Tryptophan Residues in Caldesmon Are Major Determinants for Calmodulin Binding†

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ABSTRACT: Calmodulin has been shown to interact with the COOH-terminal domain of gizzard h-caldesmon at three sites, A (residues 658–666), B (residues 687–695), and B′ (residues 717–725), each of which contains a Trp residue [Zhan et al. (1991) J. Biol. Chem. 266, 21810–21814; Marston et al. (1994) J. Biol. Chem. 269, 8134–8139; Mezgueldi et al. (1994) J. Biol. Chem. 269, 12824–12832]. To determine the contribution of each of the three Trp residues in the calmodulin–caldesmon interaction, we have mutated the Trp residues to Ala in the COOH-terminal domain of fibroblast caldesmon (CaD39) and studied the effects on calmodulin binding by fluorescence measurements and using immobilized calmodulin. Wild-type CaD39 binds with a $K_d$ of 0.13 × 10⁻⁶ M and a stoichiometry of 1 mol of calmodulin per mol of caldesmon. Replacing Trp 659 at site A or Trp 692 at site B to Ala reduces binding by 22- and 31-fold ($K_d$ = 2.9 × 10⁻⁶ M and 4.0 × 10⁻⁶ M), respectively, and destabilizes the CaD39–calmodulin complex by 1.75 and 1.94 kcal mol⁻¹, respectively. Mutation of both Trp 659 and Trp 692 to Ala further reduces binding with a $K_d$ of 6.1 × 10⁻⁶ M and destabilizes the complex by 2.17 kcal mol⁻¹. On the other hand, mutation of Trp 722 at site B′ to Ala causes a much smaller decrease in affinity ($K_d$ = 0.6 × 10⁻⁶ M) and results in a destabilization energy of 0.87 kcal mol⁻¹. To investigate the relative importance of the amino acid residues near each Trp residue in the calmodulin–caldesmon interaction, deletion mutants were constructed lacking site A, site B, and site A+B. Although deletion of site A decreases binding of CaD39 to calmodulin by 13-fold ($K_d$ = 1.7 × 10⁻⁶ M), it results in tighter binding than mutation of Trp 659 to Ala at this site, suggesting that the residues neighboring Trp 659 may contribute negatively to the interaction. Deletion of site B causes a similar reduction in binding ($K_d$ = 4.1 × 10⁻⁶ M) as observed for replacing Trp 692 to Ala at this site, indicating that Trp 692 is the major, if not the only, binding determinant at site B. Deletion of both site A and site B drastically reduces binding by 62-fold. Taken together, these results suggest that Trp 659 and Trp 692 are the major determinants in the caldesmon–calmodulin interaction and that Trp 722 in site B′ plays a minor role.

Caldesmon is an actin-binding protein present in both smooth muscle and nonmuscle cells [for reviews, see Sobue and Sellers (1991), Matsumura and Yamashiro (1993), and Marston and Redwood (1991)]. Smooth muscle h-caldesmon has a $M_r$ of 87 000 and contains three structurally and functionally distinct domains: an NH₂-terminal domain (residues 1–250), a middle domain (residues 251–400), and a COOH-terminal domain (residues 401–756). While all three domains have been shown to bind tropomyosin in vitro (Redwood & Marston, 1993; Graceffa, 1987; Watson et al., 1990), the NH₂-terminal domain also binds myosin (Velaz et al., 1990; Ikebe & Reardon, 1988), and the COOH-terminal domain interacts with actin (Bartegi et al., 1990; Mezgueldi et al., 1994) and the Ca²⁺-binding proteins calmodulin (Zhan et al., 1991; Zhuang et al., 1995; Marston et al., 1994) and caltrpin (Mani et al., 1992). Both NH₂- and COOH-terminal domains of smooth muscle caldesmon are conserved in nonmuscle caldesmon with similar primary structures and functions (Hayashi et al., 1991; Bryan & Lee, 1991). The h-caldesmon middle domain, which has a high α-helical content based on secondary prediction and circular dichroism measurements (Wang et al., 1991a), is absent in the nonmuscle isoform (Ball & Kovala, 1988). Since both smooth muscle and nonmuscle caldesmon have similar functions in vitro, it is not clear what functional role is played by the middle domain.

There is strong in vitro evidence supporting a regulatory role for caldesmon in smooth muscle contraction and in nonmuscle cell motility. The COOH-terminal domain of caldesmon inhibits actomyosin ATPase by blocking the interaction of actin and myosin and/or inhibiting a kinetic step of the actomyosin ATPase cycle (Marston & Smith, 1985; Ngai & Walsh, 1984; Valez et al., 1989; Lash et al., 1986; Horiuchi et al., 1986). The inhibition can be released by Ca²⁺–calmodulin (Horiuchi et al., 1986; Sobue et al., 1985; Ngai & Walsh, 1984). It is generally agreed that there are multiple Ca²⁺–calmodulin-binding sites at the COOH-terminal domain of caldesmon. However, there is disagreement concerning the location of the sites and the relative contribution of each site in binding to calmodulin and in the release of caldesmon inhibition of actomyosin ATPase (Wang et al., 1996; Marston et al., 1994; Zhuang et al., 1995; Huber et al., 1996).
It has been proposed that $\text{Ca}^{2+} - \text{calmodulin}$ interacts with three sites in chicken gizzard caldesmon: M658-W-E-K-G-N-V-F-S666 (site A), S687-R-I-N-E-W-L-T-K695 (site B), and G717-K-R-N-L-E-W-Q725 (site B‘) (Zhan et al., 1991; Marston et al., 1994; Mezgueldi et al., 1994). Each of the three sites contains a Trp residue, which is believed to be involved in calmodulin binding as has been shown by fluorescence measurements (Shirinsky et al., 1988). Recent NMR data by Huber et al. (1996) showed the distinct $\text{Ca}^{2+} - \text{calmodulin}$ interaction with Trp residues 692 and 722 at sites B and B‘, respectively. Another report by Wang et al. (1996) has shown that there are three calmodulin-binding determinants localized within residues 628–717, which encompasses only sites A and B. However, deletion of residues 718–756, containing site B’, had no effect on calmodulin binding. The apparent discrepancy could be due to the use of small caldesmon fragments of various lengths, which likely have conformations quite different from that of the intact protein or the COOH-terminal domain.

The aim of the present study is to determine the contribution of each Trp residue at sites A, B, and B‘ in the caldesmon–calmodulin interaction. In order to minimize disruptions to the conformation of the caldesmon COOH-terminal domain, we have mutated the Trp residue in each site to Ala and in the COOH-terminal domain of human fibroblast caldesmon (CaD39) and studied the effects on calmodulin interaction. In order to minimize disruptions to the conformation of the caldesmon COOH-terminal domain, we have mutated the Trp residue in each site to Ala and in the COOH-terminal domain of human fibroblast caldesmon (CaD39) and studied the effects on calmodulin binding. We present here fluorescence and sedimentation binding data showing that Trp 659 and Trp 692, in sites A and B, respectively, are important in $\text{Ca}^{2+} - \text{calmodulin}$ binding whereas Trp 722 in site B‘ plays a minor role. To determine the contribution of the amino acid residues neighboring the Trp residues at each site to calmodulin binding, we have constructed deletion mutants lacking sites A, B, and A+B in CaD39. Binding data using the deletion mutants suggest that amino acids near Trp 692 at site B contribute little, if at all, to calmodulin binding, whereas amino acids surrounding Trp 659 may contribute negatively to binding.

**MATERIALS AND METHODS**

**Protein Preparations.** Smooth muscle caldesmon was prepared from chicken gizzard as described by Bretschler (1984). Recombinant COOH-terminal fragment of human fibroblast caldesmon (CaD39) and all mutants were expressed in *Escherichia coli* and purified as described by Novy et al. (1993), with the following modifications: the ammonium sulfate precipitation was performed from 0 to 55%, and the pooled samples from the MonoS column were dialyzed against ammonium bicarbonate and lyophilized. Bovine brain calmodulin, which is identical in sequence to chicken gizzard caldesmon, was purified according to the method of Gopalakrishna and Anderson (1982).

All protein concentrations, except for calmodulin, were determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard. Bovine brain calmodulin concentration was determined using the absorption coefficient $A_{405}$ at 276 nm = 2.0 (Wolff et al., 1977).

**In Vitro Site-Directed Mutagenesis.** The point mutants replacing Trp with Ala were constructed by the method of Deng and Nickoloff (1992) using the “Transformer site-directed mutagenesis kit, 2nd version” (Clonetech), in the pETCaD39, a gift from Dr. J. J.-C. Lin, University of Iowa. The mutagenesis was performed in the pET-3d vector. The deletion mutants removing the calmodulin-binding sites A, B, and A+B were constructed by PCR mutagenesis using the following mutagenic primer pairs: A: 5’TCAAGAGTTTCTCGCAGCGAC3’, 5’GTGGGGGGA/ACTCTTGATGTGGCCTACA3; B: 5’GGTTTCTCTACTCCGAGATGGGAA/ACACAG3’, 5’CTGGGTGT/AGAACCCTACCTTCAAG3’. The deletion mutants removing the putative calmodulin-binding sites A and B corresponding to the chicken gizzard h-caldesmon sequences (with the corresponding human fibroblast caldesmon sequences in parentheses): A668–666 (460–468) and A687–695 (449–497). A double mutant missing both sites A and B, A658–666,687–695 (460–468,489–497), was also constructed.

DNA sequence analyses were performed to confirm the identity of the Trp-to-Ala and deletion mutations.

**Fluorescence Studies.** The binding of calmodulin to CaD39, W659A, W692A, W659,692A, or W722A was monitored by tryptophan fluorescence on a Perkin-Elmer LS-50 spectrometer at 25 °C. A stock solution of calmodulin (0–8 µM final concentration) was titrated into a 1 µM solution of caldesmon fragment such that the total volume change was less than 15%; corrections to the intensity measurements were made for dilution during the titration. The buffer was 20 mM Tris-HCl, pH 7.2, 0.5 mM CaCl2, 100 mM NaCl, 1 mM DTT, and 0.1 mM NaN3. The excitation wavelength was at 295 nm, and the excitation slit width was 5 nm. Intensity measurements were made with a 290 nm filter at 330 nm, with a slit width of 10 nm.

Binding curves were fitted to the following equation using a nonlinear regression routine:

$$
\Delta I / I_o = (\Delta I_{max} / I_o) (A - (A^2 - 4nP_TL_T)^{1/2}) / 2nP_T
$$

where $\Delta I$ is the change in fluorescence intensity in the caldesmon fragment induced by calmodulin, $I_o$ is the fluorescence of the caldesmon fragment in the absence of calmodulin, $I_{max}$ is the maximal change in caldesmon fragment fluorescence saturated with calmodulin, $A = nP_T + L_T + K_o$, $n$ is the number of moles of calmodulin-binding sites per mole of caldesmon fragment, $P_T$ is the total calmodulin concentration, $L_T$ is the free calmodulin concentration, and $K_o$ is the dissociation constant for calmodulin-caldesmon binding.
FIGURE 1: Purified mutants of recombinant COOH-caldesmon. SDS–PAGE followed by Coomassie Blue staining of approximately 1 µg of CaD39 or its mutants. The numbers in the left-hand column indicate the molecular mass in kilodaltons. Lane 1, low molecular mass markers (from top to bottom: ovalbumin, 45 000 Da; carbonic anhydrase, 31 000 Da; soybean trypsin inhibitor, 22 000 Da; lysozyme, 14 000 Da); lane 2, CaD39; lane 3, W659A; lane 4, W692A; lane 5, W659,692A; lane 6, W722A; lane 7, ΔA; lane 8, ΔB; lane 9, ΔAB.

FIGURE 2: Schematic diagram of CaD39 and mutants. CaD39 and its mutants, W659A, W692A, W659,692A, and W722A are about 100:64:62:30:65, indicating that each Trp residue contributes equally to the total fluorescence of CaD39, that is, about 32%, 35%, and 30% for Trp 659, Trp 692, and Trp 722, respectively.

Calmodulin itself does not contain Trp residues, but it increased the fluorescence intensity of all three Trp residues in CaD39 by 77%. Replacement of any one of the three Trp residues by Ala results in an augmented calmodulin-induced enhancement of fluorescence in the remaining two Trp residues as shown by the values of ΔI_{max}/I_o at 330 nm (Table 1). These data suggest that the binding of calmodulin to CaD39 affects each Trp residue to a differing degree and the effect on each Trp residue is not independent of the others.

Calmodulin binding also caused a blue shift in the emission maximum from 348 to 337 nm, indicating that calmodulin either directly or indirectly shields the Trp residues from a polar environment. Our observation is in agreement with

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RESULTS

Fragments Corresponding to the COOH-Terminal Domain of Caldesmon Used in This Study. For the present studies, we have chosen to use the recombinant fragment CaD39 which corresponds to the entire COOH-terminal domain of human fibroblast caldesmon, residues 244–538 (residues 443–736 in chicken gizzard β-caldesmon). The sequences in CaD39 are highly conserved in the corresponding region in chicken gizzard h-caldesmon; moreover, the amino acid sequences of the three putative calmodulin-binding sites are identical in the two proteins with the exception of a single replacement of Gly 717 in h-caldesmon at site B′ by a Ser in the fibroblast isoform (Bryan & Lee, 1991; Novy et al., 1991). This highly conserved domain has been shown to exist as a distinct structural fragment which retains all the known functions inherent in the COOH-terminal domain of the intact protein. For example, it binds actin, tropomyosin, and calmodulin, and it inhibits actin activated myosin ATPase activity (Novy et al., 1993; Tsuruda et al., 1995). Trp/Ala mutants, W659A, W692A, W659,692A, and W722A, and deletion mutants of CaD39 lacking the putative calmodulin-binding sites A, B, and A+B (ΔA, ΔB, and ΔAB, respectively) were expressed in E. coli and purified as described under Materials and Methods. The purified mutants were examined by SDS/PAGE as shown in Figure 1, and a schematic representation of the deletion and Trp/Ala mutants is shown in Figure 2.

Binding of Trp/Ala Mutants of CaD39 to Ca^{2+}—Calmodulin. In the absence of calmodulin, the emission spectra of CaD39 and all the Trp/Ala mutants are similar in shape to that of the chicken gizzard caldesmon and exhibit a maximum fluorescence intensity at 348 nm. The relative fluorescence intensities (I_o at 348 nm) of 1 µM of CaD39, W659A, W692A, W659,692A, and W722A are about 64:62:30:65, indicating that each Trp residue contributes equally to the total fluorescence of CaD39, that is, about 32%, 35%, and 30% for Trp 659, Trp 692, and Trp 722, respectively.

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the indole ring at the \( \beta \)-carbon of each Trp residue has been truncated and replaced by a hydrogen atom. In our case, replacing Trp 659 or Trp 692 with Ala at site A or B reduces -10 reduction in binding of almost 50-fold, respectively. -10 Replacing Trp 659 or Trp 692 with Ala at site A or B reduces -10 a shift of the emission maximum from 351 to 339 nm in a less than 5-fold reduction in binding. DE. Replacing Trp 722 with Ala shows little effect on the binding of CaD39 to calmodulin. Negligible amounts of bound protein were recovered when EGTA was included in the binding assay or BSA was used as the ligand, indicating specific binding (data not shown). These results therefore confirm the fluorescence data that Trp 659 and Trp 692 are shown to be equally important in the binding of caldesmon to calmodulin, whereas Trp 722 is less so. It should be pointed out that the margins of error in the affinity binding assays are higher than those in fluorescence measurements especially for the weak-binding mutants. This is likely due to the uncertainty of the accessibility of the immobilized calmodulin to CaD39 and mutants, and the cumulative error involved in the subsequent steps of analyses of bound protein. For this reason, no attempt was made to calculate binding parameters based on the affinity binding data. However, affinity data provide a semiquantitative analysis of direct binding and help in confirming the fluorescence results.

Interaction of Calmodulin with Deletion Mutants of CaD39 Lacking Calmodulin-Binding Sites A and B. To investigate the involvement in calmodulin binding of amino acid residues other than Trp in sites A and B, we have generated deletion mutants lacking site A (\( \Delta \Lambda A \), residues 658–666, Met-Trp-Glu-Lys-Gly-Asn-Val-Phe-Ser), site B (\( \Delta \Lambda B \), residues 687–695, Ser-Arg-Ile-Asn-Glu-Trp-Leu-Thr-Lys), and both sites A and B (\( \Delta \Lambda A B \), residues 658–666 and 687–695). As shown in Table 1 and Figure 5, calmodulin induces an increase in \( \Delta \Lambda \Delta \Delta \max /I_0 \) at 330 nm by about 62% and 77% in \( \Delta \Lambda A \) and \( \Delta \Lambda B \), respectively, and causes a shift in the emission maximum from 348 to 339 nm. Deletion of site A reduces the binding of CaD39 to calmodulin by 13-fold with a \( K_d \) of \( 1.7 \times 10^{-6} \) M; interestingly, \( \Delta \Lambda A \) has a higher affinity for calmodulin than the W659A mutant which has a \( K_d \) of \( 2.9 \times 10^{-6} \) M. It appears that the amino acid residues neighboring Trp 659 at site A contribute negatively to calmodulin binding. On the
other hand, deletion of site B causes an almost identical degree of reduction in binding as observed when Trp 692 alone is replaced by an Ala (\(K_d\) for \(\Delta B\) and W692A are both about \(4 \times 10^{-6}\) M). This result suggests that Trp 692 in site B is the predominant binding determinant with little or no contribution from the neighboring amino acid residues. Deletion of both sites A and B reduces binding by almost 70-fold, and calmodulin induces changes in \(\Delta I_{max}/I_o\) of the remaining Trp 722 by about 50%, but causes a smaller shift in the emission maximum from 348 to 343 nm.

The fluorescence data were again confirmed by direct binding analyses using calmodulin immobilized on Sepharose beads. As shown in Figure 4B, deletion of both sites A and B essentially abolishes the binding of CaD39 to calmodulin under these binding conditions. Deletion of either site A or B reduces the amount of bound caldesmon fragments by about half.

**DISCUSSION**

The general approach employed by many previous studies to localize the calmodulin-binding sites on caldesmon was to identify calmodulin-binding fragments of various sizes representing the different regions of the COOH-terminal domain of caldesmon. Thus, it was first demonstrated that the major calmodulin-binding sites reside in the chymotryptic fragments corresponding to the COOH-terminal region of caldesmon (Wang et al., 1991b). Synthetic peptides corresponding to residues 658–666 (Zhan et al., 1991) and 675–695 (Mezgueldi et al., 1994) in chicken gizzard h-caldesmon were later shown to bind calmodulin in a Ca\(^{2+}\)-dependent manner. Using recombinant fragments, Marston et al. (1994) proposed that Ca\(^{2+}\)–calmodulin interacts with three distinct caldesmon sites: residues 658–666 (site A), residues 687–695 (site B), and residues 717–726 (site B'). This strategy has been successful in providing us with crucial information about the calmodulin-binding sites, especially since the detailed three-dimensional structure of caldesmon is unknown. However, accurate interpretation of binding data using this approach relies on the assumption that the fragments retain their “native” conformations. Although such an assumption is valid for most large fragments representing functional and structural domains, smaller fragments, depending on the location of the ends, may generate false positive or negative binding results. Recently, Wang et al. (1996) have attempted to address this problem by producing caldesmon mutants with truncated COOH-terminal sequences of various lengths. Their studies showed that removing 38 amino acid residues containing the putative calmodulin-binding site B’ from the COOH-terminus did not affect calmodulin binding and thus did not demonstrate site B’ as a calmodulin-binding site as previously reported by Marston et al. (1994).

In order to minimize the disruption to the overall conformation of the COOH-terminal domain and to investigate the involvement of the Trp residues in calmodulin binding, we have replaced each Trp residue in sites A, B, and B’ with Ala and studied their binding with calmodulin. We have shown that Trp 659 and Trp 692 in sites A and B are the major binding determinants since replacement of Trp 659 or Trp 692 with Ala reduces the binding of CaD39 to calmodulin by about 20- and 30-fold, and destabilizes the CaD39–calmodulin complex by 1.75 and 1.94 kcal mol\(^{-1}\), respectively. Replacing Trp 722 in site B’ with Ala has a much smaller effect on the interaction between caldesmon and calmodulin, causing about a 4-fold reduction in binding and a destabilization energy of 0.87 kcal mol\(^{-1}\), although a slight increase in \(\Delta I_{max}/I_o\) (Table 1 and Figure 3) is observed.
These data indicate that although all three Trp residues at sites A, B, and B' contribute to the calmodulin–caldesmon interaction, Trp 659 and Trp 692 play a much greater role than Trp 722. This suggests a certain flexibility in the multiple-site interaction between caldesmon and calmodulin, such that sites A and B act as primary anchoring sites. Site B', in contrast, may act as a secondary site whose binding is easily reversible depending on the environment surrounding the thin filament. The weak interaction between site B' and calmodulin may also explain why some have observed an interaction between calmodulin and caldesmon fragments containing site B' (Huber et al., 1996; Marston et al., 1994), while others (Wang et al., 1996) did not detect significant changes in binding when the region containing site B' (residues 718–756) was truncated from caldesmon.

Deletion of the nine residues corresponding to site A (residues 658–666) from CaD39 reduces its binding to calmodulin as expected; however, calmodulin has a higher affinity for this deletion mutant than for W659A where Trp 659 is replaced by an Ala. This observation suggests that the amino acid residues near Trp 659 may have a negative effect on calmodulin binding. Since site A contains the sequence Met-Trp659-Glu-Lys-Gly-Asn-Val-Phe-Ser, it is conceivable that the charged Glu and Lys residues next to Trp 659 may interfere with hydrophobic interactions involving this Trp residue. On the other hand, amino acids near Trp 692 in site B contribute little to the caldesmon–calmodulin interaction since deletion of site B reduces binding to a similar extent as replacing Trp 692 by an Ala.

It has been shown that calmodulin binds strongly to basic, amphiphilic, and α-helical motifs of about 20 residues. These are frequently found in calmodulin-binding sites of proteins that have a high affinity for calmodulin ($K_d$ ranges from $10^{-9}$ to $10^{-8}$ M) (O’Neil & DeGrado, 1990). It is apparent that the hydrophobic and electrostatic interactions both contribute to the binding of these calmodulin-binding motifs. For caldesmon, the amino acid sequences in and neighboring sites A, B, and B' bear little resemblance to the high-affinity basic, amphiphilic α-helical motif and probably represent a low-affinity binding motif belonging to calmodulin-binding proteins with a $K_d$ in the $10^{-5}$ M range. While a typical calmodulin-binding domain is believed to consist of a continuous sequence, some calmodulin-binding proteins have multiple binding sites and bind more than 1 mol equiv of calmodulin (Goold & Baines, 1994; Menegazzi et al., 1994). Caldesmon is unusual in that it has multiple calmodulin-binding sites but binds 1 mol equiv of calmodulin. Our finding that all three sites in caldesmon are involved to differing degrees with calmodulin binding is consistent with the model suggested by Marston et al. (1994) and Huber et al. (1996), who proposed a multisite interaction between caldesmon and both NH$_2$- and COOH-terminal lobes of calmodulin.

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