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The *trans*-Golgi Proteins SCLIP and SCG10 Interact with Chromogranin A To Regulate Neuroendocrine Secretion†

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Abstract

Secretion of proteins and peptides from eukaryotic cells takes place by both constitutive and regulated pathways. Regulated secretion may involve interplay of proteins that are currently unknown. Recent studies suggest an important role of chromogranin A (CHGA) in the regulated secretory pathway in neuroendocrine cells, but the mechanism by which CHGA enters the regulated pathway, or even triggers the formation of the pathway, remains unclear. In this study, we used a transcriptome/proteome-wide approach, to discover binding partners for CHGA, by employing a phage display cDNA library method. Several proteins within or adjacent to the secretory pathway were initially detected as binding partners of recombinant human CHGA. We then focused on the *trans*-Golgi protein SCLIP (STMN3) and its stathmin paralog SCG10 (STMN2) for functional study. Co-immunoprecipitation experiments confirmed the interaction of each of these two proteins with CHGA in vitro. SCLIP and SCG10 were colocalized to the Golgi apparatus of chromaffin cells in vivo and shared localization with CHGA as it transited the Golgi. Downregulation of either SCLIP or SCG10 by synthetic siRNAs virtually abolished chromaffin cell secretion of a transfected CHGA–EAP chimera (expressing CHGA fused to an enzymatic reporter, and trafficked to the regulated pathway). SCLIP siRNA also decreased the level of secretion of endogenous CHGA and SCG2, as well as transfected human growth hormone, while SCG10 siRNA decreased the level of regulated secretion of endogenous CHGB. Moreover, a dominant negative mutant of SCG10 (Cys_{22},Cys_{24}→Ala_{22},Ala_{24}) significantly blocked secretion of the transfected CHGA–EAP chimera. A decrease in the buoyant density of chromaffin granules was observed after downregulation of SCG10 by siRNA, suggesting participation of these stathmins in granule formation or maturation. We conclude that SCLIP and SCG10 interact with CHGA, share partial colocalization in the Golgi apparatus, and may be necessary for typical transmitter storage and release from chromaffin cells.

Secretion of proteins and peptides from eukaryotic cells involves at least two distinct pathways: constitutive (wherein secretory rate is constant over time) and regulated (wherein secretion of stored transmitter occurs only after a discrete, cell type specific signal). Regulated secretion involves selection (or "sorting") of proteins to be stored in dense core secretory granules. Two models for sorting have been proposed: "sorting for entry" and "sorting by retention" (1). The sorting-for-entry hypothesis proposes that the *trans*-Golgi network (TGN)[1] is the primary operator for protein sorting and that regulated secretory proteins form insoluble aggregates in

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the TGN that subsequently bind to the membrane of the forming secretory granule, or to a sorting receptor therein, effectively excluding constitutive secretory proteins (2–4). On the other hand, the sorting-by-retention hypothesis proposes that the entry of content proteins into the regulatory pathway does not depend on the "gatekeeping" function within TGN, and instead, it considers the immature granules as important post-TGN sorting stations (5), wherein selective aggregation and condensation occur, resulting in preferential retention of regulated secretory proteins, while nonaggregated proteins are removed from the maturing granule into small, clathrin-coated vesicles destined to a constitutive-like secretory pathway.

Protein–protein interactions are likely to play a central role in both sorting-for-entry and sorting-by-retention models (1). Previous studies suggested that proteins of the granin family represented by chromogranin B (CHGB) (2,6,7), prohormone processing enzymes, including carboxypeptidase E (8–11) and sulfated proteoglycans (12–14), may act as condensation factors (or "helper" proteins) in the sorting processes. Such helper proteins may facilitate intermolecular interactions and thereby improve the efficiency of copack-aging and condensation (15).

Accumulating evidence demonstrates a key role for CHGA in the biogenesis of chromaffin granules and formation of the regulatory secretory pathway in the neuroendocrine cells. For example, antisense RNA or siRNA-mediated depletion of CHGA in chromaffin cells in vitro resulted in a substantial loss of dense core secretory granules and impairment of regulated secretion (16–18). Moreover, expression of CHGA in cells lacking a regulated secretory pathway led to generation of the phenotype of regulated secretion (16,18). The granin CHGB has also been reported to induce biogenesis of secretory granules (19). Recently, we discovered that ablation of CHGA in vivo in mice resulted in morphological diminution of regulated secretory granules (20). Consistently, it appears that downregulation of chromogranins causes disruption of the regulated secretory pathway (16,19,20) and a shift in secretion of typically regulated compounds toward the constitutive pathway (16,20). Indeed, expression of CHGA may catalyze the formation of dense core secretory granules (18,21). However, the mechanism by which CHGA enters the regulated pathway or triggers the formation of the pathway (16, 18) remains unclear.

Here we used a transcriptome/proteome-wide approach, to discover a novel binding partner for CHGA, SCLIP (STMN3) (22), a member of the stathmin family of Golgi proteins (23, 24). We determined that two members of this family, SCLIP and SCG10 (STMN2), interact with CHGA, share colocalization in the Golgi apparatus, and seem to be necessary for typical release of transmitters from the chromaffin cell.

METHODS

Construction of the pET21a(+)-hCHGA Prokaryotic Expression Plasmid

Human CHGA cDNA encoding the mature protein (1317 bp DNA lacking the signal peptide sequence and termination codon) was amplified by PCR by using the following primers: 5′-GGAATTCCATATGCTCCCTGT-GAACAGCCCTATGAATA-3′ (forward primer; *Nde*I site underlined) and 5′-CCGCTCGAGGCCCCGCCGTAGTGCCTGCAG-3′ (reverse primer; *Xho*I site underlined). The DNA fragment was subcloned between *Nde*I and *Xho*I sites of the pET21a(+) vector (Novagen). There are six codons corresponding to the His residue right after the *Xho*I site, which is followed by a stop codon in the vector. Therefore, the construct expresses the recombinant human CHGA mature protein with six His residues at the carboxy terminus

[1]Abbreviations: CHGA, chromogranin A; CHGB, chromogranin B; SCG2, secretogranin II; SCG10, superior cervical ganglion-10, a neuronal growth-associated protein (STMN2); SCLIP, SCG10-like protein (STMN3); TGN, *trans*-Golgi network.

of the protein. The correct amplification of the DNA segment and subcloning was verified by DNA sequencing. The recombinant plasmid was named pET21a(+)-hCHGA.

Generation of the SCG10 Double Cys Mutant (Cys_{22}, Cys_{24}→Ala_{22},Ala_{24}) Plasmid

Mutation of the Cys residues at the codon 22 and 24 positions of the SCG10 protein, to Ala (Cys_{22},Cys_{24}→Ala_{22},Ala_{24}) residues, was carried out by using the following oligonucleotide primer and its complementary sequence primer: 5′-GCTGTCACTGATC**GC**CTCT**GC**CTTTTACCCGGAACC-3′ (the bold and underlined nucleotides denote the mutations). The pSCG10-EGFP plasmid [human SCG10 cloned between the *Eco*RI and *Sal*I sites of the pEGFPN3 vector; a gift from R. Fisher, University of Iowa College of Medicine, Iowa City, IA (25)] was used as a template for PCR. The Stratagene QuikChange mutagenesis kit was used to perform the mutations. DNA sequencing confirmed the presence of the mutations (Cys_{22}/Cys_{24}→Ala_{22}/Ala_{24}) in the resultant plasmid that was designated as pSCG10(dmut)-EGFP.

Expression and Purification of Recombinant Human CHGA

The *Escherichia coli* BL21 *txrB*(DE3)pLysS strain (Novagen) was transformed with the pET21a(+)-hCHGA plasmid. The transformant strain was grown in LB medium containing 100 μg/mL ampicillin, 15 μg/mL kanamycin, and 34 μg/mL chloramphenicol. When Abs_{600} reached 0.6, IPTG was added to a final concentration of 1 mM. After IPTG induction for 3 h, cells were harvested. The cell pellet was suspended and lysed in “Bugbuster” reagent with benzonase nuclease. The cell lysate was processed for purification of the recombinant human CHGA using a Ni-NTA column as per the manufacturer’s protocol (Novagen). The recombinant protein was detected as the characteristic M_r ~ 70 kDa band after SDS–PAGE followed by staining with gel-code blue stain (Pierce).

Phage Display To Identify Binding Partners of Human CHGA

Recombinant human CHGA (as “bait”) was immobilized in polystyrene wells as per the Novagen protocol. In brief, 200 μL of the recombinant human CHGA {5 μg/μL in TBS [10 mM Tris-HCl (pH 8.0) and 150 mM NaCl]} was applied per well of a 48-well polystyrene ELISA plate that was prewashed five or six times with deionized water. After an overnight incubation at 4 °C, the wells were washed with 300 μL of TBS three times to remove unbound bait protein; 200 μL of 5% nonfat dry milk in water was then applied per well, and the plate was incubated overnight at 4 °C. The wells were then washed five times with deionized water followed by application of 200 μL of water to each well. The CHGA-coated plates were stored at 4 °C until they were used.

A human brain phage display cDNA library (1.5×10^7 primary cDNA clones; Novagen) in the T7Select phage vector was amplified in *E. coli* strain BLT 5615 and applied to the CHGA-coated wells for 5 h at room temperature followed by an overnight incubation at 4 °C. Phage adsorption and washing were conducted at pH 6.0 [10 mM MES buffer (100 mM KCl and 1 mM $CaCl_2$)] or pH 5.5 [10 mM MES buffer (100 mM KCl and 1 mM $CaCl_2$)] to approximate the Golgi (pH 6.0) or chromaffin granule (pH 5.5) interior compartments. Following incubation with the immobilized CHGA bait, the unbound phage clones were washed out extensively with TBST buffer (TBS with 0.1% Tween 20), and the bound phage clones were then released in 200 μL of elution buffer [10 mM Tris-HCl (pH 7.5) and 1% SDS]. The eluted “prey” phage clones were amplified in *E. coli* and subjected to binding with a fresh well coated with the bait as described above. Three successive rounds of “bio-panning” were performed for each binding condition. With each successive round of biopanning, only one of every ~10^4 phages was selected (adsorbed, retained during washes, and later eluted with SDS).

After final elution, individual/clonal selected phages were identified as plaques in "lawns" of *E. coli* strain BLT-5615. Plaques were excised and suspended in 100 μL of 10 mM EDTA (pH 8.0). The plaque suspensions were heated at 65 °C for 10 min, cooled to room temperature, and centrifuged at 14000 rpm for 3 min to clarify. Two microliters of each lysate was subjected to PCR using T7SelectUP and T7SelectDown primers (Novagen). An aliquot of each PCR product was run on a 1.2% agarose gel to check the quality of the product and to determine the size of the cDNA insert in the phage clone. The PCR products were purified using the PCR purification kit (Qiagen) and sequenced using the T7SelectUP and T7SelectDown primers (Novagen). The resulting cDNA sequences were queried against GenBank, using BLAST-N searches to identify the clones.

Cell Culture and Transfection

Rat pheochromocytoma PC12 chromaffin cells were grown in DME/high-glucose medium supplemented with 5% heat-inactivated fetal bovine serum, 10% heat-inactivated horse serum, 100 units/mL penicillin G, and 100 μg/mL streptomycin at 37 °C in 6% CO_2 as described previously (26). PC12 cells were transfected with the pSCG10-EGFP, pSCLIP-EGFP (rat SCLIP fused to EGFP, obtained from N. Mori, National Institute for Longevity Sciences, Aichi, Japan), or pGalT-CFP plasmid by using Superfect as per the manufacturer's protocol (Qiagen). pGalT-CFP (Clontech) encodes the membrane-anchoring region of the classic *trans*-Golgi/TGN marker β-1,4-galactosyltransferase fused to cyan fluorescent protein (GalT-CFP), under transcriptional control of the CMV promoter, as previously described (21,27).

Co-Immunoprecipitation of CHGA with SCG10 or SCLIP

PC12 cells grown in 10 cm cell culture plates were cotransfected with pSCG10-EGFP or pSCLIP-EGFP and pCMV-CHGA (a human CHGA expression plasmid in the pCDNA3 vector). Forty-eight hours after transfection, cells were lysed in ice-cold RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.5% deoxycholate, 1% Nonidet P-40, 6 mM $MgCl_2$, and 10 mM phenylmethanesulfonyl fluoride] followed by centrifugation at 14000 rpm for 3 min at 4 °C. The supernatants were incubated with anti-CHGA (goat polyclonal raised against C-20 amino acids of human CHGA; Santa Cruz Biotechnology) or anti-GFP antibody (rabbit polyclonal; Living Colors A.v. peptide antibody; Clontech) and protein G-plus agarose (Santa Cruz Biotechnology) overnight at 4 °C for immunoprecipitation. The precipitates were collected by centrifugation, washed three times in RIPA buffer, and boiled in 1 × NuPage sample buffer (Invitrogen) for 5 min. The protein samples were subjected to SDS–PAGE and immunoblotting. As a control for immunoprecipitation reactions, normal goat serum and normal rabbit serum (both from Santa Cruz Biotechnology) were used instead of the antibodies.

Deconvolution Microscopic Analyses for Intracellular Colocalization of CHGA, SCG10, and SCLIP

Three-dimensional (3-D) deconvolution fluorescence microscopy was performed using a DeltaVision deconvolution microscopy system (Applied Precision). PC12 cells on polylysine-coated glass coverslips were cotransfected with pGalT-CFP and pSCG10-EGFP or pSCLIP-EGFP plasmids. The cells were fixed with 2% paraformaldehyde. Coverslips were subsequently washed with PBS, mounted in buffered Celvol (Celanese), and processed for 3D imaging as described previously (18).

In another set of experiments, PC12 cells transfected with pSCG10-EGFP or pSCLIP-EGFP were fixed with 2% paraformaldehyde and labeled with Hoechst 33342 (1 μg/ mL; Molecular Probes) for visualization of nuclei. The fixed cells were then incubated with a goat anti-CHGA antibody (1:100 dilution; epitope mapping the C-20 amino acids of human CHGA; Santa Cruz Biotechnology) followed by staining with donkey anti-goat IgG AlexaFluor 594 antibody

(1:200 dilution; Molecular Probes) for visualization of endogenous CHGA. Coverslips were washed, mounted, and processed for 3D imaging as described previously (18).

Gene Silencing by siRNA: SCG10 and SCLIP

SCG10 and SCLIP siRNA oligonucleotides were designed using Dharmacon *siDESIGN* software and then synthesized and annealed by Sigma-Proligo. The siRNA design algorithm (28) uses eight criteria to facilitate knockdown while minimizing off-target oligonucleotide effects. The sequences of the siRNAs for rat SCG10 (STMN2) based on reference mRNA sequence accession number NM_053440 were as follows: sense oligonucleotide (S_SCG10_34), 5′-AAACAGCAATGGCCTACAAdTdT-3′; and antisense oligonucleotide (AS_S-CG10_34), 5′-TTGTAGGCCATTGCTGTTTdTdT-3′. Similarly, rat SCLIP (STMN3) siRNA sequences (based on reference mRNA sequence accession number NM_024346) were as follows: sense oligonucleotide (S_STMN3_312), 5′-CGAACACCATCTACCAGTAdTdT-3′; and antisense oligonucleotide (AS_STMN3_312), 5′-UACUGGUAGAUGGUGUUCGdTdT-3′. BLAST of the rat genome indicated that these two oligonucleotide sequences were unique to the intended targets, and the two sequences contained only four contiguous (ungapped) bases in common; previous studies have demonstrated that even a single base mismatch is sufficient to abrogate the effect of an siRNA (29). PC12 cells were transfected with either SCG10 or SCLIP siRNA duplex at various concentrations (50, 100, and 200 nM) and for different periods of time (48, 72, and 96 h) using RNAiFect (Qiagen). To evaluate the gene silencing effect of the siRNAs, transfected cells were lysed and expressions of the proteins were analyzed by immunoblotting (using antibodies specific to SCG10 or SCLIP). An anti-SCG10 rabbit serum was the gift of G. Grenningloh (University of Lausanne, Lausanne, Switzerland), and an anti-SCLIP rabbit polyclonal antibody was obtained from Andre Sobel (INSERM). In some experiments, where transfections of siRNA duplex and a plasmid were conducted together, “TransMessenger” transfection reagent (Qiagen) was used instead of RNAiFect. To ensure that the gene silencing effects of *SCG10* and *SCLIP* siRNAs were specific, the nitrocellulose blots were stripped [by incubating them in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris (pH 6.8) buffer at 50 °C for 30 min], and expression of actin in the siRNA-transfected cells was checked using a goat anti-actin polyclonal antibody (corresponding to the 11 C-terminal amino acids of human actin; Santa Cruz Biotechnology). To further guard against off-target oligonucleotide effects, the Silencer negative control #1 siRNA (Ambion) that has no significant sequence homology with any known gene sequences from mouse, rat, or human was used as a negative control in the transfections.

Secretion and Quantitation of a CHGA–EAP Chimera from Chromaffin Cells

PC12 cells in 24-well plates were cotransfected with 0.25 μg of pCMV-CHGA-EAP [a recombinant plasmid expressing the human CHGA gene fused to a truncated domain (EAP) of the full-length human secreted embryonic alkaline phosphatase (SEAP) (21)] per well and negative control siRNA, *SCG10* siRNA, or *SCLIP* siRNA (at the final concentration of 100 nM), or both *SCG10* and *SCLIP* siRNAs (each at 100 nM) by TransMessenger transfection reagent. In another set of experiments, PC12 cells seeded in 12-well plates were cotransfected with 0.5 μg of pCMV-CHGA-EAP per well and 2.0 μg of pSCG10-EGFP or pSCG10(dmut)-EGFP per well using Superfect transfection reagent (Qiagen). Seventy-two hours after transfection, cells were incubated in mock calcium saline buffer (CaSB) [150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 10 mM HEPES (pH 7.4)] or 2 mM Ba^{2+} containing secretion buffer (BaSB) [150 mM NaCl, 5 mM KCl, 2 mM $BaCl_2$, and 10 mM HEPES (pH 7.4)] for 20 min. A secretion assay of CHGA–EAP chimera enzymatic activity in the culture supernatant was achieved with a high-sensitivity chemiluminescence assay (Phospha-Light, Applied Biosystems) using an AutoLumat 953 luminometer (EG&G Berthold) as described previously (18,21). Cells were lysed in 10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 2 mM

$CaCl_2$, 0.1% Triton X-100 buffer, and the lysate was estimated for total protein concentration using the Bio-Rad protein assay reagent. The amount of CHGA–EAP chimera chemiluminescence in the secretion medium was expressed as relative light units per microgram of total protein.

Secretion and Quantitation of Endogenous CHGA, CHGB, or SCG2 from Chromaffin Cells

Cells seeded in 10 cm plates were transfected with SCLIP or SCG10 siRNA (100 nM) or negative control siRNA (100 nM) by RNAi-fect. Seventy-two hours after transfection, cells were treated with CaSB or secretagogue-containing BaSB buffer for 20 min. The secretion media were collected, purified on Sep C-18 columns, eluted into a 60% acetonitrile/1% trifluoroacetic acid mixture in water, and freeze-dried as described previously (30). The samples were then dissolved in 1 × NuPage sample buffer (Invitrogen) and subjected to SDS–PAGE followed by detection of CHGA by immunoblotting in the samples using a rabbit polyclonal antihuman CHGA antibody (31). Similar immunoblots probed secretion of endogenous CHGB or SCG2. The amounts of secreted CHGA, CHGB, or SCG2 were estimated by densitometric scanning of the bands using Image J from NIH.

Secretion of Transfected/Expressed Growth Hormone (GH) by Chromaffin Cells

PC12 cells seeded in 12-well plates were transfected with 1 μg of human growth hormone (GH)-expressing plasmid pXGH5 (Nichols Institute) (18) per well along with negative control siRNA or SCLIP siRNA (final concentration, 100 nM) using TransMessenger transfection reagent. Seventy-two hours after transfection, cells were exposed to mock (CaSB) or BaSB stimulation buffer for 20 min. Detection of GH in the supernatant was achieved by a colorimetric enzyme immunoassay (GH ELISA, Roche Applied Bioscience). The concentrations of GH in the samples were calculated by running a series of standards (from 0 to 400 pg/mL) in the assay. The total protein concentration in the cell lysates was measured with a Bio-Rad protein assay. The amounts of GH secreted under the indicated conditions of the experiment were expressed as picograms per microgram of total cellular protein.

Chromaffin Granule Buoyant Density: Sucrose Density Gradient Centrifugation

PC12 cells in 10 cm plates were transfected with negative control siRNA or *SCG10* siRNA. Seventy-two hours after transfection, the cells were labeled with 1 μCi/mL [^{3}H]norepinephrine for 120 min. The cells were then homogenized, and the cell suspension was subjected to sucrose density gradient centrifugation as reported previously (21,27). Each gradient fraction was assayed for [^{3}H]norepinephrine by scintillation counting and sucrose concentration by refractometry. The amount of [^{3}H]norepinephrine in the gradient fractions was expressed as the percent of maximum counts in each set of experiments. The sucrose concentration in each fraction was expressed in molarity as determined by comparing the refractometry values with that of a series of sucrose standard solutions.

RESULTS

SCLIP Is a Binding Partner of CHGA: Evidence from Phage Display

To discover proteins that interact with CHGA in the regulated secretory pathway, we performed a phage display assay using purified recombinant human CHGA as bait and a human brain cDNA expression library, expressing in-frame cDNAs fused to T7 capsid protein 10B. To mimic protein–protein interaction conditions in the regulated secretory pathway, we studied adsorption of phage to CHGA at pH 6.0, to approximate the mildly acidic environment of the *trans*-Golgi TGN (a likely site for sorting decisions into the regulated secretory pathway), or pH 5.5, to approximate the chromaffin granule interior (32). A list of binding partners of CHGA is given in Table 1. Among these CHGA-binding proteins, we chose SCLIP (which bound at

pH 5.5) for further investigation considering its already known functions and its localization to the *trans*-Golgi portion of the secretory pathway (33). Noteworthy is the fact that other proteins expressed within or adjacent to the secretory pathway were also detected by CHGA binding, such as the endoplasmic reticulum protein p180 (GenBank entry AF007575; in multiple clones), Rab-6 GTPase activating protein (GenBank entry AJ011679), microtubule-associated protein 1B (GenBank entry NM005909), chapsyn-110 (GenBank entry U32376; in multiple clones), cotomer protein complex subunit α (GenBank entry NM004371), and humanin HN1 (GenBank entry AY029066; in multiple clones). These potential binding partners were not further considered in this initial report.

Specific Binding of SCLIP and SCG10 to CHGA: Co-Immunoprecipitation with CHGA from Chromaffin Cells

To determine whether the interaction between CHGA and SCLIP observed during the phage display assay also occurs in mammalian cells, co-immunoprecipitation experiments were performed. As shown in Figure 1A, CHGA could be immunoprecipitated by either anti-CHGA (lane 2) or anti-GFP (lane 3) antibodies from the lysate of PC12 cells overexpressing CHGA and SCLIP-EGFP. More efficient immunoprecipitation by anti-GFP than by anti-CHGA antibodies may reflect the synthetic peptide immunogen used to raise goat anti-CHGA antibodies (see Methods). As a negative control for immunoprecipitation, the cell lysate was also incubated with normal rabbit serum (Figure 1A, lane 4), which could not precipitate CHGA. As a positive control, we included the cell lysate (Figure 1A, lane 1) in the SDS–PAGE showing the presence of CHGA in the “input”.

Because SCLIP is substantially homologous to and shares similar functional properties with SCG10, the two have been suggested to play complementary roles (22). Therefore, examining whether SCG10 also interacts with CHGA seemed essential. Interestingly, an anti-CHGA antibody (Figure 1B, lane 3) co-immunoprecipitated both CHGA and SCG10-EGFP from the lysates of PC12 cells overexpressing CHGA and SCG10-EGFP. In a parallel experiment, an anti-GFP antibody (Figure 1B, lane 5) co-immunoprecipitated both CHGA and SCG10-EGFP from the same cell lysate. On the other hand, when the cell lysates were incubated with normal goat serum (Figure 1B, lane 2) or normal rabbit serum (Figure 1B, lane 4), neither CHGA nor SCG10-EGFP was precipitated, indicating specificity of the immunoprecipitation reactions. As a positive control, the input cell lysate (Figure 1B, lane 1) showed the presence of CHGA and SCG10-EGFP. These results demonstrate that CHGA interacts with both SCLIP and its closest stathmin family member, SCG10.

Golgi Intracellular Colocalization of SCG10, SCLIP, and CHGA

Localizations of SCG10 and SCLIP in PC12 cells were analyzed to improve our understanding of their interaction with CHGA. When PC12 cells expressing the SCG10-EGFP protein and the Golgi marker protein GalT-CFP were examined by three-dimensional (3D) deconvolution microscopy, both GalT-CFP and SCG10-EGFP displayed a typical perinuclear Golgi distribution in the cells. One representative cell is shown in Figure 2A (first row). A similar intracellular distribution pattern of SCLIP-EGFP and overlapping of its fluorescence with that of GalT-CFP were observed in a parallel experiment (Figure 2A, second row). Consistently, endogenous CHGA detected with AlexaFluor 594 displayed substantial overlap with SCG10-EGFP and SCLIP-EGFP in the perinuclear Golgi region of the PC12 cells (Figure 2B).

Dose-Dependent Downregulation of Expression of SCG10 and SCLIP by siRNA

To understand the functional implications of the interactions between SCG10 or SCLIP and CHGA, downregulation of the expression of SCG10 and SCLIP was achieved by specific siRNA duplexes. The *SCG10* siRNA duplex (as described in Methods) dose-dependently (~68% at 50 nM, ~74% at 100 nM, and ~83% at 200 nM) reduced the SCG10 protein level in

PC12 cells (Figure 3A). Similarly, the *SCLIP* siRNA duplex (as described in Methods) decreased the level of expression of SCLIP protein in a dose-dependent fashion: ~72% at 50 nM, ~85% at 100 nM, and ~93% at 200 nM (Figure 3B). Thus, the chosen siRNA duplexes for SCG10 and SCLIP exerted a substantial gene silencing effect. To investigate the specificity of these siRNAs, in another set of experiments, PC12 cells were transfected with negative control siRNA comprised of a 19 bp scrambled sequence with 3′-dT overhangs and having no significant sequence homology with any known gene sequences from mouse, rat, or human. The negative control siRNAs did not have any effect on the expression of SCG10 protein as compared to the case of mock (where no siRNA was used), while *SCG10* siRNA exhibited consistent reduction of the level of the SCG10 protein (Figure 3C).

Secretion of the Transfected CHGA–EAP Chimera Is Diminished after Downregulation of Expression of SCG10 and SCLIP

To investigate whether the interaction of SCG10 or SCLIP with CHGA might influence trafficking of CHGA within the secretory pathway, we measured Ba^{2+}-evoked secretion of CHGA–EAP chimeric protein (2 mM Ba^{2+}, 20 min) in PC12 cells where the level of expression of SCG10 or SCLIP has been downregulated by siRNAs. Silencing of SCG10 resulted in a reduction in both basal (mock medium; 3.7 ± 0.3-fold)andBa^{2+}-stimulated release of the CHGA–EAP chimera (5.8 ± 0.5-fold), as compared to cells treated with a control siRNA (Figure 4). Similarly, silencing the expression of SCLIP diminished both basal and Ba^{2+}-evoked secretion by 3.1 ± 0.2- and 3.2 ± 0.3-fold, respectively (Figure 4). Interestingly, concurrent downregulation of both SCG10 and SCLIP did not result in a further decrease in basal and stimulated secretion (4.1 ± 0.3- and 5.8 ± 0.5-fold reduction, respectively) as compared to that in SCG10-silenced PC12 cells (Figure 4), suggesting a common pathway of action for SCG10 and SCLIP.

Secretion of Endogenous CHGA, CHGB, or SCG2 Is Inhibited by siRNA-Mediated Silencing of SCLIP or SCG10

Because downregulation of the expression of either SCG10 or SCLIP resulted in a substantial reduction in basal as well as Ba^{2+}-stimulated secretion of CHGA–EAP chimeric protein from PC12 cells (Figure 4), we questioned whether secretion of endogenous CHGA, CHGB, or SCG2 might also be affected by such gene silencing.

siRNA-mediated SCLIP downregulation (Figure 5A, top) caused a significant reduction in basal (by ~71%; lane 3 → lane 1) and Ba^{2+}-stimulated secretion (by ~49%; lane 4 → lane 2) of endogenous CHGA. Similarly, SCLIP siRNA (Figure 5A, middle) reduced basal (by ~90%; lane 3 → lane 1) and Ba^{2+}-stimulated secretion (by ~40%; lane 4 → lane 2) of endogenous SCG2.

Targeted siRNA ablation of SCG10 (Figure 5B, bottom) had little effect on basal secretion (lanes 1 and 2 → lanes 5 and 6) but substantially diminished Ba^{2+}-stimulated secretion (by ~77%; lanes 3 and 4 → lanes 7 and 8) of endogenous CHGB.

Secretion of Exogenously Expressed Growth Hormone (GH) Is Diminished after Downregulation of SCLIP

To investigate whether downregulation of SCLIP or SCG10 has an effect on the secretory pathway in general, we studied basal and Ba^{2+}-stimulated secretion of transfected human GH in SCLIP-silenced PC12 cells. Both basal and Ba^{2+}-stimulated secretion of GH were significantly (by ~3- and ~4-fold, respectively) diminished in *SCLIP* siRNA-transfected cells, as compared to cells treated with negative control siRNA (Figure 6).

Expression of a Dominant Negative Mutant of SCG10 Blocks Secretion of the Cotransfected CHGA–EAP Chimera

In view of previous studies reporting that abolition of the palmitoylation sites at the Cys_{22} and Cys_{24} amino acids of SCG10 prevents Golgi localization of the protein (33), we asked whether overexpression of a mutant SCG10 bearing Cys_{22},Cys_{24}→Ala_{22},Ala_{24} substitutions affects the secretion of the CHGA–EAP protein. As shown in Figure 7, PC12 cells expressing the mutant SCG10 protein displayed a significant reduction (~2.2-fold) in Ba^{2+}-stimulated secretion of the transfected CHGA–EAP chimera, although the basal secretion was not changed.

A Reduction in SCG10 Expression Causes a Decrease in the Density of Chromaffin Granules

Because downregulation of SCG10 expression diminished both basal and stimulated secretion of transfected CHGA–EAP protein (Figure 4), GH (Figure 6), and endogenous CHGA (Figure 5), we investigated any possible change in the granule composition by measuring the buoyant density of the chromaffin granules from PC12 cells transfected with control siRNA versus *SCG10* siRNA (Figure 8). PC12 cells were labeled with [^{3}H]norepinephrine, which served as the marker of the granules. The gradient fraction emitting maximum counts of [^{3}H] norepinephrine had a sucrose concentration of 1.19 M in the case of control PC12 cells but a sucrose concentration of 1.06 M in the case of SCG10-downregulated PC12 cells. Thus, a decreased SCG10 level leads to sedimentation of the chromaffin granules to a lighter fraction in the sucrose density gradient. This observation was confirmed by repeated experiments. Results from a representative experiment are shown in Figure 8.

DISCUSSION

Overview

CHGA (as a chromogranin/secretogranin) is closely associated with the presence of a regulated secretory pathway in neuroendocrine cells. Specifically, the granulogenic function of CHGA, as demonstrated by in vitro (16,18,21) and in vivo (20) studies, suggests CHGA to be an important determinant of the regulated secretory pathway. In this study, we set out to investigate the proteins that interact with CHGA under higher-calcium and lower-pH conditions approximating the *trans*-Golgi TGN and chromaffin granule interior (21,32). We chose SCLIP for further investigation considering its known actions and its presence in the *trans*-Golgi (33). We also included its paralog SCG10 in this study because these two proteins may play complementary roles by virtue of their shared structural domains as well as similar functional properties (22).

CHGA in the Secretory Pathway: cis and trans Mechanisms

Two hypotheses for the entry of protein into the regulated secretory pathway have been frequently articulated (1): sorting for entry (in which regulated secretory proteins interact with membrane “receptors” for such cargo) versus sorting by retention (wherein the secretory cargo condenses within the lumen in response to physical changes in the milieu, such as increasing luminal H^+ and Ca^{2+} concentrations). Our previous observations that particular domains within CHGA mediate its entry into chromaffin granules (27) and that such domains can trigger secretory granule formation (18,21) suggest that condensation alone might not account for correct protein transport into the regulated pathway. Here we searched more explicitly for protein binding partners potentially subserving regulated secretory protein traffic.

We selected the phage display protein–protein interaction discovery system for two reasons. (a) The human brain cDNA library that we screened contained ~1.5×10^7 independent recombinants, likely to represent even relatively rarely expressed transcripts. (b) Unlike the

yeast two-hybrid system (which screens bait/prey partners only under physiological conditions in cytoplasm or nucleus), the phage display system allows selection of a wider range of binding conditions, such as the lower pH and higher Ca^{2+} concentration found within the Golgi apparatus and chromaffin granules (21,32).

Consequences of CHGA Binding to SCLIP or SCG10 in Vivo: Disruption of Secretion

After detection (Table 1) and confirmation (Figure 1) of binding of CHGA to SCLIP or SCG10, we first asked about the potential for colocalization in vivo. We first showed that SCLIP and SCG10 colocalized to the Golgi apparatus in chromaffin cells (Figure 2A). We next showed that CHGA exhibited partial colocalization with SCLIP and SCG10 in the Golgi region (Figure 2B), likely reflecting the passage of CHGA through the Golgi apparatus as it is trafficked into chromaffin granules.

We next asked about the consequences of such interaction for hormone storage and secretion in chromaffin cells. First, we found that siRNA directed against either SCLIP or SCG10 disrupted secretion of a CHGA chimera targeted to the regulated secretory pathway (Figure 4). Second, we established that secretion of endogenous CHGA and SCG2 was similarly inhibited by siRNA ablation of SCLIP (Figure 5). Third, we established that secretion of an ectopic secretory protein (GH) transported to the regulated pathway was also disrupted by SCLIP siRNA (Figure 6). Fourth, we demonstrated that a mutant removing both palmitoylation sites (23) of SCG10 (Cys_{22},Cys_{24}→Ala_{22},Ala_{24}) inhibited secretion of a CHGA chimera (Figure 7).

Taken together, these results indicate that the stathmins SCLIP and SCG10 participate in developing the regulated exocytosis phenotype in chromaffin cells. Whether this effect occurs at the level of transport of regulated proteins into the granule or at a later stage of exocytosis is not certain, but the change in chromaffin granule buoyant density after SCG10 ablation (Figure 8) suggests the participation of stathmins in granule formation or maturation.

SCLIP or SCG10 siRNA knockdown had a less complete effect in blocking endogenous CHGA secretion (Figure 5) than exogenous CHGA secretion [i.e., that introduced by transient transfection of the CHGA–EAP chimera (Figure 4)]. While each molecule of exogenous (newly expressed) CHGA is transported after ablation of SCLIP (Figure 4), the very long half-life of endogenous CHGA within chromaffin cells, previously established by us to be ~79 h (34), should result in a pretransported vesicular CHGA pool that would decline in size only slowly after SCLIP siRNA. In these experiments, we studied endogenous CHGA (and CHGB and SCG2) secretion after only 72 h of SCLIP siRNA. In the future, more prolonged SCLIP ablation would be expected to achieve more complete inhibition of CHGA trafficking.

CHGA, Chromaffin Granules, and Stathmins

The stathmin protein family (35), known principally as regulators of microtubule interactions, includes the cytosolic protein stathmin (STMN1), as well as Golgi and vesicular membrane-associated SCG10 (STMN2), SCLIP (STMN3), and splice variants RB3, RB3′, and RB3″. While stathmin itself is ubiquitous, SCG10 and SCLIP display a neuronal or neural crest-derived distribution. The intracellular localization of SCG10 and SCLIP to Golgi and other vesicular membranes is mediated by at least two conserved amino-terminal domains: a general membrane-attachment domain (“m”) containing two essential Cys residues (Cys_{22} and Cys_{24}) which must undergo palmitoylation and a further Golgi-specification domain (“n”), $A_7YKEKMKEL_{15}$, whose charged amino acids are required.

CHGA and SCG10 display joint biosynthetic control, by virtue of shared transactivation by the Olf/EBF protein family (36). Other constituents of chromaffin granules may bind stathmins

and thereby influence morphology or secretion. For example, the apoptosis-associated secretory protein clusterin (37) is now recognized to be a chromaffin granule constituent, previously identified as chromaffin granule glycoprotein III (38) or HISL-19 antigen/secretogranin IV (39); the interaction of clusterin and SCLIP may promote neurite outgrowth in chromaffin cells (40) and hippocampal neurons (35). Noteworthy is the fact that, for intermolecular interactions, each of these binding partners is reported to show the features of a coiled coil in at least one domain, suggesting a mechanism of interaction (by heterodimerization of coiled coils): CHGA (41), clusterin (39), and SCLIP/SCG10 (23,42).

Protein–Protein Interactions: Potential Role of Coiled-Coil Domains

Our studies identified two members of the stathmin family (23) as proteins capable of binding CHGA: SCLIP and SCG10 (Figure 1). Intriguingly, both CHGA (41) and SCLIP (42–44) are predicted to have coiled-coil domains, as well as other unusual physical properties, including extended conformations (24,41) and heat stability (24,45). We also found evidence that CHGA, SCLIP, and SCG10 partially colocalize in the Golgi apparatus (Figure 2), consistent with an in vivo interaction (perhaps transient). How might this interaction occur? High-resolution 3D structures are not available for any of these three proteins, though we recently reported that CHGA is likely to assume a conformation of an extended coiled coil along much of its length (41). SCLIP (and other stathmin family members) also contain carboxy-terminal coiled-coil domains, by which they may interact heterodimerically with other coiled coils (42–44).

Within the *trans*-Golgi, coiled-coil domains in CHGA (41) might interact with the membrane-anchored SCLIP coiled coil, guiding CHGA into the terminal cisternae of the *trans*-Golgi network, destined to form dense core granules of the regulated pathway (e.g., chromaffin granules). After (or during) interaction with SCLIP, the physical properties of CHGA (acidic amino acid composition with a low pI of ~4.5; calciumbinding at low affinity but high capacity) (46,47) would prompt aggregation in the increasingly acidic and high-calcium *trans*-Golgi milieu (21,32). Such condensation might also promote cosorting of other regulated secretory proteins (7) while excluding constitutively secreted proteins (48). Indeed, amphipathic α-helical segments may be a general feature of prohormones and propeptides destined for transport into the regulated secretory pathway (49). Which of the approximately seven CHGA coiled-coil motifs is likely to be crucial in such interactions? By deletion analysis, we identified a small segment of human CHGA, $CHGA_{77–115}$, as being necessary for the transport of the entire molecule into the regulated pathway (18,27); this segment, which overlaps the second coiled-coil domain within CHGA (41), contains such an amphipathic domain at His_{79}–Leu_{90} (H_{79}SG**F**EDE**L**SEV**L**$_{90}$), which conforms (12/12 match) to three turns of an amphipathic α-helical motif, wherein hydrophobic residues (in bold type) occur at every fourth residue in the primary structure.

Alternatively, near-amino-terminal SCLIP trafficking domain (24) “n” (amino acids 7–15, A_7Y**K**E**K**M**K**EL_{15}, basic amino acids in bold) may be sufficiently cationic (calculated local pI of 8.47) to mediate an electrostatic interaction with the polyanionic CHGA (pI ~4.5) (46, 47). During the interaction of CHGA and SCLIP in the *trans*-Golgi, the hydrophobic disulfide-bonded CHGA loop (from Cys_{17} to Cys_{38}) would provide only initial, nonspecific, low-affinity membrane contact for CHGA (6), to localize CHGA into the vicinity of the inner leaflet of the *trans*-Golgi membrane; such a role for the $Cys_{17} \rightarrow Cys_{38}$ loop is consistent with our CHGA domain deletion studies (18,27). Nonetheless, we have not examined the topology of the CHGA–SCLIP–SCG10 interactions in great detail, nor have we systematically evaluated other members of the stathmin family, such as stathmin itself (STMN1) or splice variants RB3, RB3′, and RB3″.

We found that site-directed mutagenesis of the Cys residues whose palmitoylation is crucial to interaction of the stathmin SCG10 with the Golgi apparatus (Cys_{22}/Cys_{24}→Ala_{22}/Ala_{24})

blunts the release of the transmitter from chromaffin cells (Figure 7). Since SCG10 may homodimerize via its coiled-coil domains (23), such mutants might behave in a dominant-negative fashion. Disruption of Cys_{22} and Cys_{24} in SCG10 may actually change its subcellular localization from Golgi to cytoplasm (33,50).

Among the chromogranins and secretogranins, granular trafficking of not only CHGA but also CHGB and SCG2 was disrupted by SCLIP–SCG10 siRNA ablation (Figure 5). We have not yet studied whether CHGB or SCG2 interacts directly with SCLIP–SCG10 or if their transport is indirectly affected by the interaction of CHGA with SCLIP–SCG10. Since not only CHGA but also CHGB and SCG2 contain extended coiled-coil domains (41), direct interactions of all three granins with the stathmins are conceivable.

Limitations and Future Directions

(1) Spectrum of CHGA Binding Partners—Our phage display screening of proteins expressed in human brain revealed additional ligands for CHGA, including several proteins known to localize to aspects of the secretory pathway (Table 1). Future studies on these additional proteins might yield additional clues about the mechanism of transport of protein into the regulated pathway.

One example is the 180 kDa ribosome “receptor” (p180, RRBP1) which is an integral endoplasmic reticulum (ER) membrane protein (51). Interestingly, p180 exists in various forms. One of its forms possesses a large uninterrupted C-terminal region composed predominantly of heptad repeats predicted to form α-helical coiled coils, a motif now known to be present in CHGA (41), which may permit heterodimerization. p180 contains a 10-amino acid consensus motif, NQGKKAEGAQ (basic residues underlined), repeated up to ~54 times in tandem close to the amino terminus (52), which may render the protein polycationic. Indeed, the pI of RRBP1 can be computed to be 8.69, with a major contribution of the decad repeats (pI 9.98). In our phage display assay, eight different size forms of p180 were independently detected, though only at pH 6.0. All of these p180 forms were partial cDNA clones (350–1000 bp size), and a majority of them contained 8–15 amino-terminal decad repeats. Since CHGA both is polyanionic (with a computed pI of 4.58) and contains multiple coiled-coil domains (41), binding of p180 in vitro to CHGA is not unexpected. However, we did not pursue p180 because of its very early (ribosomal) localization in the secretory pathway.

Other CHGA binding partners included humanin (HN1), a 24-amino acid secretory peptide (53), which inhibits neuronal cell death induced by familial Alzheimer’s disease mutant genes and amyloid-β (54). HN1 interacts with insulin-like growth factor-binding protein-3 in regulating cell survival and apoptosis (55). Humanin (MAPRGFSCLLLLTSEIDLPVKRRA) is also cationic (residues underlined), with a pI of 9.49. Likewise, chapsyn-110 (DLG2) has multiple protein interaction (e.g., PDZ and SH3) domains.

(2) CHGA Binding Partners Not Found in This Assay—We did not detect CHGA itself or other granins such as chromogranin B (CHGB) as binding partners of CHGA, although granins have been reported to form homodimers, homotetramers, heterodimers, and heterotetramers under certain conditions (56–58). A likely reason for this omission is that interactions among granin molecules may not be of sufficiently high affinity to survive the stringent binding and washing conditions involved in the phage display method.

(3) Binding Domains and Conditions—We demonstrated that CHGA binds SCLIP with sufficient affinity to survive multiple rounds of biopanning, with sequential adsorption, washing, elution, transformation, and amplification; the selective enrichment at each round was ~10^4-fold. We then extended such observations to co-immunoprecipitation (with appropriate negative controls) of CHGA with either SCLIP or SCG10 (Figure 1). Nonetheless,

we have not yet characterized in great detail the molar affinity, pH, or ionic dependencies of these protein–protein interactions, nor have we probed which specific amino acid domains mediate such attachment. Deletion and substitution mutagenesis in each binding partner will be required to understand the interacting domains in further detail.

Conclusions and Perspectives

A precise understanding of the mechanism of secretory protein transport into the regulated secretory pathway has been elusive (1), despite experimental models both in cells (16,18,19) and in vivo (20) that have established a role for CHGA. Our studies took a transcriptome/proteome-wide approach to ask whether CHGA interacted with proteins in the secretory pathway. Our results with SCLIP/SCG10 indicate that such interactions occur (Figure 1) and may be necessary for the process of secretory protein transport into and exocytosis from the regulated pathway (Figure 3–Figure 7). The findings thus open up new approaches to understanding the process of regulated secretory protein traffic and suggest novel, previously unsuspected points of control in the exocytotic pathway.

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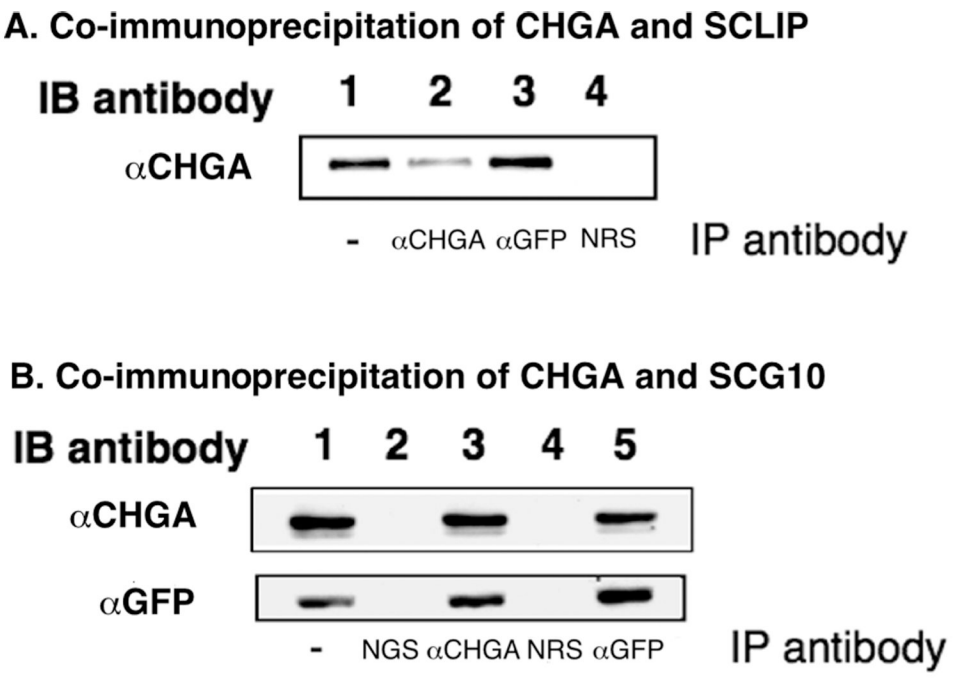

FIGURE 1.

Co-immunoprecipitation of CHGA with SCG10 or SCLIP. (A) Immunoblot showing co-immunoprecipitation of CHGA and SCLIP. Lysates of PC12 cells cotransfected with pCMV-CHGA and pSCLIP-EGFP were immunoprecipitated with anti-CHGA (lane 2) or anti-GFP (lane 3) antibodies. As a control for immunoprecipitation, the cell lysate was also incubated with normal rabbit serum (NRS, lane 4). Immunoprecipitates were subjected to immunoblotting with anti-CHGA antibodies. Lane 1 shows input cell lysate prior to immunoprecipitation. (B) Immunoblot showing co-immunoprecipitation of CHGA and SCG10. Lysates of PC12 cells cotransfected with pCMV-CHGA and pSCG10-EGFP were immunoprecipitated with anti-CHGA (lane 3) or anti-GFP (lane 5) antibodies. As controls for immunoprecipitation, the cell lysates were also incubated with normal goat serum (NGS, lane 2) or normal rabbit serum (NRS, lane 4). The immunoprecipitates were subjected to immunoblotting with anti-CHGA or anti-GFP antibodies. Lane 1 shows input cell lysate prior to immunoprecipitation.

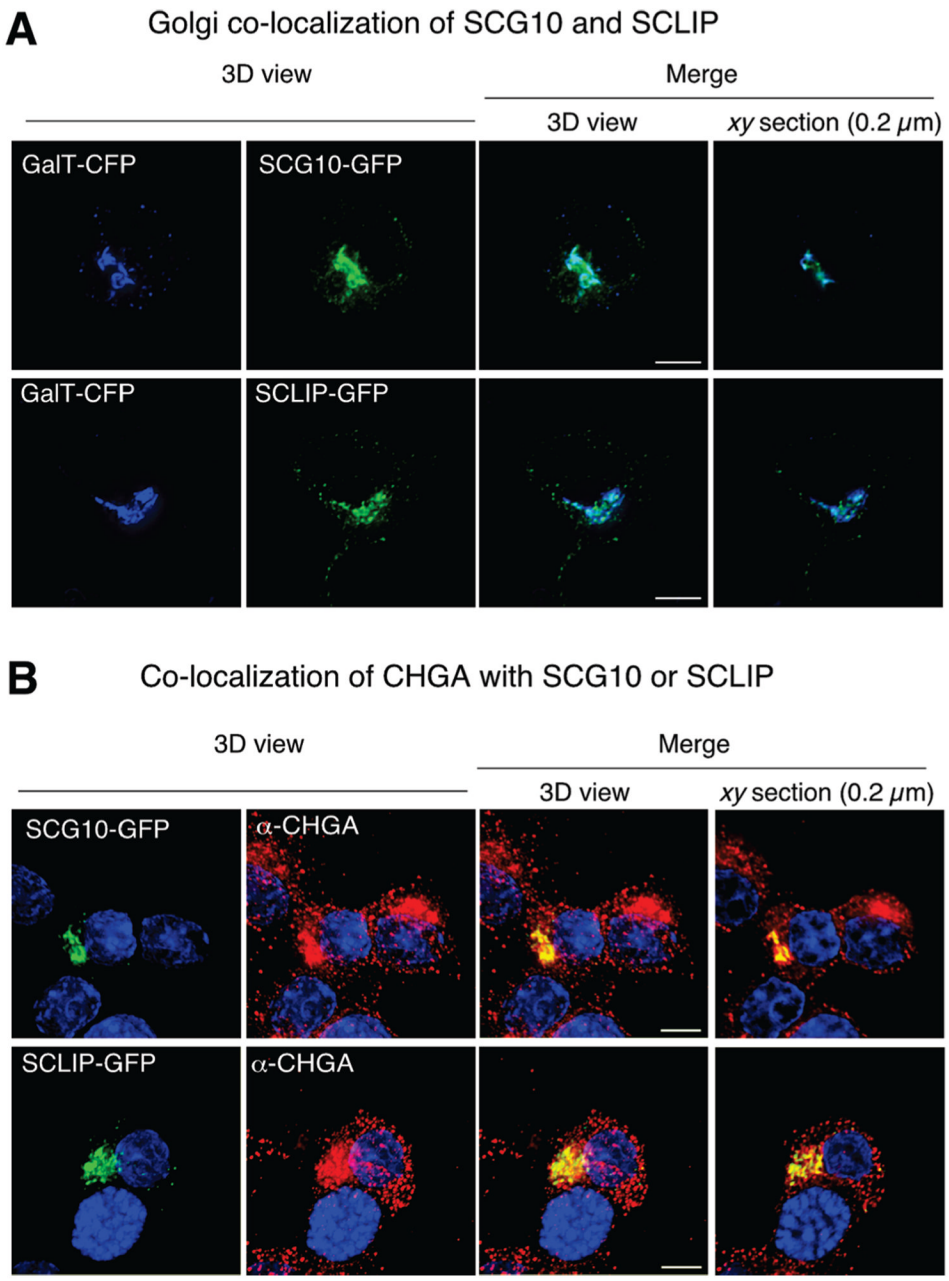

FIGURE 2.

Golgi colocalization of SCG10, SCLIP, and CHGA in chromaffin cells. (A) Colocalization of SCG10 and SCLIP with the *trans*-Golgi/TGN marker GalT-CFP. Deconvolution fluorescence microscopy analysis of PC12 cells transfected with pGalT-CFP together with either pSCG10-EGFP or pSCLIP-EGFP. Colocalization of SCG10-GFP or SCLIP-GFP (green) with GalT-CFP (blue) is shown in merged images of 3D views, and representative 0.2 μm *xy* optical sections. Cyan regions are indicative of colocalization. (B) Partial colocalization of CHGA with SCG10 and SCLIP. Deconvolution fluorescence microscopy analysis revealing partial overlap in the distribution of SCG10-GFP or SCLIP-GFP (green) with endogenous CHGA (red). SCG10-GFP- or SCLIP-GFP-expressing PC12 cells were processed for immunocytochemistry using a primary anti-CHGA antibody and an AlexaFluor 594-

conjugated secondary antibody. Colocalization of CHGA (red) with SCG10-GFP and SCLIP-GFP (green) is shown in merged images of 3D views, and representative 0.2 μm *xy* optical sections. Yellow regions are indicative of colocalization. In panel B, nuclei are stained with Hoechst 33342 (blue). The scale bar is 5 μm.

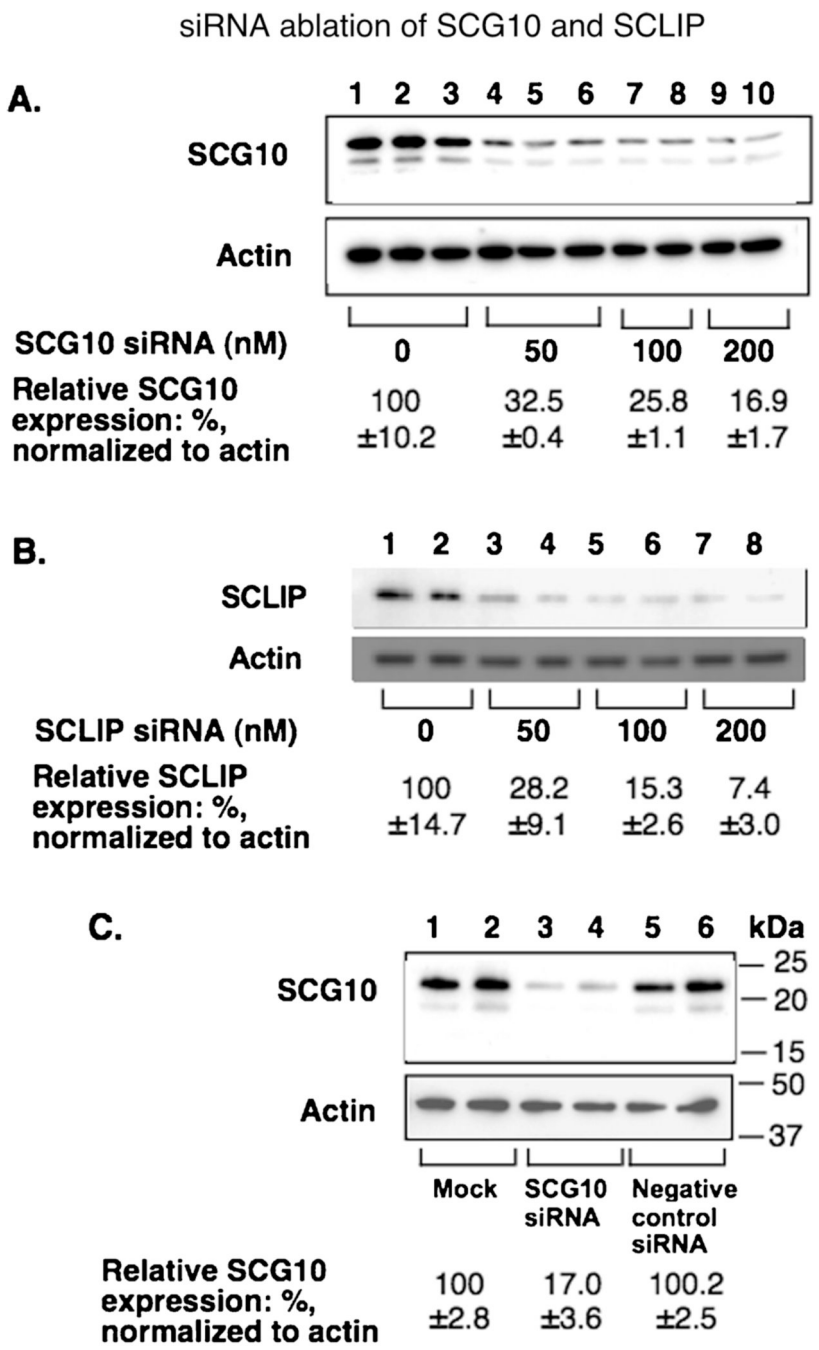

FIGURE 3.

Downregulation of the expression of SCG10 and SCLIP in PC12 cells by siRNA. Dose-dependent siRNA silencing of SCG10 protein expression. Cells were transfected with 0, 50, 100, or 200 nM SCG10 (A) or SCLIP (B) siRNA duplexes as described in Methods. Seventy-two hours after transfection, cells were lysed and subjected to immunoblot detection of SCG10, SCLIP, and actin. The level of protein expression was estimated by densitometry (NIH Image J). Means ± the standard error of the mean (from triplicate or duplicate wells) for the relative expressions of SCG10 or SCLIP (normalized to actin) are shown. SCG10 or SCLIP expression without siRNA treatment was considered to be 100%. (C) Specificity of downregulation of SCG10 expression by siRNA. Cells were transfected with mock (lanes 1 and 2) or 200 nM

SCG10 siRNA (lanes 3 and 4), or Ambion silencer negative control #1 siRNA (lanes 5 and 6), and the level of expression of SCG10 was determined after 72 h as described above. Negative control siRNA did not have any effect on SCG10 protein expression. The positions of the molecular size markers in the gel are indicated on the right-hand side of the panel.

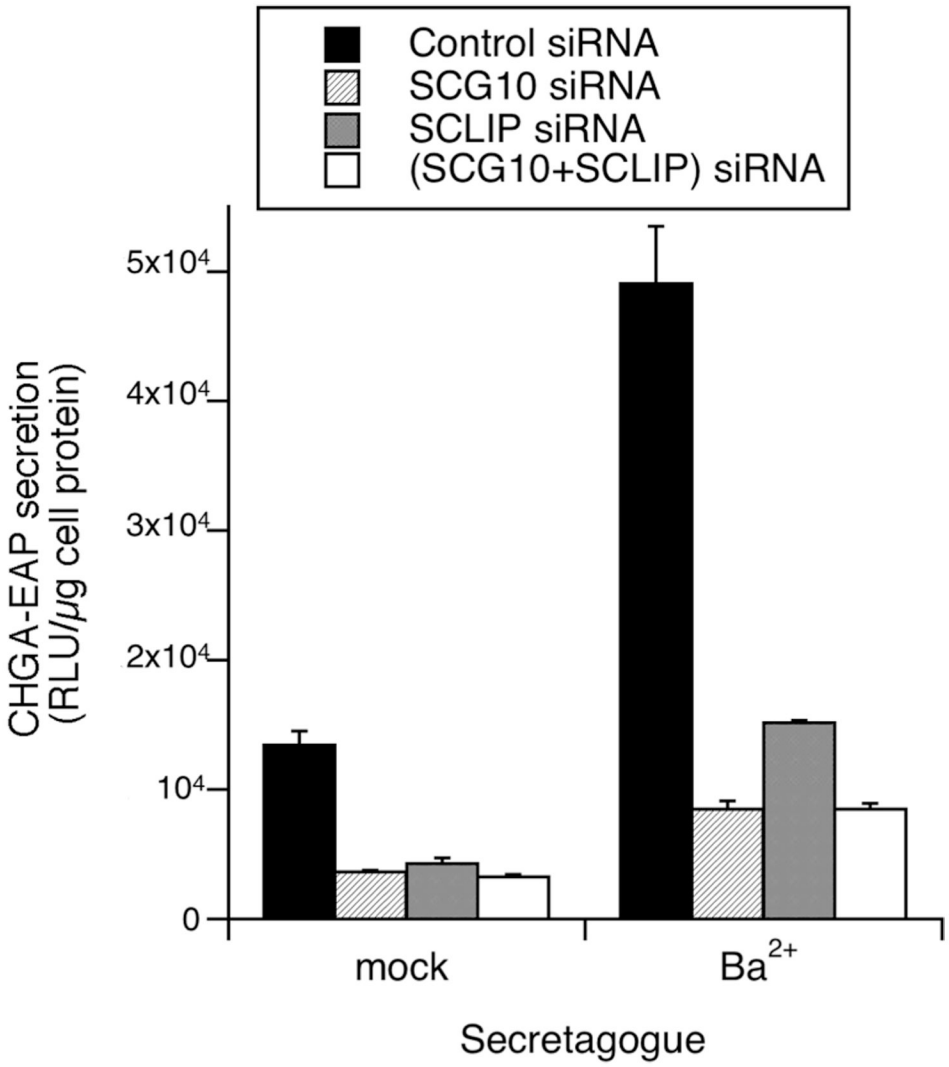

FIGURE 4.

Silencing the expression of SCLIP or SCG10 inhibits exocytotic secretion. Shown are results from a chimeric CHGA–EAP fusion protein. PC12 cells were cotransfected with pCMV-CHGA-EAP and *SCG10* siRNA (100 nM), *SCLIP* siRNA (100 nM), or both of these siRNAs (each at 100 nM). Silencer negative control #1 siRNA (Ambion) was used as a control. Seventy-two hours after transfection, cells were subjected to a secretion assay under mock vs Ba^{2+}-stimulated conditions, as described in Methods. The CHGA–EAP chimeric activity in the secretion medium was estimated by chemiluminescence (Phospha-Light, Applied Biosystems). Values are given as the means of quadruplicates ± the standard error of the mean.

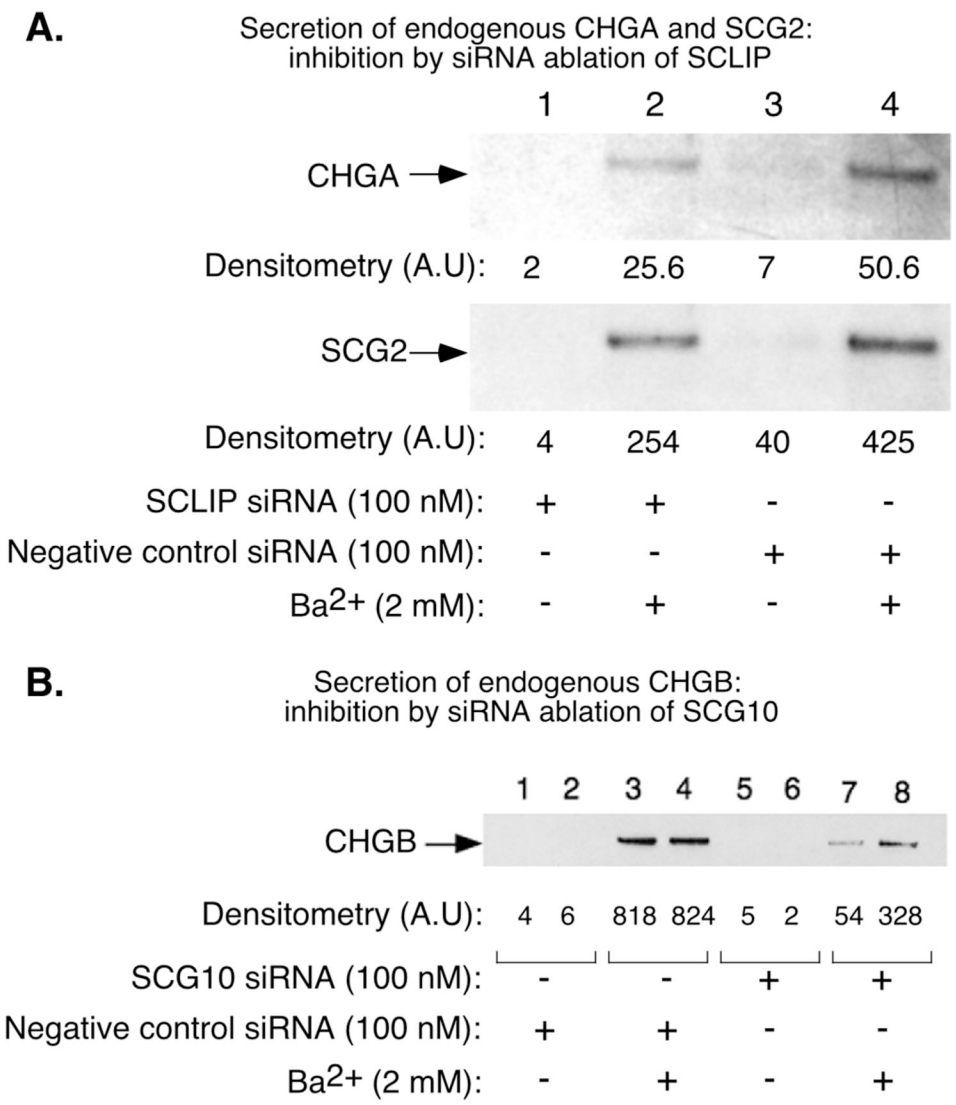

FIGURE 5.

Inhibition of secretion of endogenous CHGA, CHGB, or SCG2 in response to SCLIP or SCG10 silencing by siRNA. (A) *SCLIP* siRNA and secretion of endogenous CHGA or SCG2. PC12 cells transfected with *SCLIP* siRNA (lanes 1 and 2) or negative control siRNA (lanes 3 and 4) were stimulated with mock vs Ba^{2+} (2 mM) secretion buffer for 20 min. The secretion media were purified on SepPak C-18 columns and subjected to immunoblotting using an anti-CHGA (or anti-SCG2) antibody as described in Methods. The amounts of secreted CHGA (or SCG2) in the respective samples as shown in densitometric arbitrary units demonstrate decreased secretion of CHGA or SCG2 from *SCLIP* siRNA-transfected cells. Results of one representative experiment are shown here. (B) *SCG10* siRNA and secretion of endogenous CHGB. PC12 cells transfected with *SCG10* siRNA (lanes 5–8) or negative control siRNA (lanes 1–4) were stimulated with mock vs Ba^{2+} (2 mM) secretion buffer for 20 min. The secretion media were purified on SepPak C-18 columns and subjected to immunoblotting using an anti-CHGB antibody as described in Methods.

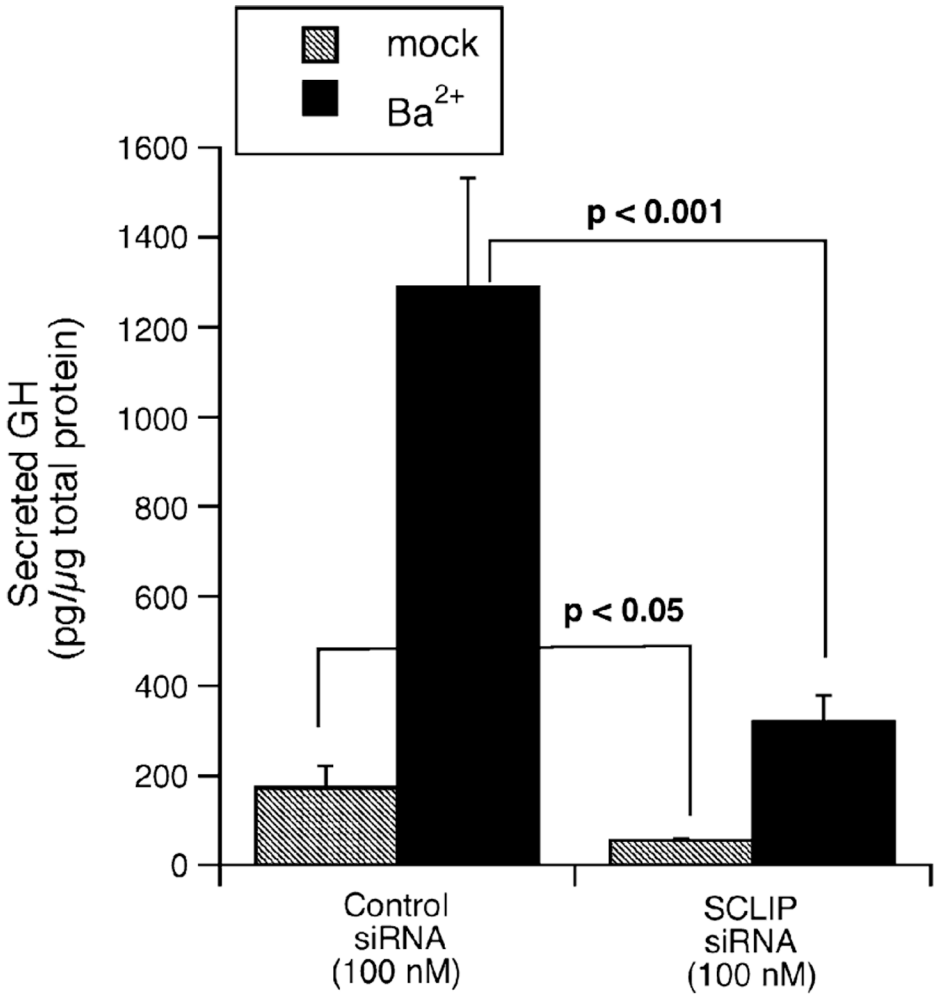

FIGURE 6.

Inhibition of secretion of growth hormone (GH) by silencing the expression of SCLIP. PC12 cells cotransfected with pXGH5 and *SCLIP* siRNA (100 nM) or Ambion silencer negative control #1 siRNA (100 nM) were treated with mock vs Ba^{2+} (2 mM) buffer for 20 min as described in Methods. Accumulation of GH in the supernatant was assayed by an ELISA (Roche Applied Bioscience) and normalized to total cell protein. Values are given as the means of quadruplicates ± the standard error of the mean and show a significant decrease in Ba^{2+}-stimulated secretion of GH from SCLIP siRNA-transfected cells.

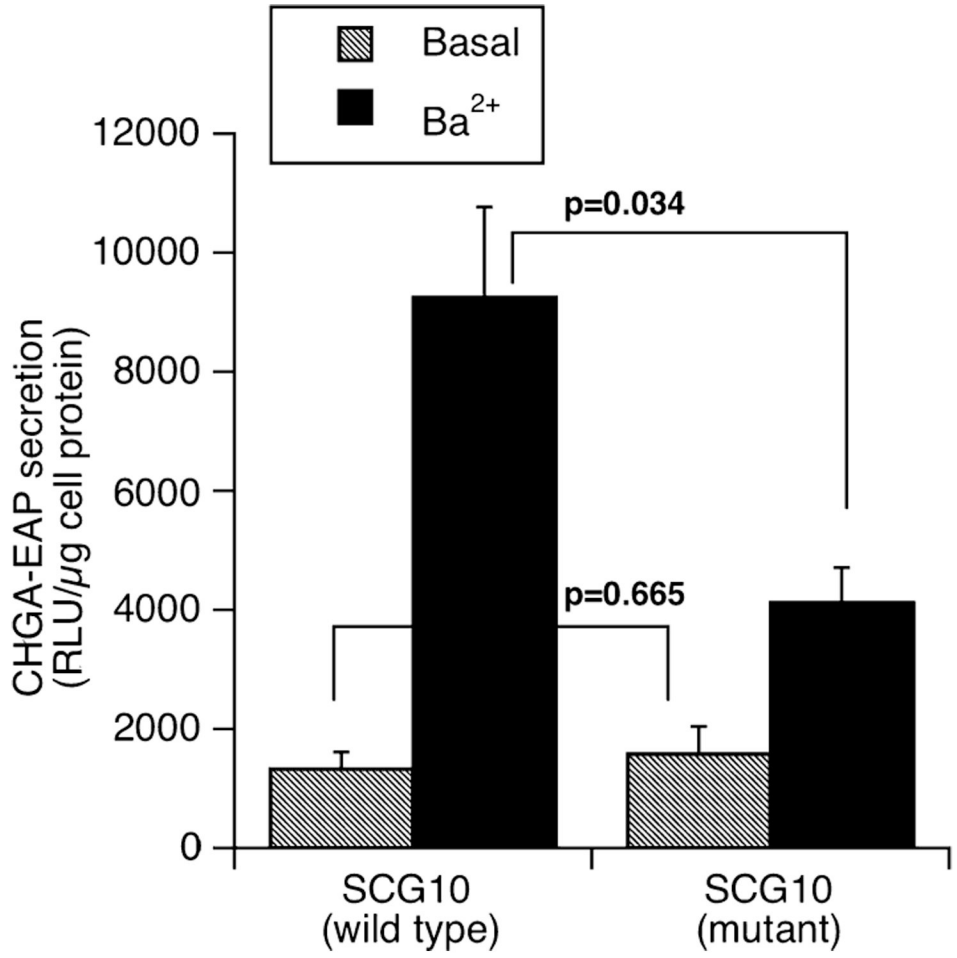

FIGURE 7.

Inhibition of regulated secretion of a CHGA–EAP chimera by overexpression of a dominant negative mutant of SCG10. PC12 cells were cotransfected with pCMV-CHGA-EAP and pSCG10-EGFP or pSCG10-Cys_{22}/Cys_{24}→Ala_{22}/Ala_{24}-EGFP, in which the palmitoylation sites, both Cys_{22} and Cys_{24} residues, were converted into Ala residues by site-directed mutagenesis. In each case, SCG10 was fused in-frame to EGFP. Seventy-two hours after transfection, cells were treated with mock or 2 mM $BaCl_2$-containing secretion buffer for 20 min as described in Methods. The enzymatic activity of the CHGA–EAP chimera in the secretion medium was estimated by chemiluminescence (Phospha-Light, Applied Biosystems). Values are expressed as relative light units (RLU) per microgram of total cell protein and determined as the means of triplicate measurements ± the standard error of the mean.

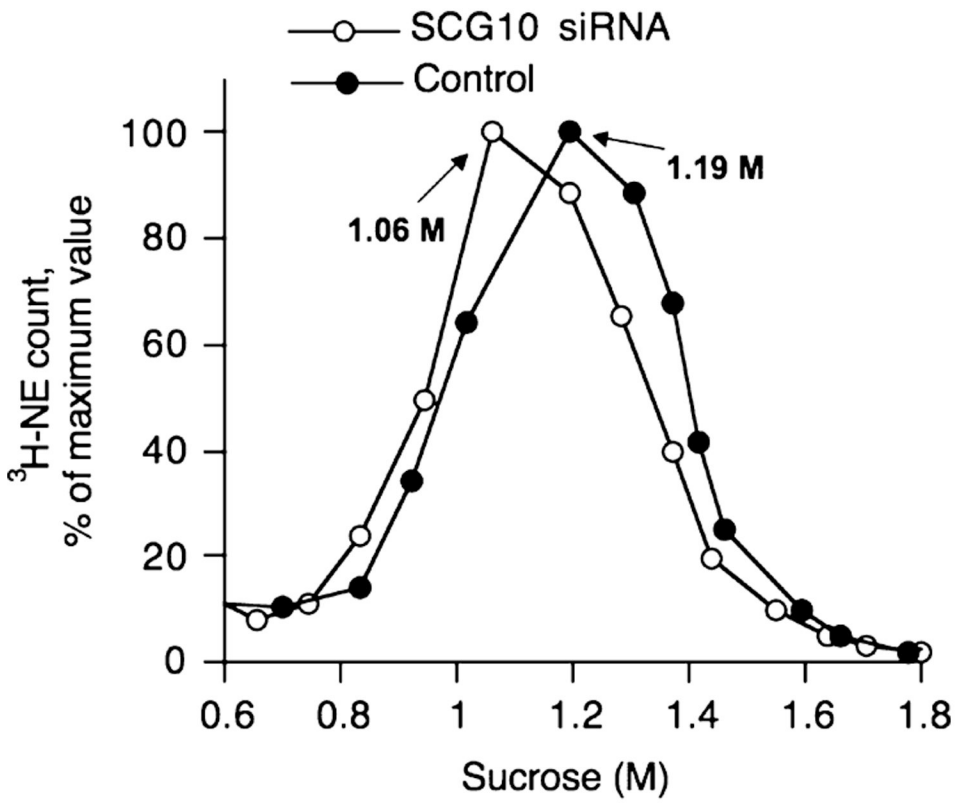

FIGURE 8.

Downregulation of SCG10 expression in PC12 cells decreases the density of chromaffin granules. Cells transfected with control (Ambion silencer negative control #1) vs *SCG10* siRNA were labeled with [^{3}H]norepinephrine for 120 min, homogenized, and centrifuged to equilibrium on sucrose density gradients. After centrifugation, the gradient fractions were assayed for [^{3}H]norepinephrine and sucrose concentration, for buoyant density. Values are given as the percent maximum of [^{3}H]norepinephrine counts vs sucrose concentrations in the gradient fractions.

Table 1

Binding Partners of CHGA in the Brain Transcriptome/Proteome[a]

Number	Sizes (bp) of (partial) cDNA inserts sequenced	Identity of cDNA clone (encoded protein)	GenBank accession number of BLAST-N match
1	350–1000	p180 (endoplasmic reticulum protein), RRBP1	AF007575
2	600	Rab-6 GTPase activating protein	AJ011679
3	550	Ser/Thr protein kinase Kp78 splice variant CTAK75a	AF159295
4	250	novel zinc finger protein	HS71L16
5	550	microtubule-associated protein 1B	NM_005909
6	600	DKFZP586A0552 protein	XM_051165
7	500	RP11-71H23 (chromosome 16, unidentified protein)	AC026475
8	650	B366024 (chromosome 4q25, unidentified protein)	AC004067
9	350	RP11-740C1 (chromosome 1, unidentified protein)	AL606752
10	500–800	chapsyn-110 (channel-associated protein of synapse)	U32376
11	225–400	humanin HN1 (secretory protein)	AY029066
12	1400	DKFZp564M1170	AL137515
13	400	CTB-105J5 (chromosome 5)	AC008600
14	850	*SLC1A3* (glutamate transporter)	XM_050270
15	450	*COPA* (cotomer protein complex, subunit α)	NM_004371
16	400	KIAA0952 protein (chromosome 20p11.23)	AL035448
17	550	FLJ13250 protein	AK023312
18	800	283E3 (chromosome 1p36.21)	AL031282
19	850	SCG10-like protein (*SCLIP*)	AF217796

[a]Proteins interacting with human CHGA were detected by screening a phage display (T7Select) human brain cDNA expression library ($1.5 \times 10\ 10^{7}$ primary cDNA clones). Results emerged after three sequential rounds of selective “biopanning” of the library phage prey against the immobilized hum human an CHGA bait, followed by washing and reamplification in *E. coli*, with ~10^{4}-fold selection/enrichment at each round. BLAST-N matches had *E* (expected per genome) values of $<10^{-9}$. A range of size inserts indicates that multiple independent clones were obtained for that binding partner.