Journal of Peptide Science
J. Pept. Sci. 2007; **13**: 237–244
Published online 31 January 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.837

JOURNAL OF PEPTIDE SCIENCE

Recombinant preparation and characterization of interactions for a calmodulin-binding chromogranin A peptide and calmodulin

SUNMI KANG, JINHO KANG, SEUNG HYUN YOO* and SUNGHYOUK PARK*

Department of Biochemistry, Center for Advanced Medical Education by BK21 project, School of Medicine, Inha University, Shinheung-dong, Chung-gu, Incheon, Korea

Received 9 November 2006; *Revised 18 December 2006*; *Accepted 20 December 2006*

Abstract: Chromogranin-derived peptides have important and varied biological activities. They affect a wide spectrum of targets such as fungal membranes, blood vessels, myocardial cells, and pancreatic cells. Despite the biological significance and the diverse activities, the molecular mechanisms of the interactions between the peptides and the target proteins have not been well understood. Here, we studied the interaction between a chromogranin A–derived peptide (CGA40–65) and its target protein, calmodulin, with NMR spectroscopy. Calmodulin was easily prepared with standard recombinant technology, but CGA40–65 posed challenges requiring multistep procedures. The recombinantly produced peptide retained the calmodulin-binding property of the full-length CGA, as shown by the HSQC binding experiment. By applying resonance assignments, we identified the residues in calmodulin involved in the CGA40–65 binding. We also found that the peak changes are close to those exhibited by the peptides having the wrap-around binding mechanism. Further analysis revealed that the CGA40–65-induced changes are more similar to those by CaMKIp peptide than those by smMLCKp peptide among the wrap-around binding peptides, suggesting that CGA40-65 can be categorized as a CaMKIp-like peptide. Our report is the first residue-resolution mechanistic study involving chromogranin peptides and their target proteins. Our approaches should be applicable to interaction studies involving other chromogranin-derived peptides and their cellular target proteins. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: chromogranin; calmodulin; refolding; binding mechanism

INTRODUCTION

Chromogranins are major Ca^{2+} storage proteins in secretory granules [1]. In addition to their storage function, chromogranins play a major role in regulating the inositol 1,4,5-trisphosphate receptors [2,3] – and therefore the cytosolic Ca^{2+} concentration – in secretory cells such as pancreatic cells, adrenal medulary chromaffin cells, and neuroendocrine cells [4]. Like other granin family proteins, chromogranins are processed by endopeptidases to form peptides exhibiting a variety of biological activities [5–7]. For example, chromogranin A (CGA) can form chromofungin with antifungal activity [8]; vasostatin-1 with vasoconstriction inhibiting activity [9], myocardial inotropic activity [10], and spontaneous colonic contracting activity [11]; pancreastatin with insulin release inhibiting activity [12], and catestatin with catecholamine releasing activity [13].

Among the various peptides formed from CGA is chromofungin, composed of residues 47–66. It is one of the few CGA peptides for which structural data are available [8]. The study suggested that the synthetic chromofungin peptide forms an amphipathic helix in the membrane-mimicking condition (water/trifluoroethanol mixture). It was also revealed that the antifungal peptide can penetrate the fungal membranes or monolayers of phospholipids. Previously, we have shown that CGA40–65, slightly longer than the chromofungin, can bind calmodulin very strongly (K_d in the nanomolar range) [14]. As chromofungin was shown to inhibit the calmodulin-dependent calcineurin activity in the Ser-Thr-protein phosphatase B [8], it is possible that the region around the CGA40–65 is important in calmodulin-dependent cellular enzyme regulation.

Although much has been studied about the biological activities of peptides from chromogranins [15,16], little is known about their characteristics or mechanisms of binding to the target proteins. As a first step to understand the molecular mechanism of the calmodulin-dependent regulation process by chromogranin-derived peptides, we studied the interaction between the CGA40–65 and calmodulin with heteronuclear NMR methods using stable isotope labeling. As the approach required recombinant expression of the peptide, we developed expression, purification, and isotopic labeling procedures of the CGA40–65 peptide. The HSQC binding experiment confirmed that the recombinant peptide

* Correspondence to: S. H. Yoo and S. Park, Department of Biochemistry, Center for Advanced Medical Education by BK21 project, School of Medicine, Inha University, Chungsuk Building, Room 505, Shinheung-dong, Chung-gu, Incheon, Korea, 400-712;
e-mail: shyoo@inha.ac.kr; spark@inha.ac.kr

retains the function of the full-length CGA in terms of calmodulin binding. The NMR binding assay and comparison with other peptides suggest that the CGA40–65 peptide interacts with calmodulin in a wrap-around binding mechanism.

MATERIALS AND METHODS

Materials

Enzymes for cloning the CGA40–65 and calmodulin gene were purchased from Takara Korea (Seoul, Korea). Primers were obtained from Bioneer (Daejun, Korea). The primers for CGA40–65 were designed to have one tyrosine in front of the coding sequence to facilitate quantification of the peptide. Both CGA40–65 and calmodulin have additional GAMA sequences due to the tobacco etch virus protease (TEV protease) cleavage site [8]. Previously, we have shown that a synthetic CGA40–64 with an additional *N*-terminal tryptophan has a K_d value in calmodulin binding very similar to the full-length CGA [14]. Therefore, the extra sequence in the peptide is not expected to affect the binding between the peptide and calmodulin. The *E.coli* host for expressing the cloned genes was Rosetta (DE3) from Novagen (Madison, WI, USA). Buffers, chemicals, and protease inhibitors were acquired from American Bioanalytical (Natick, MA, USA) or Sigma (St. Louis, MO, USA). His-TEV (pETM-11 in EMBL nomenclature) and His-GST-TEV (pETM-30 in EMBL nomenclature) vectors were obtained from Dr Ario de Marco of the Protein Expression Laboratory of the European Molecular Biology Laboratory (Heidelberg, Germany). Ni-NTA agarose was purchased from Qiagen (Valencia, CA, USA). TEV protease was produced in the lab using the gene provided by Dr David Waugh of the National Cancer Institute (Frederick, MD, USA).

Cloning of CGA40–65 and Calmodulin Genes

The CGA40–65 gene was PCR-amplified from the bovine CGA gene. Temple sequence of CGA40–65 was: GAGACACTCC-GAGGAGATGAACGGATCCTCTCAATCCTGCGACATCAGAATT TGCTGAAAGAGCTCCAAGACCTCGCT. The coding sequence for the calmodulin was amplified from the human calmodulin gene bought from the American Type Culture Collection. The sense primer for CGA40–65 has a sequence GATGACAAGC-CATGGCATATGAGACACTCCGAGGAGATGAACGG and the antisense primer CGAAACTCGGTACCTCAAGCGAGGTCTTG-GAGCTCTTTCAG. The sense primer for calmodulin has the sequence GATGACAAGCCATGGCAATGGCTGATCAGCT-GACCGAAG and the antisense primer AAACTCGGTACCT-CATTTTGCAGTCATCATCTGTACGAATTCTTCATAGTTG. The PCR products were purified, digested with *Nco* I and *Kpn* I, and then ligated into the His-GST-TEV (CGA40–65 only) and His-TEV (CGA40–65 and calmodulin) vectors using a DNA Ligation Kit (Takara Korea, Seoul, Korea). The ligation products were transformed into competent Rosetta (DE3) *E.coli*. Colonies with recombinant plasmids were selected using small-scale expression tests and colony PCR. For final confirmation, plasmids from the selected colonies were sequenced (Macrogene, Seoul, Korea).

Expression of CGA40–65 and Calmodulin

Cells expressing recombinant CGA40–65 and calmodulin were grown in LB media, with 50 μg ml^{-1} kanamycin, until their OD_{600} reached 0.4–0.5 at 37 °C. At that point, expression was initiated by the addition of IPTG at a final concentration of 1 mM. When the OD_{600} passed 1.0, the cells were harvested by centrifugation at 4427*g* for 10 min. For isotope labeling, M9 minimal media was used (0.1 mM $CaCl_2$, 5.6 mM glucose, 1 mM kanamycin, 17.2 mM K_2HPO_4, 1 mM $MgSO_4$, 8.6 mM NaCl, 56.7 mM NaH_2PO_4, 18.7 mM NH_4Cl, 0.001% thiamine). In preparation of the media, $CaCl_2$, glucose, kanamycin, $MgSO_4$, and ^{15}N-labled NH_4Cl were added by filtration into the pre-autoclaved NaH_2PO_4, K_2HPO_4, and NaCl solution. The culture procedure was the same as for the LB media culture. For CGA40–65 peptide, the pellet was resuspended in 5 ml urea lysis buffer (8 M urea, 10 mM Tris-Cl, 100 mM NaH_2PO_4, pH 8.0) and then stored at −20 °C. The stored mixture was subjected to several freeze–thaw cycles. For calmodulin, the cell pellet was frozen at −20 °C without any buffer addition.

Purification of CGA40–65 and Calmodulin

Another 30 ml of urea lysis buffer was added to the thawed pellet (35 ml final volume). Following vortexing, the total cell lysate was obtained by centrifugation at 17 000*g* for 30 min at 4 °C. The supernatant containing 6x His-tagged CGA40–65 was incubated with Ni-NTA agarose (Qiagen, Valencia, CA, USA) at 4 °C for 10 min. The resin with bound CGA40–65 was washed with step-gradient with urea wash buffer (8 M urea, 10 mM Tris-Cl, 100 mM NaH_2PO_4, pH 6.3) and native wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). The gradient wash buffer was sequentially prepared by mixing 20 ml: 0 ml, 16 ml: 4 ml, 12 ml: 8 ml, 8 ml: 12 ml, 4 ml: 16 ml, and 0 ml: 20 ml of urea wash buffer and native wash buffer, respectively. After the gradient wash, the resin was washed with another 20 ml of native wash buffer to ensure the complete removal of urea. The refolded fusion peptide was eluted with 40 ml of native elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole (pH 8.0). In the overall procedures, we did not take other auxiliary steps such as DNA disruption or addition of DTT, but could achieve good purification.

The eluted His-GST-fusion peptide was cleaved with TEV protease to release the CGA40–65 peptide. The cleavage reaction was carried out overnight at 4 °C in the native elution buffer. A protease inhibitor mixture including pepstatin, PMSF, and aprotinin was also added, which had been shown not to affect the TEV activity. The extent of cleavage was monitored with 18% SDS-PAGE electrophoresis.

The CGA40–65 peptide was separated from the other proteins in the TEV reaction mixture by a simple ethanol precipitation procedure. Five volumes of ethanol were added directly to the reaction mixture to allow precipitation of proteins including His-GST, TEV, and other impurities. The precipitated proteins were removed by centrifugation at 2800*g* for 10 min. The CGA40–65 peptide remained in the solution and the sample was dried *in vacuo*.

The dried peptide was resolubilized in H_2O and cleaned up with SEP-PAK C18 reverse phase (Waters, Milford, MA, USA). The cartridge was wetted with methanol followed by water. Then the resolubilized peptide was applied, and washed with

DOI: 10.1002/psc

water. The cleaned-up peptide was eluted with methanol and dried *in vacuo*.

The cell-growth procedures for calmodulin were the same as those used for CGA40–65. The frozen pellet was resuspended in 25 ml native lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). After adding protease inhibitors, the cells were lyzed by sonication. Total cell lysate was obtained by centrifugation at 17 000*g* for 10 min at 4 °C. The supernatant containing 6x His-tagged calmodulin was incubated with Ni-NTA agarose (Qiagen, Valencia, CA, USA) at 4 °C for 10 min with slow rotation. The resin with bound calmodulin was washed with 200 ml of native wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). The protein was eluted with 40 ml of native elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole (pH 8.0). The eluted His-tagged calmodulin was buffer-exchanged from the native elution buffer to the native lysis buffer using PD-10 desalting columns (GE Healthcare). The His tag was cleaved by TEV protease at room temperature overnight in the lysis buffer. After the cleavage, the His Tag was removed by another run of Ni-NTA chromatography. The calmodulin was further purified using gel-filtration superdex75 HiLoad 16/60 (GE Healthcare) column chromatography using 100 mM KCl, 6.1 mM $CaCl_2$, and 10 mM imidazole (pH 6.5) buffer. Purified calmodulin protein fractions were collected and concentrated with ultrafiltration.

NMR Spectroscopy

NMR spectroscopy was performed in 10 mM imidazole, 100 mM KCl, 6.1 mM $CaCl_2$, pH 6.5 buffer. The buffer condition was adapted from previous literature on calmodulin and its binding peptide [17–19]. Imidazole was added to provide higher buffer capacity at pH 6.5. The dried peptide was directly solubilized into the buffer (152 μM final concentration) containing an equivalent concentration of calmodulin. The final NMR sample had 5% D_2O for locking purposes. The spectra were recorded using a Varian Unity Inova 400 spectrometer equipped with a triple-resonance Z-gradient probe or a Bruker Avance 800 spectrometer. For diffusion coefficient measurement, a gradient spin echo pulse sequence was used. Twenty steps of gradient ramp were generated with fixed diffusion delay (170 msec). The diffusion coefficient was calculated as reported previously [20].

Chemical Shift Mapping

^{15}N-labeled calmodulin (117 μM) was titrated with an equivalent concentration of CGA40–65 peptide, and the spectra were compared with that of calmodulin alone. The residues corresponding to the peaks were identified using the reported assignments [21] (BMRB accession number 6541) and the analysis of 15N-NOESY-HSQC spectrum taken with the present sample. Chemical shift changes were calculated as described previously [22].

RESULTS

Expression and Preparation of CGA40–65 from the Insoluble Fraction

We first tried to prepare recombinant CGA40–65, as it was required for detailed interaction studies with

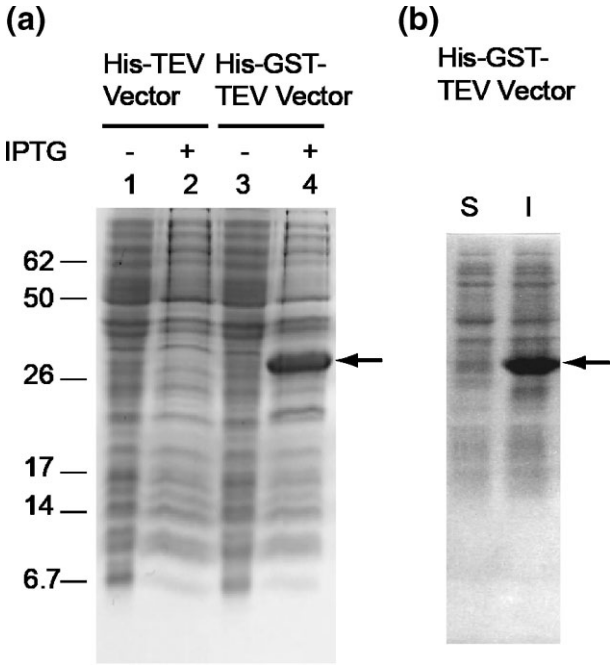

Figure 1 Expression of CGA40–65. (a) Expression of the CGA40–65 insert in His-TEV and His-GST-TEV vector with (lanes 2 and 4) and without IPTG induction (lanes 1 and 3). The arrow indicates the overexpressed His-GST-TEV fused CGA40–65. (b) Solubility test of His-GST-TEV fusion peptide of CGA40–65. The insoluble (I) and soluble (S) fractions were separated by centrifugation and analyzed by SDS-PAGE. Dilution factors are the same for the two fractions.

calmodulin by heteronuclear NMR spectroscopy. We used His-TEV and His-GST-TEV vectors with the same restriction sites (*Nco* I and *Kpn* I sites) to facilitate the cloning procedures. As shown in Figure 1(a), the His-TEV vector clone did not show any appreciable overexpression, while the His-GST-TEV clone showed much expression at the expected molecular weight. Therefore, we pursued further experiments with the His-GST-TEV fusion clone. Next, we tested the solubility of the expressed His-GST-fusion peptide. Figure 1(b) shows that it is expressed exclusively as an insoluble inclusion body. We solubilized the inclusion body in 8 M urea and tried refolding by rapid dilution or dialysis only to have much precipitation at the final step. Another trial employing the on-column refolding worked quite nicely, and we were able to obtain the fusion peptide in good amounts (see below for the yield; Figure 2, lane 1). The proper folding of the fusion peptide was confirmed by the binding of the eluted fusion peptide to the glutathione S-sepharose resin (data not shown). The fusion peptide was then cut with the TEV protease, which gave near-quantitative cleavage (Figure 2(a)). To remove the added TEV protease and the cleaved fusion protein (His-GST), we exploited the solubility difference between larger proteins and small peptides in ethanol. Proteins are generally denatured and precipitate in a high percentage of ethanol whereas peptides are

DOI: 10.1002/psc

often very soluble. The ethanol precipitation turned out to be a simple and effective method to remove larger protein impurities including the His-GST and the TEV protease (Figure 2(b)). As the peptide was purified from insoluble aggregates, we assessed the oligomeric status of the CGA40–65 by measuring the translational diffusion coefficient with pulsed field gradient spin echo. The obtained diffusion coefficient of CGA40–65 (MW = 3568) is 2.71×10^{-10} m^2 s^{-1}. We also obtained a value of 2.00×10^{-10} m^2 s^{-1} for the GB1 domain protein (MW = 7432), a very well-characterized monomeric protein. If CGA40–65 were dimeric or oligomeric, the actual molecular weight would be close to or larger than that of GB1, and CGA40–65 should have a similar or smaller diffusion coefficient than GB1. However, the measured value for CGA40–65 is significantly larger than that of GB1. Therefore, the result shows that CGA40–65 is monomeric in solution.

Preparation of Calmodulin

While the recombinant production of CGA40–65 peptide was complicated, the preparation of recombinant calmodulin was very straightforward. Calmodulin was expressed in soluble fraction with the His-TEV vector and was purified in native condition using Ni-NTA affinity chromatography. The ethanol precipitation method could not be used for calmodulin, as the protein also precipitates. We re-ran the Ni-NTA once more, after buffer exchange, to remove the cleaved His tag. The

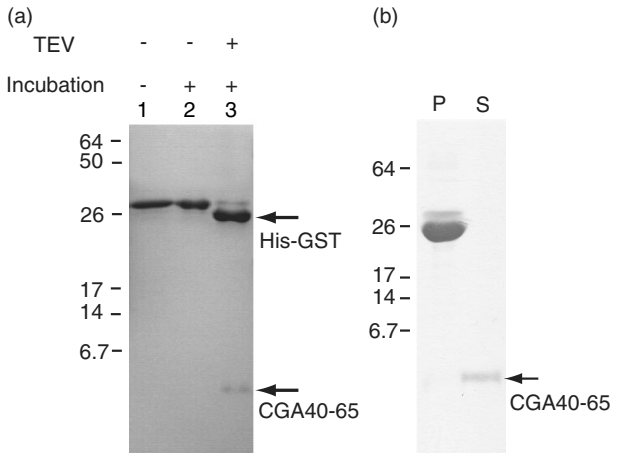

Figure 2 Final purification of the CGA40–65 fusion peptide. (a) TEV protease cleavage of the refolded CGA40–65 fusion peptide. Refolded and purified CGA40–65 fusion peptide after elution from the Ni-NTA column (lane 1). Incubation at 4 °C overnight without (lane 2) and with TEV protease (lane 3). Lane 2 shows that there is no nonspecific degradation of the fusion peptide in the absence of TEV protease. (b) Purification by ethanol precipitation. Five volumes of the ethanol were directly added to the reaction mixture after the TEV cleavage. The resulting precipitated material (lane P) and the supernatant (lane S) were analyzed by SDS-PAGE. Dilution factors are the same for the two fractions.

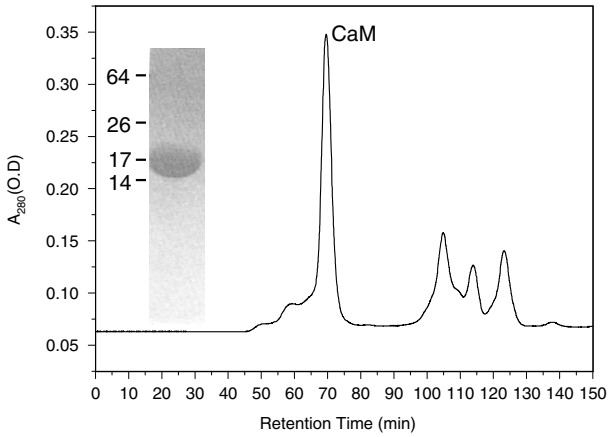

Figure 3 Purification of the recombinant calmodulin. Ni-NTA-treated fraction was applied to a Superdex 75 gel-filtration column. Calmodulin peak is marked with 'CaM'. Inset : SDS electrophoresis of the purified fraction.

calmodulin fraction was concentrated and purified by gel-filtration chromatography (Figure 3).

Stable Isotope Labeling of CGA40–65 and Calmodulin

As we were to pursue interaction studies with heteronuclear NMR, we applied the above preparation method to make uniformly ^{15}N-labeled samples. We obtained about 1.5 mg of ^{15}N-labeled CGA40–65 peptide from 4 L M9 minimal medium culture. Typically, the yield in a minimal medium culture is about one-quarter of that from an LB medium culture. The produced amount was enough to yield three to four NMR samples of very high concentration (about 800 μM). For calmodulin, about 7.5 mg was obtained from 2 L of M9 culture, enough for two NMR samples of the same concentration. For CGA40–65 peptide, MALDI-TOF mass spectrometry revealed the identity and integrity of the ^{15}N-labeled peptide (calculated mass: 3614; observed mass: 3617). The identity of the recombinant calmodulin was also confirmed by MALDI-TOF mass data (calculated mass: 17 168; observed mass: 17 174). In addition, we used HSQC spectra to verify the proper folding of the recombinant protein. The HSQC peaks of the expressed calmodulin matched very well with those reported previously [21], confirming the native folding of the protein.

Binding Assay for CGA40–65 to Calmodulin

Any recombinant peptide should be checked for its original activity. In the case of CGA40–65, it must have calmodulin-binding affinity if the recombinant production is to be appropriate. Therefore, we checked the calmodulin-binding property of the CGA40–65 using heteronuclear NMR spectroscopy. As shown in Figure 4(a), the HSQC spectrum of ^{15}N-labeled

DOI: 10.1002/psc

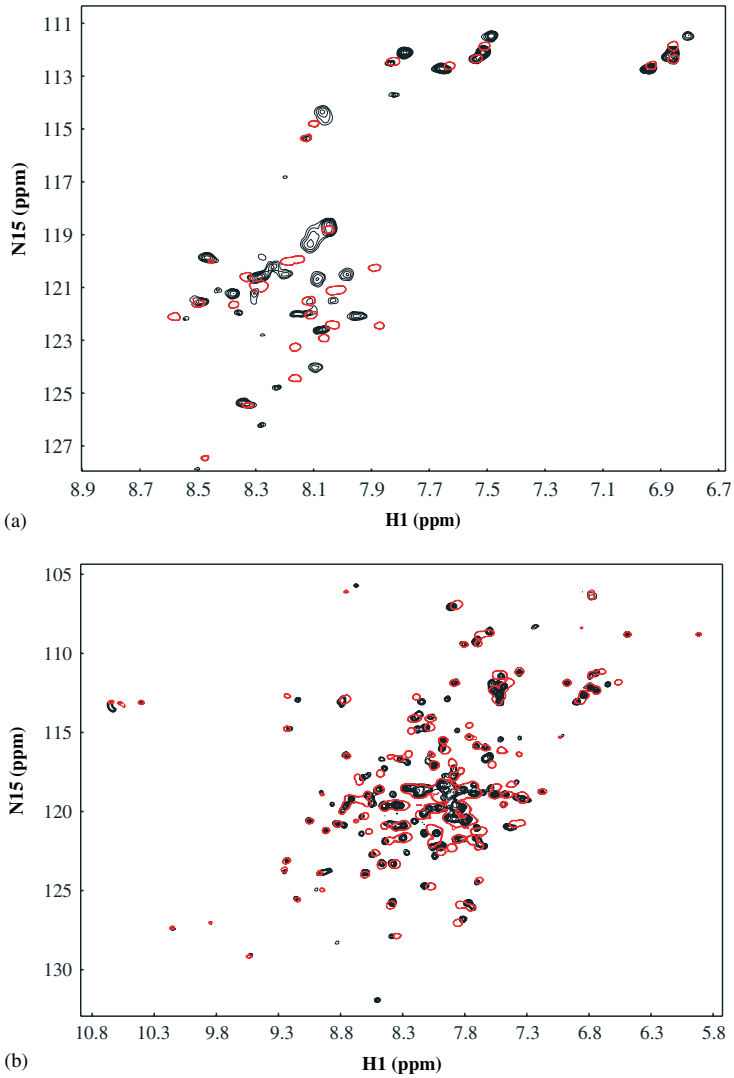

Figure 4 Binding between CGA40–65 and calmodulin. (a) HSQC spectra of ^{15}N-labeled CGA40–65 with (multiple black contours) and without calmodulin (single red contour). (b) HSQC spectra of ^{15}N-labeled calmodulin with (single red contour) and without the CGA40–65 (multiple black contours).

CGA40–65 changed significantly upon addition of unlabeled calmodulin, confirming the retention of the binding affinity. To further ensure that the changes are not nonspecific, we did the reciprocal binding experiment with ^{15}N-labeled calmodulin and CGA40–65 peptide (Figure 4(b)). The calmodulin spectrum changed significantly upon the addition of the CGA40–65, confirming the specific interaction between them.

Chemical Shift Mapping and Binding Site Identification

To characterize the binding between CGA40–65 and calmodulin at residue resolution, we carried out chemical shift mapping experiments. The HSQC spectra of the ^{15}N-labeled calmodulin with and without CGA40–65 peptide were compared. With the reported chemical shift assignments, we were able to identify the residues whose peaks were shifted significantly more than others (over 0.06 ppm, Figure 5). They are regions around residues 15–19, 25–40, 54–76, 80–92, 104–109, 121, and 145. We mapped those residues on the three-dimensional structure of a peptide–calmodulin complex having the 'wrap-around' binding mechanism (pdb code: 2BBM, solid black region in Figure 6) [23]. The results showed that the most affected residues match very well with the regions in close contact with the peptide in the model complex, indicating that CGA40–65 should have a very similar binding mechanism.

DISCUSSION

Recombinant production of peptides enables labeling with stable isotopes, which in turn enables heteronuclear NMR approaches that can significantly facilitate

DOI: 10.1002/psc

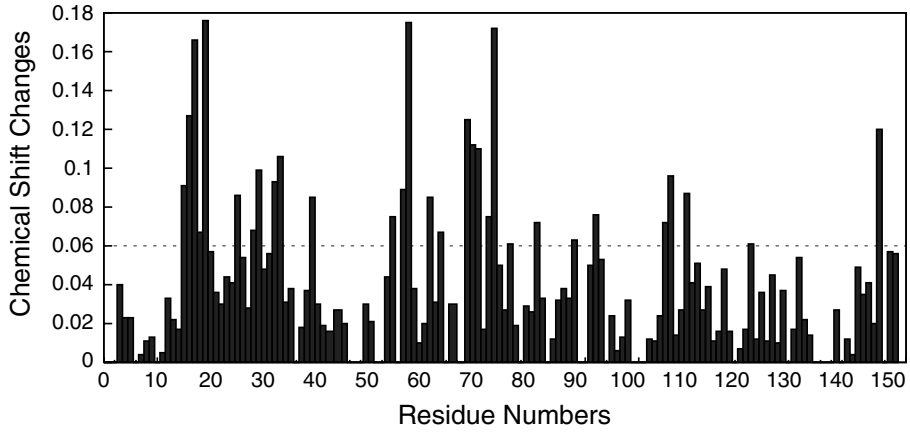

Figure 5 Chemical shift mapping analysis for the binding between calmodulin and CGA40–65. Normalized chemical shift changes after the addition of the CGA40–65 were plotted against the residue numbers. The chemical shift changes were calculated as weighted average of proton and nitrogen chemical shifts as described previously [24]. The horizontal dotted line indicates the threshold value of 0.06 ppm.

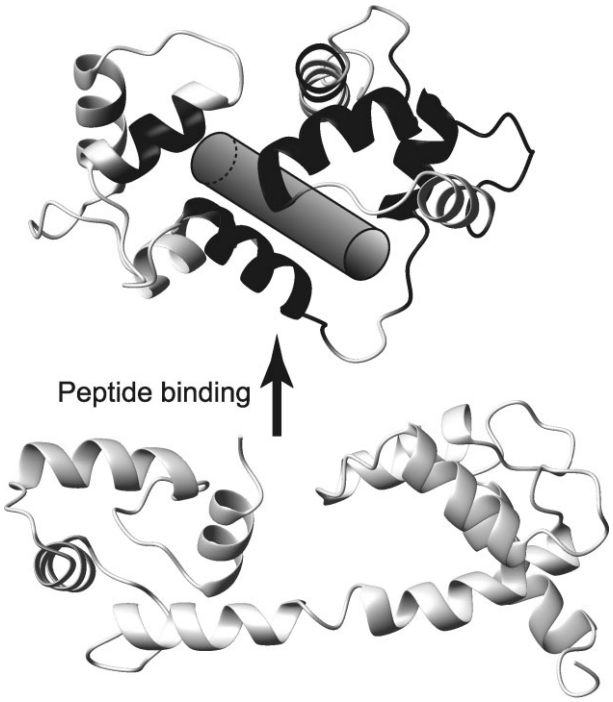

Figure 6 The wrap-around binding mechanism between calmodulin and CGA40–64. The solid black color on the upper molecule indicates the residues that shifted significantly in the chemical shift mapping analysis (chemical shift changes >0.06 ppm). The pdb codes for the calmodulin structures are 2BBM (upper) and 1X02 (lower).

mechanistic and binding studies. We performed labeling of CGA40–65 and calmodulin by recombinant technology in order to understand the interaction between them. Whereas calmodulin samples were prepared without difficulty using routine procedures, CGA40–65 required many more steps. CGA40–65 did not express appreciably with a small 6x His tag alone, but it expressed very well with a much larger His-GST-fusion tag. The use of the His-GST-fusion system made the peptide express very well, but the expression occurred entirely in the insoluble fraction. Use of an inclusion body is a good way to avoid proteolysis, which could have been the cause of the poor expression of the 6x His-tagged peptide, since the insoluble aggregate is separated from the cytosol and the proteases cannot access it. However, preparing proteins from an inclusion body necessarily involves a refolding step that often turns out to be fruitless. Nevertheless, we successfully refolded the fusion peptide using an on-column gradient refolding method, taking advantage of the presence of the 6x His tag in the fusion peptide. The 6x His tag allowed affinity purification in the denaturating condition, and the presence of GST in the same fusion peptide allowed the assessment of the folding. As green fluorescence protein fusion tags were used to rapidly screen properly folded target peptides from the soluble fraction [25,26], the GST in our case could serve as a reporter of the nativeness of the fusion peptide prepared from the insoluble fraction. Therefore, the method could be applicable to other inclusion body preparations in which the folding of the target peptide is a significant issue.

The ^{15}N HSQC spectrum of the CGA40–65 alone exhibited a very narrow spectral dispersion, reflecting the unstructured nature of the peptide. The spectrum also showed significant line broadening of CGA40–65 upon binding to calmodulin, indicating that the binding occurs in a slow-exchange regime. Previously, the affinity (K_d) of the CGA40–65 peptide for the calmodulin has been reported to be 13 nM [14]. Assuming the diffusion-controlled association between protein and peptide with $k_{on} = 10^8$ s^{-1}M^{-1} [27], the dissociation rate constant (k_{off}) can be estimated from the reported dissociation constant. The calculated value of k_{off} is 1.3 s^{-1}, which yields an average complex lifetime of 0.77 s. This slow exchange time scale is consistent with

DOI: 10.1002/psc

the observed line broadening of the peptide bound to calmodulin, owing to the significant increase in the molecular mass.

Many calmodulin-binding peptides elicit movements of the two lobes of calmodulin. Originally linked flexibly, the two lobes are brought closer by the peptide in a so-called 'wrap-around' binding mechanism [28]. With the established stable isotope labeling, we performed an HSQC binding experiment between calmodulin and CGA40–65. The data showed that many of the peaks of calmodulin were shifted, and that there were residues with much larger changes than others, indicating substantial microenvironmental changes along those residues. When we mapped those residues on the three-dimensional structure of a peptide–calmodulin complex with the wrap-around binding mode [23], they were found on the regions closest to the binding peptide (Figure 6). The analysis suggests that CGA40–65 engages equivalent residues involved in the wrap-around binding and induces similar conformation changes. The suggestion is consistent with the high sequence homology between CGA40–65 and other peptides with the wrap-around binding mechanism, such as MLCK and CaMKIV [8,29]. As there can still be differences in detailed binding mode among the wrap-around bindings, as shown for the CaMKIp and smMLCKp peptides [29], we compared the peak shift distribution by CGA40–65 with that of these two peptides. The significant changes at residues 15–19, 25–40, 54–76, 80–92, 104–109, 121, and 145 by CGA40–65 matched quite well with the changes by the CaMKIp peptide [29]. However, the pattern was significantly different from that produced by the smMLCKp peptide. Therefore, CGA40–65 can induce similar conformational changes as CaMKIp, and may be categorized, among the wrap-around binders, as a CaMKIp-type calmodulin-binding peptide. The wrap-around binding mechanism also provides a hint as to the conformation of CGA40–65 when bound to calmodulin. As peptides of the wrap-around mechanism are structured upon binding to calmodulin [8,29], it can be speculated that CGA40–65 will undergo similar structural changes by calmodulin binding.

Details on the interaction between the chromogranin-derived peptides and the cellular target proteins, despite the wide range of their biological activities, have not been explored. Here, we report the first residue-resolution studies on the interaction of a chromogranin-derived peptide, CGA40–64, with its binding partner, calmodulin. The strategy for preparing the peptide from the inclusion body, isotope labeling, and the use of the heteronuclear NMR reported in this study should be readily applicable to other chromogranin-derived peptides and facilitate the understanding of their binding mechanisms.

Acknowledgements

This work was supported by INHA University Research Grant (INHA-32729-01).

REFERENCES

1. Bulenda D, Gratzl M. Matrix free Ca2+ in isolated chromaffin vesicles. *Biochemistry (Mosc).* 1985; **24**: 7760–7765.
2. Yoo SH. Coupling of the IP3 receptor/Ca2+ channel with Ca2+ storage proteins chromogranins A and B in secretory granules. *Trends Neurosci.* 2000; **23**: 424–428.
3. Yoo SH, Oh YS, Kang MK, Huh YH, So SH, Park HS, Park HY. Localization of three types of the inositol 1,4,5-trisphosphate receptor/Ca(2+) channel in the secretory granules and coupling with the Ca(2+) storage proteins chromogranins A and B. *J. Biol. Chem.* 2001; **276**: 45806–45812.
4. Thrower EC, Choe CU, So SH, Jeon SH, Ehrlich BE, Yoo SH. A functional interaction between chromogranin B and the inositol 1,4,5-trisphosphate receptor/Ca2+ channel. *J. Biol. Chem.* 2003; **278**: 49699–49706.
5. Colombo B, Longhi R, Marinzi C, Magni F, Cattaneo A, Yoo SH, Curnis F, Corti A. Cleavage of chromogranin A N-terminal domain by plasmin provides a new mechanism for regulating cell adhesion. *J. Biol. Chem.* 2002; **277**: 45911–45919.
6. Metz-Boutigue MH, Garcia-Sablone P, Hogue-Angeletti R, Aunis D. Intracellular and extracellular processing of chromogranin A. Determination of cleavage sites. *Eur. J. Biochem.* 1993; **217**: 247–257.
7. Iacangelo AL, Eiden LE. Chromogranin A: current status as a precursor for bioactive peptides and a granulogenic/sorting factor in the regulated secretory pathway. *Regul. Pept.* 1995; **58**: 65–88.
8. Lugardon K, Chasserot-Golaz S, Kieffer AE, Maget-Dana R, Nullans G, Kieffer B, Aunis D, Metz-Boutigue MH. Structural and biological characterization of chromofungin, the antifungal chromogranin A-(47–66)-derived peptide. *J. Biol. Chem.* 2001; **276**: 35875–35882.
9. Aardal S, Helle KB. The vasoinhibitory activity of bovine chromogranin A fragment (vasostatin) and its independence of extracellular calcium in isolated segments of human blood vessels. *Regul. Pept.* 1992; **41**: 9–18.
10. Corti A, Mannarino C, Mazza R, Angelone T, Longhi R, Tota B. Chromogranin A N-terminal fragments vasostatin-1 and the synthetic CGA 7–57 peptide act as cardiostatins on the isolated working frog heart. *Gen. Comp. Endocrinol.* 2004; **136**: 217–224.
11. Amato A, Corti A, Serio R, Mule F. Inhibitory influence of chromogranin A N-terminal fragment (vasostatin-1) on the spontaneous contractions of rat proximal colon. *Regul. Pept.* 2005; **130**: 42–47.
12. Tatemoto K, Efendic S, Mutt V, Makk G, Feistner GJ, Barchas JD. Pancreastatin, a novel pancreatic peptide that inhibits insulin secretion. *Nature* 1986; **324**: 476–478.
13. Mahata SK, O'Connor DT, Mahata M, Yoo SH, Taupenot L, Wu H, Gill BM, Parmer RJ. Novel autocrine feedback control of catecholamine release. A discrete chromogranin a fragment is a noncompetitive nicotinic cholinergic antagonist. *J. Clin. Invest.* 1997; **100**: 1623–1633.
14. Yoo SH. Identification of the Ca(2+)-dependent calmodulin-binding region of chromogranin A. *Biochemistry (Mosc).* 1992; **31**: 6134–6140.
15. Helle KB, Aunis D. A physiological role for the granins as prohormones for homeostatically important regulatory peptides? A working hypothesis for future research. *Adv. Exp. Med. Biol.* 2000; **482**: 389–397.
16. O'Connor DT, Mahata SK, Taupenot L, Mahata M, Livsey Taylor CV, Kailasam MT, Ziegler MG, Parmer RJ. Chromogranin A in human disease. *Adv. Exp. Med. Biol.* 2000; **482**: 377–388.

17. Osawa M, Kuwamoto S, Izumi Y, Yap KL, Ikura M, Shibanuma T, Yokokura H, Hidaka H, Matsushima N. Evidence for calmodulin inter-domain compaction in solution induced by W-7 binding. *FEBS Lett.* 1999; **442**: 173–177.
18. Barbato G, Ikura M, Kay LE, Pastor RW, Bax A. Backbone dynamics of calmodulin studied by 15N relaxation using inverse detected two-dimensional NMR spectroscopy: the central helix is flexible. *Biochemistry* 1992; **31**: 5269–5278.
19. Ikura M, Kay LE, Bax A. A novel approach for sequential assignment of 1H, 13C, and 15N spectra of proteins: heteronuclear triple-resonance three-dimensional NMR spectroscopy. Application to calmodulin. *Biochemistry* 1990; **29**: 4659–4667.
20. Park S, Johnson ME, Fung LW. NMR analysis of secondary structure and dynamics of a recombinant peptide from the N-terminal region of human erythroid alpha-spectrin. *FEBS Lett.* 2000; **485**: 81–86.
21. Kainosho M, Torizawa T, Iwashita Y, Terauchi T, Mei Ono A, Guntert P. Optimal isotope labelling for NMR protein structure determinations. *Nature* 2006; **440**: 52–57.
22. Park HY, So SH, Lee WB, You SH, Yoo SH. Purification, pH-dependent conformational change, aggregation, and secretory granule membrane binding property of secretogranin II (chromogranin C). *Biochemistry* 2002; **41**: 1259–1266.
23. Ikura M, Clore GM, Gronenborn AM, Zhu G, Klee CB, Bax A. Solution structure of a calmodulin-target peptide complex by multidimensional NMR. *Science* 1992; **256**: 632–638.
24. Park S, Johnson ME, Fung LW. Nuclear magnetic resonance studies of mutations at the tetramerization region of human alpha spectrin. *Blood* 2002; **100**: 283–288.
25. Kawasaki M, Inagaki F. Random PCR-based screening for soluble domains using green fluorescent protein. *Biochem. Biophys. Res. Commun.* 2001; **280**: 842–844.
26. Omoya K, Kato Z, Matsukuma E, Li A, Hashimoto K, Yamamoto Y, Ohnishi H, Kondo N. Systematic optimization of active protein expression using GFP as a folding reporter. *Protein Expr. Purif.* 2004; **36**: 327–332.
27. Fersht AR. *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding.* W.H. Freeman and Company: New York, 1999.
28. Larsson G, Schleucher J, Onions J, Hermann S, Grundstrom T, Wijmenga SS. Backbone dynamics of a symmetric calmodulin dimer in complex with the calmodulin-binding domain of the basic-helix-loop-helix transcription factor SEF2-1/E2-2: a highly dynamic complex. *Biophys. J.* 2005; **89**: 1214–1226.
29. Kranz JK, Lee EK, Nairn AC, Wand AJ. A direct test of the reductionist approach to structural studies of calmodulin activity: relevance of peptide models of target proteins. *J. Biol. Chem.* 2002; **277**: 16351–16354.

DOI: 10.1002/psc