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Structures of EF-hand Ca^{2+}-binding proteins: Diversity in the organization, packing and response to Ca^{2+} Binding

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The growing database of three-dimensional structures of EF-hand calcium-binding proteins is revealing a previously unrecognized variability in the coformations and organizations of EF-hand binding motifs. The structures of twelve different EF-hand proteins for which coordinates are publicly available are discussed and related to their respective biological and biophysical properties. The classical picture of calcium sensors and calcium signal modulators is presented, along with variants on the basic theme and new structural paradigms.

Keywords: EF-hand, Ca^{2+}-binding protein, Ca^{2+} signaling, protein structure, calmodulin, signal transduction

The majority of proteins involved in transducing intracellular Ca^{2+} signals are characterized by a common helix-loop-helix structural motif in their Ca^{2+}-binding sites, termed the EF-hand. These proteins exhibit both high selectivity for Ca^{2+} (binding Ca^{2+} preferentially despite the large background of Mg^{2+}) and the ability to respond rapidly and efficiently to the modest (hundred-fold) changes in concentration associated with Ca^{2+} signals, capabilities which attest to an exquisite fine-tuning of the functional properties of EF-hand proteins. In fact, the unique relationship between the EF-hand motif and Ca^{2+} extends beyond the ability to transduce Ca^{2+} signals, as other members of this protein family have been shown to modulate cellular Ca^{2+} levels via roles in Ca^{2+} uptake, transport and buffering. This wide diversity in functions for a single family of proteins is intriguing and has prompted structural studies in a number of laboratories.

The term 'EF-hand motif' was introduced by Kretsinger based on the first three-dimensional structure of a protein from this family, that of parvalbumin (Kretsinger & Nockolds 1973). Over the subsequent twenty years, this was followed by X-ray crystal structures of other EF-hand Ca^{2+}-binding proteins (CaBPs), including troponin C, calmodulin, calbindin D_{9k} and oncomodulin. Two comprehensive treatises have been published based on the cumulative analysis of these structures, one on the general structural features of calcium-binding sites in proteins (McPhalen *et al.* 1991) and the other on the fundamental structural properties of EF-hand proteins in particular (Strynadka & James 1989). The past five years have witnessed a large acceleration in the rate of CaBP structure determination, a significant portion of which can be attributed to the development of NMR techniques for solving the three-dimensional structures of macromolecules. The ability to determine the structures of apo proteins was particularly influenced by these techniques, since the level of Ca^{2+}-loading can be readily varied in NMR experiments. In fact, most structures of apo EF-hand CaBPs are NMR solution structures.

The growing database of three-dimensional structures reveals a previously unrecognized variability in the organization and conformations of EF-hand motifs, providing tantalizing insights into the structural basis for the diverse functions of EF-hand CaBPs. The diversity in structure is most readily apparent in the organization of EF-hands into domains. EF-hand motifs almost always occur in pairs, packed together in a face-to-face manner. The

pairing of sites is presumed to stabilize the protein conformation, increase the Ca^{2+} affinity of each site over that of isolated sites, and provide a ready means for the cooperativity in the binding of Ca^{2+}, which is critical to function. There is no known EF-hand CaBP with only one EF-hand. Calbindin D_{9k}, the smallest EF-hand CaBP has two EF-hands. Several, such as calmodulin and troponin C, contain two globular domains, each consisting of a pair of EF-hands similar to those of calbindin D_{9k}. Parvalbumin has three EF-hands forming a unique structural domain, although only two are functional. There are a number of new structures with four EF-hands forming an integral structural unit, which has been achieved both by packing of a single chain containing four EF-hands (as in recoverin and sarcoplasmic calcium-binding protein) and by dimerization of a two EF-hand domain (as in the S100 proteins calcyclin and S100B). This diversity of EF-hand packing is further extended by calpain domain VI, which has five EF-hands per monomer, with the unpaired fifth EF-hand mediating dimerization to create a ten EF-hand structural unit. Recent biophysical studies of calbindin D_{28k} suggest yet another organizational scheme of six EF-hands in a single globular domain (Linse *et al.* 1997).

In addition to the diversity in the assembly of EF-hand motifs, there are substantial variations in the conformations of the EF-hands, and in the conformational changes induced by Ca^{2+}-binding. These differences are clearly related to the wide diversity of functional roles and biological activities within the EF-hand CaBP family. For example, the Ca^{2+} sensors that transduce Ca^{2+} signals into metabolic or mechanical responses must undergo a conformational change that is large enough to sequester a target interaction site in the 'off' state and expose this site in the 'on' state. Such a pronounced conformational change is not required of the members of the family that are involved in modulating as opposed to transducing Ca^{2+} signals. For these proteins, ion binding affinity, selectivity, and kinetics and protein stability are much more important than conformational change. However, some structural features of EF-hand CaBPs do seem to be conserved. One such feature, found in all EF-hand CaBP structures to date, is the short β-interaction between the two Ca^{2+}-binding loops in a pair of EF-hands. This interaction may be one means by which EF-hands achieve cooperativity in metal ion binding.

In this review, the three-dimensional structures of the twelve different EF-hand CaBPs for which there are publicly available coordinates (Table 1) are discussed and related to the biological and biophysical properties of EF-hand CaBPs. However, the discussion of each specific CaBP is necessarily limited. A list of reviews about various EF-hand CaBPs is available online (http://chazin.scripps.edu/cabp_database/general/refs/reviews.html) as part of the EF-hand CaBP Data Library (http://chazin.scripps.edu/cabp_database/), which also contains more information about the proteins discussed in this review as well as other EF-hand CaBPs.

The EF-hand motif

The canonical EF-hand consists of a 29 residue contiguous polypeptide containing helix I (helix E), a loop around the calcium ion, and helix II (helix F) (reviewed in Strynadka & James 1989, McPhalen *et al.* 1991). Residues 1, 3, 5, 7, 9 and 12 of the canonical binding loop chelate the Ca^{2+} ion, forming a pentagonal bipyramidal array of seven oxygen ligands. Residues 1, 3 and 5 provide monodentate oxygen ligands via sidechain oxygens, usually aspartate carboxylates. Residue 12 is a bidentate oxygen ligand, and is almost always a glutamate residue, which ligates calcium via both sidechain carboxlyate oxygens. Residue 7 directly coordinates Ca^{2+} via its mainchain oxygen. Residue 9 hydrogen bonds to a water molecule that provides the remaining Ca^{2+} ligand.

Most EF-hand proteins use this canonical motif. However, members of the S100 subfamily of CaBPs have a two residue insertion and a modified coordination scheme in the first binding loop, creating a motif termed the pseudo EF-hand. In this loop, residues 1, 4, 6 and 9 chelate the Ca^{2+} ion via their mainchain oxygen and residue 11 chelates indirectly via a water molecule. The side chain carboxylate of residue 14 provides bidentate ligation. As in the canonical loop, this terminal loop residue is almost always a glutamate. In fact, the terminal bidentate ligand is a conserved feature of all high affinity binding loops. Glutamate is favored over aspartate for this position because its greater length facilitates the positioning of both sidechain oxygens to ligate the Ca^{2+} ion. The role of the pseudo EF-hand loop and other atypical binding loops in modulating the structure and function of CaBPs is unclear.

The classical view of the structural response of EF-hand CaBPs to Ca^{2+} binding is based on the crystal structures of calmodulin and troponin C. These proteins consist of two largely independent domains, with two EF-hand motifs packed together in each domain, creating a bundle of four helices (numbered I-IV from the N- to C-terminus; see Fig. 1). The conformational changes associated with

Table 1. Summary of available structures of EF-hand calcium-binding proteins

Protein	Available structures PDB code (technique): description	EF-hands (coordination mode/ ion binding type)	Binding-induced conformational changes	Related proteins without structures
Calmodulin				
vertebrate	1CFC (NMR): apo 1DMO (NMR): apo 1CLL (xtal): 4Ca 3CLN (xtal): 4Ca 1DEG (xtal): 4Ca, mutant (E84Δ) 1AHR (xtal): 4Ca, mutant (T79Δ,D80Δ)	1: canonical/specifc 2: canonical/specific 3: canonical/specific 4: canonical/specific	The 'closed to open' conformational change consists of rearrangements in the packing of the helices within two EF-hands in a domain, which also causes disruption of one inter-EF-hand interhelical interface. These changes lead to the exposure of a concave hydrophobic surface.	caltractin (centrin) calcium-dependent protein kinase calmodulin-like protein squidulin
Drosophila	4CLN (xtal): 4Ca			
Paramecium	1CLM (xtal): 4Ca 1OSA (xtal): 4Ca			
vertebrate N domain	1AK8 (NMR): 2Ce			
vertebrate C domain	1CMF (NMR): apo 1CMG (NMR): 2Ca 1TRC (xtal): 2Ca			
vertebrate, complexed with peptide	2BBN (NMR): 4Ca + skMLCK peptide 1CDL (xtal): 4Ca + smMLCK peptide 1CDM (xtal): 4Ca + CaMKII peptide		Peptide binding to calcium-loaded CaM causes compaction as the two CaM domains wrap around the peptide.	
vertebrate, complexed with small molecules	1CTR (xtal) 4Ca + 1 TFP 1LIN (xtal): 4Ca + 4 TFP		Interactions of CaM and TFP cause a compaction similar to that seen in the peptide complexes.	

Table 1. *Continued*

Protein	Available structures PDB code (technique): description	EF-hands (coordination mode/ ion binding type)	Binding-induced conformational changes	Related proteins without structures
Troponin C				
skeletal	1TOP (xtal): 2Ca (in C domain) 1TNW (NMR): 4Ca 1TN4 (xtal): 4Ca 1TCF (xtal): 4Ca 1NCX (xtal): 2Cd 1NCY (xtal): 2Mn 1NCZ (xtal): 2Tb	1: canonical/specific 2: canonical/specific 3: canonical/mixed 4: canonical/mixed	Basically the same as calmodulin.	same as calmodulin
skeletal N domai n	1AVS (xtal): 2Ca 1TRF (NMR): apo 1TNP (NMR): apo 1SMG (NMR): 2Ca, mutant (E41A)			
cardiac N domain	1SPY (NMR): apo 1AP4 (NMR): 2Ca	1: non-functional 2: canonical/specific		
cardiac C domain	3CTN (NMR): 2Ca	3: canonical/mixed 4: canonical/mixed		
Parvalbumin				
alpha lineage	5PAL (xtal): 2Ca, leopard shark 1PVA (xtal): 2Ca, pike pI 5.0 1PAS (NMR): 2Ca, pike pI 5.0 1RTP (xtal): 2Ca, rat 0CPT (xtal): 2Tb, toadfish	1: non-functional 2: canonical/mixed 3: canonical/mixed	The differences between the 2Ca and 1Ca/1Mg structures are localized to the affected binding loop.	avian thymic hormone.
non-mammalian beta lineage	1PAL (xtal): 2Ca, pike pI 4.1 2PAL (xtal): 3Mn (1 non-EF-hand), pike pI 4.1 3PAL (xtal): 2Ca, 1Mg (1 non-EF-hand), pike pI 4.1 4PAL (xtal): 1Ca, 2Mg (1 non-EF-hand), pike pI 4.1 1PVB (xtal): 2Ca, pike pI 4.1 4CPV (xtal): 2Ca, carp pI 4.25 5CPV (xtal): 2Ca, carp pI 4.25 1CDP (xtal): 2Cd, carp pI 4.25			
oncomodulin (mammalian beta lineage)	1OMD (xtal): 2Ca, rat 1RRO (xtal): 2Ca, rat	1: non-functional 2: canonical/specific 3: canonical/mixed	Unknown, but presumed to be similar to other parvalbumins.	

Table 1. *Continued*

Protein	Available structures PDB code (technique): description	EF-hands (coordination mode/ ion binding type)	Binding-induced conformational changes	Related proteins without structures
Calbindin D_{9k}				
bovine	1CLB (NMR): apo (P43G) 2BCB (NMR): 2Ca (P43G) 4ICB (xtal): 2Ca 3ICB (xtal): 2Ca 1CDN (NMR): 1Cd (P43G) 5ICB (xtal): 1Mg 6ICB (xtal): 1Mn 1BOD (NMR): 2Ca, mutant (canonical EF1) 1BOC (NMR): 2Ca, mutant (A15D, P20G)	1: pseudo/specific 2: canonical/specifc	Undergoes a small calcium-induced reorganization, but remains closed. Calcium binding has a greater effect on the C-terminal EF-hand.	
porcine	1CB1 (NMR): 2Ca			
Myosin Light Chains				
scallop	1WDC: 1Mg (RLC), 1Ca (ELC) + heavy chain fragment 1SCM: 1Mg/Ca (RLC), 1Ca (ELC) + heavy chain fragment	*RLC*: 1: canonical/mixed 2: non-functional 3: non-functional 4: non-functional *ELC*: 1: novel/specifc 2: non-functional 3: non-functional 4: non-functional	RLC-N is open, ELC-N is closed, and the two C domains are semi-open in both structures. Effects of removing divalent cations and the heavy chain are unknown.	
Calcineurin B				
human *bovine*	1AUI (xtal): 4Ca, full CaN 1TCO (xtal): 4Ca, full CaN-B, CaN-A fragment + FKBP12-FK506	1: canonical/n.a. 2: canonical/n.a. 3: canonical/n.a. 4: canonical/n.a.	Calcium-induced conformational changes are unknown, but presumed to be CaM-like.	
Recoverin				
bovine, myristoylated *bovine, unmyristoylated*	1IKU (NMR): apo 1JSA (NMR): 2Ca 1REC (xtal): 1Ca	1: non-functional 2: canonical/specifc 3: canonical/specific 4: non-functional	Calcium-induced changes within the first three EF-hands, and in the packing of the EF-hands with each other. These changes allow exposure of the myristoyl group and may or may not have other functional consequences	hippocalcin neurocalcin s-modulin frequenin visinin NCS-1 NCS-2 frequenin-like ubiquitous protein visinin-like proteins 1,2,3

Table 1. *Continued*

Protein	Available structures PDB code (technique): description	EF-hands (coordination mode/ ion binding type)	Binding-induced conformational changes	Related proteins without structures
Sarcoplasmic Calcium-Binding Protein				
amphioxous	2SAS (xtal): 3Ca	1: canonical/n.a. 2: canonical/n.a. 3: canonical/n.a. 4: non-functional	Apo protein is unstrucutred	calerythrin aequorin luciferin binding protein clytin
Nereis divisicolor	2SCP (xtal): 3Ca	1: canonical/mixed 2: non-functional 3: canonical/mixed 4: canonical/mixed		
S100B				
bovine	1CFP (NMR): apo 1MHO (xtal): 2Ca	1: pseudo/n.a. 2: canonical/n.a.	Calcium-induced shift in helix III of both subunits creates a small hydrophobic patch on the surface	S100A1-A16 S100C S100P profilaggrin ictacalcin trichohyalin
human	1UWO (NMR): 2Ca			
rat	1SYN (NMR): apo 1QLK (NMR): 2Ca			
Calcyclin (S100A6)				
rabbit	1CNP (NMR): apo 1A03 (NMR): 2Ca	1: pseudo/specific 2: canonical/specific	Presumed to be similar to S100β. Current strucutres are low resolution, but show the changes in the monomer core to be similar to those in calbindin D_{9k}	same as S100β
Calpain				
domain VI	1AJ5 (xtal): apo, rat 1DVI (xtal): 3Ca, rat 1ALV (xtal): 3Ca, porcine 1ALW (xtal): 3Ca + calpain inhibitor PD150606, porcine	1: novel/n.a. 2: canonical/n.a. 3: canonical/n.a. 4: non-functional 5: non-functional	Overall, calcium-induced changes are small. They include reorganization of the packing of the EF1-EF2 linker and closer packing of EF1-EF2 and EF3-EF4. The inhibitor bound calcium-loaded strucutre is very similar to the structure with only calcium bound	calpain dIV sorcin grancalcin
SPARC				
human	1BMO (xtal): 2Ca	1: novel/n.a. 2: canonical/n.a.	Extracellular localization implies calcium is bound under most physiological conditions. Calcium seems to be required for a native-like structure	QR1 SC1 testican tsc36
human, calcium-binding domain	1SRA (xtal): 2Ca			

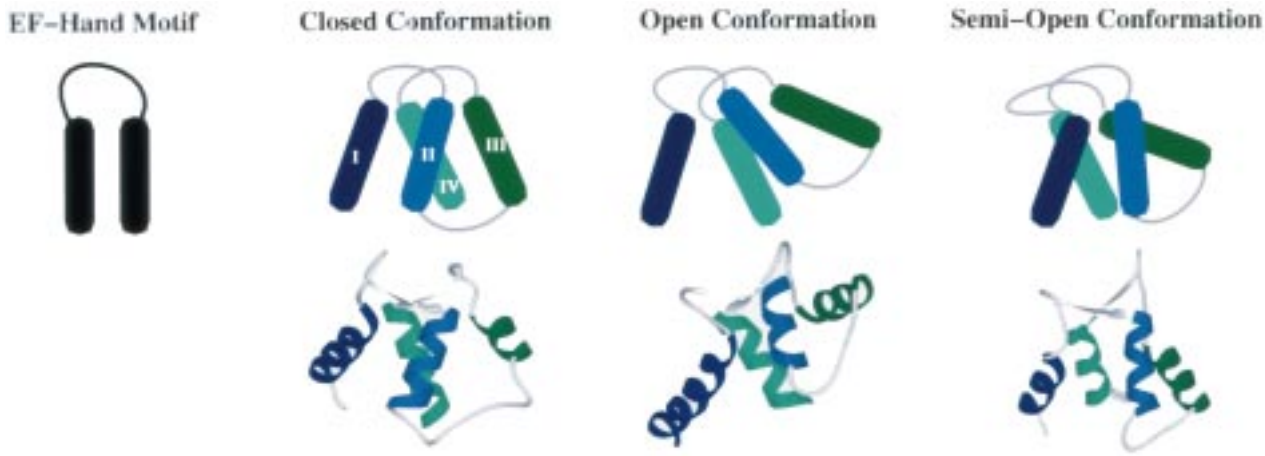

Figure 1. Schematic diagrams and molecular structures of the EF-hand motif and the three classical conformations of EF-hand domains. In both the schematics and the structures, the two helices of the first EF-hand (helices I and II) are colored dark and light blue, respectively, and the two helices of the second EF-hand (helices III and IV) are colored dark and light green, respectively. The three structures shown are: apo calbindin D_{9k} (closed conformation; PDB code 1CLB), the N-terminal domain of Ca^{2+}-loaded calmodulin (open conformation; PDB code 1CLL), and the C-terminal domain of the myosin essential light chain (semi-open conformation; PDB code 1WDC). The ribbon diagrams were generated with the PDB Ribbons module (M. Carson, A. Shaw) for AVS (Upson *et al.* 1989).

Ca^{2+} binding to these domains have traditionally been described in terms of interhelical angles, which represent more complex rearrangements of the interfaces between the four helices in a domain. Although this classical description of the Ca^{2+}-induced conformational changes is limited and proves inadequate for some proteins, it provides a useful framework within which different types of conformational changes can be examined.

The classical picture: Ca^{2+} sensors

Classical Ca^{2+} sensors such as **calmodulin** (CaM) and **troponin C** (TnC) function by undergoing a Ca^{2+}-induced conformational change, which exposes a concave hydrophobic surface in each domain of the protein. These surfaces then interact with target proteins, thereby transducing the calcium signal. The exposure of the hydrophobic surface is expected to be unfavorable, and this energetic cost seems to be 'paid for' by a reduction in the calcium affinity.

The HMJ model provided the first atomic level view of this Ca^{2+}-induced conformational change (Herzberg *et al.* 1986), by modeling the apo conformation of Ca^{2+}-free domains on the structure of the N domain of TnC (TnC), which was in the apo state in the crystal structure (Herzberg & James 1985). The model predicted that the Ca^{2+}-induced conformational change would involve rearrangements of the helices within each domain: the helices would be approximately anti-parallel in the apo state, but nearly perpendicular in the Ca^{2+}-loaded state. Although high resolution structures of $(Ca^{2+})_2$-TnC (e.g. Herzberg and James 1988; Satyshur *et al.* 1988) and $(Ca^{2+})_4$-CaM (e.g. Babu *et al.* 1988; Chattopadhyaya *et al.* 1992) became available, the HMJ model remained the only view of the Ca^{2+}-induced conformational changes until 1995, when NMR solution structures of apo and Ca^{2+}-loaded CaM and TnC allowed the first direct determination of these conformational changes in regulatory EF-hand domains (Finn *et al.* 1995; Gagné *et al.* 1995; Kuboniwa *et al.* 1995; Zhang *et al.* 1995). These and the more recent crystal structures of the Ca^{2+}-loaded N-terminal domain of TnC (Houdusse *et al.* 1997; Strynadka *et al.* 1997) have confirmed the basic tenets of the HMJ model.

Detailed comparisons of the apo and Ca^{2+}-loaded states using the new structures provide a more complete picture of the calcium-induced conformational changes in CaM and TnC. In the apo domains, all interhelical interfaces are well-packed, except the interface between helices I and III, which do not interact. In the calcium-loaded domains, the two *intra*-EF-hand interfaces (I/II and III/IV) 'swing open' into an inverted-V shape, with the Ca^{2+}-binding loop at the vertex (Fig. 1; Nelson & Chazin 1998).

A variety of studies have indicated that the two domains of CaM are structurally distinct in the absence of targets. For many years, the central helix separating the two domains in the crystal structures

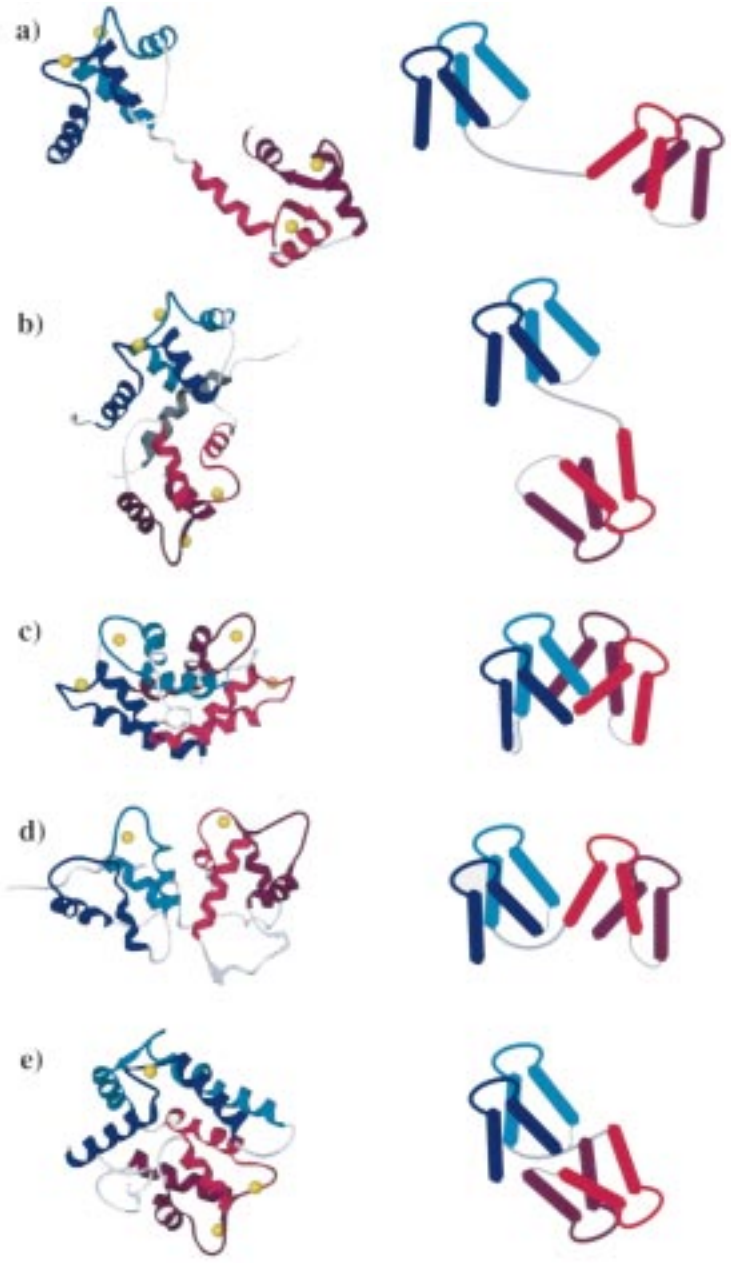

Figure 2. Variability in organization of four EF-hand domains. Ribbon diagrams of five different proteins containing four EF-hands are shown, along with simplified schematic diagrams to highlight the variation in domain organization. In all cases, EF1 is dark blue, EF2 is teal, EF3 is pink, and EF4 is burgundy. The structures shown are: *a)* Ca^{2+}-loaded calmodulin (PDB code 1CLL), *b)* Ca^{2+}-loaded calmodulin complexed with a peptide derived from the skeletal muscle myosin light chain kinase (PDB code 2BBM), *c)* Ca^{2+}-loaded S100B (PDB code 1UWO), *d)* Ca^{2+}-loaded recoverin (PDB code 1JSA), *e)* Ca^{2+}-loaded sarcoplasmic calcium-binding protein from *Nereis divisicolor* (PDB code 2SCP). The ribbon diagrams were generated as in figure 1.

caused some controversy because such a long, solvent exposed helix was not expected to be stable in solution. Fluorescence anisotropy and small angle x-ray scattering experiments implied that CaM adopted a more compact shape than seen in the crystal structures (e.g. Heidorn & Trewhella 1988). NMR experiments then showed directly that a portion of the central helix of CaM is flexible in both the apo and Ca^{2+}-loaded states (Spera *et al.* 1991; Barbato *et al.* 1992; Tjandra *et al.* 1995). The role of this linker region as a 'flexible tether' was further confirmed by the structures of Ca^{2+}-loaded CaM

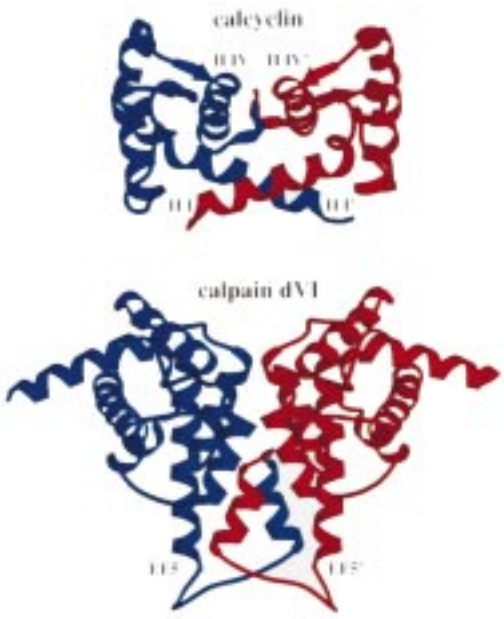

Figure 3. Two distinct dimerization modes of EF-hand proteins. Ribbon diagrams of apo calcyclin (L. Mäler, B. C. M. Potts & W.J. Chazin, in preparation) and Ca^{2+}-loaded calpain domain VI (PDB code 1DVI) are shown, with the portions of the structures that mediate dimerization labeled. In both cases, one subunit is blue and the other is red. The ribbon diagrams were generated as in Figure 1.

complexed with peptides derived from the CaM-binding domains of skeletal muscle myosin light chain kinase (MLCK) (Ikura *et al.* 1992), smooth muscle MLCK (Meador *et al.* 1992), and CaM-dependent protein kinase II (CaMKII) (Meador *et al.* 1993). In these structures, the CaM molecule compacts as the two domains wrap around the target peptide (Fig. 2b), with CaM-C interacting with the N-terminal portion of one face of the peptide and CaM-N interacting with the C-terminal portion of the other face. These conformational changes almost exclusively involve the quaternary structure of CaM, leaving the structure of the individual domains approximately the same as in the Ca^{2+}-loaded, peptide-free structures (reviewed in Crivici & Ikura 1995).

The flexible nature of the region linking the two domains is one reason for CaM's ability to interact with a wide-range of target peptides. The peptides are basic amphipathic α-helices (O'Neil & DeGrado 1990), and often have two large hydrophobic 'anchors' separated by twelve resides. However, as the structure with the CaMKII peptide demonstrated, CaM can also bind peptides in which the two hydrophobic anchors are separated by as few as eight residues. In this case, the flexible linker between the two domains simply allows the two CaM domains to come closer together. The predominance of non-specific hydrophobic interactions between CaM and the peptides, many of them mediated by the inherently flexible methionine residues in the protein's hydrophobic patches, may also contribute to the ability of CaM to accommodate a variety of targets (O'Neil & DeGrado 1990).

In contrast to CaM, TnC has only one target: troponin I (TnI). However, the atomic details of the interaction between TnC and TnI are not known. TnC-C is thought to always have either Mg^{2+} or Ca^{2+} bound *in vivo*, and appears to maintain its contact with TnI even when Ca^{2+} levels are low. Ca^{2+}-induced changes in TnC-N are thought to be the 'trigger' that transduces the Ca^{2+} signal. TnC also has some unique structural features, including a longer linker between the two domains and an additional helix at the N-terminus that is not part of an EF-hand. The exact functional roles of these features are not known.

The recent NMR structures of the apo and Ca^{2+}-loaded N domain of cardiac TnC complicate the picture of TnC function (Sia *et al.* 1997; Spyracopoukos *et al.* 1997). The binding loop of EF1 in cardiac TnC (cTnC) lacks two sidechain Ca^{2+} ligands, which drastically reduces the Ca^{2+} affinity, rendering the loop non-functional in solution studies. Therefore, only one Ca^{2+} ion is expected to bind to cardiac TnC-N. While there are some Ca^{2+}-induced conformational changes in cardiac TnC-N, the Ca^{2+}-loaded structure of this domain is not in the classical open conformation seen in TnC-C, the N domain of skeletal TnC (sTnC), and both domains of CaM. Rather, the differences between the apo and Ca^{2+}-loaded states of cTnC-N mostly involve EF2 (helices III and IV).

It is important to remember that while these structures of TnC-N present a fascinating biophysical story, they represent only a portion of the biologically relevant complex. *In vivo,* TnC-N is tethered to TnI by TnC-C. This high local concentration of target may shift the conformational equilibrium of Ca^{2+}-loaded cTnC-N, and may even allow Ca^{2+} to bind in the crippled EF1 binding loop. Studies with CaM have shown that the presence of target peptide increases the Ca^{2+} affinity of CaM (Bayley *et al.* 1996), and when CaM is covalently linked to a target peptide, the Ca^{2+} affinity is increased even further (Martin *et al.* 1996). In fact, Ca^{2+}-binding activity can be recovered in 'crippled' binding loop mutants of CaM by the presence of a target peptide (Haiech *et al.* 1991; Findlay *et al.* 1995). Therefore, although the cTnC structures and the recent studies of mutants

of sTnC and CaM in which one EF-hand has drastically reduced Ca^{2+} affinity (Evenäs *et al.* 1997; Gagné *et al.* 1997) indicate that two high affinity Ca^{2+}-binding events are required to completely populate the open conformation in the absence of target, the full biological significance of these observations will remain unclear until more is known about the *in vivo* interactions of these proteins with their targets.

The classical picture: signal modulators

Unlike the signal transducing proteins, signal modulators such as parvalbumin and calbindin D_{9k} do not require exposure of a hydrophobic target-interaction surface to function. In fact, exposing such a surface would probably lead to a reduction in these proteins' high Ca^{2+} affinities, which would presumably interfere with their functions. For instance, **parvalbumin** is thought to be involved in quenching the Ca^{2+} signal in muscle cells (allowing muscles to relax after Ca^{2+}-stimulated contractions). The function of parvalbumins in non-muscle cells is less clear, although a role in protecting against calcium toxicity, particularly in the nervous system, has been proposed. Parvalbumin may also function as a Ca^{2+} buffer, with an indirect role in signal transduction: by binding Ca^{2+}, parvalbumin removes the ion from the cytosol where it could otherwise continue to activate signal transducers such as CaM (reviewed in Pauls *et al.* 1996). All of these proposed functions for parvalbumin would require carefully tuned Ca^{2+} binding properties, but not a Ca^{2+}-induced conformational change.

Although the structure of a carp parvalbumin was the first structure of an EF-hand CaBP (Kretsinger & Nockolds 1973), parvalbumin is considered an unusual EF-hand CaBP due to its odd number of EF-hands (three). Structures of parvalbumins from the two evolutionary lineages (the alpha and beta lineages; **oncomodulin** is the mammalian beta lineage parvalbumin) are very similar (e.g. Ahmed *et al.* 1993; McPhalen *et al.* 1994). EF1 is non-functional, and its helices are nearly anti-parallel. In contrast, the two functional EF-hands have approximately perpendicular interhelical angles, similar to those seen in Ca^{2+}-loaded CaM. The non-functional EF-hand packs into the hydrophobic patch created by EF2 and EF3, preventing the energetically costly exposure of hydrophobic surface area and the potential loss of Ca^{2+} affinity. In fact, deleting the first EF-hand reduces the Ca^{2+} affinity more than 50-fold, and most of this lost affinity can be restored by adding the EF1 peptide to the EF2-EF3 fragment (Parmyakov *et al.* 1991).

Parvalbumin's binding loops are usually of the Ca^{2+}/Mg^{2+} mixed type. Like Ca^{2+} specific binding loops, mixed-type binding loops have higher Ca^{2+} affinity than Mg^{2+} affinity. However, the mixed loops bind Mg^{2+} with enough affinity to be Mg^{2+}-loaded at basal physiological conditions (mM Mg^{2+}, sub-μM Ca^{2+}). Most parvalbumins have two mixed loops, but a few have two Ca^{2+} specific loops. Only oncomodulin has one specific binding loop and one mixed binding loop (Pauls *et al.* 1996).

Because they have mixed-type binding loops, most parvalbumins will be in the $(Mg^{2+})_2$ state in resting cells. Unfortunately, there is no three-dimensional structure of a parvalbumin in this state. However, there is a structure of pike beta parvalbumin with one Ca^{2+} and one Mg^{2+} ion bound (Declercq *et al.* 1991). This structure is very similar to the fully Ca^{2+}-loaded structure. It differs mainly in the binding loop of EF3, in which the Mg^{2+} is bound. This binding loop has contracted relative to its Ca^{2+}-bound conformation, due to the smaller size of the Mg^{2+} ion. The Mg^{2+} is coordinated by an octahedral array of six oxygens, in contrast with the seven oxygens arranged in a pentagonal bipyramid around the Ca^{2+} ion. The terminal glutamate, which is bidentate in the Ca^{2+}-loaded structure, adopts a less-favorable rotamer in the Mg^{2+}-bound structure, and is monodentate. NMR studies indicate that the effects of substituting a Mg^{2+} for the Ca^{2+} in EF2 are similar (Blancuzzi *et al.* 1993), and that the $(Mg^{2+})_2$ form is similar in structure to, but significantly more flexible than the $(Ca^{2+})_2$ form (Baldellon *et al.* 1992).

Like parvalbumin, the small, two EF-hand protein **calbindin D_{9k}** does not expose a hydrophobic surface upon Ca^{2+} binding, and undergoes only minor Ca^{2+}-induced conformational changes. These very modest changes are fully consistent with the function of the protein, as calbindin D_{9k} is thought to play roles in Ca^{2+} uptake and transport. Calbindin D_{9k} requires high Ca^{2+} affinity for these activities, which would be compromised by the energetically costly exposure of a hydrophobic surface upon Ca^{2+} binding. Calbindin D_{9k} was the first EF-hand CaBP whose structure was determined both in the presence and absence of Ca^{2+}, and both structures revealed a well-packed conformation very similar to the closed conformation of the apo CaM domains (Szebenyi & Moffat 1986; Kördel *et al.* 1993; Skelton *et al.* 1995).

Calbindin D_{9k} contains the N-terminal pseudo EF-hand binding loop characteristic of the S100 subfamily. In spite of the unusual nature of this site, the binding affinities of the two sites in the protein are nearly equivalent (Linse *et al.* 1987). Furthermore, mutagenesis studies have shown that the

unusual coordination mode can not be the sole reason that calbindin D_{9k} remains in a closed conformation when bound to calcium, for the Ca^{2+}-loaded structure of a mutant with a canonical type first binding loop also reveals a closed conformation (Johansson *et al.* 1993). Indeed, the energetic and structural reasons for the different response to Ca^{2+} binding in calbindin D_{9k} relative to CaM remain unclear. The unique properties of the N-terminal EF-hand of calbindin D_{9k} are one possible explanation. The structural changes that are observed upon Ca^{2+} binding to calbindin D_{9k} are substantially greater in the C-terminal EF-hand than in the N-terminal hand (Skelton *et al.* 1994). In fact, the N-terminal EF-hand has been termed a 'pre-formed' EF-hand because it is so well poised to bind Ca^{2+}.

The asymmetry in the response of the two EF-hands to ion binding implies that the mechanisms of cooperative binding of Ca^{2+} must be different for the two binding pathways (Ca^{2+} binding first to EF1 versus EF2). The structure of calbindin D_{9k} with Cd^{2+} bound only in the C-terminal EF-hand provides insight into the mechanism for cooperativity in the pathway in which Ca^{2+} binds EF2 first, and shows that the ion-induced structural changes are largely attained upon binding of the first ion (Akke *et al.* 1995). Less is known about the mechanism for cooperativity in the other pathway, although again, the preponderance of structural changes appear to occur upon binding of the first ion (Wimberly *et al.* 1995).

The Mg^{2+}-bound X-ray crystal structure of calbindin D_{9k} also contains only a single ion in the C-terminal EF-hand. However, while Cd^{2+} binding mimics Ca^{2+} binding, the Mg^{2+} ion is hexa-coordinate in the usual manner for magnesium, with three side chain oxygens, one main chain oxygen and two water molecules serving as ligands. The differences in coordination are accompanied by differences in the ion-induced structural changes in the protein, relative to the Ca^{2+}-loaded and $(Cd^{2+})_1$ half-saturated state, providing a rationale for the negative allostery observed between Ca^{2+} and Mg^{2+} (Andersson *et al.* 1997).

Variations on the classical picture

At first glance the **myosin light chains** seem to fit neatly into the classical paradigm described above. Myosin, a Mg-ATPase which converts chemical energy into mechanical energy, is a complex of the catalytic heavy chain and the regulatory and essential light chains (RLC and ELC), which bind to a long α-helix at the C-terminal end of the heavy chain. Myosins are found in all eukaryotes, and fall into several distinct subfamilies (Sellers & Goodson 1995). However, high resolution structural information is available only for conventional myosins (myosin II) of higher eukaryotes. In most myosins, the light chains are EF-hand CaBPs that are closely related to CaM, although some unconventional myosins actually use CaM itself as a light chain (Cheney & Mooseker 1992). Not surprisingly, then, the basic features of the conventional myosin light chains bear marked similarity to those of CaM. Like CaM, both light chains consist of two domains, each with two EF-hands, connected by a linker region. Also like CaM, they bind their target (the heavy chain) with reverse polarity: the C-terminal domain of the ELC (ELC-C) binds to the most N-terminal portion of the heavy chain's α-helix, and the N-terminal domain of the RLC (RLC-N) binds to the most C-terminal portion.

The similarity to CaM breaks down at the level of ion binding. The Ca^{2+}/Mg^{2+} binding site in the RLC is the only conserved metal binding site in the light chains (Bagshaw & Kendrick-Jones 1979). There is a Mg^{2+} ion bound in EF1 of the RLC in the crystal structures of both a fragment of chicken skeletal myosin (Rayment *et al.* 1993) and a fragment of scallop muscle myosin (Houdusse & Cohen 1996). The ELC does not bind divalent cations in vertebrate myosin II, but the molluscan ELC-N has a Ca^{2+}-specific binding site, which regulates the Mg-ATPase activity. The Ca^{2+} ion is coordinated in a novel fashion, with a nine residue contiguous peptide segment providing six oxygen ligands. A seventh ligand is contributed by a water molecule, making the coordination pentagonal bipyramidal. Three of the protein ligands are aspartate carboxylates and the other three are backbone carbonyls. Although all of the protein ligands are in the ELC, Ca^{2+}-binding requires the presence of the RLC and the heavy chain (Szent-Györgyi & Chantler 1994), and particularly Gly117 of the RLC (Jansco & Szent-Györgyi 1994). This requirement is consistent with the structure of scallop muscle myosin, in which RLC Gly117 makes important hydrogen bonds, stabilizing the unusual Ca^{2+}-binding loop in the ELC EF1. Other contacts between the ELC and the RLC, and between the ELC and the heavy chain further stabilize the binding loop. However, the structure is not able to fully explain how the binding of calcium to this loop activates the Mg-ATPase, since there is no structure without the triggering Ca^{2+} bound.

The available structural information does allow detailed analysis of the conformations of the light chains*, and here again, there are important differences from the CaM paradigm. Although Ca^{2+} is

bound to ELC-N in the scallop myosin structure, it adopts a closed conformation very similar to that seen in the apo CaM domains. This difference may be due to the unusual Ca^{2+} coordination in the ELC, but there is no direct evidence for this. RLC-N, which has only one divalent cation bound, adopts an open conformation similar to that seen in fully Ca^{2+}-loaded CaM, and not in other structures with only one ion bound, such as cardiac TnC. However, the most striking difference is the 'semi-open' conformation adopted by the C-terminal domains of both light chains (Xie *et al.* 1994, Houdusse & Cohen 1996). This novel conformation is a distinct conformation, and not an intermediate between CaM-like closed and open conformations (Nelson & Chazin 1998).

Although the myosin light chains are currently the only structural example of the semi-open conformation, this conformation may be used by other EF-hand CaBPs, particularly CaM-C, to bind to targets in the apo state (Houdusse *et al.* 1996, Swindells & Ikura 1996). Structural comparisons show that apo CaM-C is not in the semi-open conformation in the absence of target (Nelson & Chazin 1998), although there is some evidence for a predisposition of CaM-C for the semi-open conformation (Swindells & Ikura 1996, Houdusse *et al.* 1997). Thus, if apo CaM does interact with these targets via the semi-open conformation, this conformation must be induced by target binding. This may also be the case for the myosin light chains, as there are no structures of the light chains without bound heavy chain to provide a basis for comparison. A structure of apo CaM complexed to an IQ-motif containing target will ultimately be required to determine if CaM can in fact occupy the semi-open conformation.

Like myosin, **calcineurin** is an enzyme complexed to a CaM-like regulatory subunit. Calcineurin (CaN) is a heterodimer of CaN-A, which is a serine/threonine protein phosphatase, and CaN-B, an EF-hand CaBP. The phosphatase activity is regulated by Ca^{2+}-CaM and by direct Ca^{2+} binding to CaN-B. Once the heterodimer is formed, it can only be disrupted by denaturing conditions. However, Ca^{2+} is required to reconstitute the complex after removing denaturant or to create the dimer from recombinant subunits. Although CaN-B is similar in sequence and structure to CaM, CaN-B and Ca^{2+}-CaM bind to different sites on CaN-A and are not interchangeable (Klee *et al.* 1988).

CaN was originally isolated from brain extracts, where it is the major soluble CaM-binding protein (Klee & Krinks 1978), but it has been most extensively studied for its essential role in T-cell activation (Clipstone & Crabtree 1992; O'Keefe *et al.* 1992). Calcineurin's phosphatase activity is inhibited by immunosuppresant drugs such as cyclosporin and FK506, and biochemical data have shown that CaN is indeed the immunosuppresive target of these drugs (Liu *et al.* 1991; O'Keefe *et al.* 1992; Clipstone & Crabtree *et al.* 1992). In fact, one of the available structures of CaN is a complex between CaN and FK506 and its binding protein, FKBP12 (Griffith *et al.* 1995). The differences between the structure of this complex and that of CaN alone seem to be restricted to an auto-inhibitory portion of the enzyme. This protein segment is bound to the phosphatase active site in the isolated CaN structure, but is not visible in the electron density for the complex of CaN and FK506/FKBP512 (Kissinger *et al.* 1995).

CaN-B consists of two CaM-like domains separated by a linker. Each domain has two EF-hands, and all four EF-hands in CaN-B are calcium-loaded in the crystal structures. CaN-B binds to a five turn amphipathic α-helix in CaN-A, which extends out from the rest of CaN-A. Unlike the CaM-peptide structures, both domains of CaN-B bind to the same face of the α-helix. However, it is important to note that CaM has already shown extraordinary flexibility in its target interactions, and there may eventually be an example in which both domains of CaM bind to the same face of a helix. Also, there are no structures of CaM complexed with an intact target protein with which to compare the calcineurin structure.

Another feature of CaN-B that distinguishes it from CaM is its N-terminal myristoylation. Although myristoylation can have many functions in proteins, it seems to play a purely structural role in CaN-B. Studies on myristoylated and unmyristoylated CaN found that myristoylation increases the thermostability of CaN, but does not affect the enzymatic activity, immunosuppresant binding, Ca^{2+} binding, or association with phospholipids (Kennedy *et al.* 1996). The effect on stability is consistent with the structures, in which the myristoyl group packs against the hydrophobic first helix of CaN-B, which would otherwise be solvent exposed.

As is so often the case, the structure of calcineurin has raised as many questions as it has provided answers. Particularly puzzling is the fact that once bound to CaN-A, CaN-B remains in a tight complex in the absence of Ca^{2+}, yet the binding mode in the structures is similar to that used by CaM to reversibly bind targets in a Ca^{2+}-dependent manner. Despite the structural information, how CaN-B binds CaN-A in the absence of calcium, and how

Ca^{2+} binding to CaN-B modulates the phosphatase activity of CaN-A remain mysterious.

New paradigms: four EF-hand globular domains

The neuronal calcium sensors are another family of EF-hand CaBPs that are fatty acid-acylated *in vivo*. However, in these proteins the myristoyl group seems to be an active partner in signal transduction, using a mechanism that has been termed the Ca^{2+}-myristoyl switch. Ca^{2+}-binding to the protein induces the exposure of the previously buried myristoyl group, leading to the translocation of the protein to membrane fractions, where it binds to targets and modulates their function (Ames *et al.* 1996).

Recoverin, a CaBP found in the retina, is currently the only member of this family for which there is structural information. It binds Ca^{2+} in its second and third EF-hands. In this Ca^{2+}-loaded form, recoverin binds to rhodopsin kinase, blocking the phosphorylation of photoexcited rhodopsin, and prolonging the photoresponse. At least *in vitro*, this function is enhanced by myristoylation of recoverin (Chen *et al.* 1995; Klenchin *et al.* 1995). Myristoylation is also required for cooperative Ca^{2+}-binding: unmyristoylated recoverin binds two equivalents of calcium, but does not exhibit cooperativity in this binding (Ames *et al.* 1995).

Recoverin does not follow the CaM paradigm of two independent domains separated by a flexible linker. It has four EF-hands, but these are arranged in one globular domain, with extensive interactions not only within the EF1/EF2 and EF3/EF4 pairs, but also between EF2 and EF3 in both the apo and Ca^{2+}-loaded structures (Fig. 2d). Recoverin also differs from CaM in that it has a much longer linker between EF3 and EF4 and an additional helix at the N-terminus, to which the myristoyl group is attached. In the apo structure (Tanaka *et al.* 1995), the myristoyl moiety is almost completely buried inside the protein, and contacts residues from EF1, EF2, and EF3. This changes dramatically in the Ca^{2+}-loaded NMR structure of myristoylated recoverin (Ames *et al.* 1997), in which the modified myristoyl group (a myristate analog was used to improve the solubility of the protein) is completely solvent exposed. This is accomplished by rotation of a 'hinge' at Gly42, which moves helix I of the first EF-hand, pulling the N-terminal helix away from the rest of the protein, and exposing the myristoyl group.

The dissimilarities with the CaM paradigm extend to the details of the conformational change. The largest Ca^{2+}-induced conformational changes in recoverin are not intra-EF-hand repacking similar to that seen in CaM, but rather, involve novel reorganizations of the packing between EF-hands, and between EF-hands and the linker loops (Ames *et al.* 1997, Nelson & Chazin, unpublished observations). It is not clear if the conformational changes in the protein only serve to expose the myristoyl group, or if they are also important in mediating interactions between recoverin and its target(s). However, it should be noted that the overall conformation seen in the crystal structure of unmyristoylated recoverin with one Ca^{2+} and one Sm^{3+} ion bound (Flaherty *et al.* 1993) is very similar to that seen in the Ca^{2+}-loaded, myristoylated recoverin NMR structure (Ames *et al.* 1997).

Sarcoplasmic calcium-binding proteins are another family of proteins which have four integrally associated EF-hands in one domain. These proteins are found in muscle cells and some neurons in invertebrates, and may be the functional analogs of vertebrate parvalbumins. The precise function of invertebrate sarcoplasmic CaBPs (SCPs) is not known, although like parvalbumins, they may be involved in protecting cells from the toxic effects of high Ca^{2+} levels and in quenching Ca^{2+} signals (Hermann & Cox 1995).

SCPs bind two or three equivalents of Ca^{2+}. EF1 and EF3 are predicted to be functional in all known SCPs, and both of these sites are occupied in the two structures of SCP. Like most SCPs, the proteins in both structures also have a third functional EF-hand. The third Ca^{2+} ion is bound to EF4 in SCP from *Nereis divisicolor* (Vijay-Kumar & Cook 1992) and to EF2 in SCP from *Amphioxus* (Cook *et al.* 1993). Despite this difference in the location of the third Ca^{2+} ion, the two structures are very similar. EF1 is clearly paired with EF2, and EF3 with EF4. However, there are extensive interactions between the two pairs, which pack almost end-to-end, positioning the binding loops from EF1 and EF2 at the opposite end of the molecule from those belonging to EF3 and EF4 (Fig 2e).

EF3 and EF4, which are both Ca^{2+}-loaded in *Nereis* SCP, adopt a conformation similar to that seen in the Ca^{2+}-loaded CaM domains, with approximately perpendicular interhelical angles. Although EF1 is Ca^{2+}-loaded in this SCP, EF2 is not, and this pair of EF-hands adopts a novel conformation. However, ion occupancy cannot be the only determinant of this conformation, because it is very similar to that seen in the SCP from *Amphioxus*, in which both EF1 and EF2 have Ca^{2+} bound. In fact, the conformations of all of the EF-hands are approximately the same in the two SCP structures.

In both structures, EF1 has an unusual substitution of an aspartate for the bidentate glutamate at the terminal loop position. However, the aspartate residue maintains bidentate Ca^{2+} coordination, leading to a contraction of the loop. It is not known if this substitution influences the Ca^{2+}/Mg^{2+} selectivity of the proteins. However, at least in *Nereis* SCP, all of the binding sites are of the Ca^{2+}/Mg^{2+} mixed type (Cox & Stein 1981). Therefore, in the resting cell, Mg^{2+} will be bound to SCP. The conformational changes associated with the exchange of Ca^{2+} for Mg^{2+} are not known, but it is known that these changes are nearly as fast as the necessary cation dissociation step, which is slower for Ca^{2+} than Mg^{2+}. In contrast, the conformational changes associated with adoption of a metal-free state are slower than the dissociation step (Engelborghs *et al.* 1990), consistent with NMR experiments that show that metal-free *Nereis* SCP is highly unstructured (Prêcheur *et al.* 1996).

The S100 proteins present a third unique manner in which four EF-hands are packed into a single domain. This subfamily of the EF-hand CaBPs has purported functions ranging from direct roles in Ca^{2+} transport to regulation of fundamental cellular processes including cell growth and proliferation. The S100 proteins have two EF-hands, but dimerize to create a four EF-hand globular domain as a basic structural/functional unit (Fig. 2c). Calbindin D_{9k}, which has been discussed separately, is the sole exception among the nearly 20 known S100 proteins, and exists as a monomer, presumably because it lacks the C-terminal residues that are critical for forming the interface between the two subunits. As previously noted, all S100 proteins are distinguished by an N-terminal pseudo EF-hand.

Apo and Ca^{2+}-loaded structures are available for the S100 proteins **calcyclin** and **S100B**. The basic fold of these S100 proteins is very similar. Each subunit consists of a CaM-like four-helix bundle. A well-packed hydrophobic interface is formed between the two subunits in the dimer, mediated primarily by interactions among helices I, I', IV and IV'. Despite this similarity in overall fold, there are significant differences between the structures of bovine apo S100B (Kilby *et al.* 1996) and rat apo S100B (Drohat *et al.* 1996), which remain somewhat of a mystery at the present time given that the sequences of the rat and bovine proteins differ by only a few residues. The rat apo S100B structure stands out among all S100 structures on the basis of a unique disposition of Helix III.

The structure of the Ca^{2+}-loaded state of S100B has been determined independently in three different laboratories. The structures of the rat (Drohat *et al.* 1998) and human (Smith & Shaw 1998) proteins were solved by NMR, while that of the bovine protein was determined by X-ray crystallography (the first crystal structure of a full-length S100; Matsumura *et al.* 1998). The structure of Ca^{2+}-loaded calcyclin has also been determined (by NMR; Sastry *et al.* 1998). Overall, the three-dimensional structures of S100 proteins are little changed by the binding of Ca^{2+}. For instance, the limited Ca^{2+}-induced conformational changes in the hydrophobic core of calbindin D_{9k} are strongly echoed in the individual subunits of the calcyclin dimer. The S100B structures show that the most significant Ca^{2+}-induced change occurs in the packing of Helix III (and III'), which leads to an increase in the exposure of hydrophobic surface. The Ca^{2+}-induced changes in calcyclin do not appear to be identical to those in S100B, although very high resolution structures will be required before firm conclusions can be drawn about these differences.

All of these structural analyses of S100 proteins support the hypothesis first proposed on the basis of the structure of apo calcyclin: the novel, integrally-packed dimeric structure binds to intracellular targets in a mode that is distinctly different from the classical Ca^{2+} sensor calmodulin (Potts *et al.* 1995). A Ca^{2+}-induced hydrophobic channel surrounded on both sides by acidic residues has been proposed as a site for binding targets based on the S100B structures (Matsumura *et al.* 1998; Smith & Shaw 1998). Studies of the interaction between S100B and peptides from target proteins, such as those of the peptide derived from the tumor suppressor p53 (Rustandi *et al.* 1998), are providing the first structural insight into the nature of this new mode of target recognition. However, three-dimensional structures are required to confirm this prediction and to fully understand target recognition in atomic detail. Such studies are underway in several laboratories.

New paradigms: EF-hand mediated dimerization

The recent crystal structures of **calpain domain VI** revealed another type of dimeric packing. Calpains are Ca^{2+}-activated cysteine proteases that exist as heterodimers between a heavy chain containing domains I-IV and a light chain containing domains V and VI, with the protease activity associated with domain II. The homologous domains IV and VI both contain EF-hand motifs, and interactions between these two domains seem to be responsible for the heterodimerization of calpain (Nishimura & Goll

1991; Crawford *et al.* 1993). Currently, the only structural information available for calpain is for homodimers of domain VI (Blanchard *et al.* 1997; Lin *et al.* 1997). This homodimerization is also seen in solution (Blanchard *et al.* 1996), so although it is not thought to be biologically relevant, it does not appear to be an artifact of crystallization. Furthermore, since the two EF-hand containing domains are very similar in sequence, domain VI homodimerization is probably a good model for the domain IV-domain VI interaction that mediates calpain heterodimerization (Blanchard *et al.* 1997; Lin *et al.* 1997).

The domain VI homodimers are formed by the pairing of two otherwise isolated EF-hands. Each monomer contains five EF-hands: EF1 and EF2 pack together, as do EF3 and EF4, leaving EF5 unpaired. These unpaired fifth EF-hands pack together to form a traditional EF-hand pair. This interaction seems to mediate dimer formation, although EF2 and EF4 from opposing subunits also interact. It has long been known that isolated EF-hands will dimerize (e.g. Shaw *et al.* 1990), however, this is the first example of the use of the strong tendency of EF-hands to associate in pairs to mediate protein dimerization.

The first three EF-hands in each domain VI monomer bind Ca^{2+}. EF2 and EF3 have canonical coordination schemes, but the coordination in EF1 is novel. Two sidechain oxygen ligands are replaced, one with a backbone carbonyl and the other by H_2O. Despite these differences, Ca^{2+} coordination is still pentagonal bipyramidal.

Both the apo and Ca^{2+}-loaded structures of the domain VI homodimers are available, allowing direct comparisons to determine the Ca^{2+}-induced conformational changes. These changes are relatively small. The largest differences between the apo and Ca^{2+}-loaded structures are in EF1 and in the positioning of the linker between EF1 and EF2, which moves closer to the EF3/EF4 pair upon Ca^{2+}-binding (Blanchard *et al.* 1997). Interestingly, in the structure of the domain VI homodimers complexed with PD150606, a non-competitive inhibitor of protease activity, residues in the linker between EF1 and EF2 make contacts with the inhibitor. Residues within EF1, EF2, and EF3 also contact the inhibitor. However, no significant differences in the structure are seen when PD150606 binds to Ca^{2+}-loaded calpain, and the mechanism by which this molecule inhibits protease activity remains unknown (Lin *et al.* 1997).

Expanded range: an extracellular EF-hand CaBP

EF-hand CaBPs have traditionally been considered a strictly intracellular protein family. However, the first unambiguously extracellular members of this family have recently been identified, and the crystal structure of the Ca^{2+}-binding domain of the extracellular protein **SPARC** (also called BM-40 and osteonectin) has been solved, revealing two functional EF-hands (Hohenester *et al.* 1996). SPARC (Small Protein, Acidic and Rich in Cysteines) consists of three domains: a short, acidic N-terminal segment, a follistatin-like domain, and a Ca^{2+}-binding domain.

The original structure of the isolated SPARC Ca^{2+}-binding domain was soon followed by a structure of a construct containing both the follistatin-like domain and the Ca^{2+}-binding domain (Hohenester *et al.* 1997). The conformation of the Ca^{2+}-binding domain is very similar in the two structures. Ca^{2+} is bound in both EF-hands. Its coordination is novel in EF1, which contains a one residue insertion and a substitution of a backbone carbonyl for one of the canonical sidechain carboxylate ligands. EF2 exhibits canonical Ca^{2+} coordination, but contains an unusual disulfide bond between Cys256, near the beginning of the binding loop, and Cys272, at the C-terminal end of the last helix.

Despite these unusual features, the SPARC EF-hand pair adopts a conformation similar to the open conformation seen in Ca^{2+}-CaM domains, although the interhelical angles are somewhat larger in Ca^{2+}-loaded SPARC (Hohenester *et al.* 1996). However, it is clear that structurally, the SPARC EF-hands are not as similar to those in the CaM domains as are the TnC, CaN-B, or even the RLC-N EF-hands (Nelson & Chazin, unpublished observations). Nor do the Ca^{2+}-loaded SPARC EF-hands expose a CaM-like concave hydrophobic target binding surface: the N-terminal portion of the Ca^{2+}-binding domain packs into the concave pocket created by the helices of the EF-hands. The first helix in this N-terminal segment interacts with the EF-hands in a manner similar to that seen in the interactions of CaM-like EF-hand CaBPs and their targets. Both EF-hands bind to the same side of this binding helix in SPARC, similar to the interaction mode observed in the calcineurin structures (Hohenester *et al.* 1997).

The biological role of Ca^{2+}-binding to SPARC is unclear, and the nature of the apo structure is not known. However, under the high Ca^{2+} concentrations found in the extracellular space, SPARC is probably always Ca^{2+}-loaded, so the EF-hands may be important for maintaining the native conformation of SPARC, and not be involved in Ca^{2+}-dependent processes. On the other hand, the Ca^{2+}-binding domain of SPARC does interact with targets, and the nature of these interactions is uncertain.

Clearly, further biological and structural studies are required to determine the relationship between the structure and function of the EF-hands in SPARC. In fact, important questions remain to be answered about all of the EF-hand CaBPs discussed in this review. Furthermore, many subfamilies are only represented structurally by one protein, and no structural information is available at all for many more subfamilies. While working from homology with known structures can yield useful insights into the structure and function of a new protein, the great diversity exhibited by the twelve EF-hand CaBPs reviewed here indicates that this large protein family can occupy a wide-range of structures, and novel conformations are sure to be discovered as the structures of more family members become available.

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Note

*The analysis was restricted to the scallop myosin structure, because only the α-carbon coordinates are available for the chicken skeletal myosin structure, precluding detailed comparisons. However, although there are some differences in the interface between the ECL and RLC and in the relative orientations of the two RLC domains in the two structures, the overall conformations of the individual light chain domains seem to be conserved between scallop and chicken skeletal muscle myosin (Houdusse & Cohen 1996).

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Appendix A: References for structures listed in Table 1

Structures are listed by PDB code, in the order in which they appear in table 1.

1CFC: Kuboniwa H, Tjandra N, Grzesiek S, Ren H, Klee CB, Bax A. 1995 Solution structure of calcium-free calmodulin. Nature Struct Biol 2, 768–776

1DMO: Zhang M, Tanaka T, Ikura M. 1995 Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. Nature Struct Biol 2, 758–767

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