Identification of the secretory vesicle membrane binding region of chromogranin A

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Abstract Since the conserved near N-terminal region (residues 18–37) of chromogranin A (CGA) has tentatively been identified as the vesicle membrane interacting region using synthetic peptides, it was necessary to confirm this finding with CGA deletion proteins. In order to address this need and to clarify the discrepancies of the published amino acid sequences of CGA, we cloned a CGA gene and produced CGA deletion proteins of various sizes. The recombinant CGA protein lacking the first 16 amino acid residues bound to the vesicle membrane as well as the full-length CGA at pH 5.5. However, the CGA protein lacking the first 39 amino acid residues, which include the conserved near N-terminal region (residues 17–38), failed to interact with the vesicle membrane at pH 5.5, clearly indicating the essential role of the conserved near N-terminal region in the pH-dependent interaction of CGA with the vesicle membrane.

Key words: Cloning; Sequence; Chromogranin A; pH; Secretory vesicle membrane

1. Introduction

Chromogranin A, one of the major proteins of the secretory vesicles of neuroendocrine cells [1,2], is known to interact with several other integral membrane proteins of secretory vesicles at pH 5.5 and dissociated from them at pH 7.5 [3]. Furthermore, several secretory vesicle matrix proteins bound to CGA at pH 5.5 and dissociated from it at pH 7.5 [4]. In addition to the acidic pH-dependent membrane binding property of CGA [5], chromogranin A has been shown to undergo pH- and Ca^{2+}-dependent aggregation [6,7] and conformational changes [7], more readily aggregating at pH 5.5 than at pH 7.5 in the presence of Ca^{2+}. The acidic pH- and Ca^{2+}-induced aggregation of CGA at pH 5.5, along with its binding to the vesicle membrane [5] and interaction with other vesicular matrix proteins [4], strongly suggested important roles of CGA in secretory vesicle biogenesis.

In our previous study, it was shown that a synthetic peptide representing the near N-terminal region of CGA, residues 18–37 (IVEVISDTLSKPSPMPVSKE, in single letter code), interacted with the secretory vesicle membrane at pH 5.5 and dissociated from it at pH 7.5 [5], raising the possibility that this conserved near N-terminal region of CGA may be its membrane binding anchor region. No other peptides representing various other regions of CGA, including the conserved C-terminal region, appeared to interact with the vesicle membrane [5]. Since the interaction of the near N-terminal region of CGA with the vesicle membrane was shown by a synthetic peptide, there was a need to confirm the suggested role of the conserved near N-terminal region of CGA in the interaction with the vesicle membrane using truncated CGA proteins. In order to identify the membrane interacting region of CGA and to clarify the discrepancies of the published amino acid sequences of chromogranin A [8–10], we have cloned a bovine CGA gene and generated several different length recombinant CGA proteins. Using the purified recombinant CGA proteins, we now show that the conserved near N-terminal region of CGA is essential for CGA binding to the vesicle membrane.

2. Materials and methods

2.1. Library screening and sequencing

Approximately 2×10^5 plaques from the bovine adrenal medullar cDNA library were screened using a bovine chromogranin A probe obtained by PCR amplification using oligonucleotide primers 5′-AGGGGAAGGGGGAGTGGGCA-3′ and 5′-TTAGATAAAGCTTCATACACA-3′. The nucleotide sequences of both strands of the chromogranin A clone were determined.

2.2. Construction of CGA clones and subcloning into expression vector

In order to construct the CGA clones, PCR was used to add a *Bam*HI site to the 5′-end and a *Hin*dIII site to the 3′-end of each chromogranin A clone. After the PCR products were digested with *Bam*HI and *Hin*dIII, each of the CGA clones was subcloned into pET28a(+) (obtained from Novagen) digested with *Bam*HI and *Hin*dIII previously. The correct size and proper orientation were confirmed by determining the nucleotide sequence of each CGA clone. The pET28a(+) vector carries an N-terminal His tag (a stretch of six His) to which nickel binds tightly.

2.3. Expression and Ni-NTA agarose affinity chromatography

E. coli BL21(DE3) was used as the host for expression. Initial purification of various deletion protein products of CGA was performed using nickel-nitrilotriacetic acid (Ni-NTA) agarose affinity chromatography from Qiagen.

2.4. Purification of recombinant CGA proteins

In order to further purify the recombinant CGA proteins phenyl-Sepharose column chromatography or phenyl-Sepharose chromatography followed by DE-52 column chromatography was employed as described below for each protein. The phenyl-Sepharose column (2.5 cm×8.5 cm) was equilibrated with 15 mM Tris-HCl, pH 7.5, 0.2 mM $CaCl_2$, 0.25 M ammonium sulfate, and a reverse ammonium sulfate gradient was used to elute the proteins. The DE-52 column (2.5 cm×7 cm) was equilibrated with 10 mM imidazole, pH 6.1, and KCl gradients were used to elute the proteins. The chromatographic procedures and buffers used for each recombinant protein purification were as follows: (1) recombinant CGA (rCGA) 1–431; the Ni-NTA column eluate was loaded onto the phenyl-Sepharose column, washed with 0.1 M ammonium sulfate (wash I) and 50 mM ammonium sulfate buffer (wash II), and eluted with a reverse gradient ammonium sulfate (50 mM–0 M) buffer. Additional buffer (15 mM Tris-HCl, pH 7.5, 0.2 mM $CaCl_2$) without ammonium sulfate was added at the end of the gradient to complete the elution. Appropriate pools from the phenyl-Sepharose column chromatography were loaded onto the DE-

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52 column, washed with 0.15 M KCl buffer, and eluted with a KCl gradient of 0.15 M–0.35 M. (2) rCGA 1–231; the eluted proteins from the Ni-NTA column were loaded onto the phenyl-Sepharose column, washed with 0.2 M ammonium sulfate buffer (wash I) and 0.1 M ammonium sulfate buffer (wash II), and eluted with a reverse gradient ammonium sulfate (0.1 M–0 M) buffer. Additional buffer without ammonium sulfate was added at the end of the gradient to complete the elution. (3) rCGA 232–431; the Ni-NTA column eluate was loaded onto the phenyl-Sepharose column, and the rCGA 232–431 was eluted with 0.1 M ammonium sulfate buffer. (4) rCGA 17–431; purification of rCGA 17–431 followed the same steps as for rCGA 1–431 purification except that the pooled fractions from the first DE-52 step were rechromatographed on the same DE-52 column equilibrated with 0.2 M KCl buffer. Elution of the second DE-52 column was carried out with a KCl gradient of 0.2 M–0.3 M. (5) rCGA 40–431; purification of rCGA 40–431 followed the same steps as for rCGA 1–431 purification.

2.5. Coupling the secretory vesicle membrane to cyanogen bromide (CNBr)-Sepharose 4B

Coupling of the vesicle membrane to the CNBr-activated Sepharose 4B was done as described previously [5].

3. Results

Bovine chromogranin A had previously been cloned and sequenced by three other groups [8–10]. However, they differed from each other in nine amino acid residues out of 431 residues (Fig. 1). In view of these differences between the clones and considering the importance of the establishment of the correct amino acid sequence of CGA, we have cloned and sequenced a bovine CGA. As shown in Fig. 1, our sequence agreed with the published sequences except for 10 amino acid residues. Interestingly, nine of these 10 residues are in the same locations where the amino acids differed from each other among the three published sequences (Fig. 1). However, at residue 275 our sequence contained serine where the two other sequences contained alanine. Although several differences in sequence were observed among the four sequences, no sequence variation was observed in the conserved near N-terminal region (residues 17–38), the calmodulin binding region (residues 40–65) [11], and the conserved C-terminal region (residues 407–431). Given the complete sequence identity of these essential regions and considering the fact that only one clone differed from the other three clones in all the positions with different amino acid residues (Fig. 1), the slight differences in sequence may be a reflection of polymorphism of the CGA gene.

Fig. 2 shows the size of recombinant CGA fragments generated and the purity of the purified CGA proteins used in the vesicle membrane binding experiments. Since a peptide representing the conserved near N-terminal region (residues 18–37) of CGA had been demonstrated previously to bind to the

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     1          94    118  136 137  141                 275  293  301      361  373       431
1:  --------Q---S---S  P--P-------------A--H--K-----G--Q------
2:  --------Q---F---P  G--A-------------A--R--E-----G--R------
3:  --------T---F---S  P--P-------------A--R--E-----R--R------
4:  --------Q---F---S  P--P-------------S--R--E-----G--R------
          (AAC) (TTT) (TCCCCA)(CCG)              (TCA)(CGT)(GAG)  (GGG)(CGG)
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Fig. 1. Alignment of the deduced amino acid sequences of bovine chromogranin A with published sequences. Sequence #1 is that of Benedum et al. [8], sequence #2 is that of Iacangelo et al. [9], sequence #3 is that of Ahn et al. [10], and sequence #4 is our sequence. Identical sequences are shown by short lines while the position and identity of the different amino acid residues are indicated by position numbers on top of the different residues (in single letter code). The nucleotide sequences in parentheses indicate the nucleotide sequences of #4 corresponding to the different amino acid residues shown.

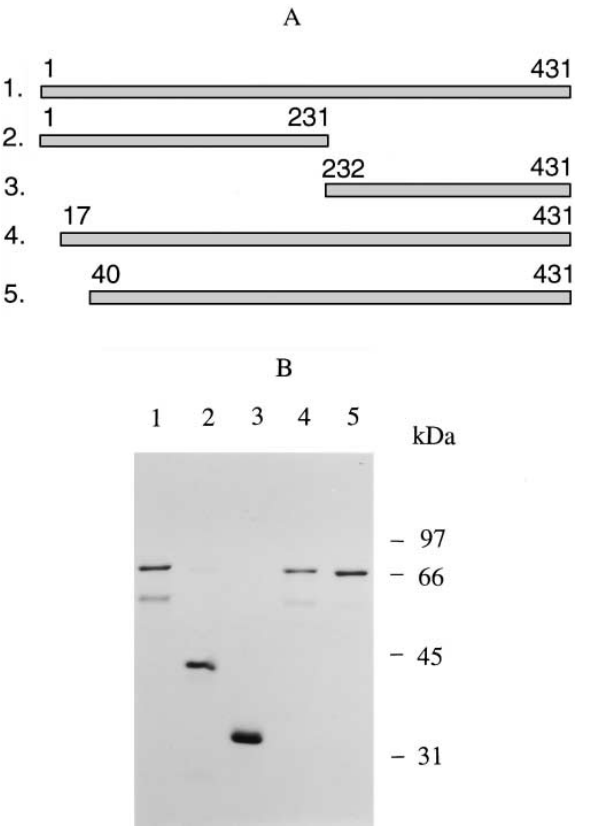

Fig. 2. Size and SDS-PAGE of the purified recombinant CGA proteins. A: Various sizes of the recombinant CGA proteins generated: (1) rCGA 1–431, (2) rCGA 1–231, (3) rCGA 232–431, (4) rCGA 17–431, (5) rCGA 40–431. B: SDS-PAGE of the five purified rCGA proteins: (1) rCGA 1–431, (2) rCGA 1–231, (3) rCGA 232–431, (4) rCGA 17–431, (5) rCGA 40–431.

secretory vesicle membrane at pH 5.5 [5], proteins 4 (rCGA 17–431) and 5 (rCGA 40–431) were constructed without the first 16 amino acids and without the first 39 amino acids, respectively.

Purified rCGA 1–431 was eluted in gel filtration chromatography in a volume indicative of a protein with a molecular mass of ~330 kDa (S.H. Yoo, unpublished result) compared to ~300 kDa shown with natural CGA [7,12], suggesting a similar physical state between rCGA 1–431 and natural CGA. To determine whether the purified recombinant CGA proteins can bind to the vesicle membrane at the intravesicular pH of 5.5, 80 μg of each rCGA protein was applied to the vesicle membrane-coupled Sepharose 4B column (0.5 ml volume) equilibrated with 20 mM sodium acetate, pH 5.5, 4 mM EGTA, 0.1 M KCl (Fig. 3). Then the column was washed with 0.1 M KCl (wash I), 1 M KCl (wash II), and again with 0.1 M KCl (wash III), all in 20 mM sodium acetate, pH 5.5, 4 mM EGTA. After thorough washes the pH of the elution buffer was changed to 7.5. As shown in Fig. 3, a pH 7.5/1 M KCl buffer released a large amount of protein from the column that had been loaded with rCGA 1–431 proteins and washed thoroughly, apparently indicating that the change of pH from 5.5 to 7.5 caused the dissociation of the bound proteins. The fractions eluted from the column were analyzed by SDS-PAGE. As shown in Fig. 4, a significant amount of rCGA 1–431 was present in the fractions eluted with the pH 7.5/1 M KCl buffer. This result indicated that rCGA 1–431

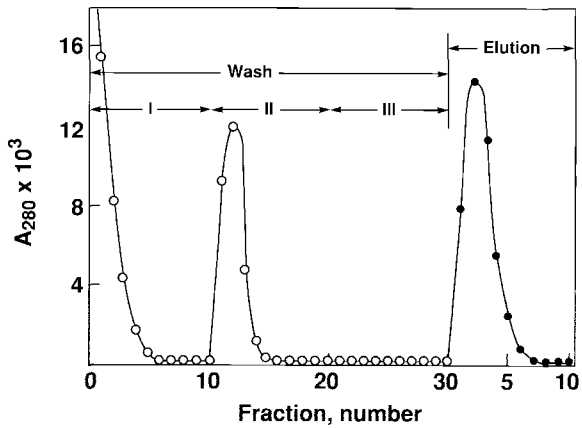

Fig. 3. Vesicle membrane-coupled Sepharose 4B chromatography of recombinant chromogranin A 1–431. 80 μg of rCGA 1–431 in 4 ml of 20 mM sodium acetate, pH 5.5, 4 mM EGTA, 0.1 M KCl was loaded onto a vesicle membrane-coupled Sepharose 4B column (0.5 ml vol) equilibrated with 20 mM sodium acetate, pH 5.5, 4 mM EGTA, 0.1 M KCl. The column was washed with 0.1 M KCl (wash I), followed by 1.0 M KCl (wash II) and 0.1 M KCl (wash III), all in 20 mM sodium acetate, pH 5.5, 4 mM EGTA. After the washes, the rCGA 1–431 was eluted with a pH 7.5 buffer (20 mM Tris-HCl, pH 7.5, 4 mM EGTA, 1 M KCl). The fraction size was 0.8 ml/fraction for the washes and 0.4 ml/fraction for elution, and the chromatography was carried out at room temperature.

bound to the vesicle membrane at pH 5.5 and was released from it at pH 7.5.

The amounts of other rCGA proteins bound to the vesicle membrane column are summarized in Table 1. Out of 80 μg of each rCGA protein loaded, 17 μg of rCGA 1–431 and 12 μg of rCGA 17–431 bound to the column compared to <2 μg of rCGA 40–431 (Table 1). The loading and elution of rCGA 1–231 and rCGA 232–431 proteins showed that only a limited amount of rCGA 1–231 (6 μg) bound to the column while virtually no rCGA 232–431 (<1 μg) bound to the column (Table 1). Although rCGA 1–231 was expected to bind well to the vesicle membrane, the lower than expected binding of rCGA 1–231 to the vesicle membrane suggests that the C-terminal half of the molecule is also important in supporting the interaction of the near N-terminal anchor region with the vesicle membrane.

4. Discussion

Chromogranin A [8–10,13–17] has two conserved regions, which are shared by chromogranin B [18–21], one being the near N-terminal region (residues 17–38) and the other being the C-terminal region (residues 407–431), implying important functional roles of these two regions. The present results identify the conserved near N-terminal region as the membrane binding anchor region of CGA, consistent with the previous results obtained with synthetic CGA peptides [5]. According to the hydrophobicity plot of CGA [8], this near N-terminal region including the two flanking cysteine residues stands out as the most hydrophobic region in the entire molecule. In this respect, the membrane binding anchor role of this hydrophobic region might have contributed to the conservation of the sequences in this region.

In spite of the fact that chromogranin A interacts with the vesicle membrane proteins through its conserved near N-terminal region, the recombinant CGA 1–231 protein that contained the N-terminal half of the molecule did not bind the vesicle membrane as well as the intact CGA, rCGA 1–431 (Table 1). This result suggests that the C-terminal half of the molecule might provide a structural stability of the whole molecule, thus facilitating the proper interaction of the near N-terminal region with the vesicle membrane. In this regard, the failure of the C-terminal half of the molecule to interact with the vesicle membrane did not come as a surprise. Since the conserved C-terminal region (residues 407–431) had been suggested to be primarily responsible for dimerization and tetramerization of CGA [22], it appeared to be a natural consequence that the C-terminal region failed to interact with the vesicle membrane.

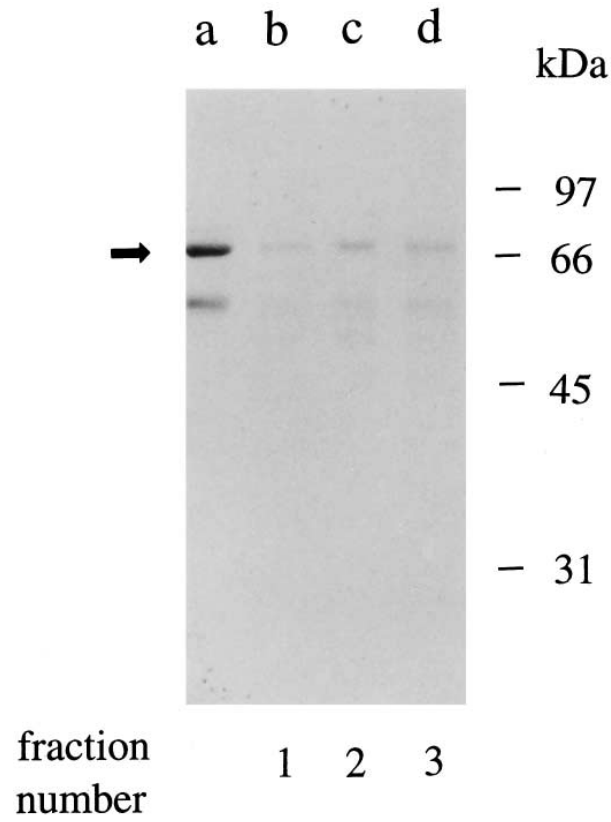

Fig. 4. SDS-PAGE of the eluted proteins from the vesicle membrane-coupled Sepharose 4B chromatography. The eluted rCGA 1–431 fractions were diluted 5-fold with water to reduce the salt concentration and concentrated 25-fold using Centricon-10 (Amicon, Beverly, MA). 25 μl aliquots of these are analyzed on a 10% SDS-polyacrylamide gel. Lane a: pre-loading rCGA 1–431 (1 μg); lanes b, c, and d: fractions 1, 2, and 3. Intact rCGA 1–431 is indicated by an arrow.

Table 1
Summary of the vesicle membrane-coupled Sepharose 4B chromatography of recombinant CGA proteins[a]

rCGA	Flow-through (μg)	Washes (μg)	Eluted (μg)
1–431	15	48	17
1–231	27	47	6
232–431	26	53	<1
17–431	15	53	12
40–431	28	50	<2

[a]80 μg of each CGA protein was loaded and the chromatography was carried out as described in Fig. 3.

In addition to the pH-induced conformational changes, chromogranin A has been known to interact with several vesicle membrane proteins including the inositol 1,4,5-trisphosphate receptor at the intravesicular pH of 5.5 and to dissociate from them at a near physiological pH of 7.5 [3]. The pH-dependent interaction of CGA with the vesicle membrane proteins was considered a pivotal step in the sorting process of both the vesicle matrix proteins and the membrane proteins during vesicle biogenesis [3]. Although it has generally been considered that the acidic pH- and Ca^{2+}-induced aggregation of vesicle matrix proteins is the first step of selective segregation of these vesicle matrix proteins of regulated secretory pathways [23,24], the question of how this vesicle matrix complex binds to the potential vesicle membrane in the trans-Golgi network and eventually ends up in the secretory vesicles is not well understood. Nevertheless, working with purified CGA and the secretory vesicle membrane, we have demonstrated that the acidic pH is the necessary and sufficient condition to cause CGA and the vesicle matrix protein aggregate to bind to the vesicle membrane [5], the key step in the sorting of vesicle matrix proteins and the vesicle membrane. In this respect, the identity of the conserved near N-terminal region as the anchor for CGA interaction with the vesicle membrane provides further insight into the protein sorting mechanism during secretory vesicle biogenesis.

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