Cell, Vol. 108, 739–742, March 22, 2002, Copyright ©2002 by Cell Press

Calmodulin in Action: Diversity in Target Recognition and Activation Mechanisms

Minireview

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Recent structural studies on calmodulin complexes with anthrax adenylyl cyclase and rat Ca^{2+}-activated K^+ channel have uncovered unexpected ways by which calmodulin interacts with target proteins.

Highly regulated changes in the concentration of cytosolic calcium ions control biological processes as diverse as muscle contraction, fertilization, cell proliferation, vesicular fusion, and apoptosis. Many of these cellular effects are modulated by the small Ca^{2+} binding protein, calmodulin (CaM). While we know that CaM interacts with a large number of proteins (the magnitude of which makes for an impressive list), even more interesting is the physiological diversity of these binding partners and the recent progress made in establishing how varied CaM's molecular recognition and substrate activation processes are.

Among these numerous target proteins, CaM-dependent serine/threonine kinases are the substrate family best characterized with respect to their structural and functional CaM interaction mechanisms. Previous studies on CaM kinase II, CaM kinase kinase, and myosin light chain kinases (reviewed in Chin and Means, 2000; Crivici and Ikura, 1995) showed that CaM could assume at least three different conformations by virtue of a flexible linker connecting two globular domains responsible for binding both Ca^{2+} ions and target proteins (Figure 1). These interactions all involve short helices (approximately 20 residues) formed by the CaM binding domain of the kinases and together suggest predictive criteria for identifying putative CaM recruitment sites (Rhoads and Friedberg, 1997; http://calcium.uhnres.utoronto.ca). From these complexes, defined CaM recruitment motifs currently fall into three groups (1–10, 1–14, and $1–16^-$), which are distinguished by their spacing of bulky hydrophobic and basic amino acids and are bracketed by aromatic residues near either end (Table 1). Another CaM binding motif is the so-called "IQ motif," corresponding to an IQxxxRGxxxR consensus sequence, which is known to appear in tandem repeats and to bind multiple CaM molecules in a predominantly Ca^{2+}-independent manner. By means of biochemical characterization, it appears that these canonical recognition motifs are used by many CaM-dependent proteins, such as calcineurin, NO synthases, IP_3 receptors, plasma membrane Ca^{2+} pump, Ras guanine nucleotide exchange factor, RAS-GRF, and rhodopsin protein phosphatase. However, sequence analysis based on these binding criteria does not always identify CaM-interacting proteins, and conversely, proteins that bind to CaM sometimes do not fit these recruitment motifs.

As the known CaM-target interactions are already quite extensive, involving a large surface of CaM given CaM's limited size (16.7 kDa), no one had probably envisioned how it could interact with a protein structure much larger than a 20 residue helix. Hence, the crystal structures of *Bacillus anthracis* adenylyl cyclase (Drum et al., 2002), the edema factor of this pathogenic bacterium, and rat Ca^{2+}-activated K^+ channel (Schumacher et al., 2001) were a total surprise to calmodulin aficionados and structural biology researchers alike.

Anthrax Adenylyl Cyclase Is a Calmodulin-Dependent Enzyme

Bacillus anthracis, a spore-forming bacterium, is the causative agent of anthrax. Virulent strains secrete two toxins composed of three proteins: the protective antigen (PA), lethal factor (LF), and edema factor (EF). PA is the common binding moiety that upon proteolytic activation forms a heptameric complex and binds to LF and EF, the intracellular enzymes that then damage the cells. EF is an adenylyl cyclase and catalyzes the conversion of ATP to cAMP. Although the extent of this effect varies according to cell type, cellular cAMP levels may reach a 1000-fold increase (representing 20%–50% conversion of cell's ATP stores), causing fluid loss in the affected tissues. Local diffusion of the anthrax adenylyl cyclase compromises the host immune response and enhances the virulence of the bacteria.

The most fascinating property of this enzyme is that it requires CaM for its activation. It has been more than two decades since the first observation that adenylyl cyclase binding of CaM, at the resting level of Ca^{2+}, can activate this bacterial enzyme 100- to 1000-fold. Given the myriad of cellular proteins to choose from, why would anthrax EF utilize this eukaryotic protein for its activation? One hint comes from CaM gene expression. CaM is one of the most abundant, ubiquitous, and conserved proteins in eukaryotic biology. In mammals, three distinct bona fide genes encode identical CaM proteins and among all vertebrates, the amino acid sequence of CaM is completely invariant. CaM, which is not present in bacteria, thereby provides a convenient, evolutionarily-constrained target for anthrax to interact with cells. Even a taxonomically distant organism, *Bordetella pertussis* (the etiological agent of the whooping cough), shares significant adenylyl cyclase homology and requires the same eukaryotic activator, providing an example of convergent evolution of this system. Secondary consequences of sequestering CaM would also be changes in calcium signaling processes. Ca^{2+} mobilization plays a central role in the host cell immune response and results in the activation of many intracellular enzymes, including the CaM-dependent phosphatase, calcineurin, and transcription factor NF-AT.

EF Activation Involves Active Site Remodeling

CaM-dependent serine/threonine protein kinases (such as CaM kinase I/II/IV, CaM kinase kinase, and myosin

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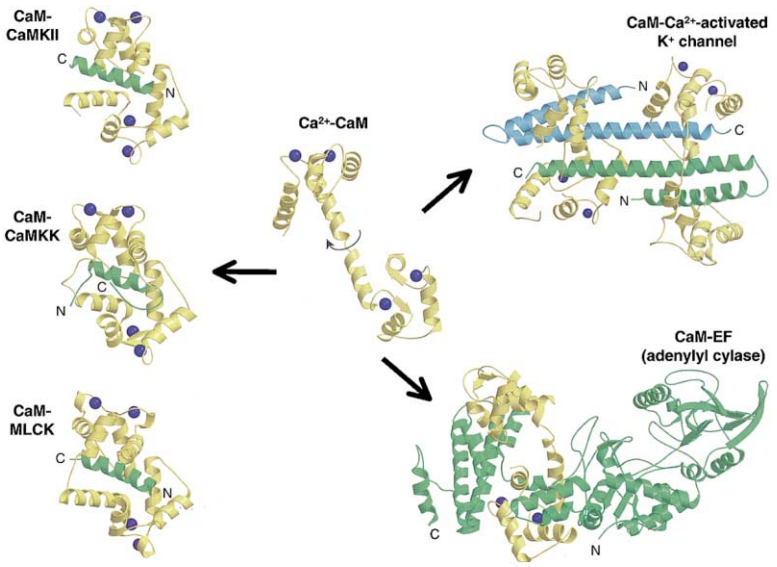

Figure 1. Comparison of Different Calmodulin-Target Structures

The Protein Data Bank accession code for Ca^{2+}-CaM alone is 1CLL, and for CaM in complex with CaMKII, CaMKK, MLCK, Ca^{2+}-activated K^+ channel, and anthrax adenylyl cyclase are 1CDM, 1CKK, 1CDL, 1G4Y, and 1K93, respectively. CaM is shown in yellow and calcium ions are depicted in blue.

light chain kinase) and the phosphatase calcineurin are all known to use a similar mechanism underlying CaM-dependent activation, namely autoinhibitory domain (AID) displacement (Figure 2). In these cases, the CaM binding site is often adjacent or within an autoinhibitory domain of the enzyme which contains a pseudosubstrate region. CaM binding to the target induces a conformational rearrangement that displaces the pseudosubstrate inhibitory domain and allows for full enzyme activity. Solved structures of these CaM/kinase peptide complexes show that the two lobes of CaM wrap around the helical AID peptide, enclosing it in a hydrophobic channel within the globular core (Figure 1).

As compared to the known mechanism of AID release from the active site of CaM-dependent kinases, the activation of EF represents a new mechanism for CaM's action (Drum et al., 2002; Table 1). Tang and coworkers beautifully showed that this process involves rearrangement of key switches to create the active site from an area that is totally solvent exposed in the CaM-free state (hence our moniker, active-site remodeling). Mammalian adenylyl cyclases constitute a membrane protein family, each member sharing the common features of an α-helical transmembrane region followed by two highly similar cytoplasmic domains that compose to catalytic core. In contrast, EF is a soluble protein comprised of three globular domains, C_A, C_B, and a helical domain, which undergo large conformational changes in response to CaM binding and its concomitant insertion between the C_A and helical domains (Figure 1). Characterized CaM binding motifs could not be superimposed on key residues for the EF/CaM interface, and this structure gives new insight to a large body of older mutagenesis studies. For instance, synthetic peptides corresponding to the switch A region, which we now know to undergo large conformational changes, were shown to interact with CaM in a Ca^{2+}-dependent manner (Munier et al., 1993). While distances in primary sequences are poor predictors for spatial distances in folded proteins, these studies did indicate distinct CaM binding sites.

What is the role of Ca^{2+} in this protein assembly? It has been previously shown that CaM can still activate EF in the presence of the Ca^{2+} chelator EGTA, albeit with significantly reduced (three orders of magnitude) efficiency (Leppla, 1984). In the reported structure, CaM binds only two Ca^{2+} ions at the C-terminal "high-affinity" lobe. This prompts speculation regarding the calcium dependency in EF binding and activation by CaM. Does EF take advantage of the onset of Ca^{2+} elevation in the cell to become activated ahead of host enzymes, which

Table 1. Classification of Calmodulin Target Recognition Modes

Class	Type	Target	Role of CaM binding
1:1 (canonical)	1–10	CaM kinase II	release AID
	1–14	MLCKs	release of AID
	1–16$^-$	CaM kinase kinase	release of AID
	IQ	unconventional myosins	CaM tethering
		Ca^{2+} channels	CaM tethering and CDI
	novel	anthrax adenylyl cyclase	active site remodeling
1:2	homo	plant GAD	dimerization and activation
	hetero	(hypothetical)	protein recruitment
2:2	homo	Ca^{2+}-gated K^+ channel	dimerization and activation
	hetero	(hypothetical)	heterodimerization

Abbreviations: CaM, calmodulin; AID, autoinhibitory domain; MLCK, myosin light chain kinase; CDI, calcium-dependent inactivation; GAD, glutamate decarboxylase.

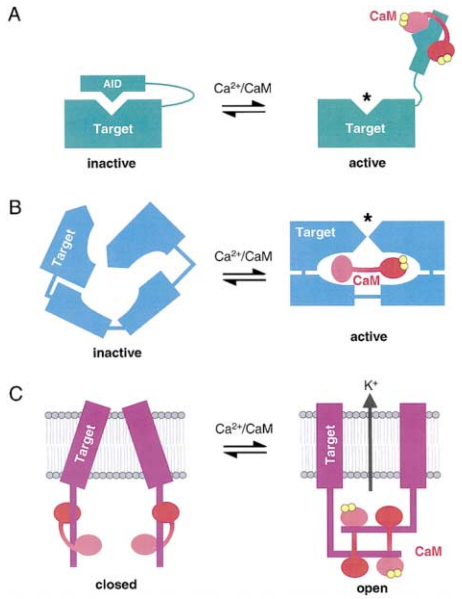

Figure 2. Protein Activation Mechanisms by Calmodulin

(A) Relieving autoinhibition. (B) Active site remodeling. (C) Dimerization. Calcium ions are depicted in yellow, and active sites by asterisks.

require four Ca^{2+} ions? These are interesting questions that await further investigation.

In general, protein-protein affinities vary in accordance with the properties of the structural interface of the interaction: the greater a buried surface, the tighter the observed binding. CaM/peptide interactions display a large buried surface (ranging from 2400–3000 Å^2) with a corresponding high affinity ($K_d = 10^{-8}$ to 10^{-9} M). By comparison, the EF/CaM complex displays a remarkably larger binding area (5900 Å^2), which may suggest an extremely high affinity with a K_d less than 10^{-10} M. However, data for the related adenylyl cyclase from *B. pertussis* showed its K_d for CaM binding equal to 10^{-10} M (Glaser et al., 1989). This may be explained at least partly by the low free energy of CaM-free, prefolded EF, unlike the case of high-energy unstructured peptides when unbound to CaM. Collectively though, this hints that CaM trapping by EF may also be a key factor in regulating its selective activity in the host cells. EF might also be more stable in the CaM-bound state as compared to the CaM-free state, thereby increasing resistance to proteolytic turnover and lengthening its half-life time in cells. Still, EF active site remodeling and CaM complex formation are likely dynamic events, and while the cues for its reversal are currently unknown, many possibilities exist. One as yet unexplored prospect is that fully calcium-loaded CaM has reduced affinity for EF or it forms a complex with EF that is less conducive to cyclase activity.

Calmodulin-Induced Ion Channel Dimerization

In addition to the release of autoinhibitory domains (for CaM-dependent kinases and calcineurin) and active site remodeling (anthrax adenylyl cyclase), a third mechanism is now known for CaM action: CaM-induced dimerization of membrane proteins as a system underlying ion channel activation.

Small conductance Ca^{2+}-activated potassium (SK) channels are found in a wide range of tissues in virtually all multicellular organisms and play a fundamental role in regulating neuronal excitability. SK channels use K^+ as their charge carrier, but are gated solely by intracellular Ca^{2+} ions at the submicromolar level, such as occurs during an action potential in many neurons. There are three known members of the SK channel family (SK1, SK2, and SK3) which show high structural homology with each other and similar calcium sensitivity with half-maximal activation at 0.3 μM (Köhler et al., 1996). The activation of SK channels causes membrane hyperpolarization that raises the threshold for further neuron firing and thereby regulates the frequency of action potentials. The afterhyperpolarization generated by these channels is maximal following an action potential and decays with a half-life on the order of hundreds of milliseconds. Hence, this reasonably rapid gating necessitates having a Ca^{2+} sensor close to the pore to quickly transduce fluctuations in ion concentration.

Correspondingly, a unique feature of SK channel α-subunits is constitutive association with CaM. This interaction is maintained both in the presence or absence of Ca^{2+}. However, channel gating also requires a Ca^{2+}-dependent interaction and structural analysis has identified distinct domains for these processes. Schumacher and colleagues have elegantly shown from the crystal of the Ca^{2+}-loaded CaM/SK2 complex that the complex presents as two CaM molecules tightly sandwiching two K^+ channel domains (Schumacher et al., 2001; Figure 1). This is the first observation of a 2:2 CaM-target complex and the active tetrameric form of the channel would likely require two sets of 2:2 dimers. CaM uses a modular strategy in which the carboxy-terminal EF hands mediate tethering to the channel and the amino-terminal EF hands are responsible for Ca^{2+}-induced dimerization leading to channel gating and direct coupling between changes in intracellular Ca^{2+} concentrations and altered membrane potential. Unlike previously determined CaM-target structures, Ca^{2+} ions were only present in the N-terminal "low-affinity" lobes. Intriguingly, the Ca^{2+}-bound and -unbound CaM lobes adopt distinct conformations, the latter being similar to that of apoCaM in solution. The overall conclusions were supported by sedimentation analysis showing that the Ca^{2+}-free CaM/SK2 complex is monomeric and dimerizes in the presence of Ca^{2+} (Schumacher et al., 2001). This example may represent the "tip of the iceberg" for CaM-induced target dimerization. Additional studies are already underway, including that of the petunia glutamate decarboxylase (GAD)-CaM complex (Yuan and Vogel, 1998; K. Yap and M.I., unpublished observations), alluding to the potential universality of this mechanism (Table 1). Are there any other structurally feasible, hypothetical arrangements awaiting future validation? CaM binding sites may not always exist on a single target protein and a 1:2 or 2:2 CaM:target heterodimerization scenario, in which CaM bridges between different proteins, would seem most probable. For example, CaM may recruit two ion channel isoforms or an ion channel and its cellular effector protein, such as a protein kinase, to a spatially proximal site in the cell.

The CaM/SK2 structure further provides detailed insight into a novel CaM-mediated target protein activa-

tion mechanism. In this 2:2 arrangement, CaM adopts a very elongated structure thereby facilitating interaction of a single CaM molecule with 3 α helices (Figure 1). Here, the SK2 N terminus is inundated by CaM, which covers more than 80% of its surface area. Some of the residues required for the molecular interface have recently been confirmed by solution NMR (Wissmann et al., 2002). As is the case with the anthrax EF/CaM complex, the CaM binding domains of SK2 do not correspond to previously identified CaM recognition motifs.

Complementary work from several laboratories extends our perspective on CaM's involvement in ion channel regulation. CaM has been also shown to play a prominent role in modulating the hallmark feedback inhibition or CDI (Ca^{2+}-dependent inactivation) of P/Q- and L-type voltage-dependent Ca^{2+} channels, and cyclic nucleotide-gated channels of the visual and olfactory systems. Acute CaM modulation of P/Q-type channels arises from CaM lobe-specific ion binding to selectively trigger CDI or Ca^{2+}-dependent channel facilitation (DeMaria et al., 2001). In the case of L-type channels, Ca^{2+}-sensitive CaM association occurs via a standard IQ motif in the pore-forming α_1 subunit of the channel. However, additional structural elements are required, and CaM function is facilitated by constitutive tethering at proximal binding sites (Pitt et al., 2001). As is the case with SK channels, binding of Ca^{2+} to only a single CaM lobe is sufficient to trigger gating, but of note, in this channel system, it is the C-terminal CaM domain which binds the Ca^{2+} ions. Although this interaction follows a 1:1 CaM:target interaction scenario (Table 1), and the exact mechanism underlying CDI remains unclear, we can envisage a relationship between CaM tethering and effector sites similar to the model established for SK channels. The picture that emerges is one in which CaM interacts in a bipartite manner with ion channels: one CaM module mediating constitutive binding and the other transmitting Ca^{2+} dependence.

Ca^{2+}-dependent facilitation, most prominent when basal Ca^{2+} is elevated or when the channels are repeatedly activated by trains of depolarizations, has also been attributed to the action of CaM kinase II (Bayer et al., 2001). Following Ca^{2+}/CaM stimulation of the CaMKII holoenzyme, CaMKII autophosphorylation traps bound Ca^{2+}/CaM and the kinase remains partially active even after the initial Ca^{2+} stimulus has subsided (Meyer et al., 1992). How might channels exploit this mechanism? Consider a scenario where increases in postsynaptic Ca^{2+} elicit only limited CaM binding to the CaMKII holoenzyme and submaximal kinase activation. CaM trapping would account for reduced CaM-CaMKII dissociation between rapid Ca^{2+} spikes and an additive increase in CaM recruitment and kinase activity with each successive oscillation. In this way, the CaM/CaMKII complex could act as a frequency to amplitude converter for intracellular Ca^{2+} transients. This complex model awaits further verification in vivo.

Perspectives

Does a single polypeptide sequence encode a single protein conformation? The case of calmodulin, a central coordinator for many proteins that are influenced by calcium dynamics, clearly indicates that the conformation of a protein changes depending on its environment. Various CaM binding partners dictate the 3D structure of CaM mainly by changing the orientation of the two CaM globular lobes. Furthermore, recent high-resolution crystallographic studies (Kurokawa et al., 2001) revealed significant rearrangement of EF-hand helices within each CaM lobe to adjust its conformation for optimal binding with a target. These phenomena occur not only in CaM but also in other proteins. For instance, prion protein undergoes conversion from a ubiquitous cellular form characterized by high α-helical content to the pathogenic "scrapie" form consisting predominantly of β sheets. The GTPase binding domain of Wiskott-Aldrich syndrome protein can also alternate between various induced structures according to the context of different binding partners (Kim et al., 2000). Moreover, a recent study on the CBP/p300-p160 nuclear receptor complex (Demarest et al., 2002) showed that even the protein folding process itself is facilitated by the protein-protein interaction (called "synergistic folding"). Hence, together with the evolution of proteomic analyses, as methods for 3D structure determination become more widely accessible, the entire complement of protein structure-function relationships will continue to be elucidated and take shape.

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