond in part to putative functional domains suspected within the cDNA-deduced primary structure of the protein (88). It is intriguing that the hydrophobic disulfide loop region is contained within exon 3 (residues +14 to +45) (88), which shares length and partial sequence homology with the disulfide loop-encoding (87) exon 3 of chromogranin B (88), suggesting a conserved structure-function relationship for this region. (Chromogranin B is also efficiently targeted to the catecholamine storage vesicles in chromaffin cells (12, 56, 70, 76).) Alternatively, several oligoglutamic acid regions are present within the primary structure of CgA. These areas contribute to the acidic nature of the protein and may interact with calcium ions and other cations (9, 45-47, 50, 76) in a process which could selectively aggregate CgA under conditions provided by the trans-Golgi milieu (76). Interestingly, one of the longest of these oligoglutamic acid regions (eight consecutive glutamic acid residues) is present in human CgA at residues +154 to +161 and is thus included in the protein chimera expressed by transfection with the plasmid prSV-hCgA122-CAT.

Further mutagenesis studies are in progress to determine whether these hydrophobic or acidic regions or other specific regions within CgA provide important targeting information, or whether some other, perhaps overall property of CgA (e.g., its acidic pI) is important for efficient targeting to the regulated pathway of secretion.

In summary, these studies demonstrate that human CgA expressed in PC-12 cells is targeted to the catecholamine storage vesicles. In addition, human CgA is capable of diverting a heterologous, ordinarily nonsecreted cytoplasmic protein into the regulated pathway of secretion. The amino-terminal half of CgA contains information sufficient for this event.

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