Chromogranin B-induced Secretory Granule Biogenesis

COMPARISON WITH THE SIMILAR ROLE OF CHROMOGRAFIN A*

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The two major proteins of secretory granules of secretory cells, chromogranins A (CGA) and B (CGB), have previously been proposed to play key roles in secretory granule biogenesis. Recently, CGA was reported to play an on/off switch role for secretory granule biogenesis. In the present study we found CGB being more effective than CGA in inducing secretory granule formation in non-neuroendocrine NIH3T3 and COS-7 cells. The mean number of dense core granules formed/cell of CGB-transfected NIH3T3 cells was 2.51, whereas that of CGA-transfected cells was 4.02, indicating the formation of 60% more granules in the CGB-transfected cells. Similarly, there were 55% more dense core granules formed in the CGB-transfected COS-7 cells than in the CGA-transfected cells. Moreover, transfection of CGA- and CGB-short interfering RNA (siRNA) into neuroendocrine PC12 cells not only decreased the amount of CGA and CGB expressed but also reduced the number of secretory granules by 41 and 78%, respectively, further suggesting the importance of CGB expression in secretory granule formation.

Chromogranins A and B are abundantly present in virtually all secretory granules of neurons, exo-endocrine, and neuroendocrine cells (1–4). They are acidic proteins containing ~25% acidic amino acid residues with isoelectric points (pI) of 5.0–5.5 (5–15). Chromogranins A and B share two conserved regions between them; one in the near N-terminal region and the other in the C-terminal region (5–15). Two cysteine residues that form a disulfide bond in each protein flank the conserved near N-terminal region of both CGA and CGB. These features are not shared by other secretory granule matrix proteins and thus are distinguished from similar acidic granule proteins secretogranins (16–20).

Moreover, chromogranins A and B share several important biochemical properties. Of these, a key determinant of the function of chromogranins is their tendency to associate with each other in a pH- and Ca2+-dependent manner (16, 21–24). This property is particularly important in secretory granule biogenesis and in the packaging of granule contents (21–26). Furthermore, a common feature of the pH- and Ca2+-dependent aggregation of chromogranins A and B is the better aggregation of the proteins at pH 5.5 than at pH 7.5 in the presence of Ca2+ (16, 21–24). In the absence of Ca2+, CGA does not aggregate even at pH 5.5 (21), but in the presence of increasing Ca2+ concentrations, it starts to aggregate at both pH 5.5 and 7.5, although the degree of aggregation at pH 5.5 is severalfold higher than that at pH 7.5 (21). Despite the seeming similarity in the aggregation property of CGA and CGB, there also exist considerable differences between the two in that the nature of CGB aggregation differs significantly from that of CGA. Unlike CGA, which does not aggregate except in the presence of near millimolar concentrations of Ca2+, CGB starts to aggregate even in the presence of micromolar Ca2+ concentrations (22) and is at least two orders of magnitude more sensitive than CGB to Ca2+ (22). The extreme sensitivity of CGB aggregation to Ca2+ is one of the many characteristics that distinguish CGB from CGA.

Chromogranins A and B also undergo pH- and Ca2+-dependent conformational changes (21, 22), and these conformational changes appear to be responsible for the interaction of chromogranins with other proteins in secretory granules. They interact not only with most of the intragranular matrix proteins but also with a number of secretory granule membrane proteins, including the IP3R, at the intragranular pH 5.5 (25). However, at a near physiological pH 7.5, CGA dissociates from most of the secretory granule proteins including the IP3R (25, 27), whereas CGB still maintains its interaction with other proteins (28, 29) including CGA and the IP3R (27), albeit at a significantly reduced degree.

Hence, given the acidic pH- and Ca2+-dependent aggregation properties (16, 21–24) and the ability to interact with other secretory granule constituents, chromogranins A and B have been proposed to play key roles in secretory granule biogenesis (21–25). Indeed, CGA has recently been proposed to play an on/off switch role in secretory granule formation (30). Using the antisense RNA technique and PC12 cells, Kim et al. (30) showed that the number of secretory granules formed is directly related to the amount of CGA expressed in PC12 cells. They further showed that secretory granule formation could be induced in non-neuroendocrine cells, which normally do not contain any secretory granules, by expressing CGA in these cells.

However, differing from the reported inability of CGB to induce secretory granule formation either in PC12 cells or in non-neuroendocrine cells (30), we have found CGB more effective than CGA in inducing secretory granule formation both in PC12 cells and in non-neuroendocrine cells. Using transfection techniques, CGB was shown to induce more secretory granule formation than CGA in non-neuroendocrine cells. Moreover, limited inhibition of CGB expression by siRNA techniques was shown to reduce the number of secretory granules in PC12 cells more than inhibition of CGA expression. In light of our recent
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**Fig. 1. Expression of bovine CGA and CGB in transiently transfected NIH3T3 cells.** The total protein extracts from the NIH3T3 cells transfected with pCI-CGA (A) or -CGB (B) were resolved on 10% SDS-gels and probed with the anti-CGA or -CGB antibody. The blots were also reprobed with the α-tubulin antibody after deprobing the first blots to check the amount of proteins loaded. The protein extracts from both the untransfected (Normal) and the pCI-neo vector-transfected (pCI-empty) cells were used as controls.

finding that chromogranin B, not chromogranin A, is localized in the nucleus and participates in transcription control (31), and that the unique biochemical properties of CGB are not shared by CGA (21, 22, 26). CGB appears to play a more direct role in secretory granule formation than CGA.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Polyclonal anti-rabbit CGA and CGB antibodies were raised against intact bovine CGA and recombinant CGB. Polyclonal anti-rabbit α-tubulin antibody was obtained from Oncogene.

**Construction of Expression Vectors**—The expression vectors for CGA and CGB were prepared by polymerase chain reaction (PCR) using bovine cDNA as a template, and the PCR products containing full-openreading frames were prepared by polymerase chain reaction (PCR) using polymerase chain reaction (PCR) with COS-7 and NIH3T3 cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum. Transient siRNA transfection was performed with 70–80% confluent cultures. The CGA-siRNA duplex sense and antisense sequences were 5'-CAACAAACAA-CACGACGCUdTdT-3' and 3'-dTdTGTicuGGUUGUUUUGUUGCUCCGA-5', respectively, and the CGB-siRNA duplex sense and antisense sequences were 5'-AUUGCcCaUUCGUAGUCCGdTdT-3' and 3'-dTdT-TLACCGGATAGUCCGUC-5', respectively. The 2-nucleotide 3'-overhang of 2'-deoxythymidine is indicated as dTdT. The cells were transfected with the siRNAs using a Silencer™ siRNA transfection kit (Ambion). Briefly, ∼1–2 × 10⁶ PC12 cells were plated on collagen type IV (BD Biosciences) coated culture dish (100 mm in diameter) in RPMI 1640 medium supplemented with 10% fetal bovine serum and were cultured for 48 h before transfection. For dose-response studies of siRNA transfection, 0.25–2 μg of the appropriate siRNA and 10 μl of siPORT Amine were used per 5 × 10⁵ cells. But for the electron microscope study, 1 μg of appropriate siRNA and 10 μl of siPORT Amine were used per 5 × 10⁶ cells. The addition of more siRNA did not reduce the number of secretory granules further. The transfection was performed for 6 h at 37 °C. After transfection, the medium was replaced with fresh prewarmed RPMI 1640 medium and was further incubated for 48 h. The transfection was monitored using a Silencer CyTM3 siRNA Labeling Kit, and electron microscope experiments using the transfected PC12 cells were performed 48 h after transfection.

**Electron Microscopy**—Cells grown on the culture dish were fixed for 1 h at 4 °C in PBS containing 2% glutaraldehyde, 2% paraformaldehyde, and 3.5% sucrose, and the cells intended for immunogold labeling were fixed in the same solution containing 0.1% glutaraldehyde, 4% paraformaldehyde. The cells were then scraped from the culture dish with a cell scraper and pelleted by centrifugation at 2000 × g for 2 min at 4 °C. The cell pellets were resuspended in warm agar (1% in PBS) and repelleted by centrifugation. After three washes in PBS, the agar-embedded cell pellets were postfixed with 1% osmium tetroxide on ice for 2 h, washed three times, and stained en bloc with 0.5% uranyl acetate, all in PBS. The cell pellets were then embedded in Epon 812 after dehydration in an ethanol series. After ultrathin sections were collected on Formvar/carbon-coated nickel grids, the grids were stained with uranyl acetate (7 min) and lead citrate (2 min) and viewed with a Zeiss EM912 electron microscope. For each grid, the ultrathin sections that were viewed in the mesh were photographed and printed at a final magnification of 75,000×. The area of the cells was measured, and the number of total dense core granules in all measured cell area was counted. The density of granules was determined by dividing the number of secretory granules by the area of the cell.

For immunogold labeling experiments, the ultrathin sections that had been collected on Formvar/carbon-coated nickel grids were floated on drops of freshly prepared 3% sodium metaperiodate (32) for 30 min. Immunogold labeling procedure was modified from Spector et al. (33) and the manufacturer's recommended protocol (Biological Cell Internationa). After etching and washing, the grids were blocked with 1% drop of solution A (phosphate-buffered saline solution, pH 8.2, containing 4% normal goat serum, 1% bovine serum albumin, 0.1% Tween 20, 0.1% sodium azide) for 30 min. Grids were then incubated for 2 h at room temperature in a humidified chamber on 50 μl droplets of anti-rabbit CGA or CGB antibody appropriately diluted in solution B (solution A but with 1% normal goat serum) followed by rinses in solution B. The grids were reacted with 10-mm gold-conjugated goat anti-rabbit IgG diluted in solution A. Controls for the specificity of CGA and CGB immunogold labeling included: 1) omitting the primary antibody and 2) replacing the primary antibody with preimmune serum. After washes in PBS and deionized water, the grids were stained with uranyl acetate (7 min) and lead citrate (2 min) and viewed with a Zeiss EM912 electron microscope.

**Extraction of Cellular Proteins and Immunoblot Analysis**—To obtain the total cell lysates from the transfected cells, ∼3–5 × 10⁶ cells were washed with ice-cold PBS and lysed in 500 μl of buffer (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, and 20 μg/ml antiprotease mix). The crude extracts were harvested by centrifugation at 22,000 × g for 10 min at 4 °C, and the supernatant was used as the total protein lysate. The proteins (15 or 30 μg each) were then resolved by SDS-PAGE and analyzed by immunoblots using either polyclonal anti-rabbit chromogranin antibodies or monoclonal α-tubulin antibody (Oncogene) and an ECL detection system (Amersham Biosciences). The protein bands were detected by autoradiography.

**Secretion Assay**—The PC12 and NIH3T3 cells that had been transfected with CGA- or CGB-siRNA and pCI-CGA or pCI-CGB, respectively, were used 48 h after transfection. For secretion assays, 1 × 10⁶ cells were washed extensively with secretions medium (150 mM NaCl, 5
mM KCl, 2 mM CaCl₂, 10 mM HEPES, pH 7.4) and subsequently exposed to 3 mL of Ca²⁺-free secretion medium containing 2 mM BaCl₂ for 15 min. Then, the medium was collected and centrifuged at 22,000 × g for 6 min to spin down any cell debris. After this step, ~2.7 mL of supernatant recovered was concentrated using centricons (Millipore) by ~50-fold, and 10 μL and 30 μL aliquots of these concentrates were separated on 10% SDS-polyacrylamide gels, the former for visualization of the protein bands and the latter for immunoblot analyses using the polyclonal CGA and CGB antibodies and the ECL detection system (Amer sham Biosciences).

RESULTS

To determine the granule-forming effect of CGA and CGB, we transfected pCI-CGA and -CGB into non-neuroendocrine NIH3T3 and COS-7 cells, respectively, and investigated the formation of secretory granules in these cells using electron microscopy. Expression of the transfected CGA and CGB in these cells was confirmed by immunoblot analysis of the protein extracts (Fig. 1). However, in untransfected cells neither CGA nor CGB was detected.

In accord with the absence of secretory granules in these cells, no dense core granules were found in untransfected NIH3T3 cells (Fig. 2A). However, the NIH3T3 cells transfected with CGA or CGB contained a number of dense core granules of varying sizes (Fig. 2, B and C). The small granules were as small as ~70 nm in diameter, whereas the large ones were as large as ~300–400 nm in diameter. In particular, the large dense core granules, ranging in size from ~300 to 400 nm, were observed primarily in the CGB-transfected NIH3T3 cells. In contrast, transfection of NIH3T3 cells with the empty vector (vector without the CGA or CGB gene) failed to produce dense core granules (Fig. 2D). Very rarely were structures that looked like aggregating vesicles observed. Nevertheless, the number of these vesicle-like structures was very few (Table I), and their morphology was clearly different from the dense core granules formed in CGA- or CGB-transfected cells. Even among the CGA- or CGB-transfected cells, it was very apparent that there were more dense core granules in CGB-transfected cells than in the CGA-transfected cells.

Therefore, to determine the number of dense core granules formed in the CGA- or CGB-transfected NIH3T3 cells, we examined 70–78 cells prepared from four different cell preparations and counted the number of dense core granules present per cell (Table I). The number of dense core granules formed/cell of CGA-transfected cells was 2.51 ± 1.07 (mean ± S.D., n = 70), of CGB-transfected cells was 4.02 ± 0.75 (mean ± S.D., n = 70, p < 0.0001 by paired t test), and of NIH3T3 cells transfected with the empty vector was only 0.11 ± 0.32 (mean ± S.D.) (Table I), clearly demonstrating the chromogranin-induced dense core granule formation. Approximately 60% more dense core granules were formed in the CGB-transfected cells than in the CGA-transfected ones.

Similar results were also observed in non-neuroendocrine COS-7 cells (Fig. 3). Despite the complete absence of secretory granules in normal COS-7 cells, the transfection of CGA and CGB into these cells caused a number of dense core granules of varying sizes (Fig. 3, A and B) to appear. The granules ranged in size from ~70 to 300 nm, although there appeared to be more of the larger dense core granules, ranging in size from 150 nm and above, than smaller ones. In contrast, the transfection of COS-7 cells with the empty vector failed to produce dense core granules.

**Fig. 2.** Electron microscopy showing the newly formed dense core granules in non-neuroendocrine NIH3T3 cells. Non-neuroendocrine NIH3T3 cells were transfected with pCI-CGA or -CGB, and the appearance of the newly formed dense core granules was examined by electron microscopy. Normal NIH3T3 cells (A) and CGA- (B) and CGB-transfected (C) and empty vector-transfected cells (D) are shown. Several of the newly formed dense core granules are indicated by arrows (large arrowheads, large granules; small arrows, small granules). Nu, nucleus; M, mitochondria; G, Golgi; er, endoplasmic reticulum. Bar = 200 nm.
granules (Fig. 3C) except for some very rare instances in which structures that looked like aggregating vesicles were observed.

To determine the number of secretory granules formed in the CGA- or CGB-transfected COS-7 cells, we examined 150–300 cells prepared from 10 different cell preparations and counted the number of dense core granules present per cell (Table I).

The results in NIH3T3 cells, —55% more dense core granules were formed in the CGB-transfected COS-7 cells than in the CGA-transfected ones.

To determine whether the newly formed dense core granules contained CGA or CGB, the two secretory granule marker proteins in neurons, exo-/endocrine cells, and neuroendocrine cells, the existence of CGA or CGB in the CGA- or CGB-transfected NIH3T3 or COS-7 cells was investigated using immunogold electron microscopy. As shown in Fig. 4A, the CGA-labeling gold particles were primarily localized in the dense core granules of CGA-transfected NIH3T3 cells. Likewise, the CGB-labeling gold particles were primarily localized in the dense core granules of CGB-transfected NIH3T3 cells. Hence, the CGA-labeling gold particles were primarily localized in the dense core granules of CGA-transfected NIH3T3 cells. Likewise, the CGB-labeling gold particles were primarily localized in the dense core granules of CGB-transfected NIH3T3 cells.

Furthermore, to determine whether the newly formed dense core granules can release the granular contents, the NIH3T3 cells transfected with CGA or CGB were stimulated with 2 mM BaCl₂, and the presence of secreted CGA or CGB in the secretion media was investigated using immunoblot analysis. As shown in Fig. 5, the amounts of proteins released from NIH3T3 cells did not differ much whether the cells were stimulated or not. However, it was apparent that CGA was secreted in response to 2 mM BaCl₂ treatment in the CGA-transfected NIH3T3 cells, whereas no CGB was detected in the control cells (Fig. 5A). Although a small amount of CGA was present in the media of the CGA-transfected cells that had not been stimulated, it was also clear that CGB was secreted in response to the secretagogue in the CGB-transfected NIH3T3 cells (Fig. 5B), further confirming the presence of CGB in the newly formed dense core granules.

In light of the granule-forming effect of CGA and CGB in non-neuroendocrine cells, we investigated the potential effect of reduced expression of CGA and CGB on secretory granule formation in neuroendocrine cells. For this purpose, we employed RNA interference technology, a recently developed method by which the translation of target genes can be inhibited selectively by transfection of siRNA duplexes (34). Using a 21-base CGA or CGB siRNA duplex sequence (see “Experimental Procedures”), a CGA- or CGB-siRNA duplex sequence was transfected into PC12 cells using siPORT Amine (Ambion), and silencing of the CGA or CGB gene was monitored 48 h after transfection. As shown in Fig. 6, the levels of CGA and CGB expression in cognate siRNA-treated PC12 cells were markedly reduced in a dose-dependent manner compared with control cells, reaching a maximum inhibition of 73 and 88%, respectively. Moreover, expression of CGB in CGA-siRNA-treated cells or expression of CGA in CGB-siRNA-treated cells was differentially inhibited; the CGB expression in the CGB-siRNA-treated cells was inhibited up to 70%, whereas the CGA expression in the CGB-siRNA-treated cells was inhibited up to 39%.

Because PC12 cells are neuroendocrine cells, they contain a number of intrinsic secretory granules (Fig. 7A). Therefore, to determine the effect of CGA- or CGB-siRNA on secretory granule formation in PC12 cells, the number of secretory granules in CGA- or CGB-siRNA-treated PC12 cells was examined. As shown in Fig. 7, B and C, the number of secretory granules in the CGA- or CGB-siRNA-treated PC12 cells was substantially decreased. However, in the cells treated with the same procedure but without the CGA- or CGB-siRNA duplex sequences, the number of secretory granules basically remained the same (Fig. 7D), further underscoring the granulogenic effects of CGA and CGB. In particular, the number of secretory granules was shown to be significantly reduced in the CGB-siRNA-treated PC12 cells as compared with CGA-siRNA-treated cells.

Therefore, to determine the relative effect of the reduced expression of CGA or CGB on the number of secretory granules formed in PC12 cells, we examined 100 cells, obtained from four different cell preparations, from each of the four experimental groups, i.e., control, empty siRNA-treated, CGA- or CGB-siRNA-treated, and tabulated the number of secretory granules found in each group (Table II). As shown in Table II, the cells treated with CGB-siRNA showed a 78% reduction in the number of secretory granules (14.99 ± 6.30/100 cells) (Fig. 7C) compared with the control cells (68.75 ± 8.50/100 cells) followed by a 41% reduction in the CGB-siRNA-treated cells (40.73 ± 7.49/cell) (Fig. 7B). The 41% reduction in the number of secretory granules in CGB-siRNA-treated PC12

| Normal | Empty transfection | CGA transfection | CGB transfection |
|--------|--------------------|------------------|------------------|
|        | Number of granules/area viewed | Number of granules/area viewed | Number of granules/area viewed | Number of granules/area viewed |
|        | Granules/cell⁶ | Granules/cell⁶ | Granules/cell⁶ | Granules/cell⁶ |
| NIH3T3⁵ | 1/9130 0 | 14/9840 0.11 ± 0.32 | 236/8205 2.51 ± 1.07⁵ | 317/7114 4.02 ± 0.75⁴ |
| COS-7 | 1/20314 0 | 61/46556 0.10 ± 0.22 | 596/14620 1.44 ± 0.89⁵ | 839/43271 2.23 ± 1.34⁴ |

⁵ An NIH3T3 cell and a COS-7 cell had 136–170 µm² and 172–203 µm² of surface area, respectively, in the central section (which crosses the center of the nucleus); therefore, “granules/cell” indicates the number of granules found in the respective average central section area of a cell in each group.

⁶ 70–8 cells sectioned from four different cell preparations were counted in each group.

" Mean ± S.D., " Mean ± S.D., p < 0.0001 by paired t test.

" Mean ± 300 cells sectioned from 10 different cell preparations were counted in each group.
cells does not appear to directly reflect the reduced level of CGA expression (which showed a 73% reduction) in these cells. On the other hand, the reduced level of CGB expression in CGB-siRNA-treated PC12 cells exhibited a more profound effect on the number of secretory granules of these cells, decreasing it by 78% (Table II).

A secretion assay of PC12 cells also indicated the reduction of the number of secretory granules in CGA- or CGB-siRNA-treated cells. As evident in the SDS-PAGE profiles of Fig. 8...
(left panels), almost identical total amounts of proteins were released from PC12 cells in response to 2 mM BaCl₂ regardless of the transfection of the siRNAs. However, it was not clear how much CGA or CGB was released from each group of cells. When the relative amounts of CGA or CGB that had been secreted were compared by immunoblot analysis, the effect of a reduced number of secretory granules was apparent. As the results shown in Fig. 8A indicate, stimulation of CGA-siRNA-treated PC12 cells with BaCl₂ released substantially less CGA and CGB than in untreated cells. Similarly,
stimulation of the CGB-siRNA-treated PC12 cells also secreted significantly less CGB than the control cells (Fig. 8B, center panel). However, secretion of CGA from the same cells did not appear to be reduced (Fig. 8B, right panel), implying a different degree of participation by CGA and CGB in secretory granule formation.

**DISCUSSION**

Although the present results show that both CGA and CGB play key roles in secretory granule formation, there were very distinct differences in the ability of each chromogranin to induce secretory granule formation. Similar to the previous results that showed the CGA-induced secretory granule formation in non-neuroendocrine cell line CV-1 (30), CGA expression in non-neuroendocrine cell lines, NIH3T3 and COS-7 cells, also induced secretory granule formation in these cells (Figs. 2 and 3). However, when CGB was expressed in these cells under identical conditions, the number of secretory granules formed was substantially increased (Table I). The number of secretory granules formed by CGA expression in NIH3T3 cells was 2.51 ± 1.07 granules/cell and by CGB expression was 4.02 ± 0.75 granules/cell (mean ± S.D., n = 70, p < 0.0001 by paired t test). The number of secretory granules formed by CGB expression was 60% higher than by CGA expression. Similarly, the number of secretory granules formed by CGA expression in COS-7 cells was 1.44 ± 0.89 granules/cell and by CGB expression was 2.23 ± 1.34 granules/cell (mean ± S.D., n = 300, p < 0.0001 by paired t test) (Table I), indicating a 55% increase in the number of secretory granules formed by CGB expression. Identification of the newly formed dense core granules as secretory granules was carried out both by the demonstration of the presence of CGA or CGB in these structures (Fig. 4) and by the secretion of CGA or CGB in these cells in response to secretagogue (Fig. 5).

Conversely, the granulogenic effects of CGA and CGB were also tested by inhibiting the expression of CGA or CGB in neuroendocrine PC12 cells (Fig. 7 and Table II). Transient transfection of PC12 cells with the CGA- or CGB-siRNA severely reduced the expression of CGA or CGB (Fig. 6). Although the level of expressed CGA and CGB proteins in the CGA- and CGB-siRNA-treated cells was reduced by 73 and 88%, respectively, the number of granules formed was reduced by 41 and 78%, respectively (Table II). However, immunoblot analysis of the CGB expression in the identical CGA-siRNA-treated cells and of the CGB expression in the identical CGB-siRNA-treated cells indicated that the CGB expression was inhibited by 70%, whereas that of CGA was inhibited by 39% (Fig. 6). These results appear to suggest that inhibition of CGB expression affected the CGA expression significantly less than the inhibition of CGA expression did on CGB expression. Analogous results were obtained in secretion assays designed to determine the amount of chromogranins secreted in each cell group. As shown in Fig. 8, the amount of secreted CGA or CGB was significantly lower in the CGA- or CGB-siRNA-treated cells compared with that of control cells. Nevertheless, it was noticed that although the amount of CGB secreted in the CGB-siRNA-treated PC12 cells was significantly reduced compared with the control, the amount of CGA secreted in the CGB-siRNA-treated PC12 cells was similar to that in the control cells (Fig. 8).

The above results apparently demonstrate the functional differences between CGA and CGB in secretory granule biogenesis. Despite the fact that CGA and CGB share several common properties, there are clear differences between CGA and CGB in the pH- and Ca\(^{2+}\)-dependence of aggregation (22), oligomerization state (35, 36), and pH-dependent interaction with the secretory granule membrane (21, 22, 26). In addition,
chromogranins also interact with most of the other granule matrix proteins at pH 5.5 (26), including secretogranin III (37), but dissociate from them at pH 7.5. Although CGA and CGB interact with the secretory granule membrane at pH 5.5, and CGA dissociates from it completely at pH 7.5 (38), CGB does not dissociate from it completely at pH 7.5 (39). It was further shown that interaction of the chromogranins with the secretory granule membrane is through chromogranin interaction with the secretory granule membrane proteins, which include the IP3Rs (21, 22, 26). Further, chromogranins A and B were recently shown to interact directly with the IP3R at pH 5.5 (26). Although CGA dissociated from the IP3R at pH 7.5 regardless of the presence of Ca2+, CGB did not dissociate completely from the IP3R at pH 7.5, and approximately one-third of the original CGB still maintained the interaction at pH 7.5 (26).

Being secretory proteins, CGA and CGB are transported to the endoplasmic reticulum (ER) of secretory cells first, where they are exposed to the physiological pH of 7.2–7.4 (40–42). However, in the ER pH of 7.2–7.4, there is neither any interaction between CGA and the IP3R (26) nor any chromogranin tetramer formation (35, 36). In view of the fact that only CGB interacts with the IP3R at pH 7.5 (26), CGB is expected to interact with the IP3R in the ER. The initial interaction of CGB with the potential secretory granule membrane proteins will in fact have the effect of earmarking these future secretory granule membrane proteins early in the ER.

The linkage between the secretory granule formation and CGA and CGB expression has also been shown previously by the finding that one PC12 cell variant that lost neurosecretion competence and secretory granules also lacked CGB expression (43). In this PC12 variant, the genes for CGA and CGB were found to be down-regulated more than 10-fold (43). Further, in a mouse pituitary cell line AtT-20 variant (HYA.15.6.T.3), which was devoid of both regulated secretion and secretory granules, no CGB expression was detected (44).

Using whole rats, the level of CGA expression in pituitary gonadotropes was shown to be closely related to the number of large secretory granules, whereas the number of small secretory granules was related to the level of secretogranin II expression (45). From these results, it appears that either CGA or CGB is capable of inducing formation of secretory granules of varying sizes. Whether additional factors are involved in chromogranin-induced secretory granule biogenesis awaits further study.

### Table II

**Distribution of dense-core granules in CGA- and CGB-siRNA-transfected PC12 cells**

|                  | Normal PC12 cell | Empty transfection | CGA-siRNA transfection | CGB-siRNA transfection |
|------------------|------------------|--------------------|------------------------|------------------------|
| Number of granules/area viewed | 12244/3222 | 12504/3283 | 7015/3187 | 2632/3069 |
| Granules/cell | 68.75 ± 8.50 | 70.19 ± 13.80 | 40.73 ± 7.49 | 14.99 ± 6.30 |
| Mean ± S.D. | 68.75 ± 8.50 | 70.19 ± 13.80 | 40.73 ± 7.49 | 14.99 ± 6.30 |

* A PC12 cell had 51–54 μm² of surface area in the central section (which crosses the center of the nucleus); therefore, “granules/cell” indicates the number of granules found in the respective average central section area of a cell in each group.

* Mean ± S.D.

* Mean ± S.D., p < 0.0001 by paired t test.

**Fig. 8.** SDS-PAGE and immunoblot analysis of the secreted proteins of PC12 cells. Proteins secreted in the media of CGA-siRNA- (A) or CGB-siRNA-transfected (B) PC12 cells in response to 2 mM BaCl₂ were resolved on 10% SDS-polyacrylamide gels (left panels). The same gels were also subjected to immunoblot analysis using CGA and CGB antibodies (right two panels). The CGA-siRNA-transfected cells (A) secreted substantially less CGA (center panel) and CGB (right panel), whereas the CGB-siRNA-transfected cells (B) secreted substantially less CGB (center) but an unreduced amount of CGB (right) into the surrounding media. Details are described under “Experimental Procedures.”
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