Presence of Secretogranin II and High-Capacity, Low-Affinity Ca^{2+} Storage Role in Nucleoplasmic Ca^{2+} Store Vesicles†

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ABSTRACT: Chromogranins and secretogranins have traditionally been known as marker proteins of secretory granules that contain the highest concentrations of cellular calcium, reaching ∼40 mM. In addition, chromogranin B was also shown to exist in the nucleus, localizing in the putative inositol 1,4,5-trisphosphate (IP_3)-sensitive nucleoplasmic Ca^{2+} store vesicles. Chromogranins A (CGA) and B (CGB) are high-capacity, low-affinity Ca^{2+} binding proteins, binding 30−90 mol of Ca^{2+}/mol with dissociation constants (Kd) of 1.5−4 mM. Yet the Ca^{2+}-binding property of secretogranins has not been studied. Here, we show the localization of secretogranin II (SgII) in the nucleus, more specifically, in the IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles along with CGB and the IP_3 receptors. We have also determined the Ca^{2+}-binding property of SgII and found that SgII binds 61 mol of Ca^{2+}/mol (910 nmol Ca^{2+}/mg) with a Kd of 3.0 mM at the intragranular pH 5.5 and 30 mol of Ca^{2+}/mol (440 nmol Ca^{2+}/mg) with a Kd of 2.2 mM at a near-physiological pH 7.5. Chromogranin B also bound 50 mol of Ca^{2+}/mol (670 nmol Ca^{2+}/mg) with a Kd of 3.1 mM at pH 7.5. Given the high-capacity, low-affinity Ca^{2+}-binding property of SgII and its presence in the IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles, these results suggest that SgII may function in the storage and control of Ca^{2+} in the nucleus through its interaction with CGB in the nucleoplasmic vesicles.

Secretogranin II[1] (chromogranin C) is a member of chromogranin−secretogranin family proteins, chromogranins A and B being the major members, which are known as marker proteins of secretory granules (*1*−*4*). Appropriate to this reputation, chromogranins A and B and secretogranin II play granulogenic roles (*5*−*7*), inducing secretory granule formation in cells in which they are expressed whether the cells are secretory or nonsecretory cells. Of the biochemical characteristics the chromogranin−secretogranin family members share, a high content of acidic amino acid residues (*8*−*13*) and the acidic pH- and Ca^{2+}-dependent aggregation property (*12*, *14*−*18*) stand out. Yet there exist very clear differences among chromogranins in the pH- and Ca^{2+}-dependent aggregation properties; CGB is by far the most sensitive to acidic pH and Ca^{2+}, followed by SgII, and CGA is least sensitive (*16*−*18*).

Moreover, chromogranins A and B and secretogranin II all interact with the secretory granule membranes at the intragranular pH 5.5 (*16*, *19*, *20*), but at a near-physiological pH 7.5 virtually all chromogranin A dissociates from the membrane, whereas chromogranin B and secretogranin II still maintain the interaction with the secretory granule membrane, indicating a much stronger interaction of CGB and SgII with the granule membranes than that of chromogranin A (*16*, *19*, *20*). In a direct comparison study of the granulogenic effects of CGA and CGB, the granulogenic effect of CGB was shown to be significantly greater than that of CGA (*6*). Therefore, the difference in the biochemical properties of CGA and CGB appears to bear upon the granulogenic effect of each molecule.

In addition to the abundance of chromogranins, secretory granules contain the most calcium in secretory cells (*21*), containing 40 mM Ca^{2+} and accounting for >60% of intracellular calcium (*22*). Most (>99.9%) of the granular Ca^{2+} is bound by chromogranins (*23*), and only a very small fraction (∼30 μM) of it stays in the free state (*23*−*26*); chromogranin A binds 55 mol of Ca^{2+}/mol with a Kd of 4.0 mM at the intragranular pH 5.5 (*27*), whereas chromogranin B binds 93 mol of Ca^{2+}/mol with a Kd of 1.5 mM (*28*). Due to the high-capacity, low-affinity Ca^{2+}-binding property, chromogranins carry out the Ca^{2+} storage function in the subcellular organelles in which they are localized. Furthermore, chromogranins A and B directly interact with the inositol 1,4,5-trisphosphate receptors (IP_3Rs) at the intragranular pH 5.5 (*29*) and modulate the IP_3R/Ca^{2+} channel activity, increasing the mean open time and the open probability of the channel, 8−42-fold and 8−16-fold, respectively (*30*, *31*). However, at a near-physiological pH of 7.5, CGA fails to interact with the IP_3R/Ca^{2+} channel (*29*), and only CGB interacts with the IP_3R/Ca^{2+} channel and activates the channel activity, increasing the mean open time and the open probability, 24-fold and 8-fold, respectively (*31*). Fitting to this coupling at pH 7.5, the IP_3R/Ca^{2+} channel-activating property of CGB has indeed been shown

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[1] Abbreviations: SgII, secretogranin II; CGA, chromogranin A; CGB, chromogranin B; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor.

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to play a key role in the IP_3-induced Ca^{2+} release from the endoplasmic reticulum (ER) (*32*).

Despite the fact that chromogranins have been considered marker proteins of secretory granules, chromogranin B, but not chromogranin A, has been shown to be present in the nucleus as well (*33*). Recently, it was further shown that CGB is present, along with the IP_3R/Ca^{2+} channels and phospholipids, in numerous small nucleoplasmic vesicles that release Ca^{2+} in response to IP_3 (*34*, *35*). The IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles have an average diameter of ~50 nm and an estimated molecular size of $\sim 2-3 \times 10^7$ Da (*34*, *35*). Given that the CGB concentration in the nucleus is estimated to be ~80 μM (*36*) and the nucleus contains ~11 mM Ca^{2+} (*34*), it appears that nuclear CGB not only plays a major role in the storage of Ca^{2+} but also modulates the IP_3R/Ca^{2+} channels in the IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles.

Hence, we have investigated in the present study the possibility of secretogranin II presence in the nucleus and found the existence of secretogranin II in the nucleus. Secretogranin II was present in the small nucleoplasmic vesicles where CGB and the IP_3Rs are localized. Further, we have also examined the Ca^{2+}-binding property of SgII, and found that SgII exhibits a high-capacity, low-affinity Ca^{2+}-binding property both at pH 5.5 and 7.5, which was also shown to be the case with CGB, thereby implying the importance of SgII and CGB in Ca^{2+} homeostasis in the nucleus.

EXPERIMENTAL PROCEDURES

Antibody Production. The polyclonal antirabbit CGA and CGB antibodies were raised against intact bovine CGA and recombinant CGB and affinity purified against purified bovine CGA and recombinant CGB, respectively. Monoclonal SgII antibody production was carried out with the secretory vesicle lysate proteins from bovine adrenal chromaffin cells as described previously (*16*). IP_3R peptides specific to terminal 10−13 amino acids of type 1 (HPPH-MNVNPQQPA) and type 2 (SNTPHENHHMPPA) were synthesized with a carboxy-terminal cysteine, and antirabbit polyclonal antibodies were raised. The polyclonal antirabbit antibodies were affinity purified on each immobilized peptide, and the specificity of each antibody was confirmed (*6*, *16*, *28*, *33*). Polyclonal antibodies for the nuclear protein histone H1 were from Upstate Biotechnology.

Preparation of Secretory Granule Lysates and the Nucleoplasm of Bovine Chromaffin Cells. The chromaffin granules from bovine adrenal chromaffin cells were prepared as described previously (*20*). The granules were lysed by resuspending in 40 volumes of 15 mM Tris−HCl, pH 7.5 and frozen and thawed twice. The lysed granules were centrifuged at 48 000*g* for 30 min to separate the lysates from the membrane, and the supernatant was collected for the soluble granule lysates. The nucleoplasm and nucleoplasmic proteins of chromaffin cells were obtained from the purified nuclei of bovine adrenal chromaffin cells as described (*34*, *35*).

Purification of the CGB- and IP_3R-Containing Vesicular Nucleoplasmic Structures. Purification of the IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles was done as described previously (*34*). Briefly, the extracted nucleoplasm (sample no. 1) from bovine chromaffin cells was first fractionated by Sephacryl S-1000 gel filtration chromatography as described (*34*). The fractions containing CGB and the IP_3-Rs were pooled and concentrated (sample no. 2). The concentrated nucleoplasmic sample no. 2 was further fractionated by sucrose gradient centrifugation. For this 7 mg of the nucleoplasmic proteins in 3 mL of buffer 3 (15 mM Tris−HCl, pH 7.5) was loaded on 28 mL of sucrose gradient solution (0.3−1.5 M sucrose in buffer 3) and centrifuged at 112 000*g* for 6 h at 2 °C. Approximately 1.1 mL per fraction was collected; every other fraction was analyzed by SDS−PAGE and immunoblots. Fractions 5−13 that contained the IP_3Rs and CGB were pooled and used as sample no. 3 after concentration.

Immunogold Electron Microscopy. For the immunogold electron microscopic study of chromaffin cells and the partially purified nucleoplasmic vesicles (sample no. 3), the tissue samples from bovine adrenal medulla as well as the nucleoplasmic sample no. 3 were prepared on Formvar/carbon-coated nickel grids as described (*34*). After etching and washing, the grids were placed on 50 μL droplets of solution A (phosphate-buffered saline solution, pH 8.2, containing 4% normal goat serum, 1% BSA, 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 monoclonal antimouse SgII 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 the 15 nm gold-conjugated goat antimouse IgG, diluted in solution A. For the double-immunogold labeling experiment, the grids that had gone through the SgII-labeling step with 15 nm particles were reacted once more with each protein (CGA, CGB, or IP_3R)-specific antibody labeled with 10 nm gold particles. Controls for the specificity of SgII-specific immunogold labeling included (1) omitting the primary antibody, (2) replacing the primary antibody with the preimmune serum, and (3) adding the primary antibody in the excess presence of purified SgII. After washes in PBS and deionized water, the grids were stained with uranyl acetate (7 min) and lead citrate (2 min) and were viewed with a JEOL JEM-1011 electron microscope.

Purification of Recombinant Secretogranin II and Chromogranin B and Ca^{2+}-Binding Study. Recombinant bovine SgII was expressed in *E. coli* and purified essentially following the procedure described for CGB (*37*). For Ca^{2+}-binding studies, purified recombinant SgII or CGB (5 mg) was coupled to 0.2 g of cyanogen bromide-activated Sepharose 4B according to the method suggested by the manufacturer (Pharmacia LKB Biotechnology). The amount of SgII or CGB coupled to Sepharose 4B was determined by extracting by a strong alkali treatment as described previously (*27*). Upon completion of the coupling, it was estimated that approximately 1 mg of protein was coupled to 1 mL (wet volume) of Sepharose 4B, and this was used for $^{45}Ca^{2+}$-binding studies. The Ca^{2+}-binding studies were carried out at pH 5.5 and 7.5 according to the procedure described for the Ca^{2+} binding to CGA (*27*).

RESULTS

To determine the possibility of the presence of secretogranin II in the nucleus, isolated nucleoplasmic proteins from

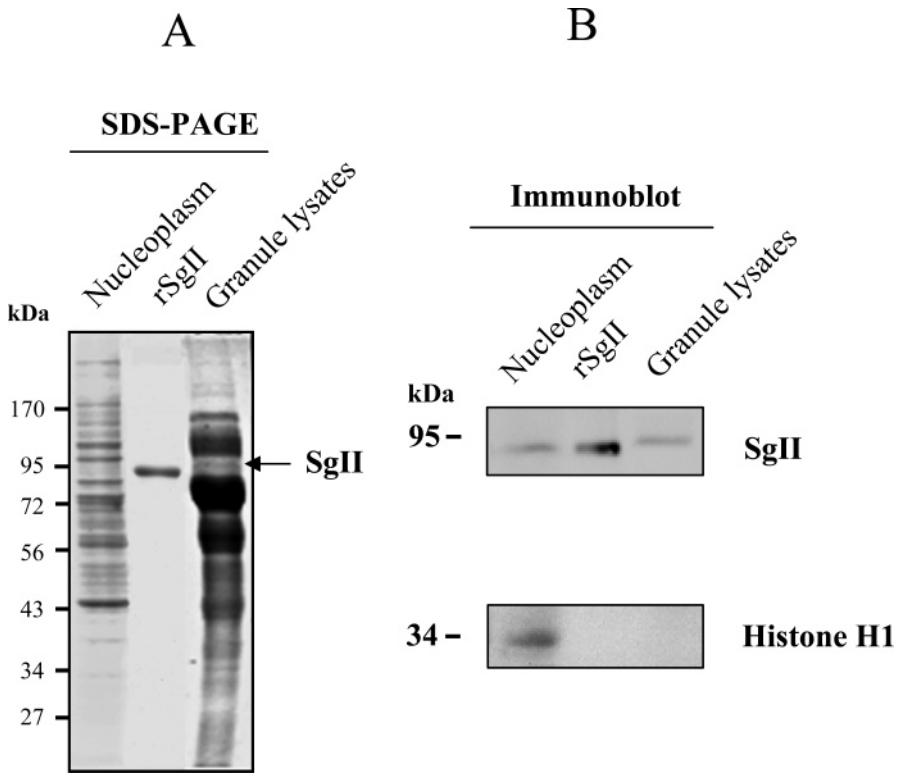

FIGURE 1: SDS−PAGE and immunoblot analysis of the nucleoplasmic proteins of bovine chromaffin cells. (A) Nucleoplasmic proteins (4 μg), purified recombinant SgII (0.2 μg), and chromaffin granule lysates (8 μg) were resolved on a 10% SDS−polyacrylamide gel. The position of the SgII band in the granule lysates is indicated by an arrow. (B) Nucleoplasmic proteins (10 μg), purified recombinant SgII (0.01 μg), and granule lysates (0.04 μg) resolved on SDS−polyacrylamide gels were subjected to immunoblot analysis using polyclonal histone H1-antibody or monoclonal SgII antibody. Details are described under the Experimental Procedures.

bovine adrenal chromaffin cells were immunoblotted with the SgII antibody (Figure 1). Figure 1A shows that bacterially expressed recombinant SgII is slightly smaller than the native SgII in the granule lysates. The immunoblot analysis of the nucleoplasmic proteins shown in Figure 1B indicates the presence of SgII in the nucleoplasmic proteins. It was nevertheless noted that the SgII position detected in the nucleoplasmic proteins was also slightly lower than that of secretory granule lysates but at the same position as that of recombinant SgII. Given that the bacterially expressed recombinant SgII is smaller than the granule lysate SgII, the presence of a slightly smaller SgII in the nucleoplasmic proteins might be due to lack of modification in the nucleoplasmic SgII. Chromogranins are known to be modified by phosphorylation, glycosylation, and sulfation (*38*, *39*) in the ER and the Golgi, whereby making the chromogranins significantly larger than the molecular sizes estimated from the amino acids. It is a plausible possibility that the smaller nuclear SgII is due to translocation of newly synthesized SgII to the nucleus without going through the usual modification steps in the ER and Golgi.

To confirm the presence of secretogranin II in the nucleus, we also used immunogold electron microscopy (Figure 2), the same technique that has been used in detecting the presence of CGB in the nucleus (*33*). As expected, the SgII-labeling gold particles were present in secretory granules and the ER but not in mitochondria (Figure 2A). Furthermore, the SgII-labeling gold particles were also localized in the nucleus (Figure 2A). In light of the absence of CGA and the presence of CGB in the nucleus, we also carried out the double-immunolabeling experiments using antibodies specific for CGA and SgII (Figure 2B) or for CGB and SgII (Figure 2C). As shown in the CGA-SgII double-labeling experiments (Figure 2B), CGA and SgII were both localized in secretory granules and the ER. In addition, although the chromogranin A-labeling gold particles (10 nm) were not present in the nucleus as reported before (*33*, *36*), the secretogranin II-labeling gold particles (15 nm) were clearly localized in the nucleus (Figure 2B).

Similar to the CGA−SgII results, the CGB−SgII double-labeling experiments (Figure 2C) also indicated the presence of both chromogranin B and secretogranin II in secretory granules, the ER and Golgi. However, unlike CGA that was absent in the nucleus, CGB is known to be present in the nucleus (*36*). In line with the previous results, CGB was present in the nucleus along with SgII (Figure 2C), the SgII-labeling gold particles (15 nm) often colocalizing with the CGB-labeling particles (10 nm). The specificity of the chromogranin− and secretogranin II−immunogold labeling was further confirmed in the absence of nonspecific gold labeling in mitochondria and in the control group (Figure 2D).

Given the localization of secretogranin II in the nucleus, we also determined the relative distribution of secretogranin II in the subcellular organelles of chromaffin cells. For this purpose, the total number of SgII-labeling gold particles found in each subcellular organelle of 45 electron micrographs from three different tissue preparations was divided by the total sum of the area occupied by each organelle (Table 1). The number of SgII-labeling gold particles/μm^2 of each organelle showed 8.23 for secretory granules and 2.61 for the ER (Table 1). The nucleus and Golgi showed 2.95 and 2.96 SgII-labeling gold particles/μm^2, respectively, whereas mitochondria showed 0.13, a level considered to

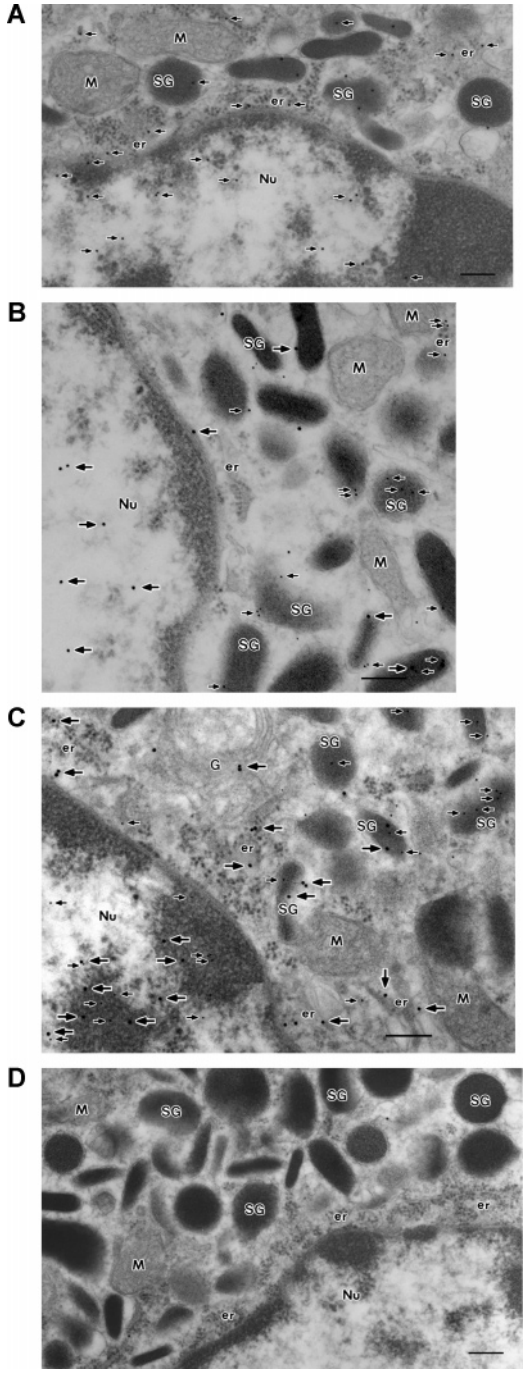

FIGURE 2: Immunogold electron microscopy showing the localization of secretogranin II in the nucleus. (A) Bovine adrenal chromaffin cells were immunolabeled for SgII (15 nm gold) with monoclonal SgII antibody. The SgII-labeling gold particles (some are indicated by arrows) are localized in secretory granules (SG), the endoplasmic reticulum (er) and nucleus (Nu), but not in mitochondria (M). (B) Chromaffin cells were also double-immunolabeled for SgII (15 nm, indicated by large arrows) and CGA (10 nm, small arrows) with monoclonal SgII antibody and the affinity-purified CGA antibody. The CGA- and SgII-labeling gold particles are localized in secretory granules (SG) and the endoplasmic reticulum (er) but not in mitochondria (M). Secretogranin II, but not CGA, is also localized in the nucleus (Nu). (C) Double-immunolabeling for SgII (15 nm, large arrows) and CGB (10 nm, small arrows) show the presence of the SgII- and CGB-labeling gold particles in secretory granules (SG), the endoplasmic reticulum (er), and the nucleus (Nu). (D) In the control experiments without the primary SgII antibody or in the excess presence of purified SgII no gold particles were seen in the cell. Bar = 200 nm.

Table 1: Distribution of the Secretogranin II-Labeling Gold Particles in Bovine Adrenal Chromaffin Cells

	no. of gold particles /area (μm^2)[a]	gold particles /μm^2	est concn (μM)
endoplasmic reticulum	232/89.04	2.61	9−12
secretory granule	919/111.61	8.23	30−40[b]
nucleus	512/173.82	2.95	10−13
Golgi	63/21.31	2.96	10−13
mitochondria	3/23.92	0.13	0

[a] A total of 45 images from three different tissue preparations were used. [b] Estimated from the immunoblot results of 2D-PAGE analysis of chromaffin granule lysates.

be background. From these results and the approximate secretogranin II concentration of 30−40 μM in secretory granules, the secretogranin II concentration in the nucleus was estimated to be ~10−13 μM and that in the ER ~9−12 μM (Table 1).

Furthermore, to identify the exact location of secretogranin II in the nucleus, we carried out the immunogold electron microscopy using the isolated nucleoplasm of bovine chromaffin cells. Our initial studies indicated the localization of SgII in small vesicular structures that looked to be similar to the IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles (*34*). Immunoblot analysis of the nucleoplasmic sample no. 2 that had been fractionated by sucrose gradient centrifugation demonstrated colocalization of SgII and CGB in the same fractions (Figure 3). These fractions are the partially purified nucleoplasmic vesicle sample no. 3 (cf., ref *34*). In addition, to determine whether SgII interacts with CGB, we carried out immunoprecipitation experiments using the vesicle lysates. As shown in Figure 4, SgII and CGB were shown to coimmunoprecipitate, thereby demonstrating the interaction between SgII and CGB.

We have further investigated the possibility of the presence of SgII in the IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles using the partially purified nucleoplasmic vesicle sample no. 3 (Figure 5). Indeed, the SgII-labeling gold particles (15 nm) localized in the lumen of the small nucleoplasmic vesicles (Figure 5), which maintained clearly visible membrane bilayer (Figure 5A). When the vesicles were double-immunolabeled with SgII and CGB (Figure 5B), both SgII (15 nm) and CGB (10 nm) were shown to colocalize in the nucleoplasmic vesicles. But when the nucleoplasmic vesicles were double-labeled with SgII and the IP_3R (Figure 5C−E), both SgII (15 nm) and the IP_3R (10 nm) colocalized to the vesicles, SgII localizing in the lumen and the IP_3R in the membrane region, thereby confirming the presence of SgII in the IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles. In our extensive EM study SgII has been exclusively observed in these vesicles. Yet it is not known at present whether or not SgII interacts with the IP_3R directly. Nonetheless, the presence of SgII in the nucleoplasmic Ca^{2+} store vesicles will be felt by the IP_3R/Ca^{2+} channels due to SgII's interaction with CGB, which in turn directly binds to the IP_3R/Ca^{2+} channels (*29*).

Considering that the small nucleoplasmic vesicles are the IP_3-sensitive Ca^{2+} stores, we also determined the Ca^{2+}-binding property of secretogranin II and found that SgII is a high-capacity, low-affinity Ca^{2+} binding protein (Figure 6). As was the case with chromogranin A (*27*), secretogranin II

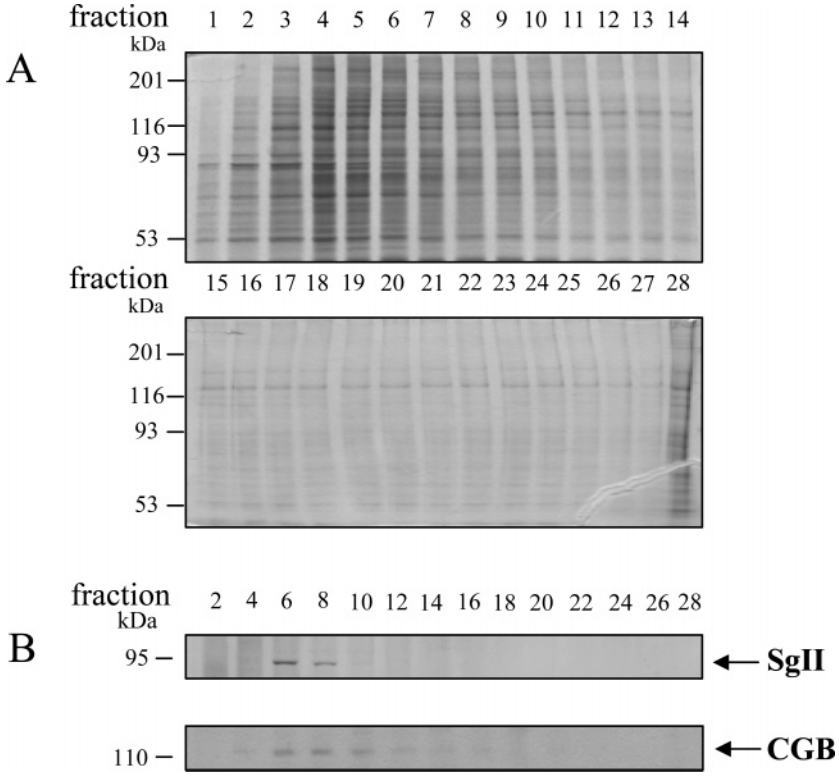

FIGURE 3: Sucrose gradient centrifugation and immunoblot analysis of the nucleoplasmic protein sample. Seven milligrams of nucleoplasmic sample no. 2 (cf., ref *34*) were separated on 28 mL of sucrose gradient (0.3−1.5 M), and 40 μL aliquots from each fraction (1.1 mL/fraction) were separated on a 10% SDS gel and visualized by coomassie blue staining (A). The same proteins were analyzed by immunoblot analysis using antibodies for SgII and CGB (B).

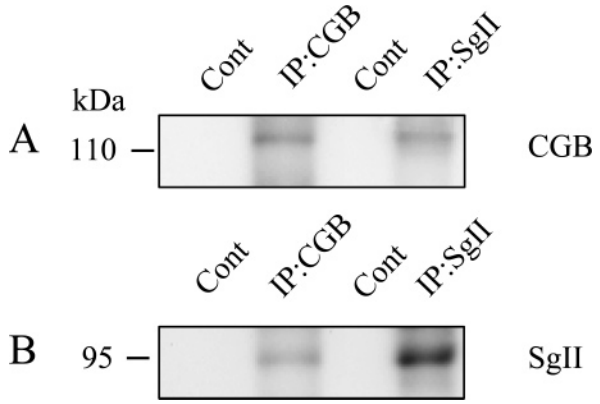

FIGURE 4: Coimmunoprecipitation of secretogranin II with chromogranin B. An amount of 200 μg of chromaffin granule lysates in 20 mM Tris−HCl, pH 7.5, 0.1 M KCl was immunoprecipitated with either CGB antibody (IP:CGB) or SgII antibody (IP:SgII). The immunoprecipitates were separated on a 10% SDS gel and were immunoblotted with either CGB antibody (A) or SgII antibody (B). Preimmune IgG was used as control antibody (Cont).

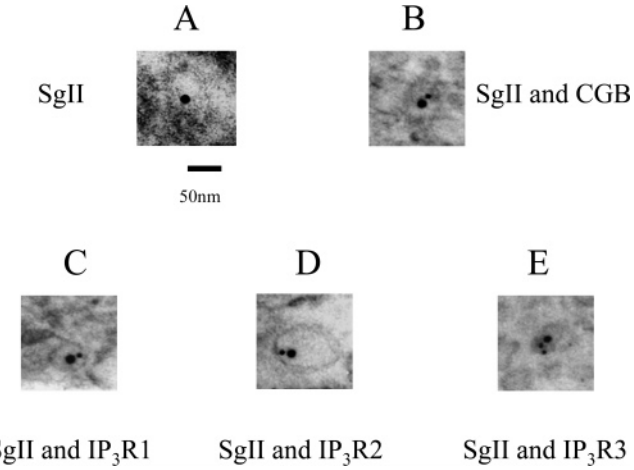

FIGURE 5: Immunogold electron microscopy with the partially purified nucleoplasmic Ca^{2+} store vesicles. Partially purified nucleoplasmic Ca^{2+} store vesicles (sample no. 3 in ref *34*) were examined by immunogold electron microscopy using antibodies for SgII, CGB, and IP_3R isoforms 1 (IP_3R1), 2 (IP_3R2), and 3 (IP_3R3). Labeling by SgII (15 nm) (A), SgII and CGB (10 nm) (B), SgII and IP_3R1 (10 nm) (C), SgII and IP_3R2 (10 nm) (D), and SgII and IP_3R3 (10 nm) (E). While SgII and CGB localized in the lumen of the vesicles, the IP_3R-labeling gold particles localized primarily in the membrane region of the vesicles. Bar = 50 nm.

bound a much larger amount of Ca^{2+} at the intragranular pH 5.5 than at a near-physiological pH 7.5, binding 61 mol of Ca^{2+}/mol (910 nmol of Ca^{2+}/mg) with a Kd of 3.0 mM at pH 5.5 and 30 mol of Ca^{2+}/mol (440 nmol of Ca^{2+}/mg) with a Kd of 2.2 mM at pH 7.5 (Figure 6). The Ca^{2+}-binding property of SgII at both pH values was similar to that of CGA, which bound 55 mol of Ca^{2+}/mol with a Kd of 4.0 mM at pH 5.5 and 32 mol of Ca^{2+}/mol with a Kd of 2.7 mM at pH 7.5 (*27*).

We have shown previously that chromogranin B also exhibits a high-capacity, low-affinity Ca^{2+}-binding property at pH 5.5, binding 93 mol of Ca^{2+}/mol with a Kd of 1.5 mM (*28*). But the Ca^{2+} binding by chromogranin B at pH 7.5 appeared to be very low compared to that at pH 5.5 so that we did not study the binding at pH 7.5 with a greater care at the time. We have therefore investigated here the Ca^{2+}-binding property of CGB at pH 7.5 as well. As shown in Figure 7, chromogranin B also bound a substantial amount of Ca^{2+} even at pH 7.5, binding 50 mol of Ca^{2+}/mol (670 nmol of Ca^{2+}/mg) with a Kd of 3.1 mM. This amount of

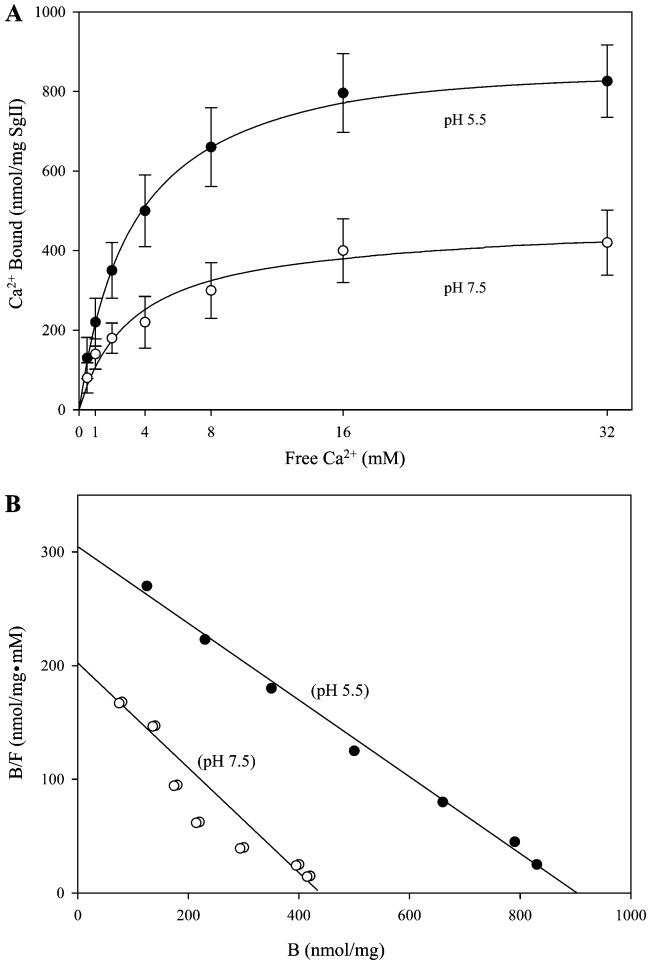

FIGURE 6: Binding of Ca^{2+} to secretogranin II immobilized on Sepharose 4B and Scatchard analysis. $^{45}Ca^{2+}$ binding to secretogranin II coupled to Sepharose 4B was measured at pH 5.5 (●) and pH 7.5 (○) and analyzed by Scatchard plot. Buffers used were either 20 mM sodium acetate, pH 5.5, 0.1 M KCl or 20 mM Tris−HCl, pH 7.5, 0.1 M KCl. Data are representative of experiments repeated four times. (A) Binding of $^{45}Ca^{2+}$ to secretogranin II immobilized on Sepharose 4B was measured as described previously (*27*). See also Table 2. (B) Scatchard analysis of the Ca^{2+} binding data in panel A. B/F, bound/free.

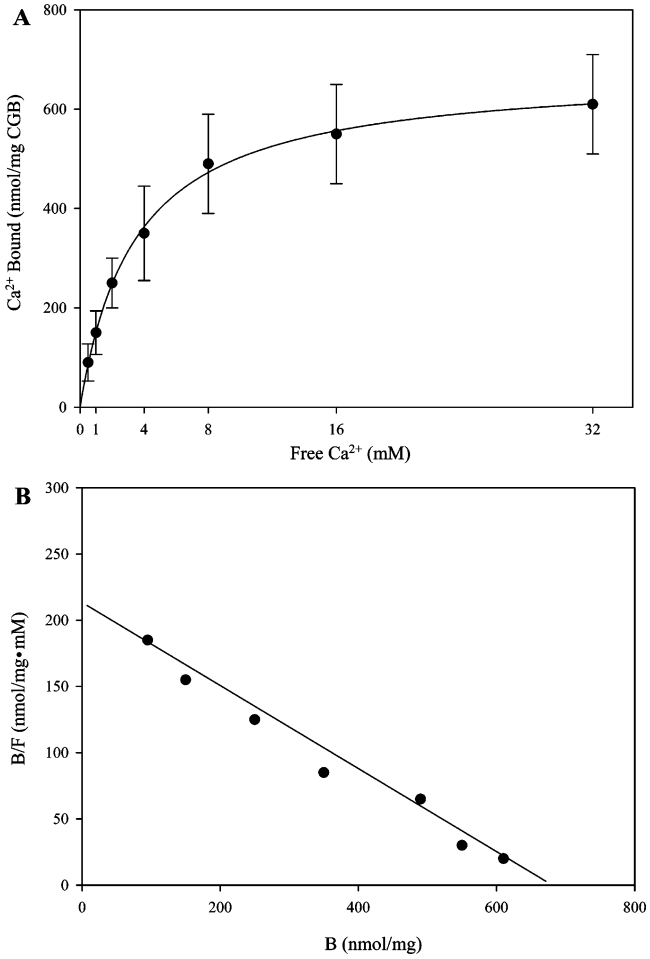

FIGURE 7: Binding of Ca^{2+} to chromogranin B immobilized on Sepharose 4B and Scatchard analysis. $^{45}Ca^{2+}$ binding to chromogranin B coupled to Sepharose 4B was measured at pH 7.5 and was analyzed by Scatchard plot. A 20 mM Tris−HCl, pH 7.5, 0.1 M KCl buffer was used. Data are representative of experiments repeated four times. (A) Binding of $^{45}Ca^{2+}$ to chromogranin B immobilized on Sepharose 4B was measured as described previously (*27*). See also Table 2. (B) Scatchard analysis of the Ca^{2+} binding data in panel A. B/F, bound/free.

Ca^{2+} is approximately half that bound by CGB at pH 5.5 (Table 2), yet it clearly suggests the high-capacity, low-affinity Ca^{2+} storage capability of chromogranin B in nonacidic organelles such as the nucleus.

DISCUSSION

The nucleus of chromaffin cells is known to contain ~11 mM Ca^{2+} (*34*), the second highest Ca^{2+} concentration found in intracellular organelles after the 40 mM Ca^{2+} found in secretory granules (*22*). The high nuclear Ca^{2+} concentration in turn strongly implied the presence of large amounts of Ca^{2+} storage proteins in the nucleus. In this respect, we have shown previously that chromogranin B exists in small nucleoplasmic vesicles that are located throughout the nucleoplasm, both in the heterochromatin and euchromatin regions, colocalizing with the IP_3Rs and phospholipids (*35*). These nucleoplasmic vesicles rapidly released Ca^{2+} in response specifically to inositol 1,4,5-trisphosphate, and no other inositol phosphates, including inositol 1,3,4-trisphosphate, inositol 1,4-bisphosphate, inositol 1,3,4,5-tetrakisphosphate, were able to induce Ca^{2+} release (*34*). These results

Table 2: pH-Dependent Ca^{2+}-Binding Properties of Secretogranin II and Chromogranin B

	secretogranin II		chromogranin B	
	Vmax, mol of Ca^{2+}/mol (nmol/mg)	dissociation constant (Kd, mM)	Vmax, mol of Ca^{2+}/mol (nmol/mg)	dissociation constant (Kd, mM)
pH 5.5	61 (910)	3.0	93 (1290)[a]	1.5[a]
pH 7.5	30 (440)	2.2	50 (670)	3.1

[a] The values are from Yoo et al. (ref *28*).

demonstrated not only the presence of large amounts of Ca^{2+} inside the vesicles but also the IP_3-sensitive Ca^{2+} store role of these vesicles.

Revealing further the identity of the molecules that may participate in the storage and control of Ca^{2+} in the nucleus, the present results demonstrate for the first time the presence of secretogranin II in the nucleus, localizing in the newly discovered IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles where chromogranin B and the IP_3Rs also exist (Figure 5). The localization of chromogranin B, the IP_3Rs, and SgII in the nucleus was exclusive to the small nucleoplasmic vesicles that are scattered throughout the heterochromatin and eu-

chromatin regions, and no other nucleoplasmic structures were shown to contain them (*34*, *35*). Therefore, colocalization of secretogranin II with CGB and the IP_3R (Figure 5) demonstrates the localization of SgII in the IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles.

It is nevertheless not evident how SgII finds its way into the nucleoplasmic vesicles. Like chromogranins A and B that interact with the secretory granule membrane at pH 5.5 (*19*, *20*), secretogranin II also interacts with the secretory granule membrane (*16*). The strength of CGA interaction with the secretory granule membrane is the weakest of the three so that CGA dissociates from the membrane by a mere change of pH from 5.5 to 7.5, a condition that can be experienced by CGA when it is released from secretory granules to the bloodstream during exocytosis. However, CGB and SgII interact with the membrane more strongly so that a mere change of pH from 5.5 to 7.5 is not sufficient to cause dissociation of these proteins from the granule membrane (*16*, *19*), implying the interaction of CGB and SgII even at pH 7.5 albeit at a reduced strength. So far, the identity of the secretory membrane target(s) to which SgII binds is not known. A possible route of targeting SgII to the nucleus could be through the participation of a nuclear localization signal (NLS) that may exist in the SgII. It is generally understood that rather than being any primary sequence of fixed amino acids NLSs consist of a certain peptide segment of proteins that meets three rules: (1) NLSs are structurally disordered in free states, (2) have overall basic character, and (3) possess a central hydrophobic or basic motif (*40*). Under this criterion, a segment in bovine SgII, ERKLKH (residues 127−132), may qualify to be a potential NLS. Secretogranin II is an unstructured protein with a high content (>75%) of random coil and β-sheet structures (*16*), and the sequence ERKLKH has a strong basic character with basic/hydrophobic residues in the center. Yet in spite of the possibility of this region being an NLS of SgII, we are inclined to think that an NLS may not be solely responsible for SgII to target to the nucleus, judging from our experience with an NLS of CGB, which did not prove to be essential for CGB to target to the nucleus (*33*).

Along with more abundantly expressed chromogranins A and B, secretogranin II is also called a secretory granule marker protein (*1*, *4*). In secretory granules of bovine adrenal chromaffin cells secretogranin II is estimated to exist in ~30−40 μM (Table 1), far less than ~1.8−2 mM of CGA and ~0.2 mM of CGB (*2*, *41*). From the SgII concentration in secretory granules and the relative distribution profile of SgII-labeling gold particles in chromaffin cells (Table 1), SgII is estimated to exist in the nucleus and the ER at 10−13 and 9−12 μM, respectively. The fact that secretogranin II exists in similar concentrations in both the ER and the nucleus contrasts with chromogranin A that exists in the ER at ~140 μM (Table 3) but is absent in the nucleus (*33*, *36*). On the other hand, chromogranin B concentration in the ER is ~120 μM, similar to that of chromogranin A in the ER, but its concentration in the nucleus is estimated to be ~80 μM (Table 3). Chromogranin B concentration in the nucleus is a bit lower than that of the ER, but it is severalfold higher than that of secretogranin II in the nucleus, implying major roles of chromogranin B in the IP_3-dependent Ca^{2+} movement in the nucleus.

Table 3: Estimated Concentrations of Chromogranins A and B and Secretogranin II in Bovine Adrenal Chromaffin Cells

	chromogranin A (μM)[a]	chromogranin B (μM)[a]	secretogranin II (μM)
endoplasmic reticulum	140	120	9−12
secretory granule	1800	200	30−40
nucleus	0	80	10−13

[a] The estimated concentrations are from Huh et al. (ref *36*).

Further, chromogranin B bound large amounts of Ca^{2+} at pH 7.5, binding 50 mol of Ca^{2+}/mol with a Kd of 3.1 mM (Figure 7). With this high-capacity Ca^{2+}-binding property and the presence of 80 μM CGB in the nucleus, CGB alone would be able to bind millimolar range of Ca^{2+}. Chromogranin B is known to directly bind to the IP_3Rs and activate the IP_3R/Ca^{2+} channel activity at both intragranular pH 5.5 and a near-physiological pH 7.5 (*29*, *31*). Moreover, the coupling between CGB and the IP_3R/Ca^{2+} channel in the ER has been demonstrated to play key roles in IP_3-dependent Ca^{2+} release from the ER in vivo (*32*). Given that the nucleoplasm also maintains a physiological pH of 7.2−7.4, it is highly likely that the nucleoplasmic CGB also modulates the IP_3R/Ca^{2+} channel activities of the nucleoplasmic vesicles.

The results in Figure 6 further show that secretogranin II is capable of binding large amounts of Ca^{2+} with low affinities not only in secretory granules but also in other organelles in which they are localized. Although SgII has been known to bind Ca^{2+} (*16*, *23*, *42*), the present results report for the first time the quantitative Ca^{2+}-binding property of secretogranin II: SgII binds 61 mol of Ca^{2+}/mol with a Kd of 3.0 mM at pH 5.5 and 30 mol of Ca^{2+}/mol with a Kd of 2.2 mM at pH 7.5 (Figure 6). It is noteworthy that the Ca^{2+}-binding property of SgII is very similar to that of its cousin but far more abundant CGA, which binds 55 mol of Ca^{2+}/mol with a Kd of 4.0 mM at pH 5.5 and 32 mol of Ca^{2+}/mol with a Kd of 2.7 mM at pH 7.5 (*27*). With the estimated concentration of SgII in the nucleus at 10−13 μM and the Ca^{2+}-binding property of 30 mol of Ca^{2+}/mol at a near-nuclear pH, SgII could also contribute significantly to the storage of Ca^{2+} in the IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles.

Given that the IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles have an average diameter of ~50 nm and that these small vesicles would occupy only a limited internal volume of the nucleus despite their widespread presence in the nucleoplasm (*34*), it is quite conceivable that the virtual concentrations of nuclear CGB and SgII in the nucleoplasmic vesicles could reach tens of fold higher than the estimated concentrations of 80 μM for CGB and 10−13 μM for SgII. In this respect, the respective CGB and SgII concentration in the nucleoplasmic vesicles could be at least severalfold higher than that in secretory granules; thus, the nucleoplasmic vesicles would be able to store substantial amounts of Ca^{2+} due to the high concentrations of chromogranin B and secretogranin II. In reality, it would be possible to store even up to tens of millimolar Ca^{2+} in the nucleoplasmic vesicles. Taking the likely CGB and SgII concentrations in the nucleoplasmic vesicles and the Ca^{2+}-binding property of these molecules at pH 7.5 into consideration, it may be

presumed that the IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles contain tens of millimolar Ca^{2+} and release Ca^{2+} through the IP_3R/Ca^{2+} channels that exist on the vesicular membranes upon IP_3 stimulation.

It is now known that chromogranins A and B interact with the IP_3R/Ca^{2+} channels through the interaction between the conserved near N-terminal regions of chromogranins and one of the intraluminal loops, L3−2 (second small loop of the third intraluminal loop), of the integral membrane protein IP_3R (*43*, *44*). Agreeing with the relative strength of the interaction between the secretory granule membrane and chromogranins A and B, the conserved near N-terminal region of CGB interacts with the intraluminal loop of the IP_3R markedly more strongly than that of CGA (*43*, *44*). However, unlike chromogranins A and B that share the conserved near N-terminal domain that participates in the interaction of chromogranins with the IP_3Rs (*43*, *44*), secretogranin II does not contain the conserved near N-terminal domain, whereby partially explaining why it is not called a chromogranin anymore.

Nevertheless, in view of the direct coupling of CGB with the IP_3Rs and modulation of the Ca^{2+} channels (*29*, *31*), and of the ability of CGB and SgII to interact with each other in the presence of Ca^{2+} even at pH 7.5 (Figure 4), it is likely that SgII plays active roles not only in the storage of Ca^{2+} inside the IP_3-sensitive nucleoplasmic Ca^{2+} store vesicles but also in the control of Ca^{2+} release through the IP_3R/Ca^{2+} channels. In spite of the presence of a high percentage of charged (20% acidic and 13% basic) amino acid residues (*8*, *11*, *12*, *45*), secretogranin II is thought to contain a significant portion of hydrophobic domains within it, being more hydrophobic than chromogranins A and B (*16*). These hydrophobic domains appear to be exposed upon binding Ca^{2+}, thereby allowing the exposed hydrophobic domains to interact with each other and leading to Ca^{2+}-dependent aggregation. The Ca^{2+}-dependent aggregation property of SgII is approximately 8-fold more sensitive to Ca^{2+} than CGA, although it is ~8-fold less sensitive to Ca^{2+} than CGB (*16*). In this respect, it is appealing to think that the highly Ca^{2+}-sensitive property of CGB and SgII could have selected out these two proteins from other members of chromogranin−secretogranin family and targeted to the nucleoplasmic Ca^{2+} store vesicles.

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