pH-Dependent Association of Chromogranin A with Secretory Vesicle Membrane and a Putative Membrane Binding Region of Chromogranin A

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ABSTRACT: Chromogranin A is a low-affinity, high-capacity Ca^{2+} binding protein, postulated to be responsible for the Ca^{2+} buffering role of secretory vesicles, and has been found only in the soluble portions of the vesicular proteins. Contrary to the generally accepted notion of chromogranin A existing as a soluble matrix protein, chromogranin A bound to the secretory vesicle membrane at the intravesicular pH of 5.5 and freed from the membrane when the pH was raised to a more physiological pH of 7.5. Trypsin digestion studies of the vesicle membrane suggested that chromogranin A interacts with the protein component(s) on the intravesicular side of the membrane. Furthermore, in a study using 14 synthetic chromogranin A peptides which represent various portions of chromogranin A, a segment in the N-terminal region (residues 18–37) was shown to bind to the vesicle membrane in a pH-dependent manner. The pH-dependent vesicle membrane binding property of chromogranin A appears to be of fundamental physiological importance with regard to the potential roles of chromogranin A in secretory vesicle biogenesis, particularly in segregating secretory vesicle membranes from others in the trans-Golgi network, and also in transmitting extravesicular signals such as inositol 1,4,5-trisphosphate or inositol 1,3,4,5-tetrakisphosphate for Ca^{2+} release or uptake to the inside of vesicles.

The Ca^{2+}-storing role of secretory vesicles which have recently been identified as a major inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3][1] -sensitive intracellular Ca^{2+} store of adrenal medullary chromaffin cells (Yoo & Albanesi, 1990a) appears to be due to the low-affinity, high-capacity Ca^{2+} binding property of chromogranin A (CGA) (Yoo & Albanesi, 1991). The Ca^{2+} binding property of chromogranin A is similar to those of the two low-affinity, high-capacity Ca^{2+} binding proteins with similar functions, i.e., calsequestrin (MacLennan & Wong, 1971; Ikemoto et al., 1972; Cozens & Reithmeier, 1984) of the sarcoplasmic reticulum (SR) and calreticulin (Ostwald & MacLennan, 1974; Treves et al., 1990) of the endoplasmic reticulum (ER). The common features of these Ca^{2+}-storing proteins are a high content of acidic amino acids (Benedum et al., 1986; Iacangelo et al., 1986; Konecki et al., 1987; Helman et al., 1988; Reithmeier et al., 1987; Fliegel et al., 1987, 1989; Scott et al., 1988; Smith & Koch, 1989), a high Ca^{2+} binding capacity with ~1000 nmol of Ca^{2+} bound/mg of protein, and a low-affinity of Ca^{2+} binding with dissociation constants (K_d) in the 1–4 mM range (Yoo & Albanesi, 1991; MacLennan & Wong, 1971; Ikemoto et al., 1972; Cozens & Reithmeier, 1984; Ostwald & MacLennan, 1974; Treves et al., 1990). Like the Ca^{2+}-storing protein of sarcoplasmic reticulum calsequestrin (Ostwald et al., 1974; Ikemoto et al., 1974; Cala & Jones, 1983), chromogranin A is also known to undergo Ca^{2+}-induced conformational changes (Yoo & Albanesi, 1990b). However, in contrast to the sarcoplasmic reticulum, the secretory vesicles in which chromogranin A exists are acidic, and pH changes have been demonstrated to change chromogranin A conformations (Yoo & Albanesi, 1991).

Furthermore, chromogranin A is the major protein of secretory vesicles of adrenal chromaffin cells, which is known to exist as a soluble intravesicular protein with no interaction with the vesicle membrane (Winkler et al., 1986). Even before its role as the major Ca^{2+}-storing protein of chromaffin vesicles was known, chromogranin A had attracted a great deal of attention due to its coexistence with catecholamine in the vesicles (Winkler, 1977; Winkler & Westhead, 1980; O'Connor & Frigon, 1984). Since its discovery in late 1960s (Helle, 1966; Smith & Winkler, 1967; Smith & Kirschner, 1967; Blaschko et al., 1967), chromogranin A has exclusively been found among the soluble portions of the vesicular proteins. Hence, it was assumed that chromogranin A exists in the acidic intravesicular milieu of chromaffin cells as a soluble protein in complex with other molecules including catecholamine (Daniels et al., 1978) and ATP (Yoo et al., 1990). However, it appears that chromogranin A does not exist as a free-floating soluble protein in vivo; rather, it stays bound to the secretory vesicle membrane at the intravesicular pH of 5.5 and is freed from the membrane when the pH is raised to a more physiological pH of 7.5.

Recently the secretory vesicles of adrenal chromaffin cells were also shown to rapidly take up Ca^{2+} in response to Ins(1,3,4,5)P_4 (Yoo, 1991). In view of the fact that Ins(1,4,5)P_3 triggers rapid release of Ca^{2+} (Yoo & Albanesi, 1990a) from the secretory vesicles and Ins(1,3,4,5)P_4 causes rapid sequestration of Ca^{2+} (Yoo, 1991), the binding of Ca^{2+} storage protein to the membrane where the receptors for Ca^{2+} release/uptake signals are located accords well with the rapid Ca^{2+} control mechanism of the chromaffin cells. Given that the pH change also changes the CGA conformation (Yoo & Albanesi, 1990b) and the change of pH from 7.5 to 5.5 loosened the overall structure of CGA such that CGA came into contact with other molecules more readily (Yoo & Albanesi, 1991), attempts were made to identify the membrane binding segment of CGA. Consequently, an N-terminal segment was found to bind to the membrane at the intravesicular pH of 5.5 and

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[1] Abbreviations: Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; CGA, chromogranin A; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; CNBr, cyanogen bromide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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released from it at a more physiological pH of 7.5. The physiological implications of the interaction, which include roles in secretory vesicle biogenesis, membrane sorting, Ca^{2+} signal transduction mechanism, and automatic untethering of the vesicle contents during exocytosis, are deemed of utmost importance. In the present study, results regarding the pH-dependent chromogranin A interaction with the chromaffin vesicle membrane are presented along with their significance.

EXPERIMENTAL PROCEDURES

Materials. Cyanogen bromide (CNBr)-activated Sepharose 4B was obtained from Pharmacia LKB Biotechnology Inc. Trypsin and soybean trypsin inhibitor were from Sigma, and acrylamide and bis(acrylamide) were from Bio-Rad.

Secretory Vesicle Membrane Preparation. To obtain the secretory vesicle membrane, the secretory vesicles were prepared as described previously (Yoo & Albanesi, 1990a), and the vesicles were resuspended in 40 volumes of 15 mM Tris-HCl, pH 7.5, and freeze-thawed to lyse the vesicles. To separate the lysates from the membrane, the lysed vesicles were centrifuged at 48000*g* for 30 min. The supernatant was collected for the soluble intravesicular lysates, and the vesicle membrane was obtained as a pellet in the centrifuge tube. The lysates were used for purification of CGA. The vesicle membrane was further washed by resuspending in 40 volumes of 15 mM Tris-HCl, pH 7.5, 0.5 M NaCl and centrifuged at 48000*g* for 30 min to remove peripheral membrane proteins. The supernatant was discarded, and the pellet was used as vesicle membrane.

Plasma Membrane Preparation. Plasma membranes from bovine adrenal medulla and rat intestine were prepared as described (Zinder et al., 1978; Pind & Kuksis, 1987).

Coupling the Secretory Vesicle Membrane to CNBr-Sepharose 4B. For the vesicle membrane coupling, 1 g (dry wt) of CNBr-activated Sepharose 4B (Pharmacia, LKB) was swollen in 50 mL of 1 mM HCl for 15 min at room temperature. The resin then was washed with ~200 mL of 1 mM HCl, 50 mL at a time, after which the resin was washed with 10 mL of coupling buffer (0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl) and mixed with 20 mg of protein of the secretory vesicle membrane in 10 mL of coupling buffer. Coupling was carried out by gently rotating the mixture for 14–18 h at 4 °C. Coupling was stopped by transferring the resin–membrane mixture to 10 mL of blocking buffer (0.2 M glycine, pH 8.0) and rotated for 2 h at room temperature, after which the resin was washed of the excess adsorbed protein by sequentially washing with the coupling buffer, with acetate buffer (0.1 M sodium acetate, pH 4.0, 0.5 M NaCl), and again with the coupling buffer, 30 mL of each. The coupled resin then was ready to be used. To determine the amount of vesicle membrane coupled to Sepharose 4B, an aliquot of the membrane-coupled Sepharose 4B was resuspended in 1 M KOH and boiled for 10 min. The boiled resin was centrifuged at 12000*g* for 5 min, and the decoupled vesicle membrane was collected in the supernatant for protein determination. Following this procedure, it was estimated that 0.66 mg of membrane protein was coupled per milliliter of Sepharose 4B (wet volume).

Chromogranin A Preparation. Chromogranin A purification was carried out as described (Yoo & Albanesi, 1990b) using the vesicle lysates (see above) as the starting material.

Chromogranin A Peptide Synthesis. Fourteen peptides, each representing 20–27 amino acids of various portions of chromogranin A, were synthesized. The synthesized peptides were purified by high-performance liquid chromatography (HPLC), and the integrity of the peptides was ensured through analyses by fast atom bombardment mass spectrometry and by amino acid composition analyses. The purity of all the peptides was 95% or higher. For peptides 1–7, 10, 12, and 14 where there was no tryptophan, a tryptophan residue was added to the N-terminus of each peptide to facilitate analysis.

Extraction of Phospholipids from the Secretory Vesicle Membrane. The phospholipids of secretory vesicle membrane were extracted using a chloroform–methanol mixture (v/v, 2:1) according to the method of Folch et al. (1957). The extracted phospholipids were quantitated by determining the phosphorus as described (Petitou et al., 1978; King, 1932). The phospholipid vesicles were prepared using extensive sonication as described (Hayden et al., 1990) except that the buffer used was 20 mM sodium acetate, pH 5.5, 0.1 M KCl.

Trypsin Treatment of the Vesicle Membrane-Coupled Sepharose 4B. One milliliter (gel volume) of the vesicle membrane-coupled Sepharose 4B suspended in 3 mL of 20 mM sodium acetate, pH 5.5, 0.1 M KCl (buffer A) was incubated with trypsin at a protein:enzyme ratio of 50:1 (w/w) by gently mixing on a rotator for 90 min at room temperature. Upon completion of the incubation, the resin was washed of the residual proteins by sequentially washing with 0.1 M KCl, 0.15 M KCl, and then again with 0.1 M KCl, all in 20 mM sodium acetate, pH 5.5, until the absorbance at 280 nm was zero. At this step, soybean trypsin inhibitor at an enzyme (original amount):inhibitor ratio of 1:1 (w/w) was added to the resin to ensure that all the remaining trypsin activity was removed, and incubated for 15 min at room temperature in buffer A in a total volume of 4 mL. Again the resin was washed of the residual proteins by sequentially washing with the sodium acetate buffers as described above, followed by a final wash with 20 mM Tris-HCl, pH 7.5, 0.1 M KCl. After the pH 7.5 buffer wash, the resin was reequilibrated with buffer A, and used for CGA chromatography.

Trypsin Treatment of the Intact Vesicles and the Vesicle Membranes. The intact vesicles were resuspended in 20 volumes of 0.3 M sucrose, 15 mM Tris-HCl, pH 7.5, and 0.1 M NaCl (buffer B) and mixed with trypsin at a vesicle protein:enzyme ratio of 200:1. The vesicle–enzyme mixture was incubated for 3 h with gentle rocking at room temperature, after which the reaction was stopped by adding soybean trypsin inhibitor at an enzyme:inhibitor ratio of 1:2 (w/w). The vesicles were then washed free of trypsin and trypsin inhibitor in buffer B by centrifugation at 55000*g* for 20 min. This washing was repeated 2 more times. Finally, the vesicles were lysed in 40 volumes of 15 mM Tris-HCl, pH 7.5, followed by two cycles of freezing and thawing, and the vesicle membranes were collected by centrifugation at 48000*g* for 30 min. The vesicle membranes in the pellet were used to test their ability to bind CGA.

For trypsin treatment of the vesicle membranes, the vesicle membranes were resuspended in 10 volumes of 15 mM Tris-HCl, pH 7.5 (buffer C), and mixed with trypsin at a membrane protein:enzyme ratio of 100:1. The vesicle–enzyme mixture was incubated for 3 h with gentle rocking at room temperature, after which the reaction was stopped by adding soybean trypsin inhibitor at an enzyme:inhibitor ratio of 1:2 (w/w). The membranes were then washed free of trypsin and trypsin inhibitor in buffer C by centrifugation at 48000*g* for 30 min. The washing was repeated a total of 3 times. The vesicle membranes in the pellet were used to test their ability to bind CGA.

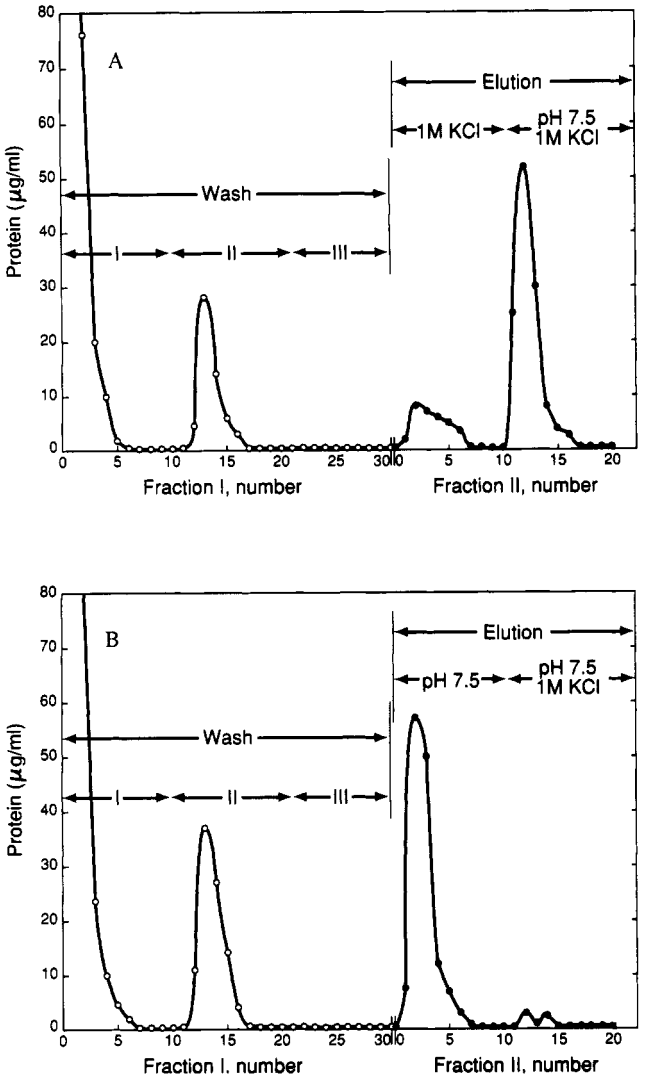

FIGURE 1: Vesicle membrane-coupled Sepharose 4B chromatography of purified chromogranin A. Two milligrams of purified chromogranin A in 2 mL of 20 mM sodium acetate, pH 5.5, was loaded onto a vesicle membrane-coupled Sepharose 4B column (1-mL volume) equilibrated with 20 mM sodium acetate, pH 5.5, 0.1 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. (A) The protein then was first eluted with 1 M KCl in the same acetate buffer. 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 the elution was continued. (B) After loading and washing of CGA as described above, the protein then was first eluted with a pH 7.5 buffer (20 mM Tris-HCl, pH 7.5, 0.1 M KCl), and followed by 1 M KCl in 20 mM Tris-HCl, pH 7.5. The fraction size was 1 mL/fraction for fraction I and 0.5 mL/fraction for fraction II, and the chromatography was carried out at room temperature.

RESULTS

In light of the fact that chromogranin A is the Ca^{2+} storage protein of secretory vesicles, a major Ins(1,4,5)P_3-sensitive Ca^{2+} store of adrenal chromaffin cells, it was of interest to examine the possibility of chromogranin A existing in direct contact with the vesicle membrane where the Ca^{2+} release/uptake signals are first received. To determine whether soluble CGA binds to the secretory vesicle membrane at the intravesicular pH of 5.5, purified CGA was loaded onto a Sepharose 4B column coupled with the secretory vesicle membrane for affinity chromatography. As shown in Figure 1A, elution of the column with a 1 M KCl buffer released a small amount of protein from the column, suggesting the possibility that a significant amount of chromogranin A was bound to the vesicle membrane at pH 5.5. In order to test the possibility of more proteins still being retained in the column after the high-salt elution, the same column was further eluted with a buffer with pH of 7.5 instead of 5.5. Indeed, the pH 7.5 buffer released a significantly larger amount of protein from the column, apparently indicating that the high-salt elution released only a portion of the bound proteins.

In order to determine whether most of these membrane-bound proteins can be released from the bound state by a simple change of pH to 7.5, the CGA-loaded column was directly eluted with a pH 7.5 buffer without a prior elution with the 1 M KCl buffer. As the result in Figure 1B showed, a large amount of protein was eluted by the pH 7.5 buffer. Further elution of the column with the pH 7.5 buffer supplemented with 1 M KCl released only a small amount of protein additionally, indicating that the pH change alone is sufficient to free most of the bound proteins from the membrane. The amount of protein released by the pH 7.5 elution alone was comparable to those eluted by the 1 M KCl buffer plus the pH 7.5–1 M KCl buffer (cf. Figure 1A), indicating that almost all the releasable proteins are freed from the membrane by the pH change alone. The presence of calcium, up to 2–4 mM, exerted very little effect on the binding and release of CGA, suggesting little or no effect of calcium on the pH-dependent interaction of CGA with the vesicle membrane. In parallel experiments, it was shown that the change of pH from 5.5 to 7.5 does not elute any vesicle membrane proteins from the column, underscoring the fact that only bound chromogranin A was freed from the column at pH 7.5.

Although a saturating amount of CGA (2 mg) was loaded onto the affinity column in the present experiments, a similar amount of CGA ($\sim$100 μg) can also be eluted from the column when as little as 500 μg of CGA is loaded. However, loadings of less than 500 μg of CGA begin to lower the amount of CGA bound and eluted. When 300 μg of CGA was loaded, approximately 65–70 μg of CGA was eluted from the same column. In addition, loading of a saturating amount of CGA and washing at pH 6.5, in place of the usual pH 5.5, followed by elution at pH 7.5 reduced the amount of CGA eluted to $\sim$20 μg, which is approximately 20% of the CGA elutable when the same amount of CGA was loaded and washed at pH 5.5.

To analyze the content of the proteins released from the column, the eluted protein fractions were analyzed by SDS–PAGE. As shown in Figure 2, CGA bands were represented in the fractions eluted with either the pH 7.5–1 M KCl buffer or the pH 7.5 buffer alone, demonstrating the fact that CGA bound the vesicle membrane at pH 5.5 and was released from it at pH 7.5. The intensities of the protein bands in the two groups appeared similar, suggesting that the pH change alone is sufficient to free almost all the bound CGA.

To determine whether CGA can bind to the vesicle membrane at pH 7.5, CGA was loaded onto a membrane-coupled column equilibrated with a pH 7.5 buffer, and eluted with a 1 M KCl buffer. Chromogranin A appeared to pass right through the column without interaction with the membrane. Elution of the column with the 1 M KCl buffer to free proteins that might have been retained in the column failed to elute any protein (not shown), indicating that CGA does not bind to the membrane at pH 7.5.

The retention of CGA by the column appears to be specifically due to the interaction of CGA with the vesicle membrane at pH 5.5, for the interaction of CGA with CNBr-activated Sepharose 4B not coupled with the vesicle membrane

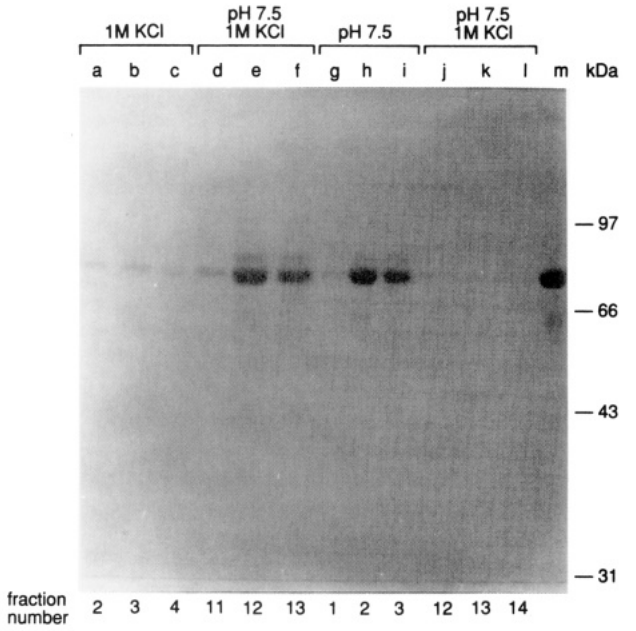

FIGURE 2: SDS–PAGE of eluted chromogranin A from vesicle membrane-coupled Sepharose 4B chromatography. One hundred microliter aliquots from fraction II of Figure 1A,B are analyzed on a 10% SDS–polyacrylamide gel (Laemmli, 1970). Lane m, purified chromogranin A; lanes a, b, and c, fractions 2, 3, and 4, respectively; lanes d, e, and f, fractions 11, 12, and 13 of Figure 1A, respectively. Lanes g, h, and i, fractions 1, 2, and 3, respectively; lanes j, k, and l, fractions 12, 13, and 14 of Figure 1B, respectively.

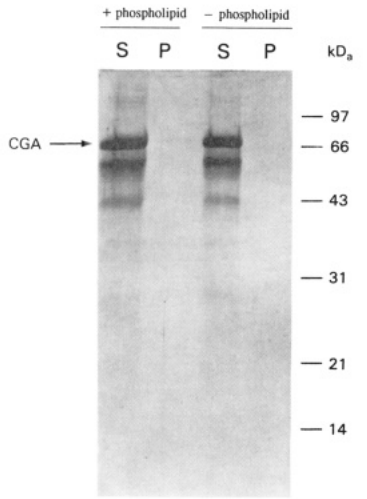

FIGURE 3: Cosedimentation analysis of chromogranin A and phospholipid vesicles. Chromogranin A (0.2 mg/mL) was added to buffer A (20 mM sodium acetate, pH 5.5, 0.1 M KCl) alone (–phospholipid) or to buffer A containing 1 mM phospholipids (+phospholipid) derived from the vesicle membranes, and was incubated for 15 min at 25 °C, followed by centrifugation at 95000*g* for 25 min at 25 °C to pellet the phospholipid vesicles. After centrifugation, liposome pellets (P) were resuspended in the original incubation volume and analyzed with supernatants (S) on 10% SDS–PAGE. Forty-microliter aliquots were loaded onto each lane. The smaller bands are CGA degradation products.

but had gone through all the coupling routine had never been observed in several control experiments. Therefore, to examine the possibility of membrane phospholipid bilayer interacting with CGA, the membrane phospholipids were extracted from the secretory vesicle membranes, and the interaction of CGA with the extracted phospholipids was tested by cosedimentation analysis (Hayden et al., 1990). As shown in Figure 3, pelleting of the phospholipid vesicles from the CGA–phospholipid mixture resulted in the complete separation of the phospholipid vesicles from CGA and its fragments. The cosedimentation technique had been successfully used previously to demonstrate myosin I binding to phospholipid vesicles (Hayden et al., 1990). In this regard, the absence of interacting CGA in the liposome pellets indicates that there is no interaction between CGA or its fragments and the phospholipid vesicles.

To further determine whether any protein components of the vesicle membrane are responsible for the interaction with CGA, the membrane-coupled resin was incubated with trypsin as described under Experimental Procedures. Following the trypsin treatment, the vesicle membrane-coupled resin failed to bind CGA at pH 5.5 as determined by chromatography as described in Figure 1, and all the loaded CGA passed through the column without any sign of interaction. Moreover, to determine whether the pH-dependent binding of CGA is due to specific characteristics of the vesicle membrane rather than those of CGA, the possibility of pH-dependent binding of other proteins to the secretory vesicle membrane was tested using soybean trypsin inhibitor and vesicle membrane-coupled Sepharose 4B chromatography. Unlike chromogranin A, soybean trypsin inhibitor failed to bind to the secretory vesicle membrane, and there was no sign of interaction with the secretory vesicle membrane (not shown), clearly demonstrating the fact that the pH-dependent binding of CGA to the vesicle membrane is not a general phenomenon involving secretory vesicle membrane and any proteins. In addition, in order to clarify whether the pH-dependent CGA binding to the vesicle membrane can also be demonstrated with other membranes, the plasma membrane from rat intestine was prepared and coupled to CNBr-activated Sepharose 4B for a pH-dependent CGA binding study. The plasma membrane coupled was 0.8 mg of protein/mL of Sepharose 4B (wet volume), similar to ~0.7 mg of vesicle membrane protein coupled to 1 mL of Sepharose 4B. The plasma membrane-coupled Sepharose 4B column bound, on a membrane protein to protein basis, approximately 20% of CGA that can be bound by a comparable vesicle membrane-coupled column (not shown), indicating that the plasma membrane cannot substitute for the vesicle membrane in exerting pH-dependent CGA binding activity.

Since the membrane proteins are attributed to the pH-dependent binding of CGA, experiments designed to determine which side of the vesicle membrane is involved in the interaction were carried out. First, to cleave the membrane proteins on the cytoplasmic side, intact secretory vesicles were subjected to trypsin treatment and lysed by hypotonic treatment followed by freezing and thawing, and the vesicle membranes were collected after centrifugation. Second, the purified vesicle membranes were subjected to trypsin treatment to remove the protein components on both sides of the vesicle membrane. These membranes were then mixed with an appropriate amount of partially purified CGA both at pH 7.5 and at pH 5.5, and the extent of CGA interaction with each membrane was determined by spectroscopy and visualized by SDS–PAGE. As shown in Figure 4, the vesicle membrane treated with trypsin on the cytoplasmic side only bound CGA as effectively as the intact vesicle membrane at pH 5.5, whereas the membrane treated with trypsin on both sides failed to bind CGA at pH 5.5, clearly indicating participation of the protein components on the intravesicular side of the membrane in the pH-dependent interaction with CGA. Unlike the vesicle membranes, the plasma membranes from adrenal medulla failed to bind CGA at pH 5.5 as shown in Figure 4.

Furthermore, to identify the CGA region which might be responsible for CGA binding to the membrane, 14 CGA peptides representing various portions of CGA were synthe-

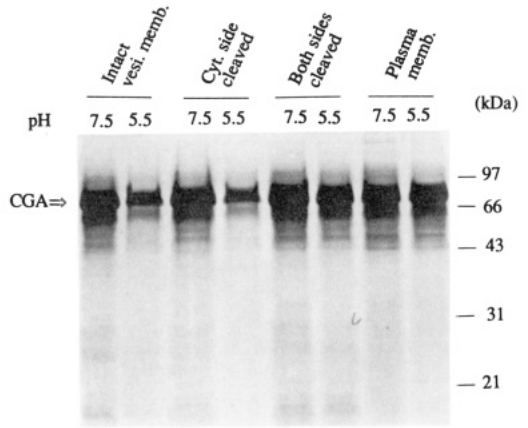

FIGURE 4: Interaction of chromogranin A with the intravesicular side of the vesicle membrane at pH 5.5. Three different kinds of vesicle membranes, i.e., (i) intact, (ii) cytoplasmic side treated with trypsin, or (iii) both sides treated with trypsin, and the plasma membranes from adrenal medulla were used for the interaction assays. These membranes were washed twice by resuspending once in 20 volumes of 15 mM Tris-HCl, pH 7.5, followed by centrifugation at 48000*g* for 5 min, and resuspending the pellets a second time in 20 volumes of 15 mM sodium acetate, pH 5.5. Then an aliquot (50 mg wet weight) of each membrane was mixed with 0.5 mg of CGA in a total volume of 0.5 mL of either 15 mM Tris-HCl, pH 7.5, or 15 mM sodium acetate, pH 5.5. After gentle mixing, the mixtures were centrifuged at 48000*g* for 5 min to pellet the membranes. Supernatants from each mixture were collected, and an aliquot (15 μL) from each supernatant was analyzed by 10% SDS–PAGE.

Peptide	Residues	Amino Acid Sequence
1	(18-37)	(W) IVEVISDTLSKPSPMPVSKE
2	(40-65)	(W) ETLRGDERILSILRHQNLLKELQDLA
3	(70-90)	(W) KERTHQQKKHSSYEDELSEVL
4	(95-117)	(W) DQAEPKEVTEEVSSKDAAEKRDD
5	(132-151)	(W) RPQASPGLGPGPKVEEDNQA
6	(156-179)	(W) EEAPSNAHPLASLPSPKYPGPQAK
7	(183-201)	(W) EGPSQGPASREKGLSAEQG
8	(213-238)	WEEAEAREKAVPEEESPPTAAFKPPP
9	(251-273)	GWPEDGAGKMGAEEAKPPEGKGE
10	(290-309)	(W) VLFHGGKSGEPKQEEQLSKE
11	(316-336)	WSKMDQLAKELTAEKRLEGEE
12	(344-364)	(W) RSMRLSFRARGYGFRGPGLQL
13	(368-390)	WRPNSQEDSVEAGLPLQVRGYPE
14	(407-431)	(W) ELESLSAIEAELEKVAHQLEELRRG

FIGURE 5: List of synthetic chromogranin A peptides. The 14 synthetic chromogranin A peptides are shown with their residue numbers indicating their positions in chromogranin A and their amino acid sequences in the single-letter code. The residue in parentheses indicates a tryptophan residue added to the N-terminus.

sized (Figure 5), and the pH-dependent binding of each peptide to the secretory vesicle membrane was tested. As shown in Figure 6, the pH dependent binding to the secretory vesicle membrane was manifested by peptide 1, representing residues 18–37 near the N-terminal end of CGA. All other peptides did not bind to the vesicle membrane under the experimental conditions, suggesting the possibility that the N-terminal segment represents a membrane binding region.

DISCUSSION

Since chromogranin A has always been found in the soluble portion of the vesicular proteins, chromogranin A has been presumed to exist in a complex form associating only with

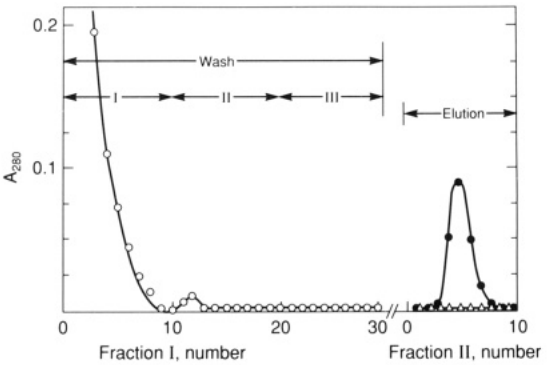

FIGURE 6: Vesicle membrane-coupled Sepharose 4B chromatography of synthetic chromogranin A peptides. For chromatography of the synthetic peptides, 1 mL of each synthetic CGA peptide (with the exception of peptides 2 and 3) at a concentration of 1 mg/mL in 20 mM sodium acetate, pH 5.5, 0.1 M KCl was loaded onto a vesicle membrane-coupled Sepharose 4B column and eluted with the pH 7.5 buffer (20 mM Tris-HCl, pH 7.5, 0.1 M KCl) after thorough washing 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. Due to difficulty in keeping peptides 2 and 3 in solution at pH 5.5, the loading pH of peptides 2 and 3 was 6.0. The circles represent the washing and elution profiles of peptide 1 (residues 18–37), whereas the triangles represent the elution profiles of all the other peptides. The washing profiles of all the other peptides are similar to that of peptide 1. All other conditions are the same as in Figure 1.

other intravesicular consitituents of the vesicle (Winkler et al., 1986; Simon & Aunis, 1989). However, present results clearly indicate that chromogranin A binds to the vesicle membrane at the intravesicular pH of 5.5. The basis of pH-dependent chromogranin A interaction with the vesicle membrane appears to be the pH-induced conformational changes of chromogranin A. We have previously demonstrated that acidic pH increases the α-helicity of chromogranin A (Yoo & Albanesi, 1990b) and trypsin proteolyzes chromogranin A much faster at pH 5.5 than at pH 7.5 (Yoo & Albanesi, 1991) despite the unfavorable pH condition for trypsin activity. It therefore appeared that the acidic pH extended the chromogranin A structure in such a way to make it more accessible for interaction with other molecules. In this regard, it appears likely that the conformational changes induced by the acidic pH provided an anchor for chromogranin A binding to the membrane.

Comparison of the amino acid sequence of bovine CGA (Benedum et al., 1986; Iacangelo et al., 1986) with other known CGA sequences, i.e., those of human (Konecki et al., 1987; Helman et al., 1988), pig (Iacangelo et al., 1988a), rat (Iacangelo et al., 1988b), and mouse (Wu et al., 1991), indicates that the N- and C-terminal regions are the most conserved regions among all the chromogranin As sequenced so far. The hydrophilicity plot of CGA (Figure 7) also indicates that residues 14–26 of the N-terminal region represent the most hydrophobic area in the entire sequence of CGA, implying a potential importance of this region. Interestingly, the conservation of the N-terminal sequence in CGA of all the organisms sequenced so far (Konecki et al., 1987; Helman et al., 1988; Iacangelo et al., 1988a,b; Wu et al., 1991) coincides with the pH-dependent binding of this segment to the vesicle membrane. Although the pH-dependent binding of CGA peptide 1 (Figure 6) implies a potential anchoring role of the near N-terminal region, it is not clear at present whether the conserved near N-terminal region is directly responsible for the pH-dependent binding of CGA to the vesicle membrane. Nevertheless, in view of the results that CGA is more exposed at pH 5.5 than at pH 7.5 as judged by the higher incidence of proteolysis by trypsin at pH 5.5

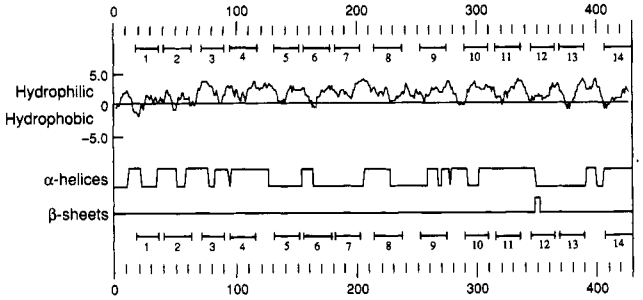

FIGURE 7: Hydrophilicity plot and secondary structure prediction of chromogranin A. The size and location of the synthetic CGA peptides are indicated as lines next to the position numbers with peptide numbers 1–14. A hydrophilicity plot of CGA according to Hopp and Woods (1981) and a secondary structure prediction according to a slightly modified method of Garnier–Osguthorpe–Robson (Garnier et al., 1978) are shown. The minimum length of a helix is six and of a β-sheet is four. Regions without adequate predictions are replaced by the conformational state of the next best probability.

(Yoo & Albanesi, 1991) and the N-terminal region represents virtually the only area with both a high degree of hydrophobicity and the predicted α-helicity (Figure 7), it is tempting to speculate that the N-terminal region extends its α-helical anchor to the membrane for interaction at pH 5.5. In a separate experiment, CGA peptide 1 was loaded onto the vesicle membrane-coupled Sepharose 4B column prior to CGA loading to see whether peptide 1 can block binding of CGA to the vesicle membrane. However, prior loading of peptide 1 did not prevent binding of CGA to the vesicle membrane (not shown). It is not currently clear why peptide 1 failed to block CGA binding to the vesicle membrane. Nevertheless, in view of the observations that intact CGA can bind to the vesicle membrane only in a tetrameric state (Yoo & Lewis, 1992) and peptide 1 exists in a monomeric state at pH 5.5,[2] one likely possibility is a potential difference in binding affinity between CGA and peptide 1, i.e., a much higher affinity of tetrameric CGA than monomeric peptide 1, for the membrane interaction sites.

Considering that chromogranin A undergoes considerable conformational changes as it binds more Ca^{2+} and the aggregation process of the Ca^{2+}-bound chromogranin A starts even at pH 7.5 (Yoo & Albanesi, 1990b), the pH-dependent interaction of chromogranin A with the vesicle membrane appears to imply an essential role of chromogranin A in the biogenesis of secretory vesicles. Moreover, in light of the fact that chromogranin A is the Ca^{2+}-storing protein of the secretory vesicles that are responsible for either release or uptake of Ca^{2+} in response to signals like Ins(1,4,5)P_3 (Yoo & Albanesi, 1990a) or Ins(1,3,4,5)P_4 (Yoo, 1991), the binding of CGA to the vesicle membrane is expected to facilitate the communication between CGA and the vesicle surface to release or sequester Ca^{2+}. In this regard, the binding seems particularly significant given that the secretory vesicles contain 35–40 mM Ca^{2+} and almost all (>99.9%) the intravesicular calcium is known to stay bound to the matrix proteins (Bulenda & Gratzl, 1985), especially to chromogranin A (Reiffen & Gratzl, 1986). Furthermore, in view of the fact that the intravesicular contents are released into the bloodstream during exocytosis, the concept of automatic untethering of chromogranin A from the vesicle membrane upon their exposure to the physiological pH of bloodstream is in line with the observed rapid release and circulation of the vesicle contents.

Questions regarding the identity of the membrane components interacting with CGA are currently under study. However, the failure of CGA to bind to the membrane phospholipids and the complete loss of CGA binding ability by the membrane-coupled column after trypsin treatment suggest that the CGA binding components on the vesicle membrane are membrane proteins. Moreover, the binding of CGA to the vesicle membrane treated with trypsin on its cytoplasmic side only and the inability of CGA to interact with the vesicle membrane treated with trypsin on both its cytoplasmic and its intravesicular sides indicate that CGA interacts with the protein components on the intravesicular side of the vesicle membrane.

[2] S. H. Yoo and M. S. Lewis, unpublished results.

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