Identification of the secretory vesicle membrane binding region of chromogranin B[1]

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Abstract In the past a synthetic peptide representing the conserved near N-terminal region of chromogranin B (CGB) (residues 17–36) has been shown to interact with the vesicle membrane. Hence, it was necessary to confirm this observation with CGB deletion proteins. In order to address this need and to confirm the published sequence of CGB, we cloned a CGB gene and produced various CGB deletion proteins. The recombinant CGB protein lacking the first 15 (rCGB 16–626) or 16 amino acid residues (rCGB 17–626) bound to the vesicle membrane as well as the full-length CGB. However, rCGB 49–626, lacking the conserved near N-terminal region, failed to interact with the vesicle membrane, indicating an anchor role for the conserved near the N-terminal region.

Key words: Sequence; Chromogranin B; pH; Secretory vesicle membrane

1. Introduction

Chromogranins A and B, the two major proteins of the secretory vesicles of neuroendocrine cells [1,2], share many common properties. Like chromogranin A (CGA), which bound to the secretory vesicle membrane at pH 5.5 and released from it at pH 7.5 [3], chromogranin B also bound to the secretory vesicle membrane [4,5]. However, chromogranin B interacted with secretory vesicle membrane much more tightly than CGA [5]. In the case of chromogranin A, a mere change of the pH of the elution buffer from 5.5 to 7.5 was sufficient to dissociate CGA from the secretory vesicle membrane [3]. However, a change of the pH of the elution buffer from 5.5 to 7.5 was not sufficient to completely dissociate CGB from the secretory vesicle membrane as evidenced by the fact that chromogranin B was not eluted from the secretory vesicle membrane-coupled Sepharose 4B column which had been previously loaded with CGB [5]. It took a combination of pH change from 5.5 to 7.5 and high salt concentration (1 M KCl) to elute CGB from this same column [5], demonstrating a tighter interaction of CGB than CGA with the secretory vesicle membrane.

In the past, using synthetic peptides, we have tentatively identified the conserved near N-terminal region (residues 16–37) of CGB as the membrane interacting anchor region of CGB [4]. Since the interaction of the near N-terminal region of CGB 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 CGB in the interaction with the vesicle membrane using truncated CGB proteins. Since chromogranins are also known to be precursors of bioactive peptides [1,2], it is important to establish accurate primary sequence of chromogranins. In order to identify the membrane interacting region of CGB and to confirm the published amino acid sequence of chromogranin B [6], we have cloned a bovine CGB gene and generated several recombinant CGB proteins. Using the purified recombinant CGB proteins, we now show that the conserved near N-terminal region of CGB is essential for CGB to bind 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 B probe obtained by PCR amplification using oligonucleotide primers 5′-GCAAACAGTATGCTCCCC-3′ (bases 1547–1564) and 5′-CTCAGGTGAAGCTTTTATTAC-3′ (complementary to bases 2344–2364) in which one base at position 2354 was substituted from T to G to create a *Hin*dIII site. Nucleotide numbering is based on the published bovine cDNA sequence [6]. The nucleotide sequences of both strands of the chromogranin B clone were determined.

2.2. Construction of CGB clones and subcloning into expression vector

In order to construct CGB clones except for CGB 49–626 and 373–626 (see below), 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 B clone. After the PCR products were digested with *Bam*HI and *Hin*dIII, each of the CGB clones was subcloned into pET28a(+) (obtained from Novagen) digested with *Bam*HI and *Hin*dIII previously. To obtain CGB 49–626 subcloned into pET28a(+), the chromogranin B full size clone (1–626) in pET28a(+) vector was digested with *Bam*HI and *Sac*I. The sticky ends of the linearized plasmid were either filled up (*Bam*HI site) or polished (*Sac*I site) and the plasmid was ligated. Likewise, to obtain CGB 373–626 subcloned into pET28a(+), pETCGB(1–626) and pET28a(+) were digested with *Xma*I and *Bam*HI, respectively, and the plasmids were digested with *Hin*dIII. The insert band and vector were eluted using the Geneclean kit (Bio 101) and ligated. The pET28a(+) vector carries an N-terminal His-tag (a stretch of six His) to which nickel (Ni) 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-nitrilo-triacetic acid (Ni-NTA) agarose affinity chromatography from Qiagen.

2.4. Purification of recombinant CGB proteins

In order to further purify the recombinant CGB 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, 0.1 mM $CaCl_2$, 0.25 M ammonium sulfate, and a reverse ammonium sulfate gradient was used to elute the proteins. The chromatographic procedures and buffers used for each recombinant protein purification are as follows: (1) recombinant CGB (rCGB) 1–626; the Ni-NTA column

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[1]The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number U88551.

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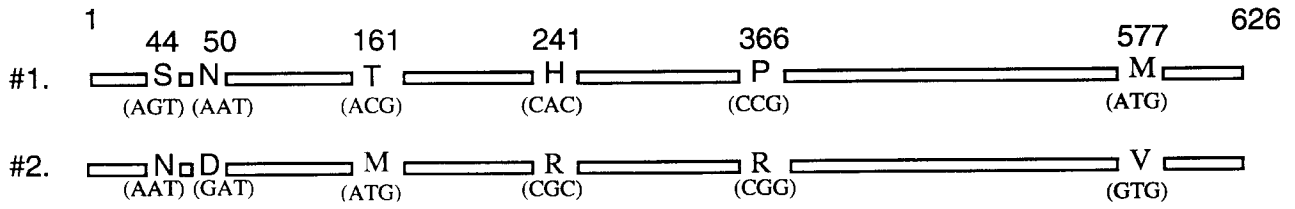

Fig. 1. Comparison of the deduced amino acid sequences of bovine chromogranin B with the published sequence. Sequence #1 is the published sequence [6], and sequence #2 is our sequence. Identical sequences are not shown while the position of the different amino acid residues are indicated by position numbers on top of the different residues (in single letter code). The corresponding nucleotide sequences are shown in parentheses.

eluate was loaded onto the phenyl-Sepharose column, washed with 0.2 M ammonium sulfate (wash I) and 0.1 M ammonium sulfate buffer (wash II), and eluted with 0 M ammonium sulfate buffer (15 mM Tris-HCl, pH 7.5, 0.1 mM $CaCl_2$). Appropriate pools from the phenyl-Sepharose column chromatography were loaded onto the DE-52 column (2.5 cm×7 cm) equilibrated with 10 mM imidazole, pH 6.1, and eluted with a KCl gradient of 0–0.4 M. (2) rCGB 1–298; 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 (0.1–0 M) buffer. (3) rCGB 1–450; purification of rCGB 1–450 followed the same procedure as for rCGB 1–298 purification. (4) rCGB 16–626; the Ni-NTA column eluate was loaded onto the phenyl-Sepharose column, washed with 0.1 M ammonium sulfate buffer (wash I) and 50 mM ammonium sulfate buffer (wash II), and eluted with a reverse gradient ammonium sulfate (50 mM–0 M) buffer. (5) rCGB 17–626; purification of rCGB 17–626 followed the same steps as for rCGB 16–626. (6) rCGB 49–626; the Ni-NTA column eluate was loaded onto the phenyl-Sepharose column, washed with 50 mM ammonium sulfate buffer, and eluted with a reverse gradient ammonium sulfate (50 mM–0 M) buffer. (7) rCGB 373–626; the Ni-NTA column elutate was 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 (0.1–0 M) buffer. In the case where reverse gradient ammonium sulfate elution is applied, additional buffer without ammonium sulfate was added at the end of the gradient to complete the elution.

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 [3].

3. Results

As shown in Fig. 1, our sequence agreed with the published sequence [6] in most cases, our sequence did differ from the published sequence in six amino acid residues in the coding region; residues 44 (S to N), 50 (N to D), 161 (T to M), 241 (H to R), 366 (P to R), and 577 (M to V). Another bovine CGB sequence that had been deposited in the Gen Bank/EMBL Data Bank (accession number, x55489)[2] differed with the published sequence [6] in eight amino acid positions (not shown), which did not share the different positions shown in Fig. 1 except one at position 44; residue 44, having Asn (N) instead of Ser (S), agreed with our sequence. The overall similarity of the sequence probably suggests polymorphism of the CGB gene.

Fig. 2 shows the size of recombinant CGB fragments generated and the purity of purified CGB proteins. Since a peptide representing the conserved near N-terminal region (residues 17–36) of CGB had previously been demonstrated to bind to the secretory vesicle membrane at pH 5.5, protein 4 (rCGB 16–626) and protein 5 (rCGB 17–626) were constructed without the first 15 amino acids and 16 amino acids, respectively. Since residues 16 and 37 of CGB are cysteines which form a disulfide bond in the intact molecules, protein 5 (rCGB 17–626), which does not contain the first cysteine residue, was especially constructed to determine whether the disulfide bond at the N-terminal region is essential in the interaction of CGB with the vesicle membrane. Protein 6 (rCGB 49–626) was constructed deleting the entire conserved N-terminal region.

In gel-filtration chromatography, purified rCGB 1–626 was eluted in a volume suggesting a protein with a molecular mass of ~220 kDa, which is the same mass as shown for the purified native bovine CGB [7] indicating that rCGB 1–626

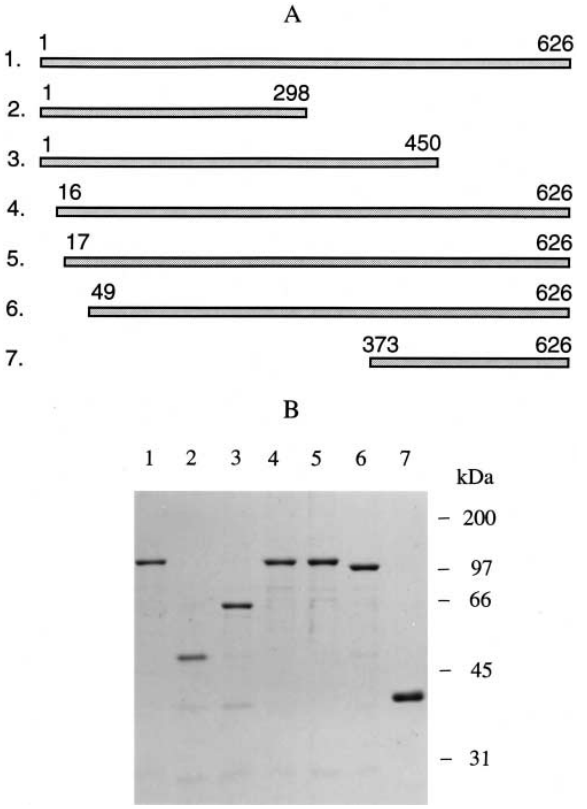

Fig. 2. Size and SDS-PAGE of the purified recombinant CGB proteins. A: Various sizes of the recombinant CGB proteins generated: 1, rCGB 1–626; 2, rCGB 1–298; 3, rCGB 1–450; 4, rCGB 16–626; 5, rCGB 17–626; 6, rCGB 49–626; 7, rCGB 373–626. B: SDS-PAGE of the seven purified rCGB proteins (0.5–1 μg each): 1, rCGB 1–626; 2, rCGB 1–298; 3, rCGB 1–450; 4, rCGB 16–626; 5, rCGB 17–626; 6, rCGB 49–626; 7, rCGB 373–626.

[2]Deposited by D.K. Grandy, R. Leduc, H. Makam, T. Flanagan, E.J. Diliberto, G. Thomas, O. Civelli and O.H. Viveros.

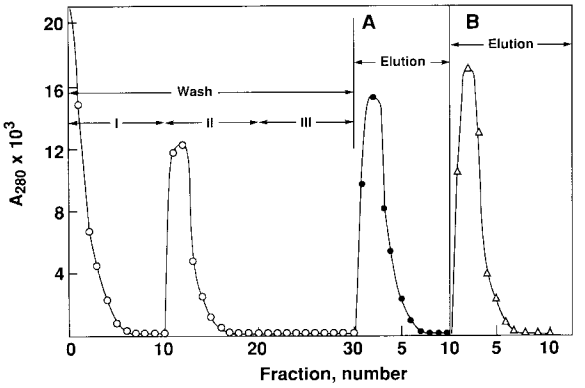

Fig. 3. Vesicle membrane-coupled Sepharose 4B chromatography of recombinant chromogranin B 1–626 and recombinant B 16–626. A: 80 μg of rCGB 1–626 in 4.0 ml of 20 mM sodium acetate, pH 5.5, 4 mM EGTA, 0.1 M KCl was loaded onto a vesicle membrane-coupled Sepharose 4B column (0.5 ml vol.) equilibrated with 20 mM sodium acetate, pH 5.5, 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 rCGB 1–626 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. B: An identical experiment that had been carried out with rCGB 16–626.

is in the same or in a similar folding state as the natural CGB. Other rCGBs also eluted in positions approximately in proportion with their respective masses (S.H. Yoo, unpublished results).

To determine whether the purified recombinant CGB proteins can bind to the vesicle membrane at the intravesicular pH of 5.5, 80 μg of rCGB 1–626 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, panel A). 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. 3A, a pH 7.5/1 M KCl buffer released a large amount of protein from the column that had been loaded with rCGB 1–626 protein and washed thoroughly, apparently indicating that the pH 7.5/1 M KCl buffer caused the dissociation of the bound proteins. An identical experiment that had been carried out with rCGB 16–626 protein exhibited a similar result (Fig. 3B).

To analyze the content of the proteins released from the column, the eluted fractions were analyzed by SDS-PAGE. As shown in Fig. 4, significant amounts of rCGB 1–626 (panel A) and rCGB 16–626 (panel B) proteins were present in the fractions eluted with the pH 7.5/1 M KCl buffer. These results indicated that rCGB 1–626 and rCGB 16–626 proteins bound to the vesicle membrane at pH 5.5 and were released from it at pH 7.5.

The amounts of rCGB proteins bound to the vesicle membrane column are summarized in Table 1. Out of 80 μg of

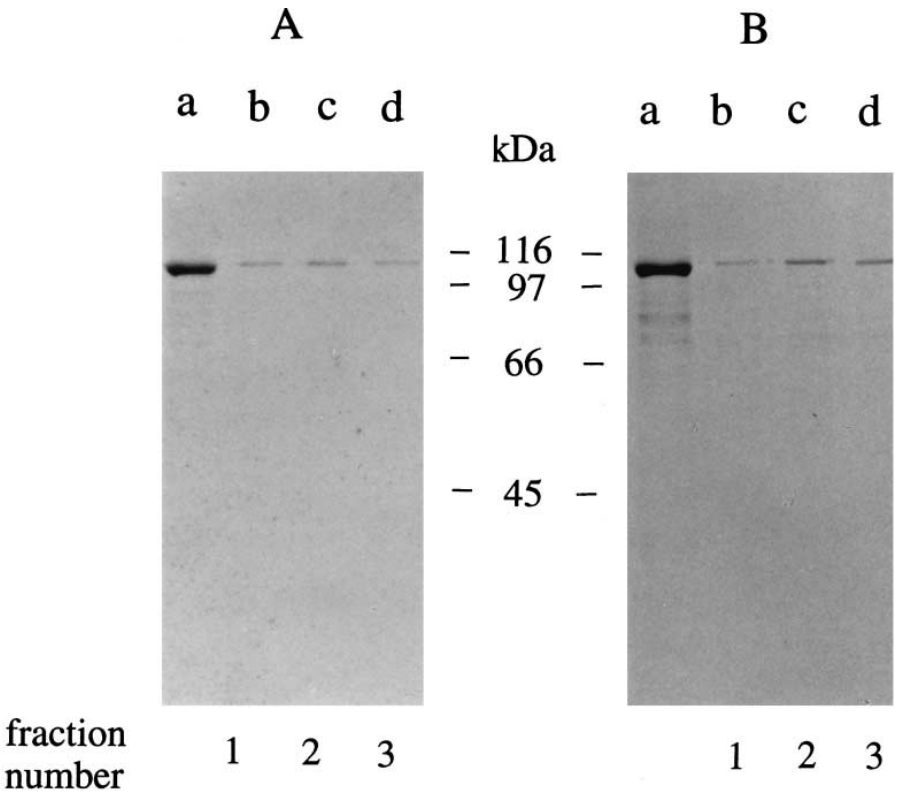

Fig. 4. SDS-PAGE of the eluted proteins from the vesicle membrane-coupled Sepharose 4B chromatography. The eluted rCGB 1–626 and rCGB 16–626 fractions were diluted 5-fold with water to reduce the salt concentration and concentrated 25-fold using Centricon-10 (Amicon, Beverly, MA). 25 μl aliquots of these were analyzed on a 10% SDS-polyacrylamide gel. A: Lane a, pre-loading rCGB 1–626 (1 μg); lanes b, c, and d, fractions 1, 2, and 3 of rCGB 1–626 elution. B: Lane a, pre-loading rCGB 16–626 (1 μg); lanes b, c, and d, fractions 1, 2, and 3 of rCGB 16–626 elution.

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

rCGB	Flow-through (μg)	Washes (μg)	Eluted (μg)
1–626	11	49	20
1–298	20	50	10
1–450	16	53	11
16–626	12	47	21
17–626	10	47	23
49–626	15	63	2
373–626	14	64	<2

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

rCGB proteins loaded, 20–23 μg of rCGB 1–626, 16–626, or 17–626 proteins bound to the column. The loading and elution of rCGB 1–298 and rCGB 373–626 proteins showed that only a limited amount (~10 μg) of rCGB 1–298 bound to the column (Table 1) while virtually no rCGB 373–626 (<2 μg) bound to the column. The loading and elution of rCGB 1–450 also gave similar results as those for rCGB 1–298 (Table 1). Although rCGB 1–298 was expected to bind well to the vesicle membrane, the lower than expected binding of rCGB 1–298 to the vesicle membrane column suggests that the C-terminal region of the molecule is also important in supporting the interaction of the near N-terminal anchor region with the vesicle membrane.

4. Discussion

Although the six amino acid difference between the published sequence [6] and our sequence (Fig. 1) might appear to be a minor difference, the difference in residue 241 has the potential to be significant in view of the fact that the presence of Arg in position 241 results in the presence of three contiguous basic residues at position 239–241 (LysArgArg). Dibasic residues (Arg and Lys) are known proteolytic processing sites for the generation of bioactive peptides from chromogranins A and B [1,2]. Whether bovine CGB is processed at this site is not known at present although processing of chromogranins at several dibasic sites have been reported [1,2]. In this regard, the study of processed CGB to identify the processing sites will shed light on the presence of tribasic residues in the region.

Bovine chromogranin B shares two conserved regions that are conserved throughout all sequenced chromogranins A and B from different species [8–17]: one at the near N-terminal region (residues 16–37) and another at the C-terminal region (residues 601–626), implying important roles for these two regions. The present results identify the conserved near N-terminal region as the membrane binding anchor region of CGB, in line with the previous result obtained with synthetic CGB peptides [4]. In spite of the fact that chromogranin B interacts with the vesicle membrane through its conserved near N-terminal region, the rCGB 1–298 or rCGB 1–450 protein that contained the conserved near N-terminal anchor region of the molecule, but without the C-terminal region, did not bind to the vesicle membrane as well as other rCGBs such as rCGB 1–626, rCGB 16–626 or rCGB 17–626 that contained the same region along with the C-terminal region (Table 1). These results appear to suggest that the C-terminal region of the molecule is essential for CGB to maintain its native conformation; without the C-terminal region, the N-terminal region of the molecule might not be able to form the proper conformation and orientation for the near N-terminal anchor to have a maximal interaction with the vesicle membrane.

Moreover, the present results indicate that a disulfide bond formed between the two cysteines (residues 16 and 37) in the conserved near N-terminal region is not required for CGB to bind to the vesicle membrane. The amounts of proteins bound and released between the two recombinant CGB proteins, rCGB 16–626 which contains a disulfide bond, and rCGB 17–626 which does not, were very similar, thereby clearly indicating that the disulfide bond at the near N-terminal region does not appear to be required for the anchor role of the conserved near N-terminal region. A hydrophobicity plot of CGB shows that the conserved near N-terminal region, including the two flanking cysteine residues, is the most hydrophobic region in the entire molecule [8]. In this regard, it appears that the membrane binding anchor role of this hydrophobic region contributed to the conservation of the sequences in this region.

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