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

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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

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

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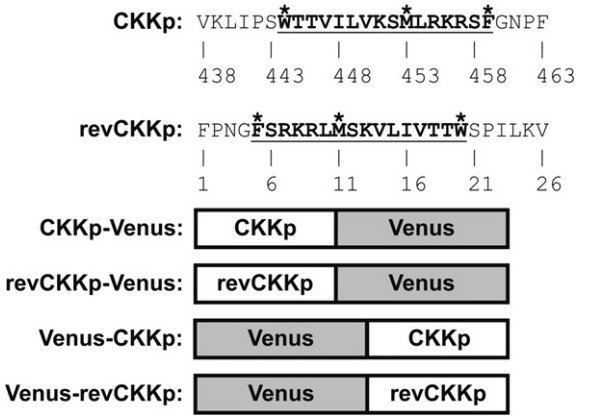

Fig. 1. Sequences and constructs of CKKp and revCKKp. The bold underlined portions of sequences are the binding core of CKKp and proposed binding core of revCKKp. Residues with asterisks are the anchoring and proposed anchoring residues on the above peptides. The schematic designs of the fusion proteins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

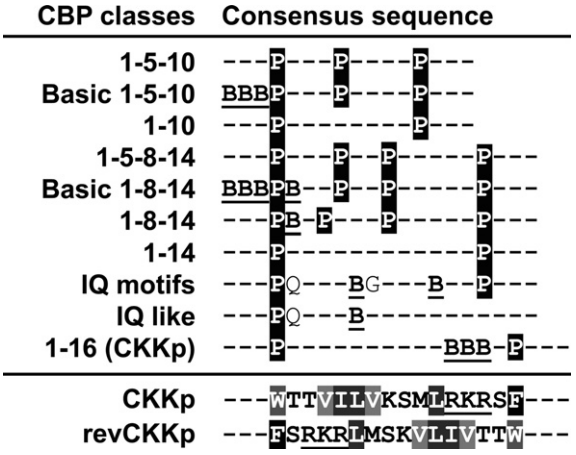

Fig. 2. Sequence comparison of revCKKp to the consensus sequences from 10 classes of CBPs. Highlighted P's are the hydrophobic residues; underlined B's are basic residues. Q's and G's from IQ and IQ like motifs are the conserved residue. Dashes are residues with no particular patterns in each class.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