Alpha II-spectrin is a major cortical cytoskeletal protein contributing to membrane organization and integrity. The Ca\(^{2+}\)-activated binding of calmodulin to an unstructured insert in the 11\(^{th}\) repeat unit of αII-spectrin enhances the susceptibility of spectrin to calpain cleavage, but abolishes its sensitivity to several caspases and to at least one bacterial derived pathologic protease. Other regulatory inputs including phosphorylation by c-Src also modulate the proteolytic susceptibility of αII-spectrin. These pathways, acting through spectrin, appear to control membrane plasticity and integrity in several cell types. To provide a structural basis for understanding these crucial biological events, we have solved the crystal structure of a complex between bovine calmodulin and the calmodulin-binding domain of human αII-spectrin (Protein Data Bank ID code 2FOT). The structure revealed that the entire calmodulin-spectrin binding interface is hydrophobic in nature. The spectrin domain is also unique in folding into an amphiphilic helix once positioned within the calmodulin-binding groove. The structure of this complex provides insight into the mechanisms by which calmodulin, calpain, caspase, and tyrosine phosphorylation act on spectrin to regulate essential cellular processes.

Spectrin αβ heterodimers are organized into filamentous structures composed of two related subunits. The alpha subunit contains 21 triple-helical homologous repeats that are further organized into five protease-sensitive domains (1). The 11\(^{th}\) repeat is the longest, and contains a unique insert that is implicated to play a critical role in spectrin integrity. The spectrin-based cytoskeleton represents a scaffold attached to both the plasma membrane and intracellular organelles by interactions involving both integral and peripheral membrane proteins as well as by direct lipid interactions (2-4). Spectrin integrity is critical for establishing organized membrane subdomains and their maintenance, supporting integral membrane protein durability (5,6). Deletions in spectrin or dominant negative blockers of spectrin disrupt membrane-protein distributions and may lead to total loss of critical signaling or transport proteins from the cell surface (7-10). Controlled spectrin proteolysis has been detected during both apoptotic and necrotic cell death processes (11-13), upon cell shape change (14), during cell differentiation (15-17), during lens development (18), and coincident with synaptic remodeling and dendrite outgrowth (19,20). It has been observed that during the early stages of apoptosis a characteristic 150 kDa fragment of αII-spectrin is generated by caspases regardless of the cell type and the apoptosis stimulus (21). In neurons, calpain catalyzed proteolysis has been linked with NMDA receptor activation and is thought to be crucial for synaptic and neuronal plasticity. Finally, the Pet toxin, a serine protease from enteropathogenic bacteria, catalyzes spectrin fragmentation and the consequent collapse of the cell membrane (22-24). In each case, the cellular response ensuing from such proteolysis is determined by cleavage at a specific site in the αII spectrin molecule.

The 11\(^{th}\) repeat of αII-spectrin contains an unusual, protease hypersensitive insert in helix C. This insert contains recognition sites for μ-calpain, caspases 2, 3, and 7, and the serine protease Pet toxin (13,23,25,26). The N-terminal side of the 11th repeat (herein termed αIIspec) is flanked by an SH3 domain while the C-terminal portion encompasses a calmodulin-binding domain that is proposed to be unstructured in solution (27). The Ca\(^{2+}\)-dependent calmodulin (CaM) binding to human αII-spectrin regulates spectrin’s proteolytic susceptibility, enhancing is cleavage by μ-calpain, while blocking caspase 2, 3, and 7 cleavage (28-30). Cleavage by calpain is also controlled by phosphorylation of residue Y\(^{1176}\) by c-Src, a kinase that binds to the flanking SH3 domain in the α10 repeat unit (31). When phosphorylated, αII-spectrin becomes resistant to calpain proteolytic activity (31). Thus, an insert in the αII repeat of the human αII-spectrin represents the convergence point of two crucial cellular signaling cascades.

The mechanism(s) of both the CaM-dependent regulation of spectrin proteolysis and the cross-talk between the two signaling networks at the site of αIIspec is not well understood. To address these...
questions we have determined the three-dimensional structure of the complex between bovine calmodulin and the proposed calmodulin-binding domain of human αII-spectrin (CaM-αIIspec) (25). The structure of this complex revealed that the CaM-αIIspec binding interface is composed entirely of hydrophobic residues, in striking contrast to previously determined CaM-peptide structures. CaM binding also induced a 23 residue stretch of αIIspec to fold into an amphiphilic α-helix with hydrophobic side-chains facing the CaM hydrophobic groove. These numerous hydrophobic interactions are reminiscent of those seen within a protein core, suggesting that complex formation is driven by an increase in solvent entropy. A detailed comparison of our structure with those of other CaM-peptide-complex structures also reveals that αIIspec is positioned uniquely in the CaM groove relative to previously known ligands (32-35). The crystal structure of the CaM-αIIspec complex thus provides a compelling structural basis for understanding how calmodulin regulates the susceptibility of αII-spectrin to proteolysis and thereby other downstream cytoskeletal signals.

Experimental Procedures

Complex preparation and crystallization - Bovine calmodulin was purchased from Sigma. The human αII-spectrin calmodulin binding domain containing cleavage sites for calpain, caspasases and Pet toxin was cloned into pGEX4T vector and expressed and purified as a GST-fusion protein from E.coli BL21(DE3) using the following procedure. An overnight culture of the E.coli BL21(DE3) using ampicillin, grown with shaking at 37 oC for 1 hour when it was induced with 1mM IPTG. The growth was continued for an additional 4 hours at 37° C. The cells were spun down at 5,000xg / 10 min. The cell pellet was resuspended in 50mM Tris-HCl pH 8.0, 50mM NaCl, 1mM EDTA, 1mM DTT, 0.1mM PMSF, 0.1mM benzamidine, 0.2 mg/mL of lysozyme and 1% Triton NaCl, 1mM EDTA, 1mM DTT, 5mM reduced glutathione. The protein was then further purified on a calmodulin-Sepharose 4B column (Pharmacia). The column was equilibrated with 50mM Tris-HCl, pH 8.0, 150mM NaCl, 5mM β-mercaptoethanol, 0.1mM PMSF, 1mM CaCl2. Prior to loading the column, CaCl2 was added to the sample to a final concentration of 10mM. The column was then washed with the above buffer until the baseline absorbance was restored. The GST-αIIspec was eluted with 50mM Tris-HCl, pH 8.0, 150mM NaCl, 5mM β-mercaptoethanol, 0.1mM PMSF and 5mM EGTA. GST was subsequently removed by thrombin cleavage and removed by absorption to glutathione-Sepharose. The αIIspec-CaM complex was formed from an equimolar solution of calmodulin and peptide. Crystals were obtained by the sitting-drop vapor diffusion method, mixing equal volumes of the protein complex and the well solution (50mM Tris-HCl, pH 8.0, 30% PEG 8000, 0.1M (NH4)2SO4) in a total volume of 40µL. The crystals belong to space group P2_12_1 (a=44.29, b=57.73, c=69.75 Å) with one molecule per asymmetric unit, and 49% solvent content.

Data collection, structure determination and refinement - Diffraction data were collected at room temperature on a Rigaku RAXIS II detector using a rotating copper anode as an X-ray source. The data were indexed in Denzo and scaled and reduced in Scalepack (36). The initial phases were determined by molecular replacement using the structure of calmodulin (PDB accession code: 1CDM). The search model was broken into two domains (residues 4-70 and 79-144) omitting the linker loop containing residues 71-80. Molecular replacement calculations were performed with Phaser 1.3 (37,38), searching for the two domains of calmodulin sequentially. The top solution had a Z score of 12 and good packing with no steric clashes. Upon a cycle of simulated annealing in CNS (39), the Fo-Fc map contoured at 2σ revealed a three-turn helix of the spectrin calmodulin binding domain. Further refinement was done in CNS (39), while electron density maps inspection and manual model rebuilding was done in Quanta. Ten percent of the data were randomly assigned to an Rfree test set for cross-validation (40). The final stages of refinement, including individual B-factor refinement, were performed against all the data. The final model yielded good geometry with 89.6% of residues in the most favored region and 10.4% in additional allowed orientations according to Procheck (41). It had an R factor 24.5% and Rfree of 26.9%. The crystallographic data and final model statistics are presented in Table 1.

CD spectroscopy - Circular dichroism studies of the purified αII-spectrin peptide alone, of CaM alone, and of the CaM-αIIspec complex, either with 0.1 mM CaCl2, or in the present of 0.1 mM EDTA, were carried out in 50 mM Tris-HCl 20 mM NaCl, pH 8.0 at RT. The molar circular dichroism was measured from 190 to 260nm, and the percentages of protein secondary structure estimated using the program K2d available at http://www.embl-heidelberg.de/%7Eandrade/k2d.html (42).
RESULTS AND DISCUSSION

Overall structure - The crystal structure of the calmodulin binding domain of αII-spectrin in complex with calmodulin was solved to 2.45Å resolution and refined to a final R factor of 24.5 % (R_free = 26.9%) (Table I). The final model contains 157 protein residues, 62 water molecules and four calcium ions. The CaM-αIIspec complex is compact and globular, like other CaM-peptide structures solved previously (Figure 2A) (32-35,43), with two calcium-binding lobes of CaM, designated as the N- and C-lobes, wrapped around the αIIspec peptide. All four calcium-binding sites are similar when compared with other CaM-ligand complex structures.

In order to crystallize the complex of CaM with αIIspec, a 42 residue peptide of the proposed calmodulin-binding domain of αII-spectrin was prepared (Figure 1A). This peptide contains cleavage sites for µ-calpain, caspases 2, 3 and 7 and the recently identified Pet toxin proteinases (23,28,30,44). A 23 residue-long stretch (residue A1198 to R1211) of the peptide was ordered and adopted an α-helical conformation. Five N-terminal residues of CaM, loop 70 (residues 74-80) and residue 116, were disordered and consequently could not be modeled into the CaM-αIIspec structure. Likewise, the 18 N-terminal residues of αIIspec, encompassing the calpain and caspase cleavage sites, and three extreme C-terminal residues, were missing from the map.

CaM-αIIspec binding is mediated entirely by hydrophobic interactions - Analysis of the CD spectra of the calmodulin binding domain of αII-spectrin as well as earlier modeling studies (27) show that it is disordered in solution (Figure 3). The structure of the CaM-αIIspec complex revealed that upon calmodulin binding αIIspec folds into an amphiphilic helix beginning with a proline residue at position 1191 (based on the human αII-spectrin numbering). The combined buried surface area at the interface between the two molecules is 2,590Å², which is a half of the αIIspec surface area. A more detailed analysis of αIIspec binding revealed a striking difference between the CaM-spectrin complex and other previously solved CaM-peptide structures.

It is well established that upon binding of Ca²⁺, two calmodulin lobes, designated as the N- and C-lobe, undergo a structural rearrangement to expose hydrophobic pockets and allow various ligands to bind. Flexibility in helix D (hD) that connects the two CaM lobes allows an additional conformational change upon ligand binding. In such a complex two CaM lobes wrap around the target ligand, stabilizing the closed conformation of the CaM-peptide complex. The typical CaM ligand is a helix of about 20 residues containing two hydrophobic side-chains separated by 8, 10, 14 or 16 residues that provide anchor points for the ligand (45,46). The proposed CaM-binding sequence of the αIIspec contains three hydrophobic residues, whereas the rest of the sequence does not bear any similarity with other known CaM ligands (Figure 1B). These hydrophobic residues put the αIIspec CaM binding domain into the 1-14 class of CaM binding peptides, or more precisely into its 1-8-14 subclass (46). The class/subclass name designates the spacing between the critical anchor residues in the CaM ligand. In all previously described CaM-peptide complexes the majority of interactions with the ligand are mediated through extensive hydrogen bonding networks that are supported with limited hydrophobic interactions.

However, unlike the other CaM-binding sequences that have been characterized, the interacting surface of the CaM-αIIspec complex is composed almost entirely of hydrophobic residues clustered into three hydrophobic pockets (Figure 4). The first CaM binding pocket is composed of the C-lobe residues from helices F (hF), H (hH), the extreme C-terminus of CaM and the loop connecting hF and helix G (hG). Two methionine residues, M124 and M144, form the first methionine cleft that binds the side-chain W1193 from αIIspec. At the bottom of the pocket are F102, L105, M108, F141 and M145 from CaM. The side-chains of F102 and M109 interact with A1195 from αIIspec, a residue positioned on the same face of the helix as W1192. The Van der Waals contacts between W1192 and the first CaM methionine cleft anchor the N-terminal end of αIIspec into the C-lobe of CaM (Figure 4A, B). Hydrophobic interactions between a tryptophan side-chain at the N-terminus of a ligand and the first hydrophobic pocket of CaM have previously been described (33,34). In those structures the tryptophan side-chain forms a hydrogen bond with the carbonyl oxygen of M124 of CaM. Conversely, in the CaM-αIIspec complex, the W1192 side-chain adopts a different rotamer conformation and interacts with the first methionine pocket via exclusively hydrophobic interactions (Figure 4B).

The second binding pocket is composed of residues from the N-lobe helix A (hA), C-lobe helix E (hE) and the loop connecting hF and hG. The αIIspec residue L1197 interacts with A15 and L18, while V1199 interacts with I85, A88 and M145. The anchor residue in the second pocket is clearly M1198, which makes contacts with V35, L39 and A112 (Figure 4C). The pocket expands further with M36 from CaM, which interacts with side-chains of T1201 and V1202 from αIIspec. This pocket represents the region where the hydrophobic surfaces of the two CaM lobes fuse into one continuous region.

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The third pocket is composed of residues from helices B, C, and D of the N-lobe of CaM. CaM side-chains F_{126}, F_{19}, I_{63}, H_{56} and F_{68} form the bottom of the pocket, while the wall is lined up with L_{32}, V_{45}, M_{51} and M_{72}. Finally, the pocket roof is composed of M_{36}, M_{51} and M_{71}. Three methionine residues M_{36}, M_{51} and M_{71} establish the second methionine cleft that interacts primarily with αIIspec residue F_{1205} (Figure 4B). Other αIIspec residues, such as T_{1201}, V_{1202}, and I_{1204} contribute with additional Van der Waals contacts. It is clear that the hydrophobic side-chains of the two αIIspec residues W_{1192} and F_{1205}, located at the opposite ends of the helix, interact with two methionine clefts located within different CaM lobes (Figure 4A,B). These residues along with M_{1198}, serve as anchor points for the αIIspec helix, playing a crucial role in CaM-αIIspec recognition.

The only two ionic interactions observed in the entire complex are the following: K_{1193} of αIIspec makes a hydrogen bond with E_{124} (N-lobe; hA), while N_{1206} forms hydrogen bonds with both the Q_{34} (N-lobe; hB) and E_{84} (C-lobe; hE) side-chains. Interestingly, these ionic interactions are contiguous to the major anchor pockets both sequentially and spatially. These small hydrogen bond networks further stabilize the orientation of the αIIspec helix. Finally, αIIspec residues from N_{1206} to the C-terminus and other polar side-chains do not contribute to the binding with CaM.

The calmodulin-binding-domain of spectrin binds differently in the CaM binding groove - We have compared our structure of spectrin’s calmodulin-binding-domain (CBD) with the following CaM-peptide complexes: CaM-CaMKII (35), CaM-MARCKS (43), CaM-NOSIII (32), CaM-smMLCK (34) and CaM-CaMKI (33). The ligands used in the comparison belong to different classes of calmodulin-binding proteins based on the motifs they contain: CaM-CaMKII is from the 1-10 class, CaM-MARCKS is from the basic class, whereas the others are from the 1-14 class of calmodulin-binding proteins (46). Further, CaM-smMLCK belongs to the basic 1-8-14, CaM-CaMKI is in the 1-14, and CaM-NOSIII is in the 1-5-8-14 subclass of the more general 1-14 class (46). The superimposition of Ca atoms of either the N-lobe (6-72) or the C-lobe residues (83-147) of CaM in our structure with those of other CaM-peptide complexes gave the following values: N-lobe r.m.s.d. = 0.77-1.31Å (67 Ca atoms superimposed) and C-lobe r.m.s.d. = 0.68-1.19Å (65 Ca atoms superimposed). These results suggest that the backbone conformation of the lobes in different CaM-peptide complexes is very similar.

Further, our data suggest that the relative orientation of the lobes differ in various complexes due to adjustments in the CaM molecule upon ligand binding. The calculated r.m.s.d. values were much higher. 2.02Å to 2.94Å for the CaM-smMLCK and CaM-MARCKS complexes, respectively when 132 Ca atoms (residues 6-72 and 83-147) were used for superimposition. Interestingly, calmodulin adopted a more open conformation upon binding to the αIIspec compared to that seen in the previously solved complexes. The N-terminal hA and the C-lobe hG are pushed apart and a shift is visible both in the second calcium binding site (loop 60) and in the N-lobe hD (Figure 5A). The flexibility of loop 60 is reflected in higher B factor values (~70Å$^2$) compared to the rest of the molecule (~46.4Å$^2$) (Figure 5D). It is possible that CaM had to open up more in order to accommodate the much longer peptide used in this study. Small spatial rearrangements of both the N- and C-lobes caused the exposure of the continuous hydrophobic binding pocket. This reflects adaptability of CaM to different ligands. Further, because of the ligand’s length, the structure of the complex between CaM and the αIIspec that was derived here may be a better representation of the physiological CaM-ligand complex. The superposition of CaM molecules derived from different CaM-ligand complexes allowed examination of the differences in the ligand positions within the CaM binding site.

When the CaM-αIIspec complex is viewed in the orientation shown in Figure 2A, it becomes evident that αIIspec binds differently in the CaM groove than do other CaM ligands (Figure 5B,C). The αIIspec is tilted upwards towards the second methionine pocket, where the side-chain of N_{1206} provides an additional hydrogen bond network stabilizing the ligand position. This tilt of the helix is present even when compared to the CaM-smMLCK complex that bears the greatest structural similarity with CaM-αIIspec. In all previously described complexes both the second and the third hydrophobic pockets are either empty (CaM-NOSIII, CaM-CaMKII, and CaM-MARCKS) or occupied by a small hydrophobic residue making a limited number of Van der Waals contacts with CaM (CaM-smMLCK). Conversely, in the CaM-αIIspec complex, there are numerous hydrophobic interactions between the ligand and the last two hydrophobic binding pockets of calmodulin. These hydrophobic interactions are reminiscent of those within the hydrophobic core of proteins, suggesting that complex formation with αIIspec is extremely stable and driven by increases in solvent entropy. The dissociation of CaM from αIIspec thus is likely to require dramatic changes in calmodulin structure, presumably the unfolding of calmodulin due to a fall in cellular Ca$^{2+}$.

Proteolysis inhibition by steric hindrance and signal transduction by domain folding - The α11 repeat, contains several proteinase recognition sites (Figure 1A). Caspases cleave at $\Delta_{1185}$, $\mu$-calpain cleaves at
Y$_{1176}$ and Pet toxin at M$_{1198}$ (13,23,25). Each of these cleavages activates distinct cellular responses such as cell shape change, neuronal growth elongation, and apoptosis. The structure of the complex between CaM and the αII-spectrin provides a structural basis for understanding many of these phenomena. It is apparent that calmodulin inhibits caspase and Pet toxin-catalyzed proteolysis of αII-spectrin by steric hindrance, while the stimulation of calpain proteolytic activity is probably accomplished through direct effects on the conformation of both the substrate and the target protease. The caspase cleavage site, 1182-DEDT-1185 is only three residues removed from A$_{1189}$ suggesting that calmodulin binding inhibits the caspase catalyzed proteolysis of αII-spectrin by steric hindrance. Once the calmodulin molecule binds to αII-spectrin, caspase cannot approach the recognition site. It is important to note that caspase cleaves the DETD sequence efficiently, suggesting that the cleavage site assumes a substrate-like conformation when αII-spectrin is not in complex with CaM. The CaM-dependent inhibition of caspase activity abolishes the degradation of αβ-spectrin heterodimer and retards the completion of the apoptotic cascade. In a fashion similar to caspase inhibition, calmodulin also inhibits the Pet toxin-catalyzed proteolysis of spectrin by steric hindrance. The target residue for Pet toxin is M$_{1198}$. In the CaM-αII-spectrin complex, M$_{1198}$ lies buried in the second hydrophobic pocket interacting with CaM residues V$_{35}$, L$_{39}$ and A$_{112}$ (Figure 4B).

The recognition site for the cysteine proteinase Milli- or μ-calpain is located adjacent to a putative PEST domain (47) at residue Y$_{1176}$, juxtaposed to helix C of the αII repeat of αII-spectrin (Figure 1A). Calmodulin binding to αII-spectrin accelerates the rate of μ-calpain cleavage of αII-spectrin, renders an adjacent βII-spectrin subunit susceptible to μ-calpain cleavage at Q$_{1441}$-S$_{1442}$, and modulates the quaternary association state of the post-cleaved spectrin heterodimer/heterotetramer (29,44,48). The consequences of calpain proteolysis of αII-spectrin are thus quite distinct from the action of the caspases, inducing the reorganization of membrane subdomains and cell shape transformation rather than cell-membrane fragmentation and blebbing (13,15-17,49). Calpain cleaves spectrin significantly only in the presence of calmodulin in vitro (30,48). Based on our structure, it is likely that these events are directly coupled to the CaM-induced folding of the αII-spectrin insertion containing the calpain cleavage site. The folding of the insert would constrain the conformation of the loop that contains the calpain cleavage site, forcing it to adopt a substrate-like conformation in which the scissile P1-P1’ bond (50) would assume the optimal orientation for calpain binding and proteolysis. These conclusions fit well with findings that the primary sequence of the natural calpain targets evolved to be suboptimal (51). The constraints in the spectrin insert presumably might also reorient the packing of helix C of the 11th spectrin repeat unit, triggering the conformational change that unmasks the adjacent β-spectrin subunit to attack by calpain.

In addition to the effects on the substrate conformation, calmodulin might affect the attacking calpain molecule through a direct interaction. The classic calpains are heterodimers consisting of large (L) and small (S) chains. The L-chain is the protease domain, while the S-chain serves as a chaperone, and the two interact through their calmodulin-like domains (52). An interesting regulatory mechanism involving dissociation of the chaperone-like S-chain has been suggested (53,54), along with the proposal that calpain activity might be regulated by binding either to endogenous activators (55) or by interaction with phospholipids (56,57) in vivo. It is plausible therefore, that CaM interacts directly with the calmodulin-like domain of the L-chain of calpain stimulating the dissociation of the regulatory S-chain. Thus, we postulate that calmodulin might stimulate calpain activity in two ways: (i) by presenting the substrate to the protease in the optimal conformation, and (ii) by tethering and stabilizing the tertiary structure of the attacking protease.

As noted above, calpain cleavage is also controlled by phosphorylation of the calpain target residue Y$_{1176}$ (31). Thus, the CaM-αII-spectrin complex represents a point of convergence of at least two cellular signaling cascades. The structure of the CaM-αII-spectrin complex presented here is the first attempt to understand the calmodulin-dependent regulation of αII-spectrin proteolysis and the downstream cytoskeletal signal transduction from a structural perspective. Further structural and biochemical studies employing progressively larger multi-subunit complexes will be necessary to decipher the precise mechanisms of the cross-talk between different signaling cascades that converge at the α subunit of spectrin.
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**FOOTNOTES**

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Table I Data collection and refinement statistics

| Crystal                                      |                  |
|----------------------------------------------|------------------|
| Space group                                  | P2₁,2₁,2₁        |
| Cell dimensions (Å)                          | a=44.29, b=57.74, c=69.75 |
| Number of crystals                           | 2                |

| Data collection                              |                  |
|----------------------------------------------|------------------|
| Resolution limit (Å)                         | 45.00 – 2.45     |
| Wavelength (Å)                               | 1.514            |
| Reflections (observed / unique)              | 260,350 / 6,253  |
| Completeness (overall / last shell; %)       | 89.8 / 87.1      |
| Rmerge (overall / last shell; %)             | 24.5 / 46.7      |
| I / σI (overall / last shell)                | 4.4 / 2.7        |
| Redundancy (overall / last shell)            | 5.5 / 2.1        |

| Refinement                                   |                  |
|----------------------------------------------|------------------|
| Number of molecules in asymmetric unit       | 1                |
| Resolution used (Å)                          | 45.00 – 2.45     |
| Effective resolution (Å)                     | 2.45             |
| Average B-factor (Å²)                        | 44.2             |
| Number of reflections (work / test)          | 5,588 / 665      |
| Number of protein atoms (amino-acids)        | 1,178 (157)      |
| Number of solvent molecules                  | 58               |
| Number of heterogen atoms (calcium ions)     | 4                |
| Rcryst (|F| > 0σ; %)                             | 24.5             |
| Rfree (|F| > 0σ; %)                              | 26.9             |
| R.m.s. deviations from ideality              |                  |
| Bond lengths (Å)                             | 0.008            |
| Bond angles (°)                              | 1.23             |
**FIGURE LEGENDS**

Fig. 1. Sequence and functional sites in αII-spec. (A) Schematic diagram of a portion of the human αII-spectrin domain structure depicting α9/10 (orange) and α11 (blue and green) repeats. The SH3 domain responsible for c-Src kinase binding is within the α9/10 repeat (light orange). A complete sequence of helix C from the α11 repeat containing an unusual insert that binds CaM is shown. The insert is in red letters, while the proposed CaM binding domain is in bold red italicized letters. A peptide (αII-spec) spanning residues E₁₁₁₇₄ to E₁₂₁₀ was used in this study. The helix symbol designates the visible part of the αII-spec peptide in the CaM-αII-spec structure presented here, showing that the proposed CaM-binding sequence fits perfectly with the structured region of the peptide. Hydrophobic residues W₁₁₉₂, M₁₁₉₈ and F₁₂₀₅, the landmarks of the 1-8-14 subclass of CaM binding proteins, are labeled with an asterisk (*). The proteinase recognition sites are labeled accordingly and the calpain cleavage site (Y₁₁₇₆) is also a target residue for c-Src kinase. (B) Sequence alignment of αII-spec with other canonical CaM ligands. Typically, there is minimal sequence similarity; the major hydrophobic residues responsible for binding CaM are shown in green and other hydrophobic anchor points are depicted in red. Sequence alignment was done using the program Multalin (58).

Fig. 2. Overall structure of the CaM-αII-spec complex. (A) Stereo view ribbon diagram of the complex between calmodulin (CaM) and calmodulin binding domain of αII-spectrin (αII-spec). The N-lobe of CaM is shown in green, the C-lobe in blue, αII-spec in red, and calcium ions are shown as gold spheres. The secondary structure elements of CaM are labeled as in the text, while the disordered regions are represented with dashed line (images were produced in Molscript (59) and rendered in Raster 3D (60)). (B) Stereo view of the simulated annealing omit |2Fo-Fc| electron density map of the N-terminal end of αII-spec (residues W₁₁₉₂-H₁₂₀₀) contoured at a level of 2σ.

Fig. 3. Circular dichroism (CD) measurements of the αII-spec at pH 8.0 reveal αII-spec to be random coil in solution. The blue line is αII-spec, the red line is CaM, and the green line is CaM-αII-spec. The helical content is calculated to be 5% for αII-spec and 30% both for CaM and CaM-αII-spec using K2d algorithm (42).

Fig. 4. The CaM-αII-spec binding surface is composed entirely of hydrophobic residues. (A) Molecular surface representations of the complex of CaM bound to αII-spec peptide. (Left) The hydrophobic residues of CaM binding groove are in yellow, while the rest of the molecule is in white. The αII-spec helix is shown as sticks with hydrophobic residues shown in green and polar, charged and uncharged, residues in blue. (Right) Surface charge distribution representation of CaM-αII-spec complex. The arrows point into the two CaM methionine clefts, labeled Met cleft 1 and 2. The N- and C-terminal residues of αII-spec are labeled as in text (produced in Grasp (61)). The view is rotated 90° clockwise in the plane relative to Figure 2A (B) Stereo-view of the major side-chain contacts between the N- (green) and C-lobes (blue) of CaM with αII-spec (red). The residues, shown as balls-and-stick, are labeled as in the text, while both methionine clefts are marked with an asterisk (*) (produced in Molscript (59) and rendered in Raster 3D (60)). (C) The second hydrophobic pocket positioned between two methionine clefts is composed of residues from both CaM lobes. The anchoring residues from αII-spec, Met₁₁₉₈ and V₁₁₉₉, interact with hydrophobic residues from both lobes of CaM. This view is rotated 90° clockwise around the horizontal axis relative to Figure 4B.

Fig. 5. The spectrin αII-spec binds differently to a more open CaM compared to ligands of previously solved CaM-peptide complexes. (A) The superimposition of the CaM lobes from different CaM-peptide complex structures revealed that CaM adopted a more open conformation once bound to αII-spectrin. The CaM-αII-spec is blue, while others are in beige (CaM-MARCKS, CaM-CaMKII, CaM-NOSIII). Only the backbones of the CaM molecules are shown. Similar results have been obtained with other CaM-peptide complexes (CaM-smMLCK and CaM-CaMKI; not shown for clarity). The superimposition using 132 Cα atoms of CaM (residues 6-72 and 83-147) was calculated using the CCP4 module Polypose (62). (B) The αII-spec calmodulin binding domain is tilted upward towards the second methionine cleft when compared to other CaM ligands. CaM is shown in an all atom representation, αII-spec is in red, the MARCKS peptide in green (PDB ID: 1IWQ), the CaMKII peptide in blue (PDB ID: 1CDM) and the NOSIII peptide in gold (PDB ID: 1NIW). The CaM molecule is in the same orientation as in A. (C) The αII-spec helix direction is different from that of other CaM ligands. The αII-spec is tilted up and twisted towards the C-terminal end of CaM. This view is rotated ~90° clockwise around the vertical axis relative to B (produced in Molscript (59) and rendered in Raster 3D (60)). The peptide ligand positions are generated upon superimposing Ca atoms of the N- and C-lobes of CaM using the CCP4 module Polypose (62). (D) B-factor (average main-chain) vs.
Residue plots for CaM-αIIspec complex reveal flexible regions in CaM. The average B-factor value for CaM is 46.4 Å$^2$ and for the αIIspec peptide is 36.2 Å$^2$. The most flexible regions in CaM are indicated on the plot and are labeled as in the text. The calculation was performed using the CCP4 module BVERAGE (63).
Figure 1

A

\[ \mu \text{-Calpain} \quad \text{Caspase} \quad \text{Pet toxin} \]

1174- EVYGMMPRDETDKTAASPWSARLMVHVATFNSIKE-1210

SH3
\[ \alpha 9/10 \quad \alpha 11-\text{hAhB} \quad \alpha 11-\text{hC} \]

B

| \text{\(\alpha llspec\)} |  | - | - | A | S | P | W | K | S | A | R | L | M | V | H | T | V | A | T | F | N | S | I | K | E |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CaMKII | L | K | K | F | N | A | R | R | K | L | K | G | A | I | L | T | T | M | L | A | T | R | N | F | S | - | - | - |
| CaMKI | I | K | K | N | F | A | K | S | K | W | K | Q | A | F | N | A | T | A | V | V | R | H | M | R | K | - | - | - |
| smMLCK | - | - | - | A | R | R | K | W | Q | K | T | G | H | A | V | R | A | I | G | R | L | S | S | - | - | - |
| NOSIII | - | - | - | R | K | K | H | F | K | E | V | A | N | A | V | K | I | S | A | S | L | M | - | - | - |
| MARCKS | K | K | R | F | S | F | K | K | S | F | K | L | S | G | F | S | F | K | - | - | - | - | - | - | - | - | - | - |

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