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Large Dense-Core Secretory Granule Biogenesis Is under the Control of Chromogranin A in Neuroendocrine Cells

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ABSTRACT

The large dense-core secretory granule is an organelle in neuroendocrine/endocrine cells, where prohormones and proneuropeptides are stored, processed, and secreted in a regulated manner. Here we present evidence that chromogranin A (CgA), one of the most abundant acidic glycoproteins ubiquitously present in neuroendocrine/endocrine cells, regulates dense-core secretory granule biogenesis. Specific depletion of CgA expression by antisense RNAs in PC12 cells led to a profound loss of secretory granule formation. An exogenously expressed prohormone, pro-opiomelanocortin, was neither stored nor secreted in a

regulated manner in CgA-deficient PC12 cells. Overexpression of bovine CgA into CgA-deficient PC12 cells rescued regulated secretion. Other secretory granule proteins, such as chromogranin B (CgB), carboxypeptidase E, and synaptotagmin, were rapidly degraded, whereas nongranule proteins were not affected in CgA-deficient PC12 cells. Unlike CgA, another granin protein CgB could not substitute for the role of CgA in secretory granule biogenesis. Thus, we conclude that CgA is a master "on/off" switch regulating the formation of the dense-core secretory granule in neuroendocrine cells.

Key Words: chromogranin A • dense-core secretory granule • secretory granule biogenesis • regulated secretion • PC12 cells

INTRODUCTION

Numerous studies have provided evidence that the large dense-core secretory granule (SG) is an organelle for storage of prohormones, proneuropeptides, processing enzymes, and other proteins required for regulated secretion in endocrine and neuroendocrine cells. SG biogenesis proceeds via two distinctive stages. First, the immature secretory granules (ISGs) are budded from the trans-Golgi network (TGN), separate from constitutive secretory vesicles.[1] GTP hydrolysis, presumably via a role of ADP-ribosylation factor 1 (ARF1), is necessary in this stage.[2,3] This is followed by the maturation step where the ISGs become mature secretory granules (MSGs). During this process, the ISGs may undergo homotypic fusion.[4] Constitutive-like vesicles are pinched off from the ISGs to remove missorted proteins such as mannose-6-phosphate receptors and furin.[5-8] Similarly vesicle-associated membrane protein-4 (VAMP4) is removed from the ISGs, while VAMP2 remains associated with the MSGs.[9] Condensation of soluble cargo proteins also seems to be required for maturation.[10]

However, although mature large dense-core secretory granules in chromaffin cells were identified more than half a century ago, the detail molecular mechanism underlying the formation of these organelles is poorly understood. Understanding these mechanisms would reveal how the sorting, packaging, and secretion of prohormones and proneuropeptides are regulated to achieve their physiological functions.

Chromogranin A (CgA), chromogranin B (CgB)/secretogranin I, and chromogranin C (CGC)/secretogranin II have been identified as the most abundant cargo proteins in SGs.[11,12] This family of granins, although they do not share much overall sequence homology, have similar biochemical properties, including isoelectric point (acidic pI), heat-stability, and Ca^{2+} binding.[13] Granins have been proposed as helper proteins in packaging other proteins (i.e., prohormones) into SGs,[14] and also serve as precursors for physiologically active neuropeptides.[15] The ubiquitous distribution of granins in neuroendocrine and endocrine tissues suggests their general role in the regulated secretion.[16-18] Moreover, the expression of CgA has been shown to be closely correlated with

the formation of large dense-core SGs from pituitary gonadotropes in rats.[19,20]

In the following study, we provide evidence that CgA is a master "on/off" switch regulating SG biogenesis in neuroendocrine cells.

CHROMOGRANIN A REGULATES SECRETORY GRANULE BIOGENESIS IN PC12 CELLS

To test the hypothesis that CgA regulates the formation of SGs, we generated CgA-deficient PC12 cells using antisense RNAs. Two antisense-RNA constructs (pcDNA3.1-CgA^{AS}-1 and -2) against two regions of mouse CgA cDNA sequence (GenBank; M64278) (Fig. 1A) were transfected into PC12 cells to generate stable clones. Clone CgA^{AS}-5, which showed a profound reduction of CgA expression, was analyzed for SGs and regulated secretion of transfected pro-opiomelanocortin (POMC). Clone CgA^{AS}-5 had almost an undetectable amount of CgA in total cell lysate compared to wild-type PC12 cells (Fig. 1B). PC12 cells stably expressing an empty vector (WT^{V}) showed no significant reduction of CgA (Fig. 1B).

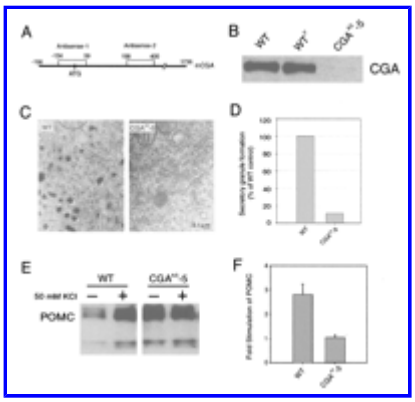

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FIGURE 1. (**A**) A diagram shows two antisense-target regions (antisense-1 and antisense-2) used for generation of clone CgA^{AS}-5. (**B**) A Western blot represents CgA levels in wild-type (WT), a stable clone expressing an empty vector (WT^{V}) and clone CgA^{AS}-5. (**C**) EM micrographs show sections from wild-type PC12 cells (WT) and clone CgA^{AS}-5. *Arrows* point out large dense-core secretory granules in WT. (**D**) A *bar graph* shows the percentage of the number of secretory granules in clone CgA^{AS}-5 (10%) compared to that of wild-type PC12 cells (100%). (**E**) A representative Western blot shows POMC secretion in the absence (-) or presence (+) of 50 mM KCl in wild-type PC12 cells (WT) and clone CgA^{AS}-5 after transfection with a POMC construct. (**F**) A *bar graph* shows fold-stimulation of POMC secretion in wild-type PC12 cells and clone CgA^{AS}-5 from (E). (Modified from Ref. 24.)

If the formation of large dense-core SG biogenesis is exclusively due to the presence of CgA, a significant reduction in the number of SGs is expected in CgA-deficient PC12 cells. Analyses by electron microscopy (EM) and morphometry of clone CgA^{AS}-5 showed a significant reduction of SG formation compared to wild-type PC12 cells (Fig. 1C and 1D). The number of SGs per unit area (μm^2) of cytoplasm counted from EM sections showed 90% reduction of SGs in clone CgA^{AS}-5 [0.18 ± 0.03

(SEM) granules/μm^2] compared to wild-type PC12 cells [1.62 ± 0.11 (SEM) granules/μm^2] (Fig. 1D).

We also determined whether the loss of SGs in clone CgA^{AS}-5 is accompanied by a loss in the regulated secretion of hormones. When a prohormone, POMC, was exogenously expressed in wild-type PC12 cells and clone CgA^{AS}-5, and assayed for their secretion in the presence of stimulation (50 mM KCl), transfected POMC was released in a stimulated manner [fold stimulation = 2.81 ± 0.44 (SEM)] in wild-type PC12 cells. In contrast, in clone CgA^{AS}-5, POMC was released in large amounts into the basal medium (no stimulation), and no significant stimulated secretion was detected in the presence of high potassium in clone CgA^{AS}-5 [fold stimulation = 1.05 ± 0.09 (SEM)] (Fig. 1E and 1F), indicating loss of hormone storage capacity consistent with the lack of SGs.

To confirm that the phenotype of clone CgA^{AS}-5 is due to the loss of CgA expression and not clonal variation, we transiently expressed a bovine CgA (bCgA) construct into clone CgA^{AS}-5 in an attempt to rescue the wild-type phenotype in these cells. The bovine CgA sequence is significantly divergent from the mouse CgA sequence at the antisense RNA target regions used for generating clone CgA^{AS}-5. The staining pattern of bCgA in transiently transfected clone CgA^{AS}-5 showed a punctate one, indicative of SGs (Fig. 2C and 2E; arrows). This staining pattern was similar to that of endogenous CgA (Fig. 2A). Little endogenous CgA was detected in clone CgA^{AS}-5 without transfection (Fig. 2B). Mock transfection with an empty vector showed no detectable bCgA signal (data not shown). Interestingly, other secretory granule proteins, such as CgB and carboxypeptidase E (CPE), showed reduced signal detected in nontransfected cells in the same field (Fig. 2D and 2F; arrowheads; see Fig. 3A). However, the level of CgB and CPE in bCgA-positive CgA^{AS}-5 cells showed upregulation of these proteins in bCgA-positive cells (arrows; Fig. 2D and 2F, respectively). The majority of bCgA puncta were overlapped with CgB as well as CPE in bCgA-positive cells (Fig. 2C-2F), indicating colocalization of bCgA with these granule proteins. Immuno-EM, showing bCgA-positive immunogold grains within the membrane-bound granules (Fig. 2G), and secretion studies showing stimulated secretion of bCgA in bCgA-transfected CgA^{AS}-5 cells (fold stimulation = 7.6) (Fig. 2H) confirmed that the transfected bCgA was packaged in SGs.

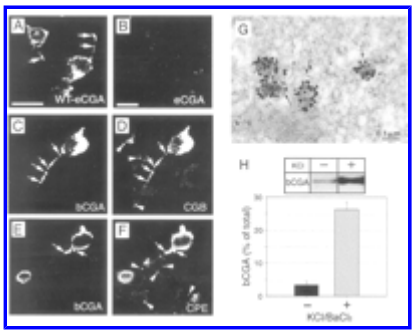

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FIGURE 2. Immunocytochemistry of wild-type PC12 cells (**A**) and clone CgA^{AS}-5 (**B-F**). (A) Wild-type PC12 cells labeled for endogenous CgA (eCgA). *Arrows* indicate a punctate staining pattern of CgA at the tips of processes in PC12 cells. *Bar* = 20 mm. (B) Staining of eCgA in clone CgA^{AS}-5. *Bar* = 20 mm. (**C, E**) Clone CgA^{AS}-5 was transfected with a bovine CgA (bCgA) construct and immunostained for bCgA using an antibody specific for bCgA. *Arrows* indicate a punctate staining pattern of bCgA in bCgA-transfected cells. (D, F) Immunostaining of endogenous CgB (D) and endogenous CPE (F) in the same field as shown in (C) and (E), respectively, is shown. *Arrows* indicate a punctate staining pattern of CgB (**D**) or CPE (F) overlapping with bCgA staining shown in (C) or (E),

respectively. *Arrowheads* indicate that cells negative for bCgA show very little staining intensity of endogenous CgB (D) or endogenous CPE (F). (**G**) An immuno-EM picture shows dense-core secretory granules containing bovine CgA in bCgA-positive clone CgA^{AS}-5. *Arrows* indicate membrane-bound granules. (H) A representative Western blot shows stimulated secretion of bCgA in bCgA-transfected clone CgA^{AS}-5. Percentages of bCgA secreted into the medium compared to total bCgA expressed in cells are shown in a *bar graph*. Bovine CgA was secreted 3.47 ± 0.98% (SEM; $n = 3$) of total bCgA in the absence (-) and 26.3 ± 2.32% (SEM; $n = 3$) of total bCgA in the presence (+) of stimulation. (From Kim *et al.*[24] Reproduced by permission.)

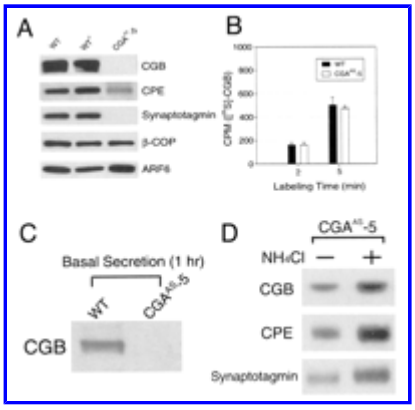

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FIGURE 3. (**A**) Western blots represent levels of secretory granule proteins (CgB, CPE, synaptotagmin) and nonsecretory granule proteins (ARF6 and ß-COP) in wild-type PC12 cells (WT), a stable clone with an empty vector (WT^V), and clone CgA^{AS}-5. (**B**) Translational activity of CgB expression in wild-type PC12 cells and clone CgA^{AS}-5. Cells were labeled with [^{35}S]methionine for 2 min or 5 min, followed by immunoprecipitation with an anti-CgB antibody. *Black and white bars* represent CPM values of radioactive CgB from wild-type PC12 cells and clone CgA^{AS}-5, respectively. (**C**) Basal secretion of CgB for 1 h (no stimulation) in wild-type PC12 cells and clone CgA^{AS}-5. (**D**) The levels of CgB, CPE, and synaptotagmin in clone CgA^{AS}-5 in the absence or presence of 10 mM NH_4Cl for 3 h. (Modified from Ref. 24.)

PROTEOLYTIC DEGRADATION OF SECRETORY GRANULE PROTEINS IN CgA-DEFICIENT PC12 CELLS

Reduced levels of CgB and CPE of clone CgA^{AS}-5 shown in Figure 2 suggest that depletion of CgA might affect the storage of other SG proteins in clone CgA^{AS}-5. Therefore we examined other granule proteins, including CgB, CPE, and synaptotagmin by Western blot analyses. As with the immunocytochemistry data in Figure 2, CgB and CPE levels were significantly reduced in clone CgA^{AS}-5 (Fig. 3A). An SG membrane protein, synaptotagmin, was also significantly decreased (Fig. 3A). Nonsecretory granule proteins such as ARF6 and ß-COP did not show any change in their expression (Fig. 3A).

To study the nature of downregulation of secretory granule proteins in clone CgA^{AS}-5, we analyzed transcriptional activities in the clone. Quantitative RT-PCR showed an approximately 2-fold downregulation of CgB mRNA in clone CgA^{AS}-5 compared to wild-type PC12 cells. Similarly, the CPE mRNA level was decreased by about 1.6-fold (data not shown). This reduction is not significant enough to explain the reduction of these proteins in clone CgA^{AS}-5. Next, we determined translational activities in clone CgA^{AS}-5. Metabolic labeling for 2 min or 5 min of clone CgA^{AS}-5 and wild-type PC12 cells followed by immunoprecipitation with an anti-CgB antibody revealed that there was no significant difference in the initial synthesis of CgB in clone CgA^{AS}-5 compared to wild-type cells (Fig. 3B).

There are two possibilities for the posttranslational loss of CgB in clone CgA^{AS}-5: (1) rapid secretion, or (2) rapid degradation. We assayed the amount of CgB secreted into the medium without stimulation from clone CgA^{AS}-5 and wild-type PC12 cells. Wild-type control cells showed a detectable amount of CgB in the medium (Fig. 3C). In contrast, no detectable amount of CgB was found in the medium from clone CgA^{AS}-5 (Fig. 3C), indicating that there was no rapid secretion of CgB from clone CgA^{AS}-5. Pulse-chase experiments confirmed that there was significant degradation occurring in clone CgA^{AS}-5 during the first 30-min chase period compared to wild-type cells (data not shown). This degradation was inhibited by 10 mM NH_4Cl in clone CgA^{AS}-5; levels of CgB, CPE, and synaptotagmin were significantly increased after this treatment (Fig. 3D). Thus, in the absence of CgA, secretory granule proteins that were synthesized normally in clone CgA^{AS}-5 were rapidly degraded in acidic compartment (s) without storage.

CHROMOGRANIN B DOES NOT RESCUE SECRETORY GRANULE BIOGENESIS IN CgA-DEFICIENT PC12 CELLS

CgB shares several biochemical properties with CgA, such as a disulfide bridge at the amino-terminus, oligomerization/aggregation,[21,22] and interaction with inositol 1,4,5-triphosphate (IP3) receptor.[23] It is plausible that CgB may function in a similar way as CgA in the formation of large dense-core SGs in PC12 cells. We generated two antisense constructs, cotransfected into PC12 cells, and isolated clones with the reduction of CgB expression. We obtained two clones (CgB^{AS}-7.4 and CgB^{AS}-10.1) with significant reduction of CgB contents (~67% and ~66%, respectively) (Fig. 4A and 4B). The expression of CgA was slightly decreased in clone CgB^{AS}-7.4 (~25%), while there is no significant change observed in clone CgB^{AS}-10.1 compared to wild-type PC12 cells (Fig. 4A and 4B). The number of SGs per unit area of cytoplasm of both clones showed slight reduction (~35% and ~26%, respectively) (Fig. 4B). The reduction of SGs is much more closely correlated with the amount of CgA, rather than CgB, content in these clones. Moreover, in contrast to clone CgA^{AS}-5, there was no significant reduction of synaptotagmin in both clones (Fig. 4A). When we tried to rescue the phenotype of CgA-deficient PC12 cells with CgB expression, we could not detect any significant overexpression of CgB (Fig. 4C). When the same

construct for CgB was expressed in wild-type PC12 cells, a significant increase of CgB levels was observed (Fig. 4D). Similarly, when COS-7 cells were transfected with the construct, CgB levels were comparable to wild-type PC12 cells (Fig. 4D). Cotransfection of CgB with a POMC construct showed only POMC expression in the clone (Fig. 4C), indicating that the protein synthesis machinery was intact in clone CgA^{AS}-5. Thus, exogenous CgB was also degraded after synthesis in clone CgA^{AS}-5 as for endogenous CgB.

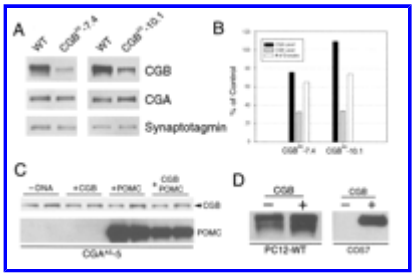

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FIGURE 4. (**A**) Western blots showing the levels of CgB, CgA, and synaptotagmin in two CgB-antisense PC12 cells (CgB^{AS}-7.4 and CgB^{AS}-10.1). (**B**) *Bar graphs* show percentage of CgA (*black*), CgB (*gray*) levels, and the number of secretory granules (*white*) in CgB antisense clones in comparison with those from wild-type PC12 cells. (**C**) Western blots show expression of exogenous CgB in clone CgA^{AS}-5. Clone CgA^{AS}-5 was transfected with no DNA, a bovine CgB construct, a POMC construct, or both CgB and POMC constructs. The levels of CgB and POMC are shown in duplicates. (**D**) Western blots showing expression of CgB in wild-type PC12 cells or COS-7 cells after transfection with a bCgB construct used for (C). (Panels A and B from Kim *et al.*[24] Reproduced by permission.)

DISCUSSION AND CONCLUSIONS

It has been proposed that granins play a role in secretory granule biogenesis. Our studies described here and reported elsewhere[24] provide strong evidence that CgA controls the biogenesis of SGs, and hence regulated secretion not only in PC12 cells but also in endocrine cells. 6T3 (a cell line derived from a mouse anterior pituitary tumor cell line AtT-20) lacking the regulated secretory pathway and CgA expressionl[25,26] showed recovery of regulated secretory phenotype when CgA was introduced exogenously.[24] Also CgA was able to initiate SG biogenesis in fibroblasts.[24]

Interestingly, although CgB shares a great many biochemical properties with CgA, it does not share the same function as CgA in regulating SG biogenesis. Likewise CgB was unable to rescue regulated secretion in 6T3 cells.[24] Any assigned function for CgB in the biogenesis of SGs is likely to be downstream of CgA.

Another function of CgA seems to be to prevent degradation of other secretory granule proteins in acidic compartment(s). This indicates that CgA may function as a protease inhibitor to prevent the loss of cargo proteins from SGs. In fact, CgA has been shown to inhibit a trypsin-like enzyme, IRCM-serine protease

1, present in microsomes.[27] That SG protein levels are regulated posttranslationally by a single-molecule CgA is most intriguing. Thus, CgA seems to be a molecule that regulates not only the formation of SGs but also the storage/stability of other granule proteins in SGs.

In conclusion, we have uncovered a very important role of CgA as a master switch regulating the biogenesis of large dense-core secretory granules in neuroendocrine cells. The exact mechanism by which CgA acts in this unique function awaits future studies.

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