The Calmodulin-binding Site in α-Fodrin Is Near the Calcium-dependent Protease-I Cleavage Site

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Fodrin (brain spectrin) binds calmodulin and is susceptible to proteolysis by calcium-dependent protease I (CDP-I, calcium-activated neutral protease I, or calpain I). Both events involve the central region of the α-fodrin subunit, and calmodulin binding enhances the sensitivity of fodrin to CDP-I mediated proteolysis. Fragments of fodrin, generated chemically or proteolytically, which retain calmodulin binding activity have been identified and analyzed by two-dimensional peptide mapping and by direct protein sequencing. Both CDP-I and calmodulin interact with the terminal portion of the eleventh repetitive unit in fodrin, which is at the center of the molecule. CDP-I cleavage occurs between Tyr354 and Gly356 and preserves the calmodulin binding activity of the carboxyl-terminal fragment. In contrast, chymotryptic cleavage at Trp120 reduces the ability of this fragment to bind calmodulin, and tryptic cleavage beyond Trp120 completely eliminates calmodulin binding activity. It is concluded that Ser-Lys-Thr-Ala-Ser-Pro-Trp-Lys-Ser-Ala-Arg-Leu-Met-Val-His-Thr-Val-Ala-Thr-Phe-Asn-Ser-Ile-Lys, a 24-residue peptide which bridges repeats 11 and 12 of brain α spectrin contains the high affinity calmodulin binding domain.

The association of nonerythroid spectrin (fodrin) with the cortical cytoskeleton and with membrane protein receptors is post-translationally regulated. Intracellular rearrangements of this protein at the membrane occur in several cell types. In lymphocytes such reorganization has been observed following ligand binding (Levine and Willard, 1983), in neurons during development (Lazarides et al., 1984), in epithelial cells with the establishment of cell polarity (Nelson and Veshnock, 1986), in endothelial cells during migration on different substrates (Pratt et al., 1984), in platelets following activation (Fox et al., 1987), and in fibroblasts following cell transformation (Burridge et al., 1982). Fodrin also appears to be involved in the regulated release of secretory vesicles in exocrine cells (Perrin et al., 1987) and may participate in the development of long-term synaptic potentiation in hippocampal neurons (Lynch and Baudry, 1984, Siman et al., 1984, 1985).

Several signals regulating the above processes probably act directly on fodrin. The regulation of glutamate sequestration in postsynaptic membranes, a postulated event in the development of long-term synaptic potentiation, requires the proteolysis of fodrin by calcium-dependent proteases (Lynch and Baudry, 1984, Siman et al., 1984, 1985). A similar proteolysis of fodrin also occurs with platelet activation (Fox et al., 1987), and the primary site of CDP-I proteolysis in vitro is near the junction of the α subunit (Harris and Morrow, 1988). A second regulatory mechanism of fodrin involves the calcium-dependent binding of calmodulin, also to a site near the center of the α subunit (Harris and Morrow, 1988). Calmodulin enhances the susceptibility of fodrin to CDP-I proteolysis (Seubert et al., 1987), and both this cleavage and calmodulin reduce the ability of fodrin to cross-link actin filaments in vitro. Despite the probable importance of these activities, and the clear association between the CDP-I susceptibility of fodrin and calmodulin binding, the precise relationship between the sites of these events in fodrin remains uncertain.

In the present report, intermediate-sized peptides of α fodrin have been prepared either by proteolytic digestion or by chemical cleavage at cysteine residues using NTCB. The position of these fragments within the parent protein has been determined by two-dimensional peptide mapping and their calmodulin binding activity measured by gel overlay using 125I-calmodulin and by their ability to bind to a calmodulin-agarose affinity column under nondenaturing conditions. Gas-phase amino acid sequencing of calmodulin-binding peptides derived from CDP-I and chymotryptic digestion identified a sequence of 24 amino acids in which the calmodulin-binding site must reside. This sequence, beginning 16 residues distal from the site of CDP-I cleavage, bridges the eleventh and twelfth repetitive units of α-fodrin.

MATERIALS AND METHODS

Protein Preparation—Human fodrin was prepared as described (Harris et al., 1986) with the following modifications. Ion-exchange chromatography was performed at 4°C on a 1 x 10-cm column of Accell-QMA (Millipore) using a 170-mI linear gradient of 25–750 mM NaCl in 20 mM Tris-HCl, 0.5 mM EDTA, 1 mM 2-mercaptoethanol; pH 7.8. The fractions containing purified fodrin (monitored by A280 and SDS-PAGE) were dialyzed overnight at 4°C versus 20 mM Tris-HCl, 25 mM NaCl, 0.5 mM EDTA, 1 mM 2-mercaptoethanol, pH 8.0 (buffer A), and concentrated using a 1 x 2-cm column of DEAE-cellulose (DE52).

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Whatman) eluted with 20 mM Tris-HCl, 1 M NaCl, 0.5 mM EDTA, 1 mM 2-me, pH 7.5. The fodrin from the column (typically at 1–2 mg/ml) was dialyzed against buffer A and stored at 0 °C. Bovine fodrin, prepared from approximately 400 g of frozen brains, was prepared as described for human fodrin (Harris et al., 1986), with the following modifications. The frozen brain was thawed and homogenized in a Waring blender for 30 s. After preparing the washed membranes, 3 M KCl was added to achieve 0.6 M KCl in a total volume of 2 liters. EGTA, dithiothreitol, and diisopropyl fluorophosphate were added from stock solutions to final concentrations of 5, 2, and 0.5 mM, respectively. The pH was adjusted to 9.0 with 3 M Tris and the protein concentration was 37 °C for 30 min. The extract was recovered by centrifugation at 30,000 g for 60 min, and precipitated by 50% saturated ammonium sulfate at 4 °C. The precipitated protein, recovered by centrifugation, was resuspended in 20 mM Tris-HCl, 150 mM NaCl, 0.5 mM EDTA, 1 mM 2-me, pH 8.0 (TBS), and centrifuged at 30,000 × g for 30 min. The supernatant was made 10% in sucrose and centrifuged for 16 h at 4 °C at 110,000 × g in a Beckman SW 27 rotor. The floating lipid was removed, and the clear supernatant was fractionated on a 5 × 95-cm Sephacryl CL-4B column equilibrated in TBS. Bovine fodrin was further purified and concentrated as described above for the human material.

A 100 × 2-cm column chromatography was performed from fresh bovine brain by the method of Burgess et al., 1980. Purified calmodulin was incubated in 10 mM diisopropyl fluorophosphate overnight at 4 °C, and dialyzed versus several changes of 20 mM Tris-HCl, 25 mM NaCl, 0.1 mM CaCl\(_2\), 1 mM 2-me, pH 7.5, prior to use.

Affinity digestion of fodrin was carried out on cysteine residues with NTCB (Eshdat and Lemay, 1979). Fodrin at 1 mg/ml in 7.5 M guanidine HCl (or 8 M urea), 200 mM Tris-HCl, 1 mM EDTA, pH 8.0, was reacted with 513 mM NTCB for 1 h at room temperature. Cleavage was initiated after the pH was raised to 9.0 with 0.5 M Tris-NaOH, pH 12, followed by incubation at 37 °C for 16 h. The reaction was terminated by the addition of 2-mec to 25 mM, and the material was dialyzed versus several changes of 10 mM Tris-HCl, 0.5 mM EDTA, 1 mM 2-me, pH 8.0, at 4 °C, and lyophilized. Proteolytic digests were done in buffer A, except as noted, at protein concentrations of 0.4–1.5 mg/ml at 0 or 23 °C. Trypsin (N-p-tosyl-l-phenylalanine chloromethyl ketone treated-trypsin, Worthington), a-chymotrypsin (Worthington), and endoprotease Lys-C (Boehringer Mannheim) or endoprotease Glu-C (Boehringer Mannheim) were added to the protein at the enzyme/substrate ratios indicated. CDP-I, prepared from bovine heart (Croll and DeMartino, 1984) was used at an enzyme/substrate ratio of 1:25 (mol/mol) at free calcium concentrations of 0.1 mM. Digestions with CDP-I were performed after overnight dialysis at 4 °C of the fodrin against 20 mM Tris-HCl, 25 mM NaCl, 0.1 mM CaCl\(_2\), 1 mM 2-me, pH 7.5, to insure a constant free calcium concentration. Digests were terminated at various times by adding diisopropyl fluorophosphate (500 mM in isopropyl alcohol) to a final concentration of 5–10 mM, followed by either the addition of gel electrophoresis buffer for SDS-PAGE or by lyophilization for isoelectric focusing/SDS-PAGE. In separate experiments, diisopropyl fluorophosphate (obtained from Sigma) was found to inhibit CDP-I as well as EGTA in these experiments, and therefore it was used as the sole protease inhibitor.

Affinity Isolation of Calmodulin-binding Peptides—NTCB-generated fodrin peptides were dialyzed against 130 mM KCl, 20 mM NaCl, 10 mM HEPES, 1 mM CaCl\(_2\), and 1 mM 2-me, pH 7.3, and loaded onto a 1.25-ml calmodulin affinity column (10 mg of calmodulin/ml of gel, Bio-Rad) equilibrated in the same buffer. After the non-adherent protein had eluted, the column was washed with 6 M urea in 20 mM NaCl, 15 mM NaCl, 7.5 mM HEPES, 1 mM CaCl\(_2\), 1 mM 2-me, pH 7.3, to remove nonspecifically bound proteins. Calcium-dependent calmodulin-binding peptides were eluted from the column with the above urea containing buffer in which the calcium was replaced with 10 mM EGTA. The fractions containing the eluted proteins were lyophilized, desalted on a 0.6 × 22-cm Sephadex G-15 column, ammonium bicarbonate buffer and lyophilized prior to two-dimensional isoelectric focusing/SDS-PAGE.

Two-dimensional Peptide Mapping—Two-dimensional cellulose 125I-peptide mapping of Coomassie Blue-stained peptides was performed using established procedures (Elder et al., 1977, Speicher et al., 1982). Briefly, the peptides were cut from polyacrylamide gels and labeled with 125I using chloramine T. After removal of the free 125I, the gel slices were digested with a-chymotrypsin (Sigma, 50 μg in 50 μM ammonium bicarbonate) for 24 h at 37 °C. Peptides were recovered in the liquid phase and lyophilized. The peptides were dissolved in electrophoresis buffer, spotted onto 20 × 20-cm cellulose plates and separated by high voltage electrophoresis in the horizontal dimension and by ascending liquid chromatography in the vertical dimension. Peptide maps were visualized by autoradiography at −70 °C using Kodak XRP film and fluorescent intensifying screens.

Amino Acid Sequencing—Digested peptides were separated by two-dimensional isoelectric focusing/SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore) according to Matsudaira (1987). Peptides were visualized by Coomassie Blue staining. The peptides to be sequenced were cut from the membrane and stored at −20 °C. Polyvinylidene difluoride membranes were not precycled and were placed directly in the cartridge block of the protein sequencer. Peptides were sequenced on an Applied Biosystems model 470A Sequencer with on-line phenylthiohydantoin analysis (Applied Biosystems 120A high pressure liquid chromatography) using the standard program (03RPTH) without modification.

GeL Electrophoresis and Other Procedures—SDS-PAGE and two-dimensional isoelectric focusing/SDS-PAGE were performed by the method of Laemmli (1970) and O'Farrell (1975), respectively, except that 3(3-chloraminopropyl)-dimethylammonio)1-propanesulfonate was substituted for Triton X-100 in the isoelectric focusing dimension. All two-dimensional gels are presented with the basic region to the left. Proteins were visualized by Coomassie Blue staining. Protein concentrations were estimated by the method of Lowry et al., (1951). Calmodulin was labeled with 125I using immobilized lactoperoxidase and glucose oxidase (Enzymobeads, Bio-Rad) as previously described (Anderson and Morrow, 1987). 125I-calmodulin overlays were performed as previously described (Carlin et al., 1985). Protein transfers to nitrocellulose or polyvinylidene difluoride membranes were performed according to Tabor et al., (1979).

RESULTS

Proteolytic Fragments of Fodrin That Bind Calmodulin—Since the calmodulin binding activity of fodrin is rapidly destroyed by trypsin (Glenney et al., 1983, Harris and Morrow, 1988), chymotrypsin, endoprotease Glu-C and endoprotease Lys-C were tested for their ability to produce fodrin fragments smaller than 150 kDa which retained the ability to bind calmodulin. The results with chymotrypsin using 125I-calmodulin gel overlays to detect calmodulin-binding peptides are shown in Fig. 1. Digestion of fodrin with chymotrypsin under mild conditions yielded a 145-kDa peptide that was active (lane 2), although the affinity of this peptide for calmodulin appeared to be diminished relative to the intact molecule or to the 150-kDa natural fragment (lane 1) of the \( \alpha \) subunit.

![Fig. 1. Digestion of fodrin with chymotrypsin produced calmodulin-binding peptides as detected by gel overlay using 125I-calmodulin. A, Coomassie Blue-stained SDS-PAGE of: (lane 1) intact fodrin (10 μg); (lane 2) fodrin digested with chymotrypsin under mild conditions (20 μg fodrin, 15 min; 1:250 mol/mol, enzyme/substrate; 25 °C; (lanes 3 and 4) more extensively digested fodrin (20 μg, 1 and 16-h, digests respectively; 1:30 mol/mol, enzyme/substrate; 0 °C). B, Autoradiogram of the gel in A after 125I-calmodulin overlay. The calmodulin-binding fragments at 150 and 32 kDa arise from the \( \alpha \) subunit, while the peptide at 25 kDa arises from the \( \beta \) subunit (see text).]
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(which is also generated by CDP-I, see below, and Harris and Morrow, 1988). More extensive digestion with chymotrypsin led to the loss of the 145-kDa peptide and the appearance of two smaller peptides of 32 and 25 kDa that both bound 125I-calmodulin in the gel overlay assay (lanes 3 and 4). Peptide mapping (data not shown) of the 32-kDa peptide demonstrated that it was derived from the 145-kDa peptide and was similar to domain III of α-fodrin (Harris and Morrow, 1988), indicating that this chymotryptic calmodulin-binding peptide was derived from the center of the fodrin α subunit. Interestingly, the peptide map of the 25-kDa peptide indicated that it was derived from the β-fodrin subunit (data not shown). This was surprising since the intact β-fodrin subunit did not bind calmodulin as determined by 125I-calmodulin gel overlay assay (Harris et al., 1986) and as shown in lane 2 where the protein remaining near 240 kDa after mild digestion is intact β-fodrin (Harris and Morrow, 1988).

Fodrin digested with endoprotease Lys-C rapidly lost all calmodulin binding activity (data not shown). Proteolytic digestion of fodrin with endoprotease Glu-C cleaved the α subunit efficiently at the hypersensitive site and yielded two peptides (145-150 kDa), the smaller of which bound calmodulin in the gel overlay assay (similar to lane 2). Prolonged digestions with this enzyme failed to generate smaller peptides that retained calmodulin binding activity (data not shown). No calmodulin binding was detected in any of the peptides in the absence of calcium (data not shown).

NTCB Cleavage of Fodrin Generates Several Related Calmodulin-binding Peptides—Chemical cleavage of fodrin at cysteine residues with NTCB yielded numerous peptides with calmodulin binding activity. An analysis of these peptides by two-dimensional IEF/SDS-PAGE is shown in Fig. 2A. An autoradiogram of an electrotransfer (of a gel identical to that shown in 2A) overlaid with 125I-calmodulin is shown in Fig. 2B. More than 80 peptides were present in this NTCB digest (Fig. 2A), and six bound calmodulin avidly enough to be detected by the overlay technique (B). Mapping of the 27-kDa calmodulin-binding peptide from the gel in A indicated that it was derived from domain III of α-fodrin. The other calmodulin-binding peptides in this experiment were not mapped since their assignment to specific Coomassie Blue-stained peptides in two-dimensional gels (A) could not be made unambiguously.

Since it was possible that the gel overlay or blotting techniques might identify peptides that did not bind calmodulin under non-denaturing conditions, it was important to confirm the calmodulin binding ability of the NTCB fragments identified in Fig. 2 by a second technique using more physiologic conditions. This was accomplished by demonstrating the ability of these peptides to bind in a calcium-dependent fashion to a calmodulin-agarose affinity column. In initial experiments, NTCB digests of human or bovine fodrin were applied to a calmodulin affinity column as described under "Materials and Methods." The results were shown in Fig. 3. The majority of these peptides failed to bind the column (Fig. 3A). In order to exclude from the analysis the peptides which associated with the column by nonspecific adsorption as noted above, the column was washed with 6 M urea in the presence of calcium as indicated in the profile shown in A. An analysis of the peptides eluted by the urea wash is shown in B. Peptide mapping of the most abundant of these peptides near 39, 55, and 89 kDa demonstrated that they were derived from the terminal portions (NH₂ and COOH) of the α subunit (data not shown). No β subunit-derived peptides were identified in the fraction. The calcium-dependent calmodulin-binding peptides were then eluted from the column in the presence of 6 M urea and 10 mM EGTA (A). Analysis of these peptides by two-dimensional IEF/SDS-PAGE (C) demonstrated that they were a unique subset of the total digest, corresponded closely to those identified by 125I-calmodulin gel overlay (cf. Fig. 2B), and were distinct from the peptides eluted in the presence of urea and calcium (cf. Figs. 3, B and C). Peptide mapping (Fig. 4) of the most prominent peptides (labeled A–F in Fig. 3C) demon-
FIG. 3. Affinity chromatography on agarose-calmodulin of a NTCB digest of fodrin resulted in the isolation of a specific subset of Ca\(^{2+}\)-dependent calmodulin-binding peptides. A, A\(_{260}\) monitor tracing of the elution profile of the digest. B, two-dimensional IEF/SDS-PAGE analysis of the peptides eluted with 6 M urea in the presence of Ca\(^{2+}\). C, two-dimensional IEF/SDS-PAGE analysis of the peptides eluted with 10 mM EGTA in the presence of 6 M urea. Labeled peptides (A–F) shared a common part of domain III of the \(\alpha\) subunit of fodrin as determined by peptide mapping (see Fig. 4). The prominent doublet at 65 kDa, identified by peptide mapping, was derived from \(\alpha\) domains II–IV (data not shown). B and C are Coo massie Blue-stained.

FIG. 4. Autoradiograms of two-dimensional cellulose \(^{125}\)I-peptide maps of calmodulin-binding peptides demonstrated that all of these peptides shared a common part of domain III despite their charge and molecular weight heterogeneity. The letters (A–F) on the maps correspond to those on the gel in Fig. 3C, and their masses by SDS-PAGE were A, 94 kDa; B, 71 kDa; C, 53 kDa; D, 45 kDa; E, 27 kDa; F, 14 kDa. The arrows identify the two common spots found in all maps from peptides that bound to the calmodulin column in a calcium-dependent manner.

strated that they all were derived from the \(\alpha\) subunit and that they all shared common features (arrows, Fig. 4) which were also present in the map of domain III (Harris and Morrow, 1988). The maps of the peptides near 60 kDa in Fig. 3C also contained these common spots (data not shown). Although several of the peptides such as those labeled B and C in Fig. 3C, typically had a range of isoelectric points (but identical molecular weight), no differences were seen in their peptide maps. By comparing these peptide maps with the domain maps of the \(\alpha\)-fodrin subunit (Harris and Morrow, 1988), the placement of these peptides within fodrin could be determined. Peptide A (94 kDa) contained portions of domains I, II and III; peptides B and C (71 and 53 kDa, respectively) were contained within domains III and IV; peptides D (45 kDa) and E (27 kDa) contained portions of domains II and III; and peptide F (14 kDa) was contained completely within domain III. Thus, each of the calmodulin-binding peptides contained all or part of domain III, which is situated at the center of the \(\alpha\) subunit. These alignments are presented schematically in Fig. 6.

Chymotrypsin and CDP-I Cleave \(\alpha\)-Fodrin at Different Re-
idues within the Hypersensitive Site—The region joining domains II and III is unusually susceptible to proteolytic cleavage (Harris and Morrow, 1988) and CDP-I rapidly and stoichiometrically cleaved the α subunit of fodrin at this hypersensitive site (Harris and Morrow, 1988). CDP-I proteolysis of α-fodrin resulted in the generation of complimentary halves of the α subunit that differed in their pl but displayed identical and anomalous molecular weights as determined by IEF/SDS-PAGE. Transfer of CDP-I-digested fodrin onto PVDF membranes resulted in quantitative transfer of the α subunit-derived peptides, but virtually no transfer of the intact β subunit (Fig. 5A). Presumably the failure of the β subunit to transfer is related to the tendency of this subunit to aggregate (Woods and Lazarides, 1986). The more basic 150-kDa CDP-I-generated peptide, which is the calmodulin-binding fragment (Harris and Morrow, 1988), was cut from the membrane and subjected to gas-phase sequencing. Reliable information was obtained for 18 of the first 23 residues; the results are presented in Fig. 6. No secondary sequences were observed in this material, indicating the cleavage by CDP-I at this site is highly specific.

Cleavage of α-fodrin at the hypersensitive site with chymotrypsin also generated two peptides which were complimentary halves of the α subunit. (These peptides are unresolved in Fig. 1, lane 2, due to their similar molecular weight).

A two-dimensional IEF/SDS-PAGE analysis of this digest revealed a pattern nearly identical to that in Fig. 5A, (Harris and Morrow, 1988). However, the cleavage site of chymotrypsin was not identical to that of CDP-I, since the chymotryptic fragments had reduced avidity of 125I-calmodulin (Figs. 1 and 5). Gas-phase sequencing of the 150-kDa calmodulin-binding chymotryptic fragment yielded reliable information for 27 residues (Fig. 6). As with CDP-I, no secondary cleavages were detected in this sequence, indicating the high specificity of chymotryptic cleavage under the conditions used. The effect of chymotrypsin and CDP-I proteolysis on the calmodulin binding activity of fodrin is quantitated in Fig. 5, B and C. Equal quantities of fodrin, either undigested (Fig. 5B, left lane), CDP-I digested (Fig. 5B, center lane), or chymotrypsin-digested (Fig. 5B, right lane) were analyzed for 125I-calmodulin binding activity by gel overlay. Cleavage by CDP-I did not significantly diminish (p > 0.05) the quantity of 125I-calmodulin bound by the molecule and its fragments; however, cleavage by chymotrypsin reduced the net binding.

The Calmodulin-Binding Site of α-Fodrin Bridges the Domain II–III Junction—The domain structure of the α-fodrin subunit as defined by proteolytic digestion with trypsin (Harris and Morrow, 1988) is shown in Fig. 6A. This domain structure is aligned with the sequence repeat structure defined by Speicher and Marchesi (1984), and as deduced from McMahon et al. (1987) and Leto et al. (1988) (Fig. 6B). Although most of the molecule is comprised of regular 106-residue repeating units, repeats 10 and 11 are abnormally short and long, respectively. Based on the data presented here the eleventh repeat must contain the site of calmodulin binding as indicated in Fig. 6, B and C. An enlargement of this region demonstrates the alignment of the three smallest calmodulin-binding peptides generated by NTCB digestion (C and D). All of the calmodulin-binding peptides span portions of repeats 11 and 12 and the amino-terminal portion of repeat 13. The 32-kDa chymotrypsin-generated calmodulin-binding fragment is also derived from this region (not shown in Fig. 6). These peptides span the domain II–III junction, the region previously associated with calmodulin binding (Harris and Morrow, 1988). The amino acid sequence of the terminal portion of repeat 11 and the first 6 residues of repeat 12 is shown in Fig. 6E for human brain spectrin, along with the cleavage sites of CDP-I and chymotrypsin (arrows, E) derived from the sequencing studies reported here. The alanine and serine immediately below the sequence shown in E represent substitutions found in the bovine brain material (that was sequenced) as compared to the human sequence. CDP-I cleaved the α subunit on the carboxyl side of tyrosine 104 while chymotrypsin cleaved on the carboxyl side of tryptophan 120. Since chymotryptic cleavage at this latter residue significantly reduces the calmodulin binding activity of fodrin, it is likely that the residues in this region contribute to the binding site. Similarly, trypsin rapidly eliminates the calmodulin activity of fodrin, presumably due to cleavage at either Lys101, Arg14, or Lys6 (of repeat 12). In conclusion, the sequence Sery-Lys-Thr-Ala-Ser-Pro-Trp-Lys-Ser-Ala-Arg-Leu-Met-Val-Thr-Val-Ala-Thr-Phe-Asn-Ser-Ile-Lys appears to represent the calmodulin-binding site within α-fodrin.

**DISCUSSION**

These results identify a single high affinity calmodulin-binding site and the specific site of CDP-I and chymotrypsin cleavage within the hypersensitive site of α-fodrin. Several lines of evidence indicate that this is a physiologically relevant...
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Fig. 6. Localization of the sites of CDP-I cleavage and calmodulin binding within fodrin. A, the proteolytic domain structure of brain spectrin as defined by tryptic digestion (Harris and Morrow, 1988). The Roman numerals indicate the domains and their sizes (kDa) as determined by SDS-PAGE as indicated. B, the corresponding 106-residue repeat structure (defined by Speicher and Marchesi, 1984, and as deduced from McMahon et al., 1987, and Leto et al., 1988). Calmodulin binds near the center of the molecule, in the carboxyl half of repeat 11. C, the alignment of the major calmodulin-binding NTCB peptides, with their apparent masses in kDa, in an expanded view of the center of the molecule (repeats 9–13). D, the positions of the cysteine residues are indicated (SH) to demonstrate the origin of the NTCB-generated calmodulin-binding peptides. The smallest calmodulin-binding NTCB fragment, N14, presumably arises from material that was previously cleaved by CDP-I in vivo and then by NTCB. E, the sequence of the terminal portion of repeat 11 and the beginning of repeat 12 of human brain spectrin is shown. (The amino acid substitutions indicated below the peptide (in parentheses) were found in the bovine fodrin protein sequence). The peptide SKTASPWKDARLMVHTVAFNSIK (from residue 114 to the carboxyl terminus of repeat 11 and the first 6 residues of repeat 12) is likely to include the actual calmodulin binding region (see text).

Table I

All sequences compared to SKM MLCK using “Bestfit.” Capital letters represent longest region that yielded the best fit, while lower case letters represent additional amino acids in the sequences identified as the calmodulin binding domains. Vertical lines indicate similar amino acids as compared to SKM MLCK.

| Protein   | 10 | 20 | 30 | % Similarity |
|-----------|----|----|----|-------------|
| SKM MLCK* | KKYLMKRRWK KNFTIAPSAAN RFKISSSGA LM | ||||| 100 |
| CaATPaseb | eIRR QGILWFRGLN RIQTQIKVKN AFssslhef | ||||| 38 |
| SKM PPKc | RVRKPVYRE IVIRDYPYALR PLRL | ||||| 38 |
| SKM PFKd | rgRSPMNNEV YKLLAHRPP APKSGSYRVA VM | ||||| 42 |
| CAM II'  | FNNRRNLK GAILTMLAT Rnf | ||||| 53 |
| B-SPECf  | rkdni1RL WSYLQELLSQ RQRALLETLA Lqk | ||||| 35 |
| Fodrin    | sktaSPWKSARLMVHVTAFNSIK | ||||| 32 |

*SKM MLCK, rabbit skeletal muscle myosin light chain kinase from Blumenthal et al. (1985).

bCaATPase, human erythrocyte Ca2+ pump from James et al. (1988).

cSKM PPK, rabbit skeletal muscle phosphorylase kinase γ subunit from Lukas et al. (1986).

dSKM PFK, rabbit skeletal muscle phosphofructokinase from Buschmeier et al. (1987).

'CAM II, rat brain type II Ca2+/calmodulin-dependent protein kinase β subunit from Bennet and Kennedy (1987).

fB-SPEC, human erythrocyte spectrin β subunit.4
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calmodulin binding domain. 1) All α-fodrin peptides that bind calmodulin in a calcium-dependent manner in solid-phase gel overlay assays also bind calmodulin in agarose affinity columns in a calcium-dependent manner under non-denaturing conditions. 2) All of these calmodulin-binding peptides encompass a common region of the α subunit. 3) The CDP-I derived 150-kDa peptide retains full calmodulin binding activity, while an overlapping chymotrypsin-generated 150-kDa peptide devoid of 16 amino-terminal residues has reduced calmodulin binding activity. 4) The sequence of the putative calmodulin binding domain is similar to other calmodulin-binding peptides. The calmodulin-binding site is in the center of the α subunit, overlapping structural repeats 11–12. This location is consistent with that determined by peptide mapping studies (Harris and Morrow, 1988), immunologic data (Glenn et al., 1983), and calmodulin binding studies on fusion proteins generated from Drosophila cDNA clones (Byers et al., 1987). The calmodulin-binding site in α-fodrin is distinct in terms of both sequence and location from that in human erythrocyte spectrin, which binds calmodulin near the amino terminus of the β subunit (Anderson and Morrow, 1987). It is difficult to reconcile these findings with the report of Takio et al. (1985), in which the calmodulin-binding site was reported to be 10–20 nm from the midpoint of a 220-nm tetrameric molecule. This translates to approximately 24–48 kDa from the amino terminus of the molecule, which would place the binding site in the middle of domain I (Harris and Morrow, 1988). We cannot demonstrate that domain I (generated by trypsin digestion) binds calmodulin, nor are we fragments that bound “specifically” to the calmodulin affinity column in a calcium-dependent manner derived solely from domain I. However, since these two studies have used dissimilar approaches to identify a calmodulin-binding site, it is possible that under different conditions additional calmodulin binding sites may become apparent. Similarly, as noted earlier (Fig. 1), a 25-kDa peptide derived from the β subunit by chymotrypsin digestion bound 125I-calmodulin in a gel overlay assay. The binding of calmodulin to this peptide is probably not physiologically relevant, since the intact β subunit (top band of lane 2, Fig. 1) does not bind 125I-calmodulin (see also Harris et al., 1986). Thus, it appears that peptides may acquire the ability to bind calmodulin in solid-phase assays after proteolysis and/or degradation by SDS. Studies determining calmodulin binding activity solely by solid-phase binding assays must therefore be interpreted cautiously.

The exceptionally high specificity of CDP-I for a single hypersensitive site in α-fodrin is remarkable, given that highly susceptible residues (X-Tyr and X-Arg, where X is large and hydrophobic), occur at least 35 other times within mammalian α-fodrin (McMahon et al., 1987, Leto et al., 1988) and given the apparent broad activity of CDP-I against both peptide substrates (Hirao and Takahashi, 1984, Sasaki et al., 1984) and other proteins (Fischer et al., 1986). Thus, the conformation of the cleavage site appears to be a greater determinant of the specificity than the specific residues within the cleavage site. The proteolytically hypersensitive site in fodrin is located in the longest repeat, 11, of the α subunit. Predictions of the secondary structure of the region encompassing the site of CDP-I cleavage based on Chou and Fasman (1978) indicate that this region will be nonhelical, similar to the predicted structure of the region of CDP-I proteolysis in vimentin (Fisch et al., 1985). The increased exposure engendered by such nonhelical secondary and tertiary structure would explain the extreme susceptibility of this area to proteolytic digestion and appears to produce the most favored conformations for cleavage at the Tyr105 and Gly106 bond by CDP-I.

The sequences of the calmodulin binding region in several calmodulin-binding proteins are aligned in Table I. While some of these peptides share limited homology, no clear consensus sequence can be appreciated, and the fodrin sequence reported here shows the least degree of similarity. It has been suggested that a common secondary structure in calmodulin-binding peptides is an amphipathic α helix flanked by basic residues (Cox et al., 1985, Malencik et al., 1986, O'Neil et al., 1987). The calmodulin binding domain of α-fodrin, while amphipathic, contains a proline residue that appears to be essential for full activity. In addition, the secondary structure predicted for this region based on the criteria of Chou and Fasman (1978) (using the program PEPtidestructure, from the Wisconsin Molecular Biology Computer Group) is that of a rigid turn connected to a weak helix and/or undefined structures and introduced the idea that an amphipathic α helix is not the only structure that can bind calmodulin with high affinity. Therefore, if the structure of fodrin is predicted, the results presented here reinforce the notion that an α helix is not necessary for a high affinity calmodulin-binding site and extends the concept to proteins that bind calmodulin.

The close proximity of the CDP-I cleavage site to the site of calmodulin binding is significant since these two activities are functionally linked. The binding of calmodulin to α-fodrin accelerates the rate of CDP-I proteolysis of both subunits of fodrin (Seubert et al., 1987). Significantly, after α-fodrin has been cleaved by CDP-I, its ability to cross-link actin filaments becomes reversibly regulated by calmodulin. Thus, calmodulin and CDP-I exert a synergistic regulatory effect on fodrin function; the observations reported here provide a firm structural basis for these functional observations. In the future, it will be of interest to identify the sites of subunit-subunit interaction, since α–β interactions are clearly altered by CDP-I proteolysis and calmodulin binding.

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