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Article Addendum

Calmodulin has the Potential to Function as a Ca^{2+}-Dependent Adaptor Protein

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KEY WORDS

calmodulin, calcium, EF-hand, adaptor protein, mitogen-activated protein kinase phosphatase.

ABBREVIATIONS

Cam	calmodulin
CaMBD	calmodulin-binding domain
KCBP	kinesin-like CaM-binding protein
CNGC's	cyclic-nucleotide gated channels
PKC	protein kinase C
GAD	glutamate decarboxylase
SK channel	small conductance Ca^{2+}-activated K^+ channels
NtMKP1	Nicotiana tabacum mitogen-activated protein kinase phosphatase NtMKP1

Addendum to:

Calcium-Dependent and -Independent Binding of Soybean Calmodulin Isoforms to the Calmodulin Binding Domain of Tobacco MAPK Phosphatase-1

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ABSTRACT

Calmodulin (CaM) is a versatile Ca^{2+}-binding protein that regulates the activity of numerous effector proteins in response to Ca^{2+} signals. Several CaM-dependent regulatory mechanisms have been identified, including autoinhibitory domain displacement, sequestration of a ligand-binding site, active site reorganization, and target protein dimerization. We recently showed that the N- and C-lobes of animal and plant CaM isoforms could independently and sequentially bind to target peptides derived from the CaM-binding domain of *Nicotiana tabacum* mitogen-activated protein kinase phosphatase (NtMKP1), to form a 2:1 peptide:CaM complex. This suggests that CaM might facilitate the dimerization of NtMKP1, although the dimerization mechanism is distinct from the previously described simultaneous binding of other target peptides to CaM. The independent and sequential binding of the NtMKP1 peptides to CaM also suggests an alternative plausible scenario in which the C-lobe of CaM remains tethered to NtMKP1, and the N-lobe is free to recruit a second target protein to the complex, such as an NtMKP1 target. Thus, we hypothesize that CaM may be capable of functioning as a Ca^{2+}-dependent adaptor or recruiter protein.

Calcium (Ca^{2+}) is a dynamic secondary messenger that regulates many signaling events in both plant and animal cells. Intracellular Ca^{2+} transients and oscillations (Ca^{2+} signals) are decoded by a large superfamily of calcium-binding proteins, the most important of which is calmodulin (CaM).[1-3] The prototypical CaM protein consists of four tandem helix-loop-helix "EF-hand" Ca^{2+}-binding motifs that are divided into distinct N- and C-terminal globular lobes connected by a flexible linker. CaM proteins from all species including the single mammalian CaM and the many different plant CaM isoforms each undergo similar Ca^{2+}-induced conformational changes involving a rearrangement of the position of its α-helices that opens distinct hydrophobic target protein-binding patches on the surface of each lobe; known as the "open" conformation (Fig. 1B). These hydrophobic patches can interact with numerous different target proteins including protein kinases, protein phosphatases, cytoskeletal proteins and other cell signaling enzymes, to regulate their activity. The closed or semi-open conformations adopted by the N- and C-lobes of Ca^{2+}-free CaM (apo-CaM) (Fig. 1A) can also interact with another subset of proteins, to target CaM to certain cellular locations or facilitate Ca^{2+}-independent regulatory events.[1-3]

The CaM-dependent regulation of target proteins can occur through numerous different mechanisms. For example, Ca^{2+}-CaM can relieve autoinhibition by binding to a short (20–25 residue) calmodulin-binding domain (CaMBD) sequence that is adjacent to or within an autoinhibitory region of the enzyme (Fig. 2A).[3] Numerous structures of these Ca^{2+}-CaM-CaMBD complexes have been reported, which reveal a characteristic "wrap-around" binding mode (Fig. 1C). Typically the CaM C-lobe binds with high affinity to a Trp residue within the N-terminal part of the target sequence, and the flexible central linker allows the N-lobe to pivot and bind to a second bulky hydrophobic "anchor" residue within the C-terminal part of the target sequence.[3] Truncation of this second anchor residue can lead to binding of only one CaM domain and an extended CaM conformation (Fig. 1D).[4,5] Studies with plant CaM isoforms having mutations to non-CaMBD-coordinating residues have also suggested that a secondary binding interface exists on the opposite surface of the CaM protein which also contributes to the activation of some of these target enzymes.[6,7]

Another regulatory mechanism involving Ca^{2+}-CaM-binding to a single contiguous CaMBD sequence may occur with the potato kinesin-like CaM-binding protein (KCBP)[8] as well as some plant cyclic-nucleotide gated channels (CNGC's).[9] In both cases the

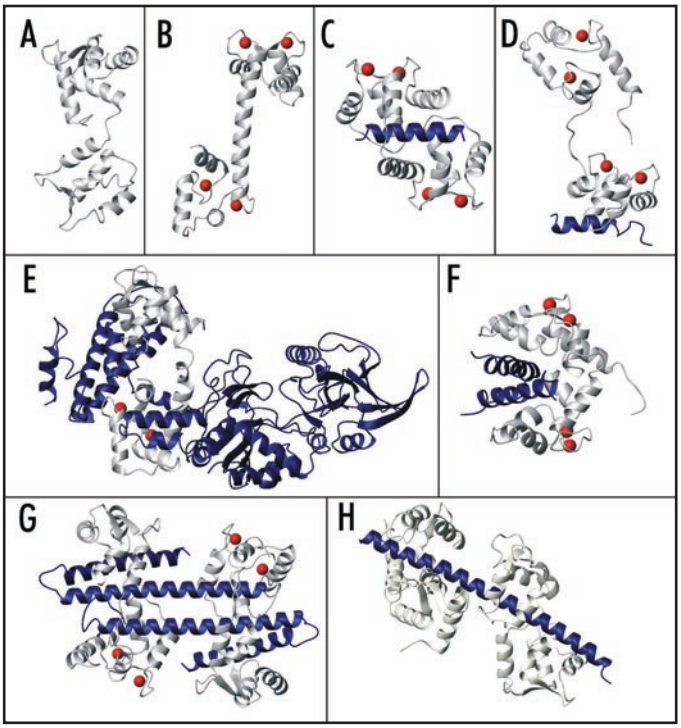

Figure 1. Structures of CaM and CaM-target complexes. (A) apo-CaM (PDB:1DMO), (B) Ca^{2+}-CaM (PDB:1CLL). Complexes of CaM bound to (C) CaMBD of smooth muscle myosin light chain kinase (PDB:1CDL), (D) partial CaMBD of plasma membrane Ca^{2+}-pump C20W (PDB:1CFF), (E) the adenylyl cyclase protein from *Bacillus anthracis* (PDB:1K93), (F) 2 glutamate decarboxylase CaMBD's (PDB:1NWD), (G) 2 CaM proteins bound to 2 small conductance Ca^{2+}-activated potassium channel (SK channel) CaMBD's (PDB:1G4Y), (H) 2 apo-CaM proteins bound to 2 tandem IQ motifs from murine myosin V (PDB:2IX7). In each panel CaM is shown in ivory, the target molecule is shown in blue and the Ca^{2+} ions bound to the N- and/or C-lobes of CaM are represented by red spheres.

Ca^{2+}-CaM binding site on the target protein overlaps with the respective ligand binding site, and thus the binding of KCBP to microtubules or the binding of cyclic nucleotide monophosphates to CNGC's may be prevented by interaction with Ca^{2+}-CaM (Fig. 2B). In a variation on this mechanism, CaM can bind to the cytoplasmic juxtamembrane region of the human epidermal growth factor receptor and sequester a threonine residue which is a specific phosphorylation target of protein kinase C (PKC). CaM-binding inhibits PKC phosphorylation of this threonine, and PKC phosphorylation inhibits CaM-binding.[10]

There are also several examples of CaM-target interactions where the N- and C-lobes bind to noncontiguous target protein regions, and play distinct roles in target regulation. The structures of a CaM-activated adenylyl cyclase from *Bacillus anthracis* with and without bound CaM shows how the N- and C-lobes of CaM can bind two distant regions of the adenylyl cyclase enzyme and induce a conformation reorganization that creates the enzyme's active site (Figs. 1E and 2C).[11] An interesting feature of this interaction is that the CaM N-lobe remains Ca^{2+}-free and in a closed conformation, while the C-lobe is in a canonical Ca^{2+}-bound open conformation. Indeed, Ca^{2+}-binding to the C-lobe but not N-lobe is required for activation of the adenylyl cyclase.[12]

The N- and C-lobes of Ca^{2+}-CaM can also each simultaneously bind to identical peptides derived from the petunia glutamate decarboxylase (GAD) enzyme to form a 1:2 Ca^{2+}-CaM:GAD complex (Fig. 1F).[13,14] This suggests that Ca^{2+}-CaM-induced target protein dimerization may be another way in which CaM can regulate target proteins (Fig. 2D). CaM-dependent dimerization has also been shown to regulate the activity of small conductance Ca^{2+}-activated K^+ channels (SK channel), although in this case a novel 2:2 CaM:SK channel complex is formed (Figs. 1G and 2E).[15] This structure is also unique because Ca^{2+} is bound to the "lower affinity" N-lobe EF-hands, but not to the "higher affinity" C-lobe EF-hands of CaM.

In addition to the SK channel, CaM can regulate voltage-gated sodium channels, voltage-gated calcium channels, as well as ryanodine-sensitive calcium release channels.[16] With these channels CaM typically binds in complex Ca^{2+}-dependent and Ca^{2+}-independent ways to several noncontiguous target sequences in the same protein, and often to so-called IQ motifs (IQXXXRGXXXR). IQ motifs are generally thought to be constitutive apo-CaM binding sites which retain CaM under resting (low [Ca^{2+}]) cellular conditions to ensure a rapid response to Ca^{2+}-stimuli.[17] However many IQ motifs can also bind specifically to Ca^{2+}-CaM or to both apo-CaM and Ca^{2+}-CaM. Structures of some Ca^{2+}-CaM-IQ domain complexes have revealed wrap-around binding modes, albeit with differences in lobe and peptide orientation compared to other complexes.[18-20] For a discussion about the mechanisms of CaM-dependent ion channel regulation (see ref. 16). A very recent crystal structure of apo-CaM bound to an IQ domain from myosin V (Fig. 1H) has also revealed yet another variation on the wrap-around binding mode, where the apo-C-lobe of CaM adopts a semi-open conformation and forms numerous interactions with the target sequence, while the apo-N-lobe adopts a closed conformation and forms weaker interactions with the IQ domain.[21]

Using several biophysical techniques we recently characterized the interaction between CaM isoforms (mammalian CaM, soybean CaM isoforms SCaM-1 and SCaM-4) and a novel CaMBD derived from the *Nicotiana tabacum* mitogen-activated protein kinase phosphatase (NtMKP1).[22] The NtMKP1 protein was initially identified as a CaM-binding protein by Ohashi and coworkers,[23] and the same group recently showed that CaM-binding NtMKP1 homologs are also present in other plant species as well.[24] We found that each CaM isoform was capable of binding to the NtMKP1 CaMBD in the absence of Ca^{2+} using only the apo-C-lobe, with the primary binding site consisting of NtMKP1 residues N438 - S449, and additional C-terminal residues G450 - K460 enhancing the overall binding affinity (K_d ~10^{-5} M). In the presence of Ca^{2+}, a 1:1 complex could be formed with the CaM C-lobe having significantly increased affinity for the N438 - S449 region of NtMKP1 (K_d 10^{-7} - 10^{-10} M). However, the Ca^{2+}-loaded CaM N-lobe interacted only very weakly with the C-terminal NtMKP1 sequence in this 1:1 complex, despite an abundance of seemingly suitable hydrophobic "anchor" residues in this region. Interestingly, the addition of more peptide triggered the independent binding of a second NtMKP1 peptide to the Ca^{2+}-CaM N-lobe (Kd 10^{-5} - 10^{-6} M) to form a 1:2 Ca^{2+}-CaM:NtMKP1 complex. As with GAD, these results suggest that CaM is capable of facilitating the dimerization of NtMKP1, although the independent and sequential NtMKP1 peptide binding to the C- and N-lobes markedly distinguishes the CaM-NtMKP1 interaction from the simultaneous high-affinity binding of 2 GAD CaMBD's to CaM.

While our NtMKP1 study was ongoing, Ohashi and coworkers reported that CaM is incapable of stimulating the phosphatase activity of the NtMKP1 enzyme, thereby implying that the CaM-NtMKP1 interaction is necessary for something other than direct enzyme regulation.[25] The independent and sequential binding of the NtMKP1 fragments to the Ca^{2+}-saturated C- and then

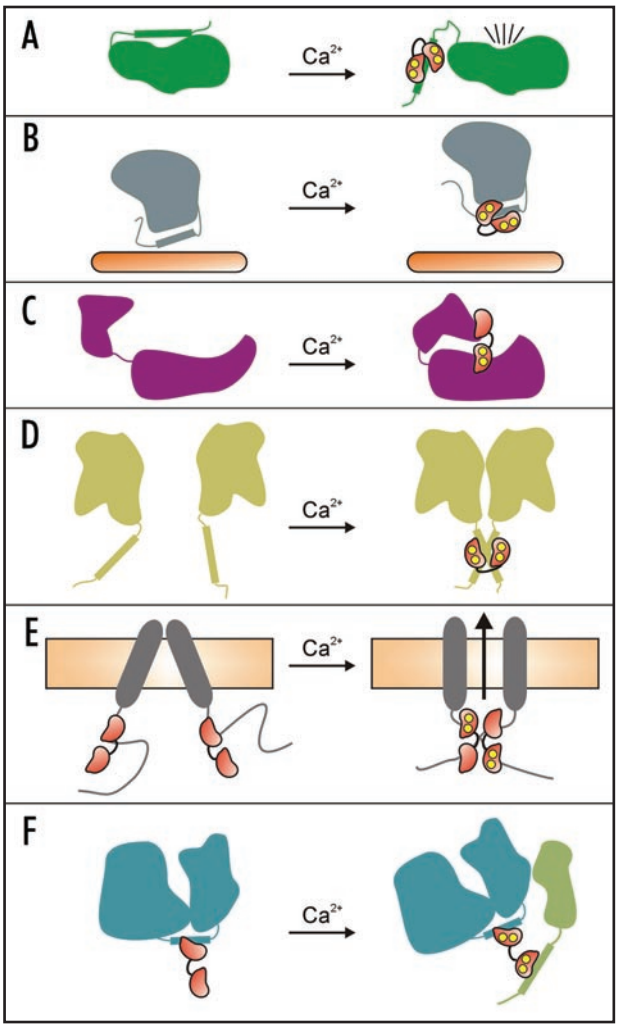

Figure 2. Schematic model for the various mechanisms of CaM-dependent target regulation. (A) autoinhibitory domain displacement, (B) sequestering of a ligand binding site, (C) active-site reorganization, (D) CaM-induced target protein dimerization (1:2 complex), (E) CaM-induced target protein dimerization (2:2 complex), (F) hypothesized model for CaM acting as an adaptor/recruiter protein. In each panel CaM is shown as a red dumbbell shaped molecule with Ca^{2+} ions represented by yellow circles, and the target proteins are shown in various colors. See the text for details on each model.

N-lobes of CaM observed in our study suggests a plausible situation in which the C-lobe of CaM is tightly bound to NtMKP1, leaving the N-lobe free to recruit a different target protein to the complex, for example, a NtMKP1 protein substrate. Therefore, CaM may be capable of acting as an adaptor or recruiter protein, which would add yet another mechanism of target regulation to CaM's repertoire (Fig. 2F). In addition to NtMKP1 peptides, the isolated N-lobe of CaM is capable of binding to other CaMBD peptides[26,27] as well as intact target proteins,[28] increasing the likelihood that the N-lobe could serve as a recruiter domain. The pre-association of the apo-C-lobe of CaM with NtMKP1 under resting conditions would also ensure a rapid response response to Ca^{2+}-stimuli, since CaM would only need to recruit one rather than both protein targets.

Although the ability of CaM to act as an adaptor protein in vivo has not yet been demonstrated, there are examples of related EF-hand proteins acting as adaptor proteins, including centrin[29] and calcium- and integrin-binding protein 1.[30] With the abundance of poorly characterized CaM-binding proteins in plants, many of which have CaMBD's with little sequence resemblance to the better characterized motifs in animals[1] it seems likely that sequences will be identified which bind preferentially to the CaM N-lobe. Considering the incredible assortment of known CaM interaction modes and regulatory mechanisms, many of which have only been identified within the last decade, it is likely only a matter of time before CaM is proven to function as an adaptor protein in vivo.

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