Purification, pH-Dependent Conformational Change, Aggregation, and Secretory Granule Membrane Binding Property of Secretogranin II (Chromogranin C)†

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ABSTRACT: Secretogranin II (SgII) is one of the three major proteins, the other two being chromogranins A (CGA) and B (CGB), of secretory granules of neuroendocrine cells. The Ca^{2+} storage proteins CGA and CGB not only are coupled to the IP_3 receptor (IP_3R)/Ca^{2+} channels that exist on the secretory granule membrane but also are known to play key roles in secretory granule biogenesis. Unlike the better studied CGA and CGB, secretogranin II has never been completely purified in the native state and studied. We have therefore purified SgII in native form from bovine adrenal medulla and subjected it to biochemical characterization. Secretogranin II consisted of largely β-sheet and random coil structures with a low level of α-helicity. Like CGA and CGB, it also underwent pH-dependent conformational changes, showing 9.5% α-helicity at pH 7.5 and 17.0% α-helicity at pH 5.5. Secretogranin II also underwent acidic pH- and Ca^{2+}-dependent aggregation, and it was approximately 8-fold more sensitive than CGA to Ca^{2+} in its pH-dependent aggregation but was 8-fold less sensitive than CGB. Further, similar to CGA and CGB that had interacted with the secretory granule membrane at the intragranular pH 5.5, SgII also interacted with the secretory granule membrane at pH 5.5 and dissociated from it at near-physiological pH 7.5, implying similar roles of SgII in the cell as those of CGA and CGB. Secretogranin II hence appeared to actively participate in secretory granule biogenesis as has been proposed for CGA and CGB.

The secretory granules of neurons and neuroendocrine cells contain hormones, neurotransmitters, peptides, proteins, and many ions including Ca^{2+}, Zn^{2+}, and Mg^{2+} (*1*). Among the many proteins found in the secretory granule, which is the major IP_3-sensitive intracellular Ca^{2+} store of secretory cells (*2*−*5*), chromogranins account for the majority of the intragranular matrix proteins (*6*−*10*). Chromogranins consist of chromogranins A and B and secretogranin II (also called chromogranin C). The two most dominant proteins of secretory granules chromogranins A and B share two conserved regions, one near the N-terminal region (residues 17−38 of bovine CGA[1] and 16−37 of bovine CGB) and the other the C-terminal region (residues 401−431 of CGA and 606−626 of CGB) (*11*−*21*). Besides the two conserved regions in CGA and CGB, the amino acid sequences of these three chromogranin proteins show little homology except that they all contain very high acidic amino acid residues, containing 20−25% acidic residues with isoelectric points of 4.5−5.5 (*11*−*26*) and an amino acid sequence motif 'LExLxxxxxE' (in the single-letter code) in the C-terminal region.

In addition, chromogranins A and B have also been demonstrated to undergo acidic pH- (*27*−*29*) and Ca^{2+}-dependent aggregation and conformational changes (*28*, *30*). Unlike the cytoplasm, the secretory granules maintain an intragranular pH of 5.5 (*31*, *32*), and this acidic milieu appears to be pivotal in controlling functions of many intragranular proteins. At this acidic pH, the intragranular chromogranins not only aggregate (*27*−*29*) but also interact with many secretory granule membrane proteins, including the IP_3R/Ca^{2+} channel (*33*). The acidic pH-dependent aggregation was observed only in the secretory granule matrix proteins but not in nonsecretory granule proteins (*22*, *34*), implying the importance of the acidic pH-dependent aggregation in the selective segregation process during secretory granule biogenesis (*8*, *27*−*29*, *34*). In addition to the selective aggregation of the secretory granule matrix proteins, the interaction of secretory granule membrane proteins with the matrix proteins will be of equal importance in segregating the potential secretory granule membrane proteins in the *trans*-Golgi network (TGN) where the secretory granule biogenesis takes place. Thus, we and others have proposed the essential role of chromogranins in the biogenesis of secretory granules (*8*, *27*−*29*, *34*, *35*). Indeed, CGA was recently shown to be directly responsible for formation of secretory granules (*36*). Using neuroendocrine PC12 cells, nonneuroendocrine fibroblast cells, and the antisense RNA technique, Kim et al. (*36*) demonstrated that CGA functions as an on/off switch for secretory granule formation.

Despite the importance of chromogranins A and B in secretory granule biogenesis (*8*, *34*, *36*) and in the IP_3-sensitive intracellular Ca^{2+} store role of secretory granules (*2*, *5*), the cellular function or relative importance of another

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[1] Abbreviations: SgII, secretogranin II; CGA, chromogranin A; CGB, chromogranin B; IP_3R, inositol 1,4,5-trisphosphate receptor; CD, circular dichroism; CNBr, cyanogen bromide; TGN, *trans*-Golgi network.

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major secretory granule matrix protein, secretogranin II (chromogranin C), has not been studied well. Although a small amount of SgII had previously been purified utilizing a boiling step and electroelution from SDS−polyacrylamide gel slices (*22*), secretogranin II has never been completely purified in the native state and studied. Exposure of proteins to SDS−PAGE is known to denature the proteins, changing the native structures. For this reason, the proteins that had been exposed to SDS may not be suitable for biochemical characterization. Moreover, we have also found in our previous study that boiling of CGA changed the native conformation (*28*). Therefore, it appears likely that boiling of SgII may also change the native conformation of SgII although both CGA and SgII are heat-stable. We have hence purified secretogranin II from the secretory granules of bovine adrenal medullary chromaffin cells in native form and subjected it to biochemical characterization.

EXPERIMENTAL PROCEDURES

Materials. DE-52 was obtained from Whatman, and phenyl-Sepharose and cyanogen bromide (CNBr)-activated Sepharose 4B were from Pharmacia LKB Biotechnology Inc. Chelex 100 was from Bio-Rad, and imidazole, acrylamide, bis-acrylamide, Tris, SDS, and EGTA were from Sigma.

Antibody Production. The polyclonal anti-rabbit 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 according to the published procedure (*37*). Briefly, BALB/c mice (4−6 weeks old) were immunized with the secretory vesicle lysate proteins from bovine adrenal chromaffin cells after mixing with Freund's complete adjuvant. The spleen cells from the immunized BALB/c mice were later used to fuse with myeloma cells (P3-NS1/1-Ag 4.1). The antibody-producing hybridoma clones were then screened, and a mouse hybridoma clone producing the antibody for bovine SgII was obtained. The monoclonal antibody was obtained from the cellular fluid of this clone.

Secretory Vesicle Lysate and Vesicle Membrane Preparation. The chromaffin granules from bovine adrenal chromaffin cells were prepared as described previously (*2*). The vesicles were lysed by resuspending in 40 volumes of 15 mM Tris-HCl, pH 7.5, and frozen and thawed twice. Separation of the vesicle lysates and vesicle membranes, and coupling of the vesicle membrane to the CNBr-activated Sepharose 4B were done as described previously (*38*).

DE-52 Column Chromatography. Eighty milligrams of vesicle lysate proteins was loaded onto a DE-52 column (2.6 × 12 cm) equilibrated with 10 mM imidazole, pH 6.1, and the column was washed with 3 bed volumes of the equilibration buffer. Elution was carried out with a 500 mL KCl gradient (0.1−0.4 M) in 10 mM imidazole, pH 6.1. The flow rate was 2.5 mL/min, and 10 mL fractions were collected. Fractions eluted at 0.22−0.31 M were pooled and subjected to phenyl-Sepharose column chromatography.

First Phenyl-Sepharose Column Chromatography. The pooled DE-52 fractions (23.4 mg) were adjusted to 0.3 M $(NH_4)_2SO_4$, 15 mM Tris-HCl, pH 7.5, 0.2 mM $CaCl_2$, and were loaded on a phenyl-Sepharose column (1.6 × 10 cm). The proteins were eluted with a 320 mL reverse $(NH_4)_2SO_4$ gradient (0.3−0 M) in 15 mM Tris-HCl, pH 7.5, 0.2 mM $CaCl_2$. The flow rate was 2 mL/min, and 8 mL fractions were collected. An additional 80 mL of buffer with no ammonium sulfate was added to complete the elution. Secretogranin II-containing fractions were obtained primarily in the additional elution without ammonium sulfate.

Second Phenyl-Sepharose Column Chromatography. The SgII-containing fractions (0.73 mg) from the first phenyl-Sepharose column were adjusted to 0.3 M $(NH_4)_2SO_4$ in 15 mM Tris-HCl, pH 7.5, 0.2 mM $CaCl_2$, and loaded on a second phenyl-Sepharose column (1.0 × 4 cm). The proteins were eluted with 100 mL of 50 mM ammonium sulfate in the same buffer. The flow rate was 2 mL/min, and 4 mL fractions were collected. Pure SgII was eluted in fractions 16−25.

Two-Dimensional PAGE and Amino Acid Sequencing. One-dimensional isoelectric focusing was carried out in the pH range of 4−7 using a Bio-Rad protein IEF cell, and two-dimensional PAGE was carried out on a 10% SDS-gel. The amino acid sequences were determined by the Edman degradation method using an Applied Biosystem 491 Procise protein sequencer, and seven amino acids from the N-terminus were determined.

Circular Dichroism (CD) Spectroscopy. Circular dichroism spectra were recorded using a Jasco 715 CD spectrophotometer (Jasco Inc., Easton, MD) using a cell with a path length of 0.1 cm and a protein concentration of 0.08 mg/mL in either 7.5 mM sodium acetate, pH 5.5, or 7.5 mM MOPS, pH 7.5. All spectra were taken at 22 °C and are the average of at least four scans. Analysis of spectra was carried out using the CD reference spectra provided by Jasco Inc. (prepared by J. T. Yang).

Aggregation Experiments. Chelex 100-treated CGA, CGB, or SgII in either 15 mM sodium acetate, pH 5.5, or 15 mM MOPS, pH 7.5, was titrated with concentrated $CaCl_2$. Aggregation was monitored at 320 nm by measuring the turbidity change with a Beckman spectrophotometer. All the measurements were done at 22 °C.

RESULTS

Purification. To purify SgII from the secretory vesicle lysates of bovine adrenal medulla, the chromaffin granule lysates were fractionated on a DE-52 column as shown in Figure 1A. Fractions containing SgII in the middle of the elution were pooled and further fractionated by hydrophobic chromatography on phenyl-Sepharose (Figure 1B). Secretogranin II was eluted in the absence of ammonium sulfate at the end of a reverse $(NH_4)_2SO_4$ gradient (0.3−0 M) (Figure 1B). As shown in Figure 1B,C, chromogranin A started to elute from the hydrophobic column at 0.2−0.25 M ammonium sulfate concentration, followed by CGB. However, SgII was not eluted until the elution buffer contained no ammonium sulfate (Figure 1C), indicating a higher hydrophobicity of SgII compared to that of CGA and CGB. The SgII-containing fractions from the first phenyl-Sepharose column chromatography still contained substantial amounts of CGA, and were further fractionated on a second phenyl-Sepharose column as described under Experimental Procedures. In the second phenyl-Sepharose column chromatography, CGA was eluted first, but it was followed by pure intact SgII (cf. Figure 2A). Very rarely some CGA still eluted

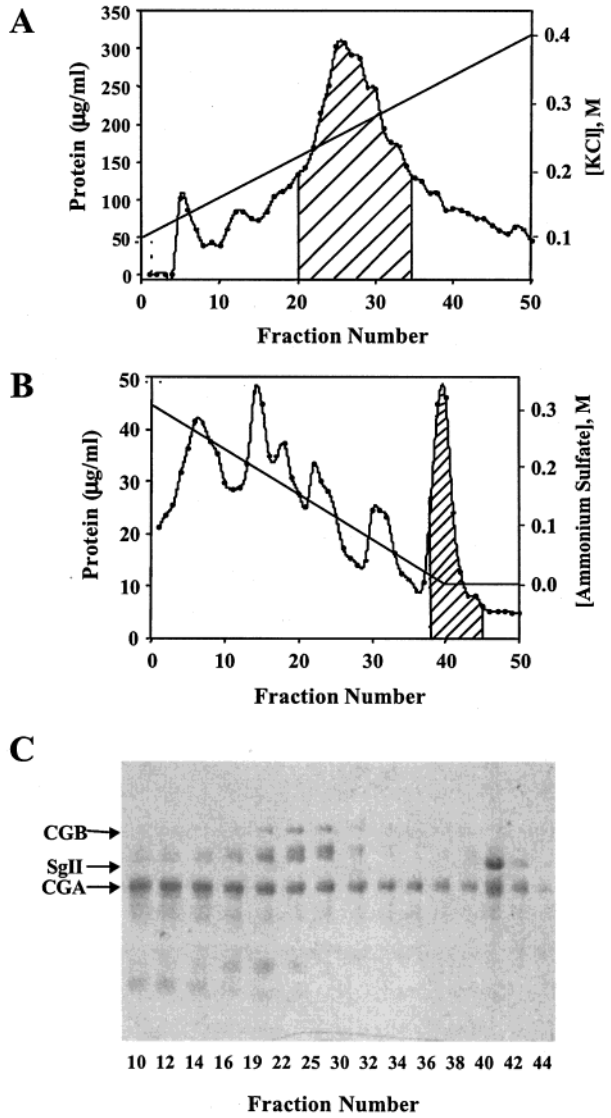

FIGURE 1: Chromatography and SDS−PAGE of the secretory vesicle lysate proteins. (A) DE-52 column chromatography: the secretory vesicle lysate proteins were directly loaded onto a DE-52 column and eluted with a KCl gradient. The pooled (shaded area) fractions were further purified on a phenyl-Sepharose column. (B) First phenyl-Sepharose column chromatography: the pooled fractions from the DE-52 column were loaded and eluted with a reverse ammonium sulfate gradient. The SgII-containing fractions (shaded area) were subjected to the second phenyl-Sepharose column chromatography. (C) Appropriate fractions from the first phenyl-Sepharose column chromatography were analyzed on a 10% SDS−polyacrylamide gel stained with Coomassie blue. The location of CGB, SgII, and CGA is indicated by arrows. Details are described under Experimental Procedures.

with SgII in this final step. In that case, the second phenyl-Sepharose 4B column step was repeated to remove the remaining CGA completely. The SgII obtained after the second phenyl-Sepharose column chromatography step consisted of a single protein as shown in the 2D-polyacrylamide gel (Figure 2B). The identity of this protein was further confirmed by amino acid sequencing of the protein (Figure 2B). Quantification of the SgII during the purification procedures was carried out after electroelution of the SgII band excised from the SDS−PAGE gels. With these purification steps, 0.17 mg of pure intact native SgII was obtained in three column steps from 80 mg of vesicle lysates, recovering 22% of the original SgII contained in the lysates

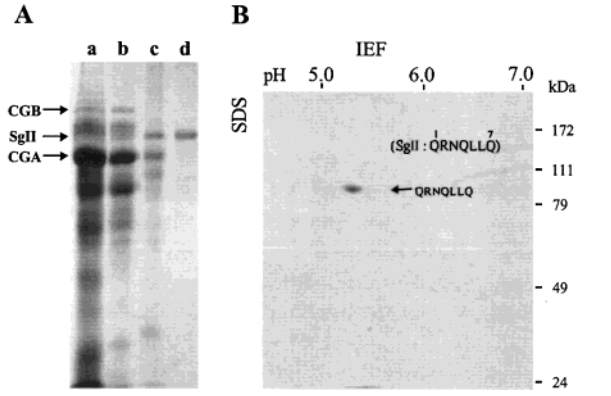

FIGURE 2: SDS−PAGE of secretogranin II fractions and 2D-PAGE of purified SgII. (A) The vesicle lysate proteins from each purification step were visualized on a 10% SDS−polyacrylamide gel: vesicle lysate (17 μg, lane a); protein after the DE-52 step (6 μg, lane b); protein after the first phenyl-Sepharose step (2 μg, lane c); purified SgII after the second phenyl-Sepharose step (0.8 μg, lane d). The location of CGB, SgII, and CGA is indicated by arrows. (B) Two-dimensional PAGE was carried out on a 10% SDS-gel using 2 μg of purified SgII. The amino acid sequences were determined by the Edman degradation method. The gels were stained with Coomassie blue.

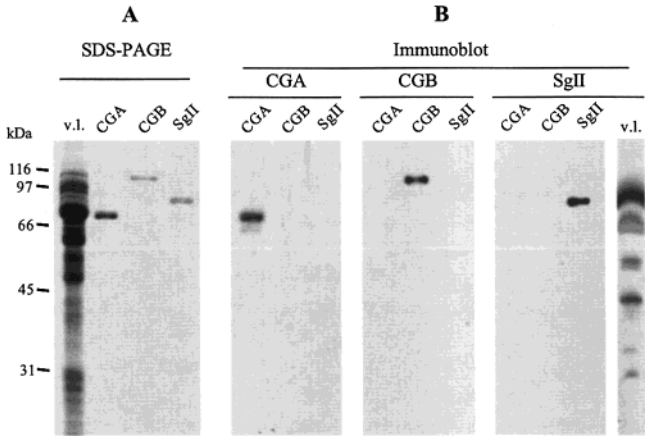

FIGURE 3: SDS−PAGE and immunoblot analysis of purified CGA, CGB, and SgII. (A) The vesicle lysate proteins (v.l., 20 μg), purified CGA (1 μg), CGB (0.6 μg), and SgII (0.6 μg), all from bovine adrenal chromaffin cells, were separated on a 10% SDS−polyacrylamide gel and visualized with Coomassie blue staining. (B) The purified CGA, CGB, and SgII, separated on a 10% polyacrylamide gel as shown in the left panel, were immunoblotted with polyclonal CGA, CGB, and monoclonal SgII antibodies. The vesicle lysate proteins were also immunoblotted with the monoclonal SgII antibody using the enhanced chemiluminescence technique as shown in the rightmost lane (v.l.).

Table 1: Summary of Secretogranin II Purification from the Secretory Vesicle Lysate of Bovine Adrenal Medulla

	total protein (mg)	% recovery	% purity	secretogranin II (mg)
vesicle lysate	80.00	100.0	0.95	0.76
DE-52	23.40	82.9	2.69	0.63
first phenyl	0.73	36.8	38.67	0.28
second phenyl	0.17	22.4	100.00	0.17

(Table 1). Immunoblot analysis of the purified SgII by CGA, CGB, and SgII antibodies also confirmed that the purified SgII is free from any potentially contaminating CGA or CGB (Figure 3). Moreover, it further indicated that the vesicle lysate proteins contain several minor SgII fragments in addition to the major intact SgII (Figure 3B, lane v.l.).

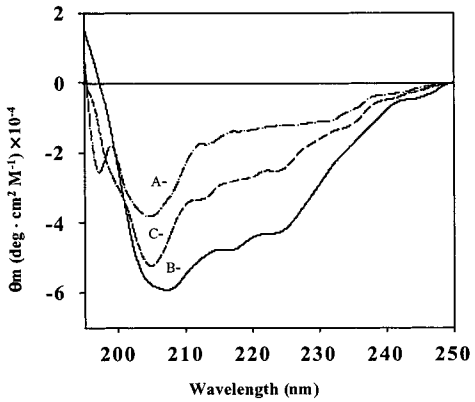

FIGURE 4: CD spectra of secretogranin II at pH 5.5 and 7.5. (A) SgII in 7.5 mM MOPS, pH 7.5, and 0.2 mM EGTA; (B) SgII in 7.5 mM sodium acetate, pH 5.5, and 0.2 mM EGTA; (C) SgII in 7.5 mM sodium acetate, pH 5.5, and 0.2 mM $CaCl_2$. Secretogranin II concentration was 0.08 mg/mL.

Table 2: Effects of pH and Ca^{2+} on Secretogranin II Conformation

pH	[Ca^{2+}] (mM)	% α-helix[a]	% β-sheet	% turn	% random coil
7.5		9.5	52.0	13.3	25.3
5.5		17.0	37.2	7.2	38.6
	0.2	7.4	48.8	1.5	42.3

[a] CD spectra of SgII obtained under various conditions as described in Figure 4 were analyzed according to the reference spectra provided by Jasco Inc.

Conformation. The nature of the pH-induced conformational changes of SgII was studied by circular dichroism at pH 5.5 and 7.5. As shown in Figure 4, the CD spectra of SgII at pH 5.5 and 7.5 are very different. In the absence of Ca^{2+}, the α-helicity of SgII at pH 7.5 was 9.5%, but it increased to 17.0% at pH 5.5 (Table 2). Moreover, the random coil structure increased from 25.3% at pH 7.5 to 38.6% at pH 5.5. However, both β-sheet and turn structures decreased from 52.0% and 13.3% at pH 7.5 to 37.2% and 7.2% at pH 5.5, respectively. Further, the presence of Ca^{2+} changed the conformation of SgII at pH 5.5, decreasing the α-helicity from 17.0% to 7.4% and the turn structure from 7.2% to 1.5%, and at the same time increasing the β-sheet and random coil structures. However, unlike the effect of Ca^{2+} on SgII conformation at pH 5.5, the presence of up to 15 mM Ca^{2+} at pH 7.5 did not cause any detectable change of the SgII CD spectra (not shown), suggesting the failure of Ca^{2+} to change the SgII conformation at pH 7.5.

In gel-filtration chromatography, SgII was eluted at a position indicative of a globular protein with a size of 85 kDa at pH 7.5 and 74 kDa at pH 5.5 both in the presence (2 mM Ca^{2+}) and in the absence (4 mM EGTA) of Ca^{2+} (Figure 5). Given that the actual molecular mass of bovine SgII is 67 kDa, the elution sizes of 74−85 kDa suggest that the purified SgII exists in a monomeric state regardless of the presence of Ca^{2+} at both pH 5.5 and pH 7.5.

pH-Dependent Secretory Vesicle Membrane Binding. In light of the pH-dependent secretory vesicle membrane binding of CGA (*38*) and CGB (*39*), it was of interest to determine whether purified SgII binds to the vesicle membrane. For this purpose, 80 μg of purified SgII in 20 mM sodium acetate, pH 5.5, 0.1 M KCl was applied to the vesicle membrane-coupled Sepharose 4B column (0.6 mL volume) equilibrated with 20 mM sodium acetate, pH 5.5, 0.1 M KCl.

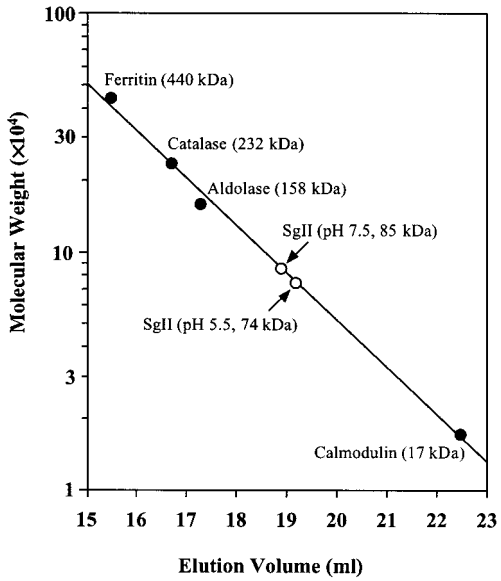

FIGURE 5: Gel filtration on Superose 6. Secretogranin II (50 μg) was chromatographed on a Superose TM 6 column (1.0 × 31 cm) equilibrated with either 20 mM Tris-HCl, pH 7.5, or 20 mM sodium acetate, pH 5.5, both in 0.15 M KCl, 4 mM EGTA, and eluted at 24 mL/h. The column was calibrated with the molecular size markers ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and calmodulin (17 kDa). The elution locations of SgII at pH 7.5 and 5.5 are indicated by arrows with estimated molecular sizes in parentheses. Identical results were obtained in the presence of 2 mM $CaCl_2$ (added in place of 4 mM EGTA).

As shown in Figure 6A, elution of the column with a 1 M KCl buffer released a small amount of protein from the column, suggesting the possibility that SgII bound to the vesicle membrane at pH 5.5. Further elution of the same column with a buffer of pH 7.5 instead of pH 5.5 released a significantly larger amount of protein from the column, indicating that the change of pH from 5.5 to 7.5 caused the dissociation of the bound proteins. Given the possibility that both the intragranular and cytoplasmic sides of the secretory granule membranes were coupled to the Sepharose 4B resin and from the fact that CGA interacts only with the intragranular side of the granule membrane (*38*), the amount of SgII bound to and released from the granule membrane is presumed to be the SgII that had interacted only with the intragranular side of the membrane.

To analyze the content of the eluted proteins, the protein fractions were analyzed by SDS−PAGE. As shown in Figure 6B, there was very little SgII in the fractions eluted with the 1 M KCl buffer with no change in pH, but a significant amount of SgII was present in the fractions eluted with the pH 7.5 1 M KCl buffer. The amount of SgII eluted was ∼17 μg, accounting for 21% of the loaded SgII. This result clearly indicated that SgII bound to the vesicle membrane at pH 5.5 and was released from it at pH 7.5. The pH-dependent binding of SgII appeared to be a specific interaction between the vesicle membrane and SgII because the same CNBr-activated Sepharose 4B resin that had gone through all the coupling steps but without the vesicle membrane was not able to bind any SgII.

Since it has previously been shown that a large portion of the bound CGA can be released from the vesicle membrane-coupled column by changing the pH of the elution buffer to 7.5 without changing the salt concentration (*38*), the possibility of elution of SgII from the vesicle membrane-coupled

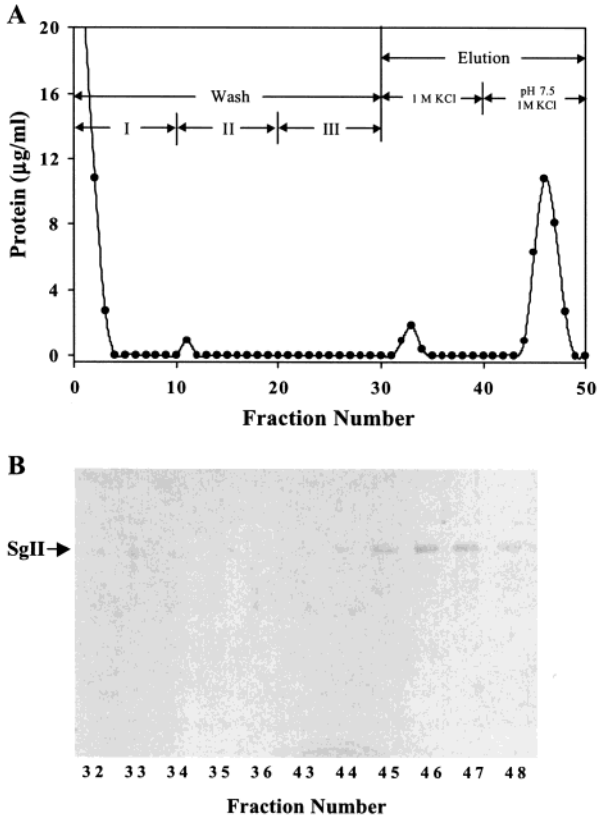

FIGURE 6: Vesicle membrane-coupled Sepharose 4B chromatography and SDS−PAGE of the eluted secretogranin II. (A) 80 μg of SgII in 0.3 mL of 20 mM sodium acetate, pH 5.5, 0.1 M KCl was loaded onto a vesicle membrane-coupled Sepharose 4B column (0.6 mL volume) equilibrated with 20 mM sodium acetate, pH 5.5, 0.1 M KCl. The column was washed with 0.1 M KCl (Wash I), followed by 0.15 M KCl (Wash II) and 0.1 M KCl (Wash III), all in 20 mM sodium acetate, pH 5.5. The protein was first eluted with 1 M KCl in the same acetate buffer (Elution I). After the high-salt elution, the pH of the elution buffer was changed to 7.5 (20 mM Tris-HCl, pH 7.5, 1 M KCl) and further eluted (Elution II). The fraction size was 1 mL/fraction for Washes I−III, and 0.5 mL/fraction for Elutions I−II. The chromatography was carried out at room temperature. (B) SDS−PAGE of the eluted proteins from (A). 50 μL aliquots from the eluted fractions are analyzed on a 10% SDS−polyacrylamide gel visualized with Coomassie blue staining. Secretogranin II eluted is indicated by an arrow.

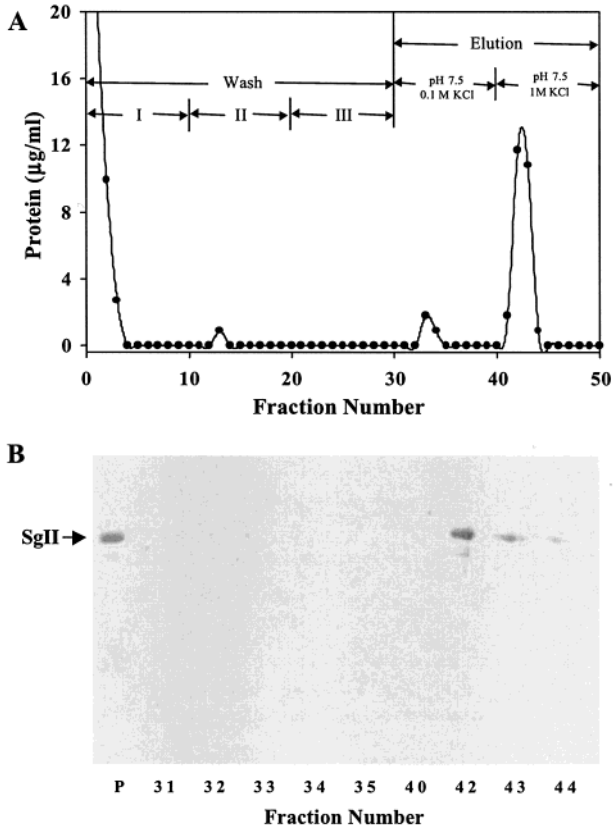

FIGURE 7: Vesicle membrane-coupled Sepharose 4B chromatography and SDS−PAGE of the eluted secretogranin II. (A) 80 μg of SgII in 0.3 mL of 20 mM sodium acetate, pH 5.5, 0.1 M KCl was loaded onto a vesicle membrane-coupled Sepharose 4B column (0.6 mL volume) equilibrated with 20 mM sodium acetate, pH 5.5, 0.1 M KCl. The chromatography was carried out as described in Figure 5 except that the first elution was carried out with a pH 7.5 buffer (20 mM Tris-HCl, pH 7.5) with no added salt. The second elution was carried out with 1 M KCl in 20 mM Tris-HCl, pH 7.5. Other conditions are the same as for Figure 5. (B) SDS−PAGE of the eluted proteins from (A). 50 μL aliquots from the eluted fractions are analyzed on a 10% SDS−polyacrylamide gel visualized with Coomassie blue staining. Secretogranin II eluted is indicated by an arrow. P stands for loaded purified SgII (0.5 μg).

column by the pH change from 5.5 to 7.5 was tested (Figure 7). Hence, 80 μg of SgII was loaded onto the same vesicle membrane-coupled column, washed with the pH 5.5 sodium acetate buffers as had been done in Figure 6, and eluted with a pH 7.5 buffer without changing the salt concentration (Figure 7A). As shown in Figure 7A, elution with a pH 7.5 buffer alone released only a small amount of protein. However, elution of the same column with the pH 7.5 1 M KCl buffer released a large amount of protein, indicating that the pH change alone is not sufficient to release the bound protein from the column. Analysis of the eluted proteins by SDS−PAGE (Figure 7B) showed that the bound SgII is primarily eluted by the pH 7.5 1 M KCl buffer, suggesting a stronger interaction of SgII with the vesicle membrane than CGA.

Aggregation Property. The two major secretory granule matrix proteins CGA and CGB have been shown to undergo pH- and Ca^{2+}-dependent aggregation, aggregating to a far greater extent at pH 5.5 than at pH 7.5 (*28, 30*). Hence, to determine whether SgII also undergo pH- and Ca^{2+}-dependent aggregation, the aggregation property of SgII was determined at both pH 5.5 and 7.5 in the presence of increasing concentrations of Ca^{2+} (Figure 8). The results in Figure 8 show that the extent of SgII aggregation at pH 5.5 is much higher than at pH 7.5; even at maximal aggregation, the extent of aggregation at pH 7.5 is less than one-tenth that at pH 5.5, demonstrating a marked pH-dependent aggregation property of SgII. In addition, Figure 9 shows that the pH- and Ca^{2+}-dependent aggregation pattern of SgII is dependent upon the protein concentration; i.e., the higher the SgII concentrations, the lower the Ca^{2+} concentrations that were needed to induce aggregation. Moreover, the extent of aggregation was also strongly dependent on the Ca^{2+} concentration at each protein concentration (Figure 9). When the SgII concentration was low, the maximal level of aggregation was also low, thus clearly indicating a critical dependence of aggregation on SgII concentration. This pH- and Ca^{2+}-dependent aggregation property of SgII is similar to that of CGA and CGB (*28, 30*).

Although both CGA and CGB have been shown to exhibit pH- and Ca^{2+}-dependent aggregation properties (*28, 30*), the extent of CGB aggregation was far greater than that of CGA

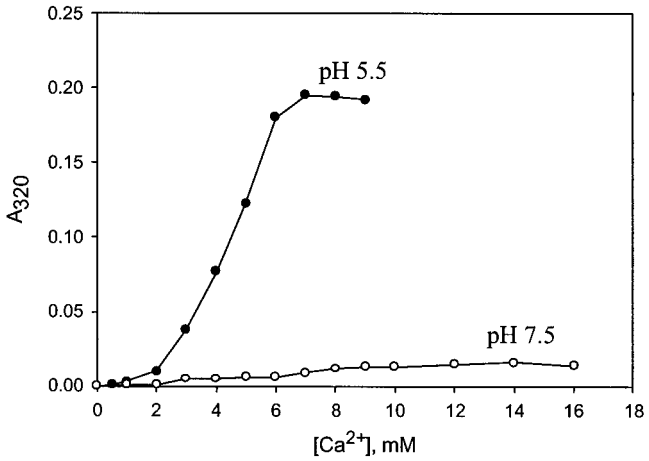

FIGURE 8: Effect of pH on secretogranin II aggregation. Aggregation of SgII (0.4 mg/mL) at pH 5.5 (●) and 7.5 (○) was measured as a function of Ca^{2+} concentration. The buffers were either 20 mM sodium acetate, pH 5.5, or 20 mM MOPS, pH 7.5. The A_{320} values are those taken 2 min after the addition of Ca^{2+}.

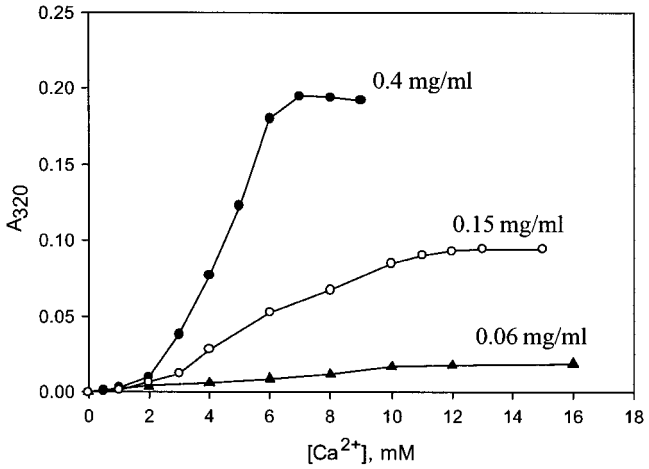

FIGURE 9: Aggregation of secretogranin II as a function of Ca^{2+} concentration at pH 5.5. Secretogranin II concentrations are 0.4 (●), 0.15 (○), and 0.06 mg/mL (▲) in 20 mM sodium acetate, pH 5.5. The A_{320} values are those taken 2 min after the addition of Ca^{2+}.

aggregation (*30*), and the CGB aggregation was at least 2 orders of magnitude more sensitive to Ca^{2+} than CGA aggregation. In this regard, it became of immediate interest to determine both the extent and the sensitivity to Ca^{2+} of SgII aggregation in comparison with those of CGA and CGB. As shown in Figure 10, the maximal level of SgII aggregation at the protein concentration of 0.15 mg/mL was similar to that of CGA aggregation at the CGA concentration of 0.6 mg/mL. However, the Ca^{2+} concentrations required to induce the maximal aggregation of CGA and SgII were different; i.e., the maximal level of SgII aggregation at 0.15 mg/mL required 12−13 mM Ca^{2+} whereas that of CGA aggregation at 0.6 mg/mL required ∼25 mM Ca^{2+} (Figure 10). Taking the protein and Ca^{2+} concentrations into account, the aggregation of SgII was estimated to be 8-fold more sensitive to Ca^{2+} than that of CGA. Further comparison of the aggregation property between SgII and CGB at 0.15 mg/mL showed that the maximal level of CGB aggregation is approximately 3-fold higher than that of SgII. Moreover, the Ca^{2+} concentration required to induce the maximal aggregation of each protein was different; 5 mM Ca^{2+} was required

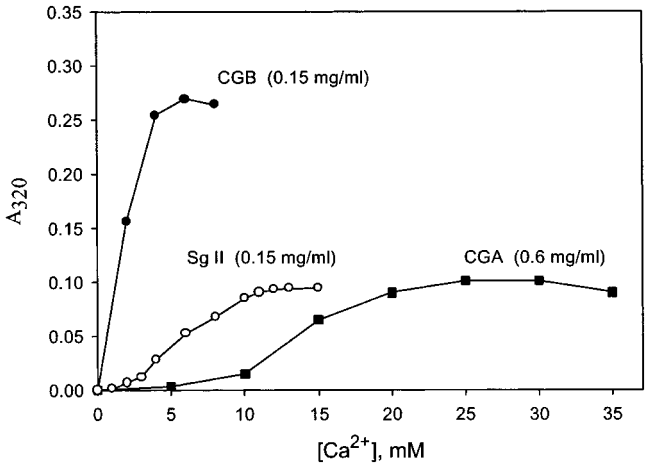

FIGURE 10: Comparison of the aggregation properties of chromogranins A and B, and secretogranin II as a function of Ca^{2+} concentration. Aggregation of CGA (0.6 mg/mL), CGB (0.15 mg/mL), and SgII (0.15 mg/mL) in 20 mM sodium acetate, pH 5.5, was measured as a function of Ca^{2+} concentration. The A_{320} values for CGB (●), SgII (○), and CGA (■) are those taken 2 min after the addition of Ca^{2+}.

to induce the maximal level of CGB aggregation whereas 12−13 mM Ca^{2+} was required for SgII aggregation, indicating that the CGB aggregation is 7−8-fold more sensitive to Ca^{2+} than SgII. Taken together, these results show that CGB is most sensitive to Ca^{2+}, followed by SgII and CGA, in its pH-dependent aggregation.

DISCUSSION

Purification. The present study reports the purification and biochemical characterization of secretogranin II for the first time. From the elution position of secretogranin II in the phenyl-Sepharose column (Figure 1B,C), SgII appeared to be more hydrophobic than either CGA or CGB. Despite the high content of hydrophilic amino acid residues in CGA and CGB (*11*−*21*), chromogranins A and B both contained hydrophobic areas in the molecules (*28*, *30*), and this hydrophobic property proved to be crucial in purifying CGA and CGB previously (*28*, *39*). Likewise, SgII which consists of 33% hydrophilic (20% acidic and 13% basic) amino acid residues (*22*−*26*) exhibited a hydrophobic property, which also proved to be useful in purifying SgII (cf. Figure 1B,C).

In pulse−chase experiments using PC12 cells, Rosa et al. (*40*) have previously observed the existence of newly synthesized SgIIs in different stages of posttranslational modifications, thus resulting in the presence of multiple SgII spots on 2D-gels. Unlike the newly synthesized SgIIs shown in the pulse−chase experiments (*40*), the SgII present in the secretory granule bovine adrenal chromaffin cells from which we purified SgII appeared to be in a mature form, existing in one dominant mature SgII plus several minor proteolyzed fragments as has been demonstrated in the immunoblot analysis of the secretory vesicle lysates by SgII antibody (Figure 3B, lane v.l.).

Conformation. Like CGA and CGB, which have shown pH-dependent conformational changes (*28*, *30*), SgII has also undergone pH-dependent conformational changes (Figure 4 and Table 2). The CD results indicated that secretogranin II was mostly composed of β-sheet and random coil structures and contained a low level of α-helicity, containing 9.5%

α-helix at pH 7.5 and 17.0% α-helix at pH 5.5 in the absence of Ca^{2+} (Figure 4 and Table 2). The high content of β-sheet and random coil structures in SgII explains the larger molecular size (~80 kDa) on the SDS−polyacrylamide gel than the actual size of 67 kDa (*23*). Similarly, chromogranins A and B have also been shown to contain high (60−70%) random coil structures (*28, 30*), and the high random coil structures were considered to contribute to the slower migration of these proteins on the SDS−polyacrylamide gel, migrating at the 75 kDa position for CGA compared to its actual size of 48 kDa (*11*−*16*) and at the 110 kDa position for CGB despite its actual size of 72 kDa (*17*−*21*).

In gel-filtration chromatography, SgII eluted at positions indicative of a protein with a molecular size of 74 kDa at pH 5.5 and 85 kDa at pH 7.5 (Figure 5), thereby indicating a monomeric existence of SgII at both pH values. Similarly, CGB has also been eluted previously at positions indicative of a globular protein with a molecular size of ~200 kDa at both pH 5.5 and 7.5 (*30*), indicating a monomeric existence. However, CGA eluted at positions suggesting a globular protein with a size of 300 kDa at pH 7.5 and $\sim 1 \times 10^6$ Da at pH 5.5 due to the dimeric and tetrameric existence of CGA at pH 7.5 and at pH 5.5, respectively (*28*). In our previous studies, the α-helicity of CGA and CGB was shown to increase as a result of the pH change from 7.5 to 5.5, increasing from 25% to 40% α-helicity for CGA (*28*) and from 15% to 20% α-helicity for CGB (*30*). The increase in α-helicity of SgII at pH 5.5 appears to reflect the more compact structures of CGA, CGB, and SgII at pH 5.5. In line with the structural features of these proteins, monomeric CGB and SgII eluted in gel-filtration chromatography at positions implying smaller overall sizes at pH 5.5 than at pH 7.5 regardless of the presence of Ca^{2+} (Figure 5).

Secretory Vesicle Membrane Interaction. Like CGA and CGB, which interacted with the secretory vesicle membrane at the intravesicular pH of 5.5 and dissociated from it at a near-physiological pH of 7.5 (*38, 39*), SgII also interacted with the secretory vesicle membrane at pH 5.5 and dissociated from it at pH 7.5 (Figures 6 and 7). In addition to CGA, CGB, and SgII, most of the secretory vesicle matrix proteins were also shown to bind to the vesicle membrane at pH 5.5 and to dissociate from it at pH 7.5 (*41*). From the fact that the elution of SgII from the secretory granule membrane required both the change of pH from 5.5 to 7.5 and high salt concentration (1 M KCl) (Figure 7), the interaction between SgII and the secretory granule membrane appears to be stronger than that between CGA and the granule membrane (*38*). Similarly, elution of CGB from the secretory granule membrane has also been shown to require the combination of pH change and high salt concentration (*39*), suggesting a stronger interaction of CGB with the membrane than CGA. In this respect, it was indeed shown that CGB interacts with the secretory granule membrane protein, IP_3R/Ca^{2+} channel, far more strongly than CGA (*42*).

Furthermore, the acidic pH-dependent interaction of SgII with the secretory vesicle membrane appears to reflect the well-known characteristics of secretory vesicle matrix proteins as has been studied in detail with the two major secretory vesicle matrix proteins CGA and CGB (*38, 39*). During secretory vesicle biogenesis, the potential secretory vesicle matrix proteins and the vesicle membrane proteins need to be selected out from the rest of the nonsecretory vesicle components in the *trans*-Golgi network. Given that the pH of the TGN is maintained at 6.4 (*29*), the acidic pH-dependent interaction of SgII with the vesicle membrane suggests that SgII has the intrinsic ability to bind to the potential secretory vesicle membrane in the TGN, thus ensuring its entry into the secretory vesicle during secretory vesicle biogenesis. Nevertheless, unlike CGA and CGB which contain the well-conserved N- and C-terminal regions that are deemed to play key roles in sorting of chromogranins (*38, 43*), SgII does not share these conserved regions with CGA and CGB. Hence, the regions of SgII that interact with the vesicle membrane remain to be determined. Once inside the secretory vesicles, SgII will stay bound to the vesicle membrane as is the case for CGA and CGB, but it will dissociate from the secretory vesicle membrane during exocytosis due to the exposure of SgII to extracellular pH 7.4, and enter the bloodstream. In light of the observations that SgII is processed to a potentially bioactive peptide, secretoneurin (*44, 45*), the SgII that enters the bloodstream is expected to serve as the precursor for the SgII fragments which may exert their effects on various targets in the body.

Aggregation. Moreover, analogous to CGA and CGB which have shown pH- and Ca^{2+}-dependent aggregation properties, SgII also exhibited pH- and Ca^{2+}-dependent aggregation properties (Figure 8). This result is in line with the reported aggregation property of SgII that had been purified using a non-native condition like SDS−PAGE (*22*). As shown in Figure 10, the SgII aggregation appeared to be 8-fold more sensitive to Ca^{2+} than that of CGA, but it was approximately 8-fold less sensitive than CGB, placing SgII between CGB and CGA in terms of its Ca^{2+}-dependent aggregation property. These results suggest that binding of Ca^{2+} to CGB or SgII exposes far more hydrophobic regions of CGB than it does with SgII although there may be more intrinsic hydrophobic regions in SgII than in CGB, allowing the newly exposed hydrophobic regions of CGB to come in contact with each other to form larger CGB aggregates. The fact that the aggregation readily occurs at pH 5.5 points to the pH-induced conformational changes of the proteins to conformations more conducive to Ca^{2+} binding. In line with the aggregation results shown in Figure 10, the pH- and Ca^{2+}-induced conformational changes of CGA and CGB have been shown before (*28, 30*). Further, most other secretory vesicle matrix proteins have also been shown to aggregate in a Ca^{2+}- and acidic pH-dependent manner (*34*). Taken together, the pH- and Ca^{2+}-dependent aggregation appears to be the property shared by most of the intravesicular matrix proteins as has been individually demonstrated for CGA, CGB, and SgII.

The pH- and Ca^{2+}-dependent conformational changes as well as the aggregation properties of CGA, CGB, and SgII appear to highlight the close relationship between the conformation and the Ca^{2+}-binding property of these proteins (*46*−*48*); i.e., the conformation assumed by the proteins at pH 5.5 is substantially more favorable than at pH 7.5 for Ca^{2+} binding. Consistent with this possibility, CGA has been shown to bind 55 mol of Ca^{2+}/mol at pH 5.5 while binding 30 mol of Ca^{2+}/mol at pH 7.5 (*48*). Likewise, CGB also bound 93 mol of Ca^{2+}/mol at pH 5.5 while little or no Ca^{2+} was shown to bind CGB at pH 7.5 (*49*). These results suggest that the extent of Ca^{2+} binding to CGA and CGB is closely related to the conformation of these proteins, which in turn

influences the Ca^{2+}-induced aggregation property of these proteins. Although no detailed study has been done regarding the Ca^{2+}-binding property of SgII, it would not be surprising to find SgII binding more Ca^{2+} at pH 5.5 than at pH 7.5 as has been the case for CGA and CGB. That SgII also aggregates far more readily at pH 5.5 than at pH 7.5 (Figure 6), requiring lower concentrations of Ca^{2+}, appears to favor this possibility.

REFERENCES

1. Winkler, H., and Westhead, E. (1980) *Neuroscience 5*, 1803−1823.
2. Yoo, S. H., and Albanesi, J. P. (1990) *J. Biol. Chem. 265*, 13446−13448.
3. Gerasimenko, O. V., Gerasimenko, J. V., Belan, P. V., and Petersen, O. H. (1996) *Cell 84*, 473−480.
4. Nguyen, T., Chin, W.-C., and Verdugo, P. (1998) *Nature 395*, 908−912.
5. Yoo, S. H. (2000) *Trends Neurosci. 23*, 424−428.
6. Simon, J.-P., and Aunis, D. (1989) *Biochem. J. 262*, 1−13.
7. Helle, K. B. (1990) *Neurochem. Int. 17*, 165−175.
8. Huttner, W. B., Gerdes, H.-H., and Rosa, P. (1990) *Trends. Biochem. Sci. 16*, 27−30.
9. Winkler, H., and Fischer-Colbrie, R. (1992) *Neuroscience 49*, 497−528.
10. Iacangelo, A. L., and Eiden, L. E. (1995) *Regul. Pept. 58*, 65−88.
11. Benedum, U. M., Baeuerle, P. A., Konecki. D. S., Frank, R., Powell, J., Mallet, J., and Huttner, W. B. (1986) *EMBO J. 5*, 1495−1502.
12. Iacangelo, A., Affolter, H.-U., Eiden, L. E., Herbert, E., and Grimes, M. (1986) *Nature 323*, 82−86.
13. Iacangelo, A. L., Fischer-Colbrie, R., Koller, K. J., Brownstein, M. J., and Eiden, L. E. (1988) *Endocrinology 122*, 2339−2341.
14. Konecki, D. S., Benedum, U. M., Gerdes, H.-H., and Huttner, W. B. (1987) *J. Biol. Chem. 262*, 17026−17030.
15. Helman, L. J., Ahn, T. G., Levine, M. A., Allison, A., Cohen, P. S., Cooper, M. J., Cohn, D. V., and Israel, M. A. (1988) *J. Biol. Chem. 263*, 11559−11563.
16. Wu, H.-J., Rozansky, D. J., Parmer, R. J., Gill, B. M., and O'Connor, D. T. (1991) *J. Biol. Chem. 266*, 13130−13134.
17. Bauer, J. W., and Fischer-Colbrie, R. (1991) *Biochim. Biophys. Acta 1089*, 124−126.
18. Benedum, U. M., Lamouroux, A., Konecki, D. S., Hille, A., Baeuerle, P. A., Frank, R., Lottspeich, F., Mallet, J., and Huttner, W. B. (1987) *EMBO J. 6*, 1203−1211.
19. Pohl, T. M., Phillips, E., Song, K., Gerdes, H. H., Huttner, W. B., and Rüther, U. (1990) *FEBS Lett. 262*, 219−224.
20. Linard, C. G., Mbikay, M., Seidah, N. G., and Chretien, M. (1990) *Nucleic Acids Res. 18*, 1298.
21. Depreitere, J., Wang, Z., and DePotter, W. P. (2001) *Eur. J. Biochem. 268*, 235−242.
22. Gerdes, H.-H., Rosa, P., Phillips, E., Baeuerle, P. A., Frank, R., Argos, P., and Huttner, W. B. (1989) *J. Biol. Chem. 264*, 12009−12015.
23. Fischer-Colbrie, R., Gutierrez, J., Hsu, C. M., Iacangelo, A., and Eiden, L. E. (1990) *J. Biol. Chem. 265*, 9208−9213.
24. Gerdes, H.-H., Phillips, E., and Huttner, W. B. (1988) *Nucleic Acids Res. 16*, 11811.
25. Schimmel, A., Braunling, O., Rüther, U., Huttner, W. B., and Gerdes, H.-H. (1992) *FEBS Lett. 314*, 375−380.
26. Anouar, Y., Jegou, S., Alexandre, D., Lihrmann, I., Conlon, J. M., and Vaudry, H. (1996) *FEBS Lett. 394*, 295−299.
27. Gorr, S.-U., Shioi, J., and Cohn, D. V. (1989) *Am. J. Physiol. 257*, E247−E254.
28. Yoo, S. H., and Albanesi, J. P. (1990) *J. Biol. Chem. 265*, 14414−14421.
29. Chanat, E., and Huttner, W. B. (1991) *J. Cell Biol. 115*, 1505−1519.
30. Yoo, S. H. (1995) *J. Biol. Chem. 270*, 12578−12583.
31. Johnson, R. G., and Scarpa, A. (1976) *J. Biol. Chem. 251*, 2189−2191.
32. Casey, R. P., Njus, D., Radda, G. K., and Sehr, P. A. (1977) *Biochemistry 16*, 972−977.
33. Yoo, S. H. (1994) *J. Biol. Chem. 269*, 12001−12006.
34. Yoo, S. H. (1996) *J. Biol. Chem. 271*, 1558−1565.
35. Pimplikar, S. W., and Huttner, W. B. (1992) *J. Biol. Chem. 267*, 4110−4118.
36. Kim, T., Tao-Cheng, J.-H., Eiden, L. E., and Loh, Y. P. (2001) *Cell 106*, 499−509.
37. Galfre, G., and Milstein, C. (1981) *Methods Enzymol. 73*, 3−46.
38. Yoo, S. H. (1993) *Biochemistry 32*, 8213−8219.
39. Yoo, S. H. (1995) *Biochemistry 34*, 8680−8686.
40. Rosa, P., Mantovani, S., Rosboch, R., and Huttner, W. B. (1992*) J. Biol. Chem. 267*, 12227−12232.
41. Yoo, S. H. (1993) *Biochim. Biophys. Acta 1179*, 239−246.
42. Yoo, S. H., and Lewis, M. S. (2000) *J. Biol. Chem. 275*, 30293−30300.
43. Cowley, D. J., Moore, Y. R., Darling, D. S., Joyce, P. B. M., and Gorr, S.-U. (2000) *J. Biol. Chem. 275*, 7743−7748.
44. Leitner, B., Fischer-Colbrie, R., and Winkler, H. (1996) *J. Neurochem. 66*, 1312−1317.
45. Liang, F., Li, J. Y., Lovisetti-Scamihorn, P., Coen, E., Depreitere, J., Claeys, M., Wechsung, E., Dahlstrom, A., Winkler, H., and De Potter, W. P. (1999) *Brain Res. 818*, 459−467.
46. Bulenda, D., and Gratzl, M. (1985) *Biochemistry 24*, 7760−7765.
47. Reiffen, F. U., and Gratzl, M. (1986) *FEBS Lett. 195*, 327−330.
48. Yoo, S. H., and Albanesi, J. P. (1991) *J. Biol. Chem. 266*, 7740−7745.
49. Yoo, S. H., Oh, Y. S., Kang, M. K., Huh, Y. H., So, S. H., Park, H. S., and Park, H. Y. (2001) *J. Biol. Chem. 276*, 45806−45812.

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