Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 352 (2007) 932–935
BBRC
www.elsevier.com/locate/ybbrc

Sequence reversed peptide from CaMKK binds to calmodulin in reversible Ca^{2+}-dependent manner

Isaac T.S. Li [a], Ranjith K.R. [a], Kevin Truong [a,b,*]

[a] *Institute of Biomaterials and Biomedical Engineering, University of Toronto, 164 College Street, Toronto, Ont., Canada M5S 3G9*
[b] *Edward S. Rogers Sr. Department of Electrical and Computer Engineering, University of Toronto, 10 King's College Circle, Toronto, Ont., Canada M5S 3aG4*

Received 18 November 2006
Available online 4 December 2006

Abstract

Calmodulin (CaM) is a highly versatile Ca^{2+} signaling transducer known to regulate over a hundred proteins. In this paper, we further demonstrate the versatility of CaM binding by showing that it binds to a synthetic peptide (revCKKp) made by reversing the amino acid sequence of the CaM-binding peptide (CKKp) from CaM-dependent protein kinase kinase (CaMKK) (residues 438–463). Sequence comparison between revCKKp and other CaM-binding peptides (CBPs) from the CaM target databank showed that revCKKp does not resemble any existing classes of CBPs, except CKKp [M. Zhang, T. Yuan, Molecular mechanisms of calmodulin's functional versatility, Biochem. Cell Biol. 76 (1998) 313–323; S.W. Vetter, E. Leclerc, Novel aspects of calmodulin target recognition and activation, Eur. J. Biochem. 270 (2003) 404–414]. Furthermore, computational modeling showed that revCKKp could bind CaM in a similar manner to CKKp. Lastly, we experimentally showed that our synthetic revCKKp binds to CaM in a reversible Ca^{2+}-dependent manner.

Keywords: Calmodulin; Sequence reverse; Calmodulin-binding peptide; Calmodulin-dependent protein kinase kinase; Ca^{2+}-dependent binding; Protein modeling; Calmodulin affinity chromatography

Calmodulin (CaM) is an important Ca^{2+} signaling transducer found in eukaryotic cells that regulates many cellular processes such as muscle contraction, cell proliferation, metabolism, and gene transcription [3–5]. CaM is composed of four EF-hand domains, each capable of binding to one Ca^{2+} ion. Upon binding to Ca^{2+} ions (concentration of 0.1–1 μM), CaM undergoes a conformational change, which allows it to bind to target proteins with high affinity (K_d of 0.01–10 nM). This binding induces a subsequent conformational change in the target protein that modulates its function. The target recognition of CaM is highly versatile as it is known to regulate over a hundred proteins [1,6]. Its versatility is contributed by two factors. First, the high abundance of methionine residues in the hydrophobic binding pocket that allows sequence-independent peptide binding [7]. Second, the flexibility of linkers between its N- and C-terminal domains that allows its hydrophobic residues to be separated at different lengths while binding to the CaM-binding peptides (CBPs) [1]. While the CaM-binding regions themselves have no sequence homology, they do share certain key structural features such as the relative locations of hydrophobic and basic residues [2,6].

In this paper, we further demonstrate the versatility of CaM binding by showing that it binds to a synthetic peptide (revCKKp) made by reversing the amino acid sequence of the CaM-binding peptide (CKKp) from CaM-dependent protein kinase kinase (CaMKK)[1] (residues 438–463). We chose to study the reversed sequence of CKKp because its binding mode is more elaborate than

* Corresponding author. Fax: +1 416 9784317.
E-mail addresses: isaac.li@utoronto.ca (I.T.S. Li), kr.ranjith@utoronto.ca (Ranjith K.R.), kevin.truong@utoronto.ca (K. Truong).

[1] CaMKK, calmodulin-dependent protein kinase kinase.

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.11.123

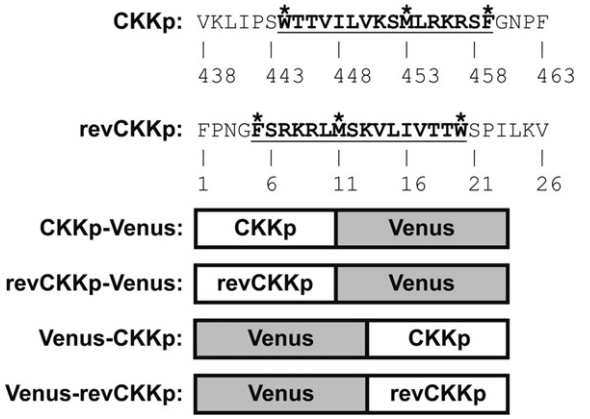

Fig. 1. Sequences and constructs of CKKp and revCKKp. The bold underlined portions of sequences are the binding core of CKKp and proposed binding core of revCKKp. Residues with asterisks are the anchoring and proposed anchoring residues on the above peptides. The schematic designs of the fusion proteins are shown. Venus is a mutant of green fluorescent protein [12].

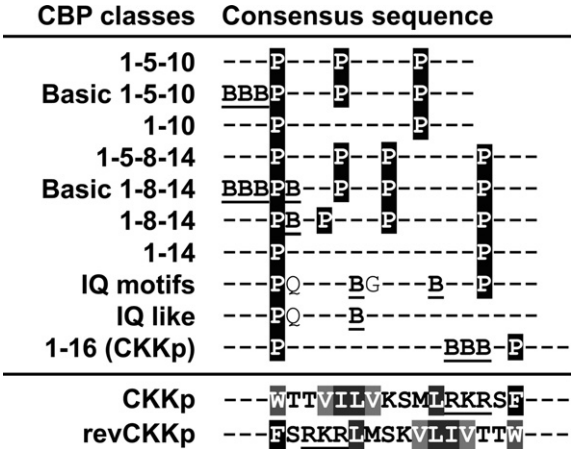

Fig. 2. Sequence comparison of revCKKp to the consensus sequences from 10 classes of CBPs. Highlighted P's are the hydrophobic residues; underlined B's are basic residues. Q's and G's from IQ and IQ like motifs are the conserved residue. Dashes are residues with no particular patterns in each class. The hydrophobic residues in both CKKp and revCKKp are highlighted; darker residues are more hydrophobic than lighter ones. The basic residues in both CKKp and revCKKp are underlined.

other CBPs. The structure of the CaM-CKKp complex is distinct in the following three aspects. First, CKKp anchors to CaM at Trp-444, Met-453, and Phe-459, making it the longest binding element (16 residues) among all CBPs [8] (Fig. 1). Second, unlike other CBPs, CKKp comprises of both the helix (residues 444–454) and the hairpin loop (residues 455–463) [8]. Lastly, the binding orientation of CKKp is reverse to other known CBPs as the N-terminal end of CKKp is bound to N-terminal lobe of CaM rather than the C-terminal lobe [8] (Fig. 1).

Materials and methods

Sequence comparison between revCKKp and other CBPs. The hydrophobic and basic residues were identified from the 10 binding mode classes of CBPs found in the CaM target database [6] (Fig. 2). This was then compared against the hydrophobic and basic residues found on revCKKp.

Computational modeling using MODELLER. The revCKKp model was constructed by first isolating CKKp from the CaMKK-CaM complex (PDB Accession No. 1CKK), and then editing the PDB file to invert the N- and C-terminals of each amino acid in the CKKp. Next, the revCKKp model was recombined with CaM with the aid of MODELLER 7.2 using the original CaM-CKKp complex as a modeling template [9,10].

Plasmid construction. The DNA sequences for the 26-residues CKKp and revCKKp were constructed by standard PCR techniques using overlapping primers, the two underlined portions (from 5′ to 3′) on each primer mark the enzymatic digestion and overlap annealing sequences: CKKp forward: 5′-CGGGATCCGTGAAACTGATTCCGAGCTGGACCACC GTGATTCTGGTGAAATCTATGCTG-3′ CKKp reverse: 5′-CTAGC TAGCGAACGGGTTGCCGAAGCTACGTTTACGCAGCATAGATT TCACCAGAATCAC-3′ revCKKp forward: 5′-CGGGATCCTTCCC GAACGGCTTCAGCCGTAAACGTCTGATGTCTAAAGTGCTGAT TGTG-3′ revCKKp reverse: 5′-CTAGCTAGCCACTTTCAGAATCG GGCTCCAGGTGGTCACAATCAGCACTTTAGACATCAG-3′.

The human codon preference was used for both CKKp and revCKKp. The PCR products were then individually subcloned into pCfvtx plasmid cassette between the *Bam*HI and *Nhe*I restriction cut sites, making the pCKKpVTx and prevCKKpVtx plasmids, which express CKKp-Venus and revCKKp-Venus fusion proteins, respectively [11]. As an intermediate step to create a fusion with Venus [12] (a mutant of green fluorescent protein) at the N-terminal of the peptides, Venus from the above plasmids were excised by *Pme*I digestion followed by self-ligation to create pCKKptx and prevCKKptx [11]. pCKKptx and prevCKKptx were combined with pVentx using the cassette-based strategy to create pVCKKptx and pVrevCKKptx, which express the Venus-CKKp and Venus-revCKKp fusion proteins [11].

Protein expression and CaM-sepharose chromatography assay. The plasmids expressing CKKp-Venus, Venus-CKKp, revCKKp-Venus, Venus-revCKKp, and Venus were each transformed in DH5α competent cells and grown in LB + 100 μM/mL ampicillin at 37 °C for 24 h. The proteins were produced by leak expression and extracted from the cells by sonication in the protein buffer (50 mM Tris buffer, pH 7.5, and 100 mM NaCl). The concentration of the protein samples were normalized by their fluorescence intensity. CaM-sepharose affinity chromatography resin (Stratagene) was used to determine the Ca^{2+} dependency of CBP binding to CaM. Two sets of each protein sample (100 μL) were separately incubated with CaM-sepharose (20 μL) for 10 min at room temperature, with one set in the presence of 2 mM Ca^{2+} while the other set in 2 mM EDTA. After incubation, the CaM-sepharose assays were washed with protein buffer containing either 2 mM Ca^{2+} or 2 mM EDTA, corresponding to the incubation condition. The amount of protein bound to the CaM-sepharose was then determined by Venus fluorescence at 488 nm excitation.

To further test the reversibility of the Ca^{2+}-dependent binding of the peptides to CaM, multiple Ca^{2+} and EDTA loading cycles were performed in the following steps. First, 100 μL of revCKKp-Venus, Venus-revCKKp, CKKp-Venus and Venus-CKKp were separately incubated with 20 μL of CaM-sepharose in 1 mM Ca^{2+} for 10 min at room temperature. Second, we added EDTA to each of the above solutions to achieve the final concentration of 2 mM and then incubated them for a further 10 min at room temperature. The above two steps were repeated with 2 mM Ca^{2+} and EDTA such that the effective Ca^{2+} concentration is 1 mM and 0 mM (i.e. 1 mM excess EDTA) in every cycle.

Results and discussion

Sequence comparison between revCKKp and other CBPs

CBPs are classified by their hydrophobic and basic residue patterns. In particular, CKKp belongs to the 1–16 class and is the only member in its class. Our sequence analysis showed that revCKKp does not resemble any

known classes of CBPs but only the reverse pattern of CKKp itself (Fig. 2). Hence, a plausible binding mode for revCKKp to CaM is similar to CKKp but opposite in orientation. This inverted binding direction would be favourable because the basic residues (Arg-455, Lys-456, Arg-457) on the peptide can form salt bridges with the acidic residues at the C-terminal lobe of CaM, as is evident in the binding patterns of all known classes of CBPs (Fig. 2). Furthermore, in this inverted orientation, the positions of hydrophobic residues on revCKKp match that of CKKp. Hence, by maintaining the hydrophobic interactions, revCKKp can achieve a structure similar to CKKp when bound to CaM.

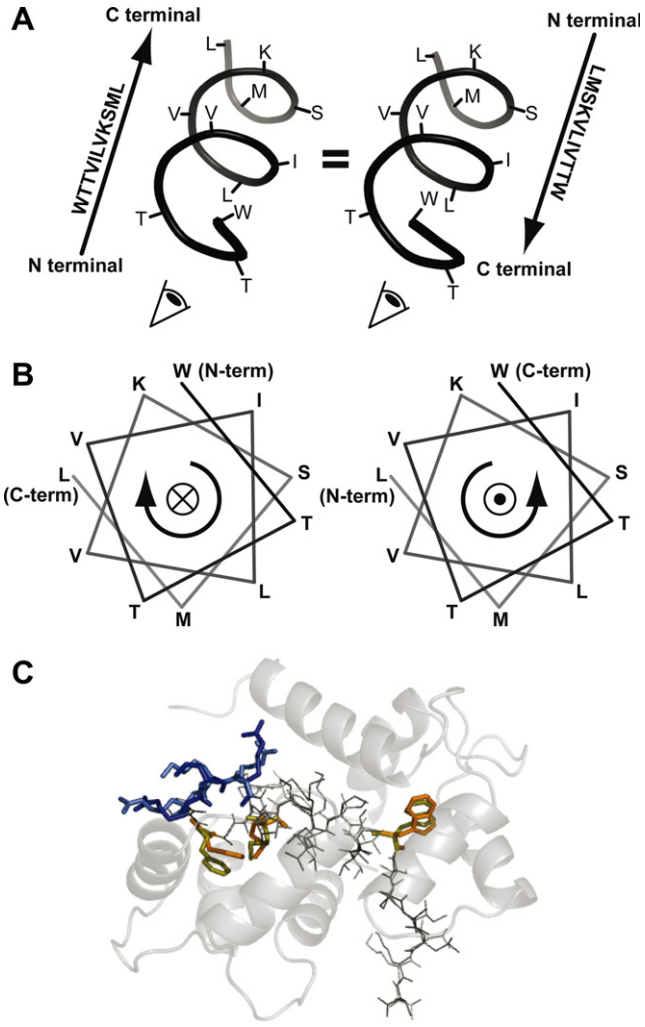

Fig. 3. (A) The identical side chain positions of CKKp (left) and revCKKp (right) after forming α-helices. (B) Helical wheel projection of the side chain positions for CKKp (viewed in N- to C-terminal direction) and revCKKp (viewed in C- to N-terminal direction). (C) The ribbon structure of CKKp (1CKK) and revCKKp (modeled) bound to CaM. Dark and lighter blue indicate basic residues from revCKKp and CKKp, respectively. Orange and yellow indicate anchoring hydrophobic residues from revCKKp and CKKp, respectively. CaM is shown in transparent grey ribbons. The picture is rendered using POV-Ray. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Side-chain positions of revCKKp

In principle, revCKKp can have the same Ca^{2+}-dependent binding behaviour as CKKp due to their nearly identical side-chain configurations. Both CKKp and revCKKp contain right-handed α-helices because they are composed of naturally occurring L-amino acids (Fig. 3A). By symmetry, the amino acids in both helices appear clockwise in the same order when viewed in N- to C-terminal direction for CKKp and in C- to N-terminal direction for revCKKp (Fig. 3A). This implies that the side-chain branching positions of CKKp and revCKKp are identical, despite their opposite orientations as shown in the helical wheel projections (Fig. 3B). Since CaM binding to its natural target peptides is known to involve only side-chain interactions [1], we expect revCKKp to bind CaM with similar side chain interactions as CKKp. Next, the structure of the CaM-revCKKp complex was modeled using MODELLER [9,10] with spatial constraints from the CaM-CKKp complex (Fig. 3C). The model suggested that revCKKp can indeed bind to CaM in the predicted inverted fashion with only minimal changes to the side-chain positions including the anchoring residues (Phe-5, Met-11, and Trp-20, see Fig. 1) with no steric strains (Fig. 3C).

Ca^{2+}-dependent reversible binding of revCKKp to CaM

The CaM-sepharose binding assay showed that both Venus-revCKKp and revCKKp-Venus bind to CaM-sepharose in the presence of Ca^{2+} but not in its absence (Fig. 4). Venus alone was used as a negative control since it should not bind CaM-sepharose, while Venus-CKKp and CKKp-Venus as positive controls since their Ca^{2+}/CaM-dependent binding is known. In the presence of free Ca^{2+}, the CaM-sepharose beads recruited Venus-CKKp, CKKp-Venus, Venus-revCKKp, and revCKKp-Venus as indicated by the fluorescence intensity at the beads. In

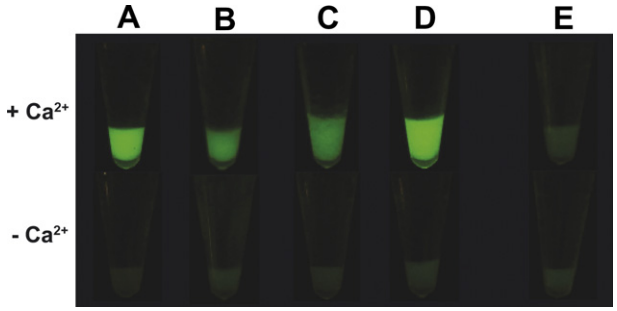

Fig. 4. The CaM-sepharose assay. The top row shows the Venus fluorescence on the CaM-sepharose beads in the presence of Ca^{2+} for (A) revCKKp-Venus, (B) CKKp-Venus, (C) Venus-revCKKp, (D) Venus-CKKp and (E) the Venus control. The bottom row shows the Venus fluorescence when Ca^{2+} was removed. The samples were excited by 488 nm light and the pictures were taken were taken behind 510 nm high-pass filter using a Canon-A75 digital camera under the same aperture and exposure time.

the absence of free Ca^{2+} by addition of the Ca^{2+} chelator EDTA, these fusion proteins dissociated from the beads. The reversibility of this activity was confirmed by several cycles of the Ca^{2+} and EDTA loading to alter the presence or absence of free Ca^{2+}. As expected, Venus fluorescence migrated from the supernatant to the CaM-sepharose beads in the Ca^{2+} loading cycle and back into the supernatant in the EDTA loading cycle. This phenomenon was not observed in the control Venus protein. Thus, the Ca^{2+}/CaM-dependent binding was attributed to CKKp or revCKKp.

Conclusion

We have demonstrated the versatility of CaM in its ability to bind to a variety of target proteins by showing that CaM even binds to the reversed sequence of CKKp, in the same Ca^{2+}/CaM-dependent and reversible manner as the native sequence of CKKp. We further deduced that the binding direction of revCKKp is opposite to that of CKKp, such that the side-chain interactions between revCKKp and CaM closely resemble that between CKKp and CaM. Further NMR or X-ray crystallography studies on the structure of the CaM-revCKKp complex are needed to precisely determine the binding mode.

Acknowledgments

This work was supported by grants from the Canadian Foundation of Innovation (CFI), Canadian Institutes of Health Research, and the National Science and Engineering Research Council (NSERC).

References

[1] M. Zhang, T. Yuan, Molecular mechanisms of calmodulin's functional versatility, Biochem. Cell Biol. 76 (1998) 313–323.
[2] S.W. Vetter, E. Leclerc, Novel aspects of calmodulin target recognition and activation, Eur. J. Biochem. 270 (2003) 404–414.
[3] M. Ikura, M. Osawa, J.B. Ames, The role of calcium-binding proteins in the control of transcription: structure to function, Bioessays 24 (2002) 625–636.
[4] K.P. Hoeflich, M. Ikura, Calmodulin in action: diversity in target recognition and activation mechanisms, Cell 108 (2002) 739–742.
[5] A. Crivici, M. Ikura, Molecular and structural basis of target recognition by calmodulin, Annu. Rev. Biophys. Biomol. Struct. 24 (1995) 85–116.
[6] K.L. Yap, J. Kim, K. Truong, M. Sherman, T. Yuan, M. Ikura, Calmodulin target database, J. Struct. Funct. Genomics 1 (2000) 8–14.
[7] S.H. Gellman, On the role of methionine residues in the sequence-independent recognition of nonpolar protein surfaces, Biochemistry 30 (1991) 6633–6636.
[8] M. Osawa, H. Tokumitsu, M.B. Swindells, H. Kurihara, M. Orita, T. Shibanuma, T. Furuya, M. Ikura, A novel target recognition revealed by calmodulin in complex with Ca^{2+}calmodulin-dependent kinase kinase, Nat. Struct. Biol. 6 (1999) 819–824.
[9] M.A. Marti-Renom, A.C. Stuart, A. Fiser, R. Sanchez, F. Melo, A. Sali, Comparative protein structure modeling of genes and genomes, Annu. Rev. Biophys. Biomol. Struct. 29 (2000) 291–325.
[10] A. Sali, T.L. Blundell, Comparative protein modelling by satisfaction of spatial restraints, J. Mol. Biol. 234 (1993) 779–815.
[11] K. Truong, A. Khorchid, M. Ikura, A fluorescent cassette-based strategy for engineering multiple domain fusion proteins, BMC Biotechnol. 3 (2003) 8.
[12] T. Nagai, K. Ibata, E.S. Park, M. Kubota, K. Mikoshiba, A. Miyawaki, A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications, Nat. Biotechnol. 20 (2002) 87–90.