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Chromogranin A, an "On/Off" Switch Controlling Dense-Core Secretory Granule Biogenesis

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Summary

We present evidence that regulation of dense-core secretory granule biogenesis and hormone secretion in endocrine cells is dependent on chromogranin A (CGA). Downregulation of CGA expression in a neuroendocrine cell line, PC12, by antisense RNAs led to profound loss of dense-core secretory granules, impairment of regulated secretion of a transfected prohormone, and reduction of secretory granule proteins. Transfection of bovine CGA into a CGA-deficient PC12 clone rescued the regulated secretory phenotype. Stable transfection of CGA into a CGA-deficient pituitary cell line, 6T3, lacking a regulated secretory pathway, restored regulated secretion. Overexpression of CGA induced dense-core granules, immunoreactive for CGA, in nonendocrine fibroblast CV-1 cells. We conclude that CGA is an "on/off" switch that alone is sufficient to drive dense-core secretory granule biogenesis and hormone sequestration in endocrine cells.

Introduction

Formation of dense-core secretory granules at the trans-Golgi network (TGN) is an essential step for the regulated secretion of prohormones and their processed products in endocrine and neuroendocrine cells. Prohormones synthesized at the rough endoplasmic reticulum (ER) are transported to the Golgi complex. Upon arrival at the TGN, they are sorted with high fidelity into the regulated secretory pathway. Prohormones are processed into active hormones through the immature and mature secretory granules in the regulated secretory pathway, and later secreted from mature secretory granules upon calcium influx triggered by extracellular stimulation. Thus, biogenesis of secretory granules at the TGN is a critical step to provide an intracellular vehicle for prohormones and proneuropeptides to be properly sorted, processed, and released for their physiological functions.

The biochemical mechanism controlling the initiation and regulation of dense-core secretory granule biogenesis is unknown. This process may involve one master control molecule. A member of the granin family is a plausible candidate to serve such a regulatory role for secretory granule biogenesis. The granin family of acidic glycoproteins includes chromogranin A (CGA), chromogranin B (CGB/secretogranin I), and chromogranin C (CGC/secretogranin II). They are quantitatively the major constituents of secretory granules in endocrine and neuroendocrine cells and have long been proposed to be involved as a driving force in the physical process of secretory granule formation, due to their pH- and calcium-dependent aggregation property (Fischer-Colbrie et al., 1987; Gerdes et al., 1989; Glombik and Gerdes, 2000; Hendy et al., 1995; Huttner et al., 1991; Iacangelo and Eiden, 1995; Jain et al., 2000; Ozawa and Takata, 1995; Winkler and Fischer-Colbrie, 1992; Yoo, 1995; Yoo and Lewis, 1996). Until a master molecule, if any, which can drive secretory granule biogenesis is identified, the relationship between secretory granule formation and the downstream events of hormone sequestration and regulated secretion cannot be fully understood in a cellular context.

In this study we have examined the role of chromogranins CGA and CGB, in dense-core secretory granule biogenesis. We analyzed the effect of specific depletion of either CGA or CGB, using an antisense RNA strategy, on dense-core secretory granule formation in rat pheochromocytoma (PC12) cells, a model neuroendocrine cell line. We also expressed CGA in a pituitary cell line (6T3) lacking the regulated secretory pathway and nonendocrine fibroblast cells to determine its effect on induction of dense-core secretory granule biogenesis and regulated secretion. Finally, we determined whether CGA could regulate the level of other secretory granule proteins in neuroendocrine and endocrine cells, PC12 and 6T3. These studies identified CGA as a key regulator of dense-core secretory granule biogenesis and storage of other granule proteins in endocrine cells.

Results

Downregulation of CGA Expression in PC12 Cells Leads to Reduction of Large Dense-Core Secretory Granules

To determine the role of CGA in dense-core secretory granule biogenesis in neuroendocrine PC12 cells, CGA-deficient PC12 cells were obtained by generating stable clones expressing antisense RNAs against two regions of CGA mRNA. Two antisense constructs, pcDNA3.1-CGA^{AS}-1 and pcDNA3.1-CGA^{AS}-2 (see Experimental Procedure), were cotransfected into PC12 cells (Figure 1A). Among the 47 G418-resistant CGA antisense (CGA^{AS}) clones analyzed, 5 clones showed significant reduction of CGA (CGA^{AS}-2, 5, 17, 20, and 33) (data not shown). Clone CGA^{AS}-5 showed $\leq$3% and clone CGA^{AS}-17 showed $\sim$5% expression of CGA compared to wild-type cells (WT) (Figure 1B, lanes 3 and 4). Clone CGA^{AS} -20 showed $\sim$50% reduction of CGA expression compared to wild-type cells (Figure 1B, lane 5). As a control, 50

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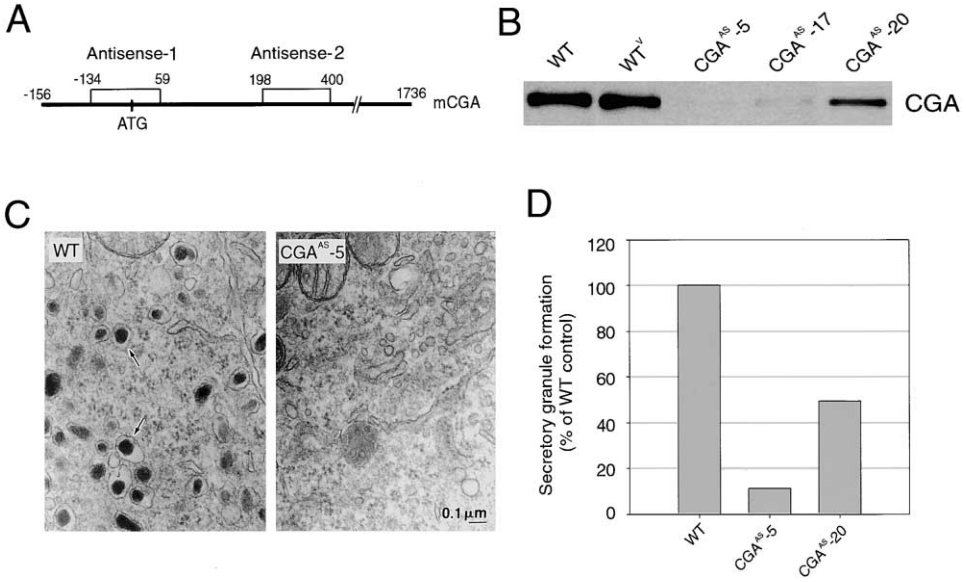

Figure 1. Analysis of CGA-Depleted PC12 cells

(A) Antisense constructs against CGA sense sequences. Two sequences were selected for antisense target regions from mouse CGA cDNA (GenBank accession number: M64278). Antisense-1 and -2 target fragments were from −134 to 59 (193 bp) and from 198 to 400 (203 bp) of CGA cDNA sequence, respectively.

(B) Western blotting analyses of CGA expression in wild-type (WT), vector-only control (WT^V) PC12 cells, CGA^{AS}-5, CGA^{AS}-17 and CGA^{AS}-20 clones.

(C) Electron micrographs of wild-type PC12 cells (left panel) and clone CGA^{AS}-5 (right panel). Dense-core secretory granules (arrows) are abundant in the wild-type PC12 cells (left panel) and very scarce in clone CGA^{AS}-5 (right panel).

(D) Percentages of the number of dense-core secretory granules in unit area (μm^2) of cytoplasm in clones CGA^{AS}-5 (11%) and CGA^{AS}-20 (49%) are represented in the bar graph in comparison with that of wild-type PC12 cells (100%). The number of dense-core secretory granules in each thin-sectioned cell was counted and divided by the area of cytoplasm (less area of nucleus).

clones of PC12 cells stably transfected with the empty vector only were selected and analyzed. None of these 50 clones showed significant reduction of CGA expression compared to wild-type PC12 cells. One example of these clones (WT^V) is shown in Figure 1B, lane 2.

If CGA plays an essential role in regulating secretory granule biogenesis, the depletion of CGA should lead to a decrease in the number of secretory granules in the CGA^{AS} clones. Analysis of large dense-core secretory granule density in wild-type PC12 cells and CGA^{AS} clones was carried out using electron microscopy and morphometry. Clone CGA^{AS}-5 and clone CGA^{AS}-20 were chosen for study to determine if there was a correlation of the amount of CGA and secretory granule biogenesis. A total of 61 wild-type, 54 CGA^{AS}-5, and 24 CGA^{AS}-20 cell sections were subjected to quantitation of dense-core secretory granules. As shown in Figure 1C, the quantity of dense-core secretory granules in the CGA^{AS}-5 clone was strikingly diminished compared to those in wild-type PC12 cells. When the number of dense-core secretory granules in the cytoplasm (less area of the nucleus) was quantitated, wild-type PC12 cells had 1.62 ± 0.11 (SEM) granules/μm^2, whereas clone CGA^{AS}-5 had 0.18 ± 0.03 (SEM) granules/μm^2, representing an ~9-fold decrease of dense-core secretory granules. Clone CGA^{AS}-20 had 0.80 ± 0.11 (SEM) granules/μm^2, representing a 2-fold reduction. In both CGA^{AS} clones, the number of secretory granules in a unit area (μm^2) was significantly reduced compared to wild-type cells (Figure 1D), and this decrease appears to be correlated with the amount of CGA in the CGA^{AS} clones shown in Figure 1B.

Regulated Secretion of POMC Is Impaired in CGA-Deficient PC12 Clones

Since CGA^{AS} clones showed a striking reduction in their ability to form dense-core secretory granules, we hypothesized that expression of a foreign secretory granule component, such as a prohormone, proopiomelanocortin (POMC), in these cells would result in its diversion from the regulated secretory pathway to the constitutive secretory pathway. To test this hypothesis, a bovine POMC construct was transfected into wild-type PC12 cells, clones CGA^{AS}-5 and CGA^{AS}-17. Secretion of POMC was assayed upon stimulation of the transfected cells with 50 mM KCl. In wild-type PC12 cells, the secretion of transfected bovine POMC was stimulated 2.81 ± 0.44 (SEM) fold by 50 mM KCl (Figures 2A and 2B). In contrast, bovine POMC expressed in clones CGA^{AS}-5 and CGA^{AS}-17 showed high basal secretion in the absence of stimulation, and showed no significant increase in secretion after stimulation with 50 mM KCl (fold stimulation = 1.05 ± 0.09 [SEM] and 1.28 ± 0.25 [SEM], respectively) (Figures 2A and 2B). A 23 kDa fragment of processed POMC was also detected, which showed a secretion pattern similar to POMC in all cases (Figure 2A, arrow). The lack of stimulated secretion and the high basal constitutive secretion of exogenously transfected POMC indicate that POMC was not packaged for secretion via the regulated secretory pathway in CGA-deficient cells

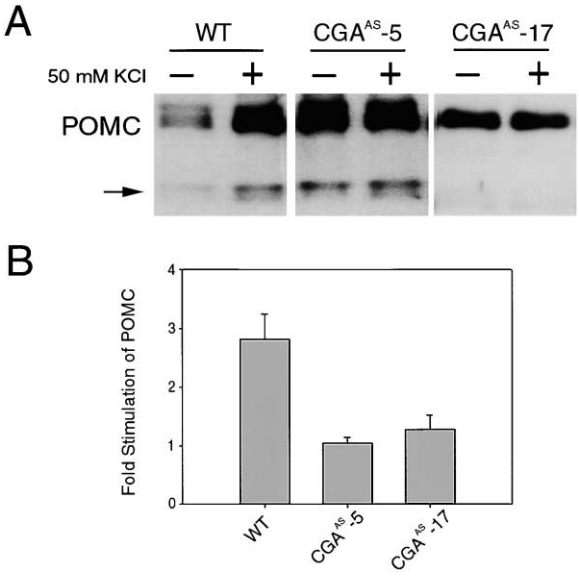

Figure 2. Analysis of Secretion of Transfected POMC from Wild-Type and CGA^{AS} PC12 Cells

(A) Bovine POMC construct was transfected into wild-type, CGA^{AS}-5, and CGA^{AS}-17 PC12 cells. The Western blot shows secretion of POMC into the medium, in the presence (+), or absence (−) of 50 mM KCl. A representative blot of at least 3 different secretion assays is shown. A 23 kDa processed form of POMC (arrow) was secreted with the same pattern as POMC, in all cases.

(B) Bar graphs show the mean ± SEM fold stimulation of POMC secretion from wild-type and CGA^{AS} PC12 cells. Fold stimulation of POMC from wild-type PC12 cells was 2.81 ± 0.44 (n = 6), and for clones CGA^{AS}-5 and CGA^{AS}-17 was 1.05 ± 0.09 (n = 3) and 1.28 ± 0.25 (n = 4), respectively.

due to their poor ability to form dense-core secretory granules.

Downregulation of CGA in PC12 Cells Profoundly Reduces Levels of Other Secretory Granule Proteins without a Corresponding Loss in Their mRNA Expression

To determine the effect of CGA depletion on the expression of other secretory granule proteins, CGB, carboxypeptidase E (CPE), and synaptotagmin in wild-type PC12 cells and CGA^{AS} clones were compared. In clone CGA^{AS}-5, CGB expression was almost completely (≤3% of wild-type control) downregulated relative to wild-type PC12 cells and a clone with the vector only (WT^{V}) (Figure 3A, lane 3). Similarly, CGB levels were reduced to ~55% and to ~72% of wild-type cells in clones CGA^{AS}-17 and CGA^{AS}-20, respectively (Figure 3A, lanes 4 and 5). Another secretory granule protein, CPE, was reduced in clones CGA^{AS}-5, CGA^{AS}-17, and CGA^{AS}-20 to ~49%, ~30% , and ~72% of wild-type, respectively (Figure 3A). Finally, we examined synaptotagmin as an example of membrane-bound proteins in secretory granules and found that it was significantly reduced to ≤5%, ~34%, and ~64%, relative to wild-type cells, in clones CGA^{AS}-5, CGA^{AS}-17, and CGA^{AS}-20, respectively (Figure 3A). To determine if CGA depletion only affects secretory granule proteins, expression of two coat proteins, ADP-ribosylation factor 6 (ARF6), a small GTPase attached to secretory granules at the cytoplasmic face (Galas et al., 1997), and β-COP, a coatamer protein present in the ER and Golgi, was analyzed. Neither ARF6 nor β-COP was significantly reduced in any CGA^{AS} clones compared to wild-type PC12 cells (Figure 3A). Thus, depletion of CGA in PC12 cells leads to a proportional reduction of secretory granule proteins such as CGB, CPE, and synaptotagmin, but not other nongranule proteins, such as ARF6 and β-COP.

To investigate whether the reduction of other secretory granule protein levels in CGA^{AS} clones is a transcriptional or posttranscriptional event, we amplified CGB and CPE mRNAs from clone CGA^{AS}-5 using specific primer sets. Quantitative RT-PCR results showed that CGB and CPE mRNAs in clone CGA^{AS}-5 were only 2.09- and 1.60-fold less, respectively, than wild-type PC12 cell mRNA levels, indicating that transcription of these genes in the CGA-depleted clones was not significantly impaired.

Metabolic labeling with [^{35}S]methionine of clone CGA^{AS}-5 for 2 and 5 min, followed by CGB immunoprecipitation, showed that the initial rate of CGB biosynthesis in both clone CGA^{AS}-5 and wild-type PC12 cells was very similar (Figure 3B). This result indicates intact translation of CGB in clone CGA^{AS}-5 at 2 and 5 min.

Pulse-chase experiments indicated a faster rate of disappearance of newly synthesized CGB in CGA^{AS}-5 cells versus wild-type control cells (data not shown). To test the possibility that disappearance of CGB may be due to rapid secretion of granule proteins from CGA^{AS}-5 cells, the amount of CGB secreted into the medium over a 1 hr period without stimulation (basal secretion) was assayed. Western blot analysis of CGB in the media collected after 1 hr incubation showed secretion of CGB from wild-type PC12 cells. In contrast, the medium from CGA^{AS}-5 cells showed undetectable amounts of CGB (Figure 3C), indicating no significant secretion of CGB. We therefore tested for possible degradation of granule proteins in acidic compartment(s) to account for the disappearance of granule proteins in CGA^{AS}-5 cells. CGA^{AS}-5 cells treated with 10 mM NH_4Cl for 3 hr showed a significant increase of CGB, CPE, and synaptotagmin levels compared to those in untreated CGA^{AS}-5 cells (Figure 3D). Therefore, we conclude that downregulation of CGA in PC12 cells leads to enhanced posttranslational degradation of other endogenous secretory granule proteins.

Expression of Bovine CGA in Clone CGA^{AS}-5 Rescues Secretory Granule Formation and Secretory Protein Levels

The preservation of gene expression of neuroendocrine cell proteins (Figure 3) strongly supports the contention that secretory granule loss and decreased secretory granule protein levels are a specific consequence of downregulation of CGA in PC12 cells in which CGA antisense mRNA is expressed. This is in contrast to nongranule expressing PC12 cell variants PC12-27 (Corradi et al., 1996) and A35C (Pance et al., 1999) in which the PC12 cell transcriptome is profoundly altered, and in particular the transcription of a variety of secretory granule proteins is severely downregulated, indicating that these variants are in fact no longer neuroendocrine cells

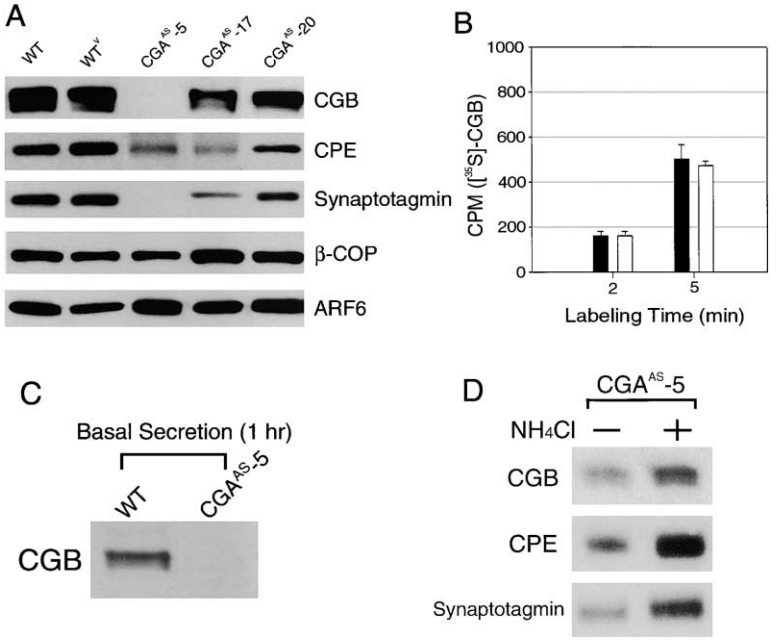

Figure 3. Synthesis of Secretory Granule Proteins in CGA^{AS} PC12 Cells

(A) Western blots of secretory granule proteins (CGB, CPE, and synaptotagmin) and nongranule proteins (ARF6 and β-COP) in wild-type PC12 cells and CGA^{AS} clones.

(B) Metabolic labeling followed by CGB immunoprecipitation. Wild-type (black bar) and CGA^{AS}-5 (white bar) PC12 cells were incubated with [^{35}S]methionine for 2 (n = 3) and 5 (n = 3) min and immunoprecipitated with a goat anti-CGB antibody. CPM values on the graph were obtained after normalization against total protein concentration. Error bars show the SEM.

(C) Basal secretion (1 hr in DMEM/0.01% BSA) of CGB from wild-type and CGA^{AS}-5 PC12 cells. No detectable amount of CGB is present in the medium from CGA^{AS}-5 cells.

(D) Western blots of secretory granule proteins (CGB, CPE, and synaptotagmin) from CGA^{AS}-5 cells with or without NH_4Cl treatment. After 3 hr incubation with 10 mM NH_4Cl, the levels of CGB, CPE, and synaptotagmin were significantly increased compared to untreated CGA^{AS}-5 cells. The blot is a representative of three experiments.

per se. To further substantiate that the effect of CGA antisense transfection was indeed wholly attributable to loss of CGA expression in CGA^{AS} PC12 cells, we attempted to rescue the secretory granule phenotype lost from clone CGA^{AS}-5 by transfecting a bovine CGA cDNA. Bovine CGA was chosen because its mRNA is sufficiently divergent from the CGA antisense RNA sequences used for generation of CGA^{AS} clones that it would not be likely to be a target for CGA antisense RNAs. Bovine CGA (bCGA) plasmid (pcDNA3.1-bCGA) transiently transfected into clone CGA^{AS}-5 showed a transfection efficiency of <10%. Cells expressing bCGA were determined using a guinea pig polyclonal antibody raised against purified bovine CGA. The guinea pig anti-bCGA antibody detects only bovine CGA and does not cross-react with CGA from other species, including rat (data not shown). Double-label immunocytochemical analysis showed that cells that were positive for bCGA (guinea pig anti-bCGA antibody) had a punctate staining pattern throughout the cell body and in processes (Figures 4A, 4D, 4G and 4J; arrows), characteristic of secretory granule localization. This immunostaining pattern was very similar to that of endogenous CGA in wild-type PC12 cells detected by a rabbit anti-CGA antibody (Figures 4M and N). Little endogenous CGA staining by a rabbit anti-CGA polyclonal antibody was evident in clone CGA^{AS}-5 (Figure 4O). The bCGA-positive CGA^{AS}-5 cells were also intensely immunostained for endogenous CGB (Figures 4B and 4E) and CPE (Figures 4H and 4K), indicating restoration of CGB and CPE levels. In contrast, untransfected bCGA-negative CGA^{AS}-5 cells in the same field showed a low intensity staining for these secretory granule proteins (Figures 4B and 4H; arrowheads) due to their degradation (see Figure 3). The composite images confirmed that cells with intense staining for CGB or CPE were also immunopositive for bCGA, and these proteins were therefore colocalized in the same cells (Figures 4C and 4I). Images at higher magnification showed colocalization of CGB and CPE with bCGA in the same granules (Figures 4F and 4L; arrows). No immunostaining of bCGA was observed in CGA^{AS}-5 cells transfected with the empty vector alone (data not shown). To verify that the bCGA punctate immunostaining represents localization in dense-core secretory granules, EM-immunocytochemistry was carried out on bCGA-expressing CGA^{AS}-5 cells with the anti-bCGA guinea pig antibody. The presence of immunoreactive bCGA in large dense-core secretory granules is shown in Figure 4P. In addition, stimulated secretion of bCGA with 50 mM KCl and 2 mM $BaCl_2$ further confirmed that the punctate staining in rescued CGA^{AS}-5 clone represents bCGA in dense-core secretory granules (Figure 4Q). Without stimulation, 3.47 ± 0.98% (SEM; n = 3) of total bCGA was released (Figure 4Q). With stimulation, 26.3 ± 2.32% (SEM; n = 3) of total bCGA was secreted. Thus, transfection of bCGA into the CGA^{AS}-5 clone was able to rescue the wild-type phenotype, in that secretory granule biogenesis, regulated secretion, and levels of the secretory granule proteins CGB and CPE were restored.

Depletion of CGB in PC12 Cells Has No Effect on Dense-Core Secretory Granule Biogenesis

Since CGB shares structural and biochemical features with CGA (Benedum et al., 1987; Gorr et al., 1989; Winkler and Fischer-Colbrie, 1992; Yoo, 1995), it is possible that CGB can also function in a similar manner as CGA in regulating dense-core secretory granule biogenesis. To address this question, we generated CGB antisense PC12 clones using antisense RNAs against two different target regions of CGB full-length mRNA (Figure 5A). Among clones stably expressing CGB antisense RNAs, clone CGB^{AS}-7.4 showed a substantial reduction (~65%) of CGB and a much smaller reduction (~25%) of CGA (Figure 5B). The number of dense-core secretory granules (0.66 ± 0.04 [SEM] granules/μm^2; n = 112) in CGB^{AS}-

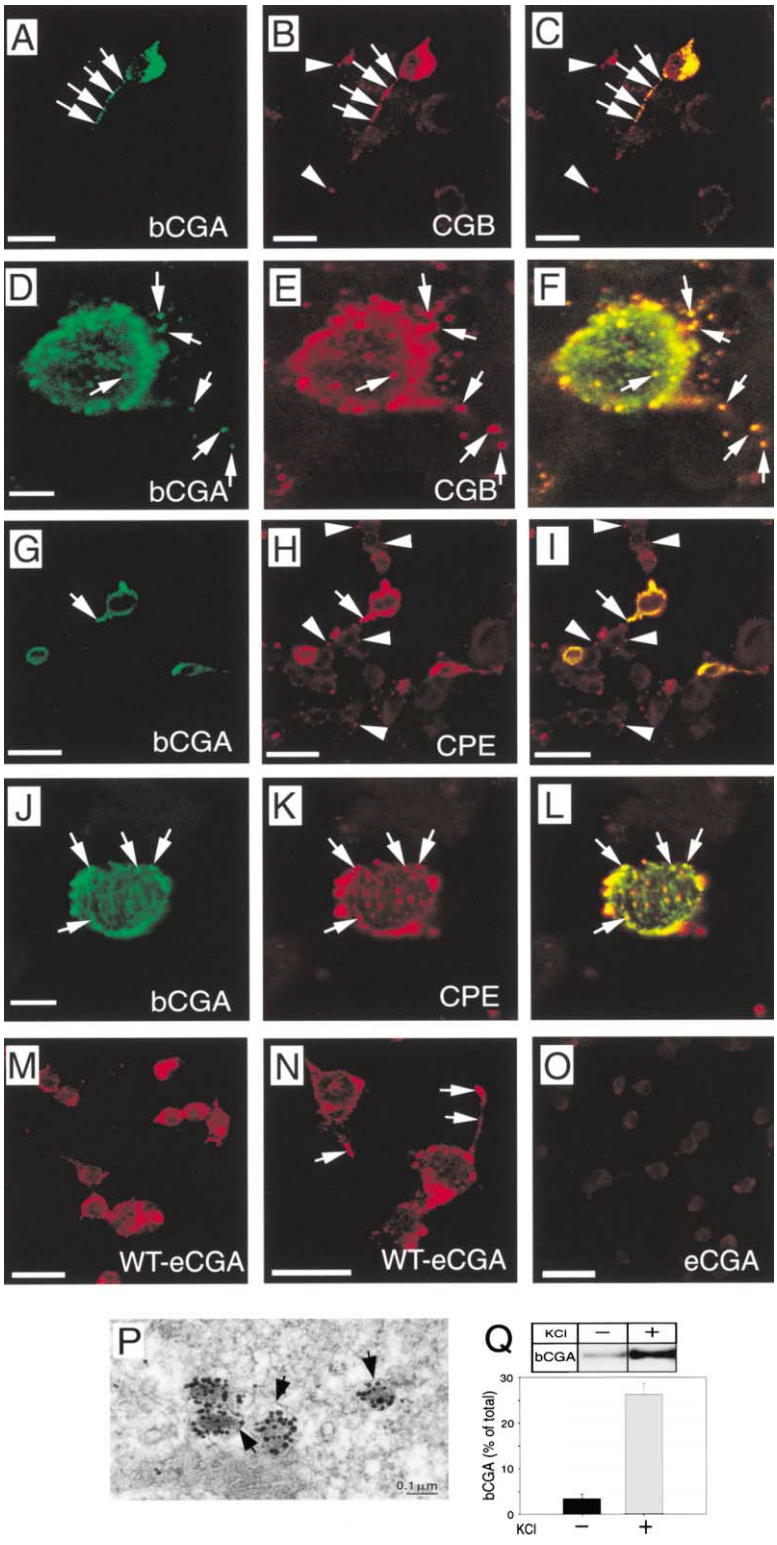

Figure 4. Rescue of the Regulated Secretory Phenotype by Expressing Bovine CGA in CGA^{AS}-5 Clone

(A–L) Double-label immunocytochemical analyses of bovine CGA (FITC; A, D, G and J) and endogenous CGB (Cy3; B, E) or endogenous CPE (Cy3; H, K) in bCGA-transfected CGA^{AS}-5 cells are shown. Composite images of bCGA and CGB immunostaining in C and F, and bCGA and CPE in I and L. Note the punctate staining of bCGA in the cell process of a bCGA-positive CGA^{AS}-5 cell indicated by the arrows. Untransfected, bCGA-negative CGA^{AS}-5 cells in the same field showed a low-intensity punctate staining for these secretory granule proteins (Figures 4B and 4H; arrowheads).

(M–O) Endogenous rat CGA (WT-eCGA; Cy3) detected by a rabbit anti-CGA antibody is observed in wild-type PC12 cells (M, N), but little immunostaining is present in CGA^{AS}-5 cells (eCGA; O). Punctate staining of endogenous CGA is evident along the cell process and the tips in wild-type PC12 cells (arrows; N), but not in CGA^{AS}-5 cells (O). (Scale bars: 5 μm for D–F and J–L; 10 μm for A–C; 20 μm for G–I and M–O).

(P) Immuno-EM analysis of bCGA-transfected CGA^{AS}-5 clone showed localization of bCGA in membrane-bound dense-core secretory granules (arrows).

(Q) Secretion assay on bCGA after transient transfection of bCGA plasmid into CGA^{AS}-5 cells. Western blot and the bar graph show that bCGA was stimulated 7.6-fold compared to basal secretion.

7.4 showed an ~35% reduction when compared to wild-type PC12 cells (1.02 ± 0.05 [SEM] granules/μm^2; n = 91). This reduction in dense-core secretory granules was correlated more closely with the levels of CGA in this clone than to the level of CGB (Figure 5C). Similar results were also obtained in another clone (CGB^{AS}-10.1; Figure 5C). Thus, unlike CGA, the significant depletion of CGB in CGB^{AS} PC12 cells was not matched by a similar decrease of secretory granule formation. In addition, in contrast to CGA-depleted clones, expression of synaptotagmin was not significantly changed in CGB-depleted clones CGB^{AS}-7.4 and CGB^{AS}-10.1 (Figure 5B).

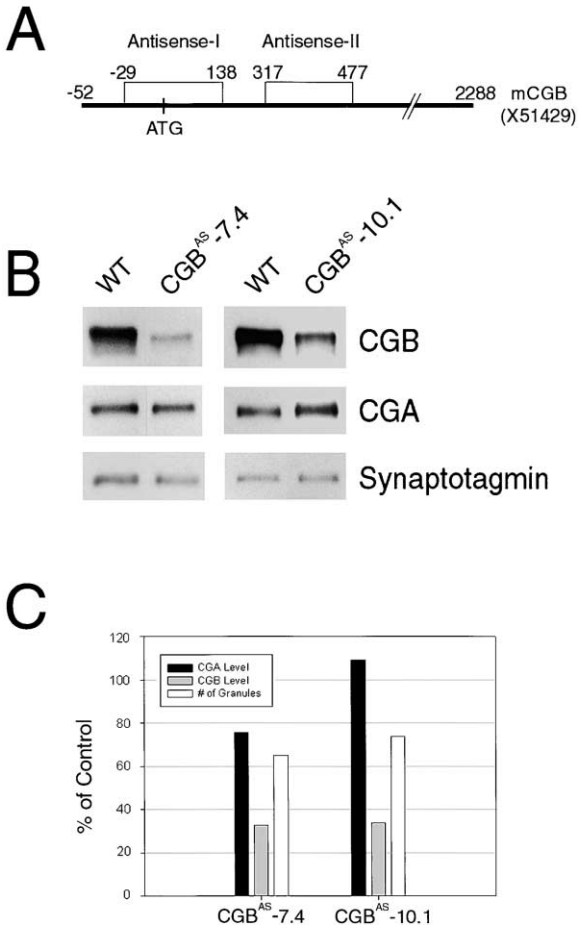

Figure 5. Analysis of CGB^{AS} PC12 Cells

(A) Two CGB antisense RNA target regions were used for stable CGB^{AS} clones. Antisense I and II were amplified using primer sets obtained from the sequence of mouse CGB (GenBank accession no. X51429). (B) Western blotting analysis of CGA, CGB, and synaptotagmin from wild-type (WT), clone CGB^{AS}-7.4, and clone CGB^{AS}-10.1 PC12 cells. (C) CGA levels in clones CGB^{AS}-7.4 (76%) and CGB^{AS}-10.1 (109%), and CGB levels in clones CGB^{AS}-7.4 (33%) and CGB^{AS}-10.1 (34%) were compared with percentages of the number of dense-core secretory granules in clones CGB^{AS}-7.4 (65%) and CGB^{AS}-10.1 (74%). All numbers were expressed as percentages of wild-type control.

Expression of CGA Rescues Regulated Secretion of POMC in 6T3 Cells Lacking the Regulated Secretory Pathway

To complement the "knockout" experiments in PC12 cells, we tested whether CGA transfected into 6T3 cells, a variant AtT-20 pituitary endocrine cell line, could rescue regulated secretion in these cells. 6T3 cells lack dense-core secretory granules, a regulated secretory pathway, and expression of secretory granule content proteins, including CGA and CGB (Matsuuchi and Kelly, 1991). 6T3 cells were stably transfected with bovine CGA cDNA. Restoration of the regulated secretory pathway was determined biochemically by secretion assays. After mock transfection, CGA was not detected in the cells and media with or without 50 mM KCl stimulation (data not shown), confirming that this cell line lacks endogenous expression of CGA (Matsuuchi and Kelly, 1991). When bovine POMC was transiently transfected into these cells (6T3-bCGA), it was secreted in a nonregulated manner, showing high basal secretion levels and no stimulated secretion with 50 mM KCl (Figure 6B). This confirms that 6T3 cells lack a regulated secretory pathway, as reported by Matsuuchi and Kelly (1991). However, when CGA was stably transfected into 6T3 cells (6T3-bCGA), CGA secretion was significantly stimulated by 50 mM KCl, while basal secretion was low (Figure 6A), indicating that CGA expression in these mutant endocrine cells was able to restore the regulated secretory pathway. Similarly, regulated secretion of exogenous POMC was evident after stimulation of CGA-transfected 6T3 cells (Figures 6B and 6C). Wild-type 6T3 cells transfected with POMC showed no stimulation with 50 mM KCl (fold stimulation = 0.9 ± 0.08 [SEM], n = 4), whereas the secretion of POMC in 6T3-bCGA was significantly stimulated. The fold stimulation was 2.11 ± 0.19 (SEM; n = 4, $p < 0.001$ versus wild-type control), similar to the wild-type AtT-20 cells (Castro et al., 1989; Heisler and Reisine, 1984) (Figure 6C). Thus, CGA alone was sufficient to rescue regulated secretion in a CGA-deficient mutant corticotroph endocrine cell line lacking the regulated secretory pathway.

We also tested possible involvement of CGB in controlling secretory granule biogenesis by transfecting a bovine CGB construct into 6T3 wild-type cells. Interestingly, we could not obtain any detectable levels of CGB (Figure 6D, left panel) in these transfected cells, whereas there was robust expression of CGB in COS7 cells using the same construct (data not shown). To verify whether this was due to a rapid degradation of CGB in 6T3 cells, these transfected 6T3 cells were treated with 10 mM NH_4Cl for 90 min, 24 hr posttransfection. NH_4Cl-treated 6T3 cells showed significant amounts of CGB immunoreactivity, whereas untreated cells did not (Figure 6D). These data indicate that transfected CGB is degraded in 6T3 cells that lack CGA, and that CGB does not play the same role as CGA in driving secretory granule biogenesis in these cells.

Overexpression of CGA Induces Large Dense-Core Granule Formation In Fibroblasts

To further determine the ability of CGA to ultimately induce dense-core secretory granule biogenesis across cell lineages, we examined the effect of exogenous CGA expression in a nonneuroendocrine fibroblast cell line, CV-1, which contains no CGA or dense-core secretory granules. As shown in Figure 7, expression of CGA at moderate levels resulted in the appearance of dense-core granules in CV-1 cells (Figure 7B, arrows; Figures 7C–7E). These dense-core granules varied in size from around 80 to 200 nm, similar to that of large dense-core secretory granules in PC12 cells. Immuno-EM analysis of the CGA-transfected CV-1 cells revealed several dense-core granules near the Golgi complex stained specifically for CGA, indicating that CGA was packaged into membrane-bound structures at the Golgi complex in these cells (Figure 7F). CGA-positive dense-core granules were also present throughout the cytoplasm, examples of which are shown in Figures 7B (arrows), 7G, and 7H. Table 1 indicates the relative number of dense-core granules formed in CV-1 cells transfected with or without

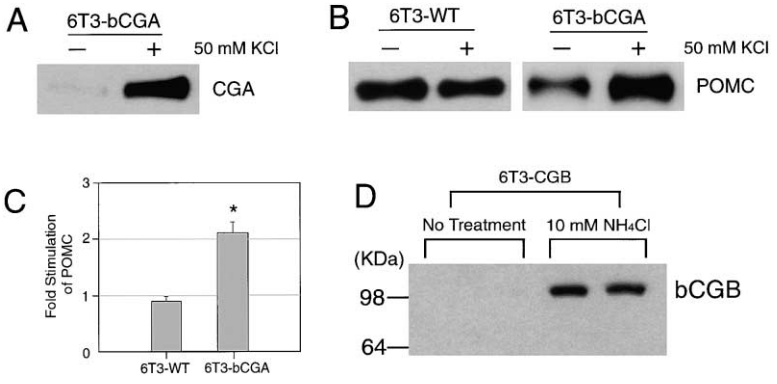

Figure 6. Restoration of Regulated Secretion of POMC by CGA Expression in CGA-Deficient 6T3 Endocrine Cells

(A) Western blot of release medium in the presence of 50 mM KCl in 6T3 cells stably transfected with CGA (6T3-bCGA) shows that CGA secretion was significantly stimulated.

(B) Western blots of release media from wild-type (6T3-WT) and CGA transfected 6T3 cells (6T3-bCGA). In 6T3-WT cells, transfected bovine POMC was secreted at high basal levels, and no stimulation was detected with 50 mM KCl. In 6T3-bCGA cells, stimulated secretion of exogenous POMC was restored.

(C) Bar graphs show the mean ± SEM fold stimulation of POMC secretion from 6T3-WT and 6T3-bCGA. Fold stimulation of POMC was 0.90 ± 0.08 (n = 4) for 6T3-WT and 2.11 ± 0.19 (n = 4) for 6T3-bCGA. (*: $p<0.001$)

(D) Degradation of transiently transfected CGB (6T3-CGB) was rescued by treatment with NH_4Cl. 6T3 cells after transfection with bovine CGB construct did not show any detectable CGB immunoreactivity without NH_4Cl treatment. Treatment with 10 mM NH_4Cl (90 min) restored the level of expressed CGB. Results are shown in duplicates.

CGA. Cells containing 1–3 dense-core granules were considered as background, and their percentages were higher in the CGA-transfected CV-1 cells (14.2%) than the mock-transfected cells (6.1%). Most significantly, the percentage of cells containing more than 3 dense-core granules after mock transfection was extremely low (0.7%). This percentage was increased to 15.6% in CGA-transfected CV-1 cells. These results show that CGA expression alone was able to generate dense-core granules in a nonendocrine cell line.

Discussion

CGA Controls Secretory Granule Biogenesis and Regulated Secretion of Hormones

Granins have long been proposed to be involved in dense-core secretory granule formation in endocrine and neuroendocrine cells. In the present study, we have provided pivotal evidence that dense-core secretory granule biogenesis is controlled by CGA, and that in the absence of CGA-driven granule biogenesis, expression/storage of other granule components is diminished in endocrine cells.

Depletion of CGA expression in neuroendocrine PC12 cells by antisense RNA led to (1) a profound loss of dense-core secretory granules with the amount of decrease being proportional to the level of CGA; (2) the obliteration of regulated secretion of POMC; and (3) the lack of storage of other secretory granule proteins such as CGB, CPE, and synaptotagmin (see Table 2). Transfection of bovine CGA into these CGA-depleted PC12 cells rescued dense-core secretory granule biogenesis and secretory granule protein levels. Transfection of CGA in a variant AtT-20 endocrine cell line 6T3, lacking

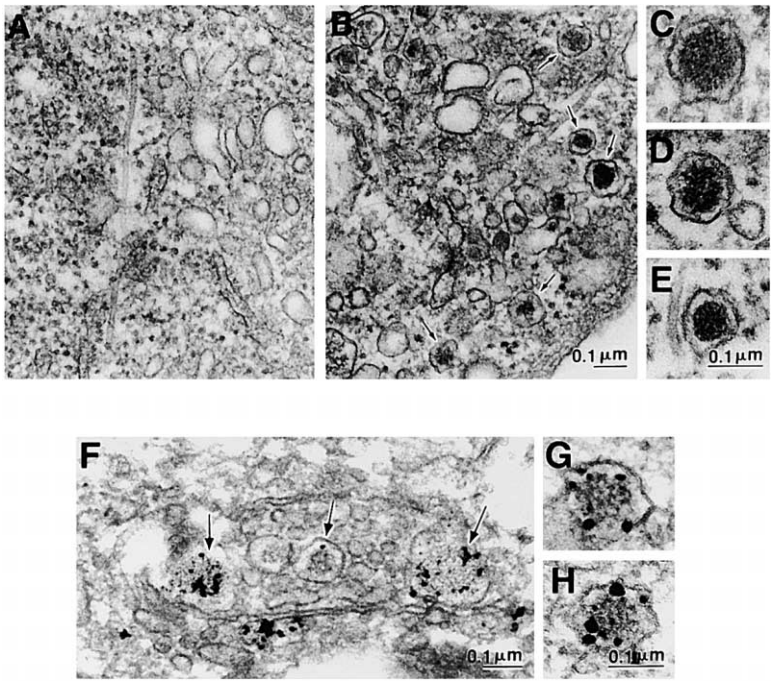

Figure 7. Electron Microscopical Appearance of CV-1 Cells Transfected with the CGA Expression Plasmid

(A) CV-1 cells were transfected with pGEM2 and infected with vaccinia virus alone (mock transfection). No apparent dense-core granule was detected.

(B) CV-1 cells were transfected with pGEM2 containing the CGA open reading frame. Arrows indicate granules containing an electron-dense core.

(C–E) Enlarged images of dense-core granules in CGA-expressing CV-1 cells.

(F) Immuno-gold labeling of CGA in CGA-expressing CV-1 cells. The Golgi complexes are positive in CGA-transfected CV-1 cells. The silver enhanced immunograins are distinctly contained in membrane-bound vesicles (arrows).

(G and H) Enlarged images of dense-core granules exhibiting immunogold labeling of CGA.

Table 1. Relative Number of Large Dense-Core Granules in CGA-Transfected CV-1 Fibroblasts Compared to Mock Transfected Cells

	Mock Transfection	CGA Transfection
# Cells with dense-core granules*/Total # cells examined	1/148	22/141
% Cells with 4–6 dense-core granules (# of cells)	0.7% (1/148)	2.8% (4/141)
% Cells with >7 dense-core granules (# of cells)	0% (0/148)	12.8% (18/141)

* Cell profiles containing more than 3 dense-core granules were counted as unambiguously positive in double-blind rating. Those containing 3 or fewer secretory granule-like profiles (6.1% [9/148] for mock transfection and 14.2% [20/141] for CGA-transfected cells) were not included in the results above.

CGA and the regulated secretory pathway, restored regulated secretion of exogenously introduced POMC in these cells. Furthermore, CGA transfected into nonendocrine CV-1 cells induced the formation of large dense-core granules. Thus, our data demonstrate that CGA is a regulatory switch, and its presence and absence can turn on and off, respectively, secretory granule formation in endocrine cells. This switch also controls regulated secretion and expression/storage of other secretory granule proteins.

Interestingly, although the expression level of CGB is higher than CGA in PC12 cells (Fischer-Colbrie and Schober, 1987), and despite the similar biochemical and structural properties shared with CGA (Benedum et al., 1987; Gorr et al., 1989; Winkler and Fischer-Colbrie, 1992; Yoo, 1995), CGB, when downregulated by antisense RNA, did not significantly affect dense-core secretory granule biogenesis in these cells. Furthermore, transfection of bCGB into 6T3 cells (Figure 6D) and PC12 CGA^{AS}-5 cells (unpublished data) lacking CGA did not rescue secretory granule biogenesis in these cells, but instead CGB was rapidly degraded. A lack of effect of CGB on secretory granule formation in PC12 cells was also observed by Corradi et al. (1996). Exogenously introduced human CGB in a mutant PC12 clone that lacks secretory granules was not able to rescue the regulated secretory pathway and secretory granule biogenesis. Thus, we conclude that another granin, CGB, does not play a direct role in inducing dense-core secretory granule biogenesis and does not substitute for such a role of CGA in PC12 and 6T3 cells.

Our data demonstrate a direct and specific correlation of the reduction of secretory granule proteins such as CGB, CPE, and synaptotagmin with downregulation of expression of CGA in PC12 CGA^{AS} clones (see Table 2), whereas expression of other nonsecretory proteins such as ARF6 and β-COP was unaffected. Quantitative RT-PCR and degradation analyses of CGB in CGA-depleted PC12 cells indicate that the regulation of these secretory granule protein levels by CGA is at the posttranslational level through degradation. Indeed, the ability of one granule constituent to markedly affect the expression of others at the posttranslational level has also been observed in mast cells in vivo. Transgenic mice lacking fully sulphated heparin, a major constituent of the secretory granules of mast cells, were shown to have a loss of metachromatic granules and exhibited a failure to store other granule content proteins (Humphries et al., 1999).

A correlation between CGA expression and large dense-core secretory granule biogenesis has been observed in whole animals in vivo. Estradiol treatment of castrated rats resulted in the complete elimination of both CGA expression and large dense-core secretory granule formation in pituitary gonadotropes, while testosterone treatment of these animals restored CGA expression and large dense-core granules to wild-type levels in these cells (Watanabe et al., 1998). Given our specific genetic CGA "knockout" and "knockin" data from endocrine cells, the changes in large dense-core secretory granule formation with different steroid treatments in this animal model are likely causally attributed to the up- and downregulation of expression of the molecular switch, CGA, that controls dense-core secretory granule biogenesis. Indeed, the CGA gene contains an estrogen response element (Iacangelo et al., 1991; Wu et al., 1991), and CGA mRNA is regulated by several hormones and second messengers (Iacangelo and Eiden, 1995).

In conclusion, we have identified an on/off switch, CGA, that controls dense-core secretory granule biogenesis in endocrine cells. The synthesis of CGA itself can be regulated by hormones. Thus, CGA serves an important higher-order physiological role in controlling hormone secretion through regulating secretory granule biogenesis in endocrine and neuroendocrine cells.

Table 2. Comparison of CGA Expression Level, Secretory Granule Protein Expression, POMC Secretion, and Secretory Granule Number among Wild-Type and CGA^{AS} PC12 Cells

	% of Wild-Type			
	PC12 Wild-Type	CGA^{AS}-5	CGA^{AS}-17	CGA^{AS}-20
CGA level	100	≤3	5	50
CGB level	100	≤3	55	72
CPE level	100	49	30	72
Synaptotagmin level	100	5	34	64
Regulated secretion of POMC (Fold stimulation)	2.81 ± 0.44	1.05 ± 0.09	1.28 ± 0.25	ND
# Secretory granules	100	11	ND	49

ND = not determined

Experimental Procedures

Cell Culture

PC12 cells obtained from ATCC were grown in DMEM supplemented with 7.5% fetal bovine serum (FBS; Life Technologies), 7.5% donor horse serum (Quality Biological), and penicillin/streptomycin (Life Technologies). PC12 cells were grown on plates coated with type I rat tail collagen (100 μg/ml; Becton Dickinson). Monkey kidney fibroblast CV-1 cells were grown on collagen-coated dishes in DMEM supplemented with 10% FBS and antibiotics. A variant AtT-20 cell line, 6T3 (Matsuuchi and Kelly, 1991), was obtained from L. Matsuuchi (University of British Columbia, Canada) and grown in 10% FBS/DMEM supplemented with antibiotics.

Plasmid Constructs and Transfection

Two antisense sequences targeting 5′-UTR and the start codon (antisense-1: -134→33) and the middle of the coding region (antisense-2: 194→363) of mouse CGA cDNA (Wu et al., 1991) were designed. Messenger RNA of AtT-20 cells grown in 10% FBS in DMEM was purified using an mRNA isolation kit (Boehringer Mannheim). Two sets of primers (all 25 bp) flanked with XhoI (5′) and NheI (3′) sites were utilized for RT-PCR (Titan One tube RT-PCR kit, Roche). Two amplified PCR fragments (antisense-1 and antisense-2, Figure 1A) were subcloned into pcDNA3.1 plasmid (Invitrogen) digested with XhoI and NheI. Both antisense constructs (pcDNA3.1-CGA^{AS}-1 and pcDNA3.1-CGA^{AS}-2) were cotransfected into PC12 cells using LipofectAMINE 2000 reagent (Life Technologies). Briefly, 17.5 μg per each plasmid was mixed with 56 μl of LipofectAMINE 2000 in Opti-MEM (Life Technologies) and added to PC12 cells grown in 100 mm culture plate. Stable CGA antisense (CGA^{AS}) clones were selected (1 mg/ml of G418; Life Technologies) and analyzed for expression of CGA (total 47 clones). Stable CGA^{AS} clones were maintained in 500 μg/ml of G418. For a negative control, pcDNA3.1 (no insert) was transfected into PC12 cells and selected as above. For CGB antisense clones, two antisense target sequences were chosen (antisense-I: -29→138; antisense-II: 317→477, based on mouse CGB full-length mRNA sequence [GenBank X51429]). Both antisense sequences were also flanked with XhoI (5′) and NheI (3′) sites and subcloned into pcDNA3.1-neo as above. Transfection and selection procedure for stable CGB antisense clones were the same as for CGA antisense clones.

For generation of bovine CGA-expressing CGA^{AS} cells, a plasmid containing a full-length cDNA of bovine CGA (pcDNA3.1-bCGA) was transiently transfected into CGA^{AS}-5 clone as described above. For generation of CGA-expressing 6T3 stable clones, pcDNA3.1-zeo plasmid (Invitrogen) containing a full-length cDNA of bovine CGA (pcDNA3.1-bCGA-zeo) was transfected as above and selected with Zeocin (500 μg/ml; Invitrogen). A full-length bovine CGB cDNA was subcloned into pcDNA3.1 and transfected as described above.

Western Blotting Analysis

Cells were washed with 1× PBS and lysed in 20 mM Tris (pH 7.5)/1% TX-100/1 mM EDTA/1 mM DTT/protease inhibitor cocktail (Complete, Roche) and protein concentration was determined (Dc protein assay kit, Bio-Rad). Ten μg of total protein lysate of each sample was loaded onto 8%–16% or 4%–12% polyacrylamide precast gels after 10 min of boiling and transferred on nitrocellulose membrane. Equal loading was confirmed by staining blots with 0.02% Ponceau S (Sigma). Blots were blocked in 5% nonfat milk in PBS containing 0.1% of Tween-20 for 1 hr and incubated with primary antibodies in dilutions described above. Detection of protein signals was performed using chemiluminescent reagents (SuperSignal West Pico, Pierce). To quantitate the intensity of the detected bands, the radiographs were scanned using a digital scanner (MicroTek), Photoshop 5.0 (Adobe), and a Macintosh computer. The digitized images were analyzed with ImageQuant 1.2 (Molecular Probe).

Antibodies

CGA was detected using either a rabbit anti-CGA polyclonal antibody (a gift from S. Yoo) or a rabbit anti-CGA/CGB polyclonal antibody (Biodesign International) at 1:3000. CGB was detected using either an anti-CGB goat polyclonal antibody (1:3000; Research Diagnostics) or the above rabbit anti-CGA/CGB polyclonal antibody. An anti-CGA guinea pig antibody was generated against purified bovine CGA. This antibody recognizes only bovine CGA, but not other species, in Western blotting and immunocytochemistry (unpublished data). Rabbit anti-CPE polyclonal antibodies (against N-terminal and C-terminal of CPE; 1:2500; Cool et al., 1997) and a rabbit anti-POMC polyclonal antibody (DP4; 1:3000; Loh et al., 1985) were used after preincubation in 1% BSA in blocking buffer for 30 min. A mouse anti-synaptotagmin monoclonal antibody (Transduction Laboratories), a rabbit anti-ARF6 polyclonal antibody (a gift from J. Donaldson), and a rabbit anti-β-COP antibody (Affinity Bioreagents) were used at 1:1000.

Secretion Assay

For secretion studies on wild-type PC12 and 6T3 cells, CGA^{AS} clones, and 6T3-bCGA clones, cells were preincubated for 30 min in DMEM. After preincubation, cells were incubated with DMEM without stimulation at 37°C for 30 min and the media were collected. Cells were incubated in sequence for another 30 min with DMEM supplemented with 50 mM KCl. For basal secretion assay of CGB from wild-type and CGA^{AS}-5 PC12 cells (Figure 3C), cells were incubated in DMEM/0.01% BSA for 1 hr. All collected media were mixed with trichloroacetic acid (TCA; 10% final) and a carrier protein (50 μg of BSA or RNase A) overnight at 4°C. TCA precipitates were centrifuged, and the resulting pellets were washed in acetone. Pellets were resuspended in an equal volume of SDS-PAGE sample buffer. Equal volume of samples was loaded onto 8%–16% polyacrylamide gels, and secretion of CGA was determined by Western blotting (see above). For secretion studies of POMC, cells (wild-type PC12 cells, CGA^{AS} clones, and 6T3 cells) were transiently transfected with a plasmid containing a full-length bovine POMC cDNA (pcDNA3.1-bPOMC) using LipofectAMINE 2000 reagent (Life Technologies). 24 hr post-transfection, cells were washed with DMEM and stimulated as described above. The secretion of POMC was analyzed by Western blotting. Secretion assay for bCGA from bCGA-transfected clone CGA^{AS}-5 was performed as described in Cowley et al. (2000). Briefly, 24 hr post-transfection with pcDNA3.1-bCGA-neo into CGA^{AS}-5, cells were preincubated in Krebs-Ringer-Hepes buffer for 1 hr, followed by sequential basal and stimulated secretion (30 min each).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% TX-100 in PBS. An anti-CGA guinea pig antibody (1:100) in 3% normal goat serum/PBS was used for bCGA. Endogenous CGA was detected by an anti-CGA rabbit antibody (1:1000) donated by S. Yoo (KAIST, Korea). An anti-rat CGB rabbit antibody (1:200) was a gift from P. Rosa (CNR, Italy). An anti-CPE antibody (Cool et al., 1997) was used in 1:1000. An anti-rabbit IgG-Cy3 (1:1000) and an anti-guinea pig IgG-FITC (1:1000) were used as secondary antibodies. Fluorescent images were taken using a confocal microscope (Bio-Rad) and processed using Confocal Assistant and Adobe Photoshop® 5.0.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from approximately 5×10^6 wild-type PC12 or CGA^{AS}-5 cells as a frozen pellet using the RNAqueous 4PCR kit (Ambion) and the manufacturer's instructions. Five μg aliquots of total RNA were reverse transcribed via priming with random hexamers and SSII reverse transcriptase (GIBCO), followed by RNase H digestion. Real-time PCR was performed on 5% of the cDNA obtained in the reverse transcriptase reaction, using forward and reverse primers and FAM/TAMRA-labeled detection probes chosen with PCR Express (Perkin Elmer) based on the sequences of rat CGA and CPE, and VIC/TAMRA-labeled probe for rat GAPDH. Relative concentrations of each mRNA were inferred from the ratio of threshold cycle number for the message of interest, corrected for threshold cycle number for GAPDH in the same sample (using the fluoresence tag VIC in place of FAM) for mRNA quantitation by QC-RT-PCR using the Perkin Elmer 7700 Sequence Detection System.

Ammonium Chloride Treatment of Cells

CGA^{AS}-5 PC12 cells and CGB-transfected 6T3 cells were treated with 10 mM NH_4Cl in culture media for 3 hr and 90 min, respectively.

Cells were harvested in lysis buffer after treatment and analyzed by Western blotting.

Metabolic Labeling and Immunoprecipitation

Cells were incubated in 10% dialyzed FBS/methionine-deficient DMEM (Biofluids, Inc.) for 1 hr, labeled for 2 or 5 min in the same medium supplemented with [^{35}S]methionine (100 μCi/ml, Amersham), and washed once with DMEM supplemented with cold methionine (1 mM) and twice with ice-cold PBS on ice. All cells were extracted in lysis buffer (1% TX-100, 50 mM Tris-HCl [pH 7.4], 300 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 10 mM iodoacetamide, protease inhibitor cocktail [Complete, Roche]). After preclearing with Protein A-sepharose beads (Boehringer Mannheim) in PBS/0.1% BSA/0.02% NaN_3, a goat anti-CGB antibody (Research Design) was used for immunoprecipitation (1:50). Immunoprecipitates were counted for radioactivity and the specific counts per minute of each immunoprecipitate were normalized against total protein concentration of each sample.

Electron Microscopy and Morphometry of PC12 Cells

Cells were fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 hr, washed with buffer, and treated with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hr. Samples were washed and en bloc mordanted with 0.5% uranyl acetate in acetate buffer at pH 5.0, dehydrated in ethanol, and embedded in epoxy resin. Thin sections were counterstained with lead citrate.

For each grid of thin sections, ~10 openings from 4–5 different areas were randomly chosen, and every cell in these openings was photographed. Measurements were made on micrographs printed at a final magnification of 20,000. All dense-core secretory granules were counted. The area of cytoplasm was calculated by measuring the area of the entire cell minus the area occupied by the nucleus using a digitizing tablet and the NIH Image 1.61 program. The density of secretory granules was then calculated by dividing the number of secretory granules by the area of cytoplasm.

Expression of CGA in CV-1 Fibroblasts

The plasmid pCHRG4A, containing the complete open reading frame (ORF) of bovine CGA, was modified to include the T7 bacteriophage λ promoter immediately upstream. This plasmid was transfected into CV-1 cells, which were then infected with vaccinia virus expressing the T7 polymerase under the control of the CMV promoter (Erickson and Eiden, 1993). As a control, cells previously transfected with expression plasmid (pGEM2) lacking the CGA ORF were examined.

EM Morphometry in CV-1 Cells

Typically, samples were blinded for morphometry. Randomly selected areas of thin sections from each experimental group were scored for the number of dense-core granules per cell profile. Every cell encountered in these grid openings was scored. Dense-core granules were defined as membrane-bound spherical structures with a diameter of 80–200 nm containing a dense core with a halo separating the core and the vesicular membrane. Cells containing more than three dense-core granules by these criteria were counted as dense-core granule-positive (Table 1). Percentages of cells with dense-core granules were calculated by dividing the number of cells with dense-core granules by the total number of cells examined.

EM Immunocytochemistry

Cells were grown in plastic chamber slides (Labtek, Miles Laboratory), fixed with 4% EM grade paraformaldehyde (Electron Microscopy Science) in 0.1 M phosphate buffer at pH 7.4 at room temperature for 45 min, and washed. Some samples were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer and postfixed with 1% osmium tetroxide for better preservation of the ultrastructure. Preembedding EM ICC was carried out as described before (Tanner et al., 1996). Briefly, the fixed and washed samples were blocked and permeabilized with 5% goat serum plus 0.1% saponin in PBS for 1 hr, incubated with an anti-CGA (anti-WE-14) antibody (Schafer et al., 1994) (1:1000) or an anti-bCGA guinea pig antibody (1:100) in the blocking solution for 1 hr, washed and incubated with a 1.4 nm gold-conjugated secondary antibody (Nanogold, Nanoprobe) for 1 hr, washed and fixed with 2% glutaraldehyde in PBS, washed in water, and silver enhanced (HQ silver enhancement kit, Nanoprobes). Samples were postfixed with 0.2% osmium tetroxide in buffer, en block mordanted with 0.5%–1% uranyl acetate, dehydrated and embedded in epoxy resins. Thin sections were counterstained with lead citrate. Control for immunostaining was carried out by omitting the primary antibody.

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