Interaction of smooth muscle caldesmon with calmodulin mutants

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Abstract The interaction of avian smooth muscle caldesmon with calmodulin (CaM) was investigated by studying the ability of selected mutant calmodulins to induce fluorescence changes in caldesmon. Different types of CaM mutants were used including point charge mutants, cluster mutations, and mutations which alter the calcium binding of CaM. The caldesmon binding properties were only slightly affected by E84K-CaM or by the double mutation E84Q/E120Q-CaM. Affinity of calmodulin to caldesmon was decreased 2–4 times by point mutation G33V-CaM, double mutation E84K/E120K-CaM, deletion of residues 82–84, and by cluster mutations DEE118-120 → KKK or EEE82-84 → KKK. Mutations of the first (E31A-CaM) and the second (E67A-CaM) calcium binding sites reduced the affinity of calmodulin to caldesmon by at least 5-fold; in addition these calmodulin mutants exhibited smaller changes in the fluorescence spectra of caldesmon. Simultaneous mutation of the two negatively charged clusters of calmodulin EEE82-84 → KKK and DEE118-120 → KKK resulted in a more than 15-fold decrease in the affinity of calmodulin for caldesmon. The data indicate that charged and uncharged amino acids in both halves of CaM play an important role in the binding of calmodulin to caldesmon, and that Ca^{2+} binding must be maintained in the amino-terminal sites for maximal interaction with caldesmon.

Key words: Caldesmon; Calmodulin; Mutant; Protein electrostatics

1. Introduction

Caldesmon is a ubiquitous actin-binding protein which seems to be involved in the regulation of smooth muscle and non-muscle motility [1–3]. Caldesmon interacts with a number of calcium-binding proteins such as calmodulin [1–3], S-100 protein [4,5], caltropin [6] and troponin C [7]. Of all these proteins, calmodulin seems to be the most probable regulator of caldesmon function in smooth muscle [1–3]. The carboxyl-terminal actin- and tropomyosin-binding domain of chicken gizzard caldesmon contains at least two calmodulin-binding sites encompassing residues 651–666 and 680–695 [8,9]. Although an initial report on the localization of the caldesmon-binding sites on calmodulin has appeared in the literature [10], the sites on calmodulin that interact with caldesmon are not well characterized. The structural basis of the binding of calmodulin by caldesmon is poorly understood because it belongs to a group of lower affinity calmodulin-binding proteins and may be different from that of the well-characterized, high-affinity calmodulin-binding proteins such as myosin light chain kinase (MLCK). The aim of the current study was to characterize the interaction of several calmodulin mutants with caldesmon to obtain information which contributes towards our understanding of the mechanism underlying the calmodulin–caldesmon interaction.

2. Materials and methods

Duck gizzard caldesmon and bovine brain calmodulin were isolated as described earlier [11,12]. Site-directed mutagenesis was used to generate the various mutant calmodulins [13,14]. The mutations are described in Table 1. Mutants of this series have been referred to by using the prefixes 'SYNCAM' or 'VU' [13–15]. All calmodulins were homogeneous as assessed by SDS-gel electrophoresis. The concentration of caldesmon and bovine brain calmodulin was determined spectrophotometrically using $E^{0.1\%}_{280}$ equal to 0.33 and 0.20, respectively [16,17]. The concentration of calmodulin mutants was determined by the dye-binding method of Spector [18] using bovine brain calmodulin as a standard.

The interaction of caldesmon with calmodulin was determined by measuring the calmodulin-induced increase in Trp fluorescence of caldesmon [19–21]. A single concentration (200 nM) of caldesmon was used throughout these experiments to allow facile comparison of the data derived from several CaM mutants, and avoid problems associated with caldesmon self-association [20]. Briefly, 200 nM of caldesmon in 10 mM Tris-HCl, pH 7.4, containing 100 mM KCl, 0.1 mM $CaCl_2$ and 4.2 mM β-mercaptoethanol, was titrated with increasing quantities of calmodulin. The excitation wavelength was set at 290 nm (xenon lamp, slit width 5 nm) and fluorescence measured at 322 nm (slit width 1.5 nm) on a Hitachi F-3000 spectrofluorimeter. The fluorescence cell (optical path 1 cm) containing 1 ml of caldesmon solution was maintained at 26°C by a constant-temperature water bath and illuminated for only short periods of time (less than 30 s) for making measurements. Calmodulin was added in small portions by microsyringe, and the total volume of solution added was not more than 25 μl. Therefore, the dilution caused by calmodulin additions was negligible. In separate control experiments the incubation buffer without caldesmon was titrated with calmodulin, and the fluorescence caused by calmodulin was subtracted from the experimental values. Sample fluorescence spectra and titration data are shown in Fig. 1. Data were fit to a non-linear binding equation:

$$F = F_0 + F_{max} \cdot \left[\frac{(N + C + K)}{2N} - \sqrt{\left(\frac{(N + C + K)}{2N} \right)^2 - \frac{C}{N}} \right]$$

where F_0 is the initial caldesmon fluorescence in the absence of calmodulin added; F_{max} is the maximal increase in the fluorescence induced by calmodulin; C, total calmodulin concentration; N, total concentration of calmodulin binding sites; and K, the apparent dissociation constant, K_d. F_{max}, N and K can thus be calculated from the fluorescence titration data. In all cases we found that N was essentially equal to the caldesmon

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Abbreviations: CD, caldesmon; CaM, calmodulin; MLCK, myosin light chain kinase; SDS, sodium dodecyl sulfate.

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concentration. Therefore, the binding constants (K_d) were calculated based upon the binding of 1 mol of CaM per mol of caldesmon [8,20].

Under the experimental conditions used, the K_d values for the low affinity (K_d > 400 nM) calmodulin mutants can not be determined precisely. These data are clearly noted in Table 1.

3. Results

Calmodulin itself does not contain Trp residues [17], therefore, following the Trp fluorescence of calmodulin-binding proteins one can obtain information on the interaction of the target proteins with calmodulin. Indeed titration by Ca^{2+}-saturated calmodulin results in a 1.5–1.8 fold increase in tryptophan fluorescence of caldesmon accompanied by a 10–15 nm blue shift of the fluorescence maximum (from 355–360 nm to 340–345 nm) [19–21] (Fig. 1A). These properties make fluorescence titration the method of choice for investigation of interaction of intact caldesmon and its fragments with calmodulin [8,9,19–21]. Under the conditions used, bovine brain calmodulin and bacterially expressed calmodulin (VU-1) induced a 1.8–1.9 fold increase in Trp fluorescence of caldesmon and a significant blue shift of the fluorescence maximum (Table 1, Fig. 1A). The apparent K_d calculated for the two wild-type calmodulins was about 70 nM. These results agree well with the data of Malencik et al. [20] obtained under similar conditions.

The use of CaM mutants for studying the interaction of CaM with target proteins is well documented [13–15,22–24]. Many of the mutants have differences in charged amino acids which affect their ability to activate selected CaM-dependent enzymes such as MLCK [14,23,24], CaM-dependent protein kinase II [14], phosphorylase kinase [15], and plasma membrane Ca^{2+}-ATPase [22]. In our study of CaM mutants we found that the results with the CaM mutants can be divided into three groups, as shown in Table 1. The distinction among the groups is based upon the relative effects on caldesmon interaction, as revealed by the fluorescence titrations. The first group includes mutations which had little or no effect on the CaM–caldesmon interaction. These mutants contained single point mutations in the central helix (E84K-CaM, VU-28) or in the third Ca-binding loop (S101F-CaM, VU-17) or a mutant where two negatively charged residues (Glu-84 and Glu-120) are replaced by the neutral Gln residues (VU-65). The group 1 mutants also induced significant blue shift of the caldesmon fluorescence maximum, similar to wild-type CaMs.

A second group of CaM mutants (group 2) showed decreased interaction with caldesmon, as evidenced by a 2–4 fold increase in the K_d values (Table 1). Included in this group are: G33V-CaM (VU-5), containing a point mutation flanking the first Ca^{2+}-binding site; a deletion mutant (VU-64C), where three Glu residues in the central helix have been deleted; and a charge cluster mutant (VU-8), where residues 82–84 have been replaced by three Lys residues. These CaMs exhibited about a twofold increase in the K_d value compared to wild-type CaM. The increase in caldesmon fluorescence (F_{max}) and degree of blue shift produced by these group 2 mutants, however, was comparable to that induced by wild-type calmodulin (Table 1). On the other hand, changes in F_{max} and/or decreased blue shifts accompanied the increased K_d values of caldesmon interaction with two other group 2 calmodulin mutants, VU-12A and VU-40. The latter mutant contains two charge changes (E84K and E120K), while VU-12A CaM has the charge cluster, DEE(118-120) changed to lysine residues. Moreover, this mutant induced only a 20% increase in caldesmon fluorescence at 322 nm and marginally shifted its fluorescence maximum (Table 1). As the common mutation in VU-40 and VU12A CaMs is Glu120 → Lys, it appears that caldesmon may be quite sensitive to electrostatic perturbation in this region of CaM.

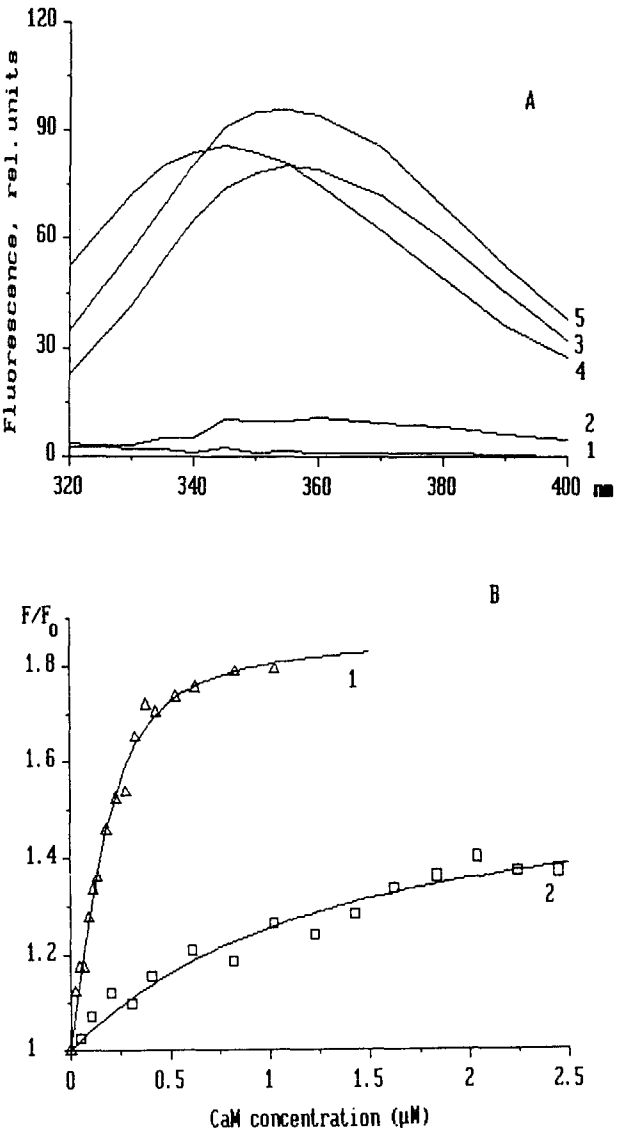

Fig. 1. Interaction of duck gizzard caldesmon with calmodulin mutants. (A) Fluorescence spectra of isolated VU-1 CaM (1), VU-60 CaM (2), caldesmon (3) and its complexes with calmodulin mutants VU-1 (4) and VU-60 (5). The final concentration of caldesmon was 200 nM, that of calmodulin mutants was 400 nM (VU-1) or 800 nM (VU-60). (B) Titration of caldesmon by mutant calmodulins VU-1 (1) and VU-60 (2). The relative fluorescence (F/F_0) was measured at 322 nm. The smooth curve through the data points was calculated for the binding of 1 mol of calmodulin/mol caldesmon with K_d = 65 nM and F_{max}/F_0 = 0.86 for VU-1, and K_d = 1200 nM and F_{max}/F_0 = 0.57 for VU-60.

The third group of calmodulin mutants studied were those with very poor interaction with caldesmon. For example, VU-18A calmodulin, in which negatively charged clusters in the central helix (EEE82-84 and DEE118-120) were simultaneously

changed to lysines induced only a very small increase in caldesmon fluorescence (F_{max}) and only slightly blue-shifted the wavelength of the fluorescence maximum. Caldesmon binding parameters were estimated only from the titration so that the K_d value in Table 1 represents a lower limit of the affinity of caldesmon for this calmodulin. In addition to the mutant with dramatically altered charge, we also analyzed two mutants which have defects in the first (E31A-CaM, VU-60) and in the second (E67A-CaM, VU13) Ca^{2+}-binding loops [25]. We found that both of these mutants had rather low affinity for caldesmon, induced only slight blue shifts, and increased the fluorescence intensity of caldesmon to values consistently lower than wild-type CaM (Table 1). The calculated K_d values for VU-13 and VU-60, therefore, represent lower limits. All experiments with VU-13 and VU-60 mutants were performed under standard conditions in the presence of 0.1 mM $CaCl_2$ where these proteins are fully saturated by Ca^{2+} [25], even in the absence of calmodulin binding proteins. Complete interpretation of these results, however, will require titration of caldesmon with VU-13 and VU-60 at different Ca^{2+} concentrations. Nevertheless, the data presented indicate that the Ca^{2+}-binding capacity in the first and second Ca^{2+}-binding domains of CaM dramatically affects the interaction of calmodulin with caldesmon.

4. Discussion

Recently published results [10] indicate that a synthetic peptide analogue of avian caldesmon (residues G651–S667) binds to calmodulin in an α-helical conformation. This agrees well with the fact that many calmodulin-binding peptides interact with calmodulin in the form of an amphiphillic α-helix [26]. For example, the amphiphillic α-helix of a peptide analog of the calmodulin-binding domain of smooth muscle/non-muscle myosin light chain kinase (MLCK) is located in a tunnel formed by a bent calmodulin molecule [27]. Both amino-terminal and carboxyl-terminal globular parts of calmodulin and its central α-helix are involved in the complex formation. Similar results were obtained with calmodulin-binding peptides based upon the skeletal muscle isoform of MLCK [28] and calmodulin-dependent protein kinase II [29]. If this model is applicable to caldesmon one can expect that a number of residues located in different parts of the calmodulin molecule will be involved in caldesmon binding.

Table 1
Interaction of calmodulin mutants with avian gizzard caldesmon

Name, mutation	K_d (nM)	$(F_0+F_{max})/F_0$	Blue shift of the maximum of fluorescence (nm)
Wild-type CaM	82 ± 18	1.82	11–13
VU-1	65 ± 22	1.84	10–12
Group 1			
VU-17, S101F	101 ± 23	1.77	10–12
VU-28, E84K	80 ± 22	1.97	11–13
VU-65, E84Q E120Q	70 ± 14	1.75	10–12
Group 2			
VU-5, G33V	140 ± 28	1.96	11–13
VU-8, EEE82-84KKK	150 ± 20	1.87	11–13
VU-64C, del (82-84)	150 ± 21	1.81	11–13
VU-12A, DEE118-120KKK	210 ± 90	1.20	2–4
VU-40, E84K E120K	270 ± 40	1.71	5–7
Group 3			
VU-13, E67A	>400	1.48	5–7
VU-60, E31A	>800	1.59	2–4
VU-18A, EEE82-84KKK DEE118-120KKK	>2000	1.24	5–7

K_d, concentration of calmodulin-induced half-maximal increase in tryptophan fluorescence, $(F_0 + F_{max})/F_0$, increase in the fluorescence at 322 nm induced by addition of saturating concentrations of calmodulin.

Our data with calmodulin mutants supports this sort of model for caldesmon interaction in that amino acid residues in both halves of CaM appear to be important for the interaction with caldesmon. In the carboxyl-terminal domain the negatively charged cluster DEE118-120 contributes to the interaction with caldesmon, a property shared with a number of other target proteins (such as Ca^{2+}-calmodulin-dependent protein kinase II, MLCK, phosphorylase kinase and Ca-ATPase [13–15,22,27]). This also correlates with the fact that the backbone amide resonances of Asp-118 and Val-121 of calmodulin undergo a small chemical shift after binding to a caldesmon fragment [10]. In the central helix, residues 82–84 may have different contributions towards interaction with caldesmon, as mutant E84K-CaM was nearly identical to wild-type CaM but the cluster mutant VU-8 (Glu82-84 → Lys) or deletion mutant VU- 64C exhibited decreased interaction. These results differ from MLCK where Glu-84 is more important for interaction with CaM than Glu-82 or -83 [23,24]. Therefore, the sensitivity of caldesmon to changes in residues 82–83 of CaM may be similar to that of the catalytic subunit of phosphorylase kinase [15], which is affected by mutations at these two amino acids in CaM.

The amino-terminal domain of calmodulin is also involved in caldesmon binding. Mutation of Gly-33 to Val (VU-5) in the helix following the first Ca^{2+}-binding loop, significantly affects the binding of calmodulin to caldesmon. In addition, alteration of the first (VU-60) or of the second (VU-13) Ca^{2+}-binding loops of CaM, decreased binding to caldesmon and altered calmodulin-induced changes in the fluorescence spectra of caldesmon (Table 1). Additional experiments directed towards determining the Ca^{2+} occupancy of these proteins when bound to caldesmon may reveal how caldesmon affects Ca^{2+} binding to CaM in the complex. Altogether the results support models of caldesmon interaction where a linear peptide segment of the protein, with the potential to form an amphiphillic helix, is a major contributor to the interaction with CaM.

It has been proposed that caldesmon has two closely located calmodulin-binding sites [8,9]. The first site (residues 651–667 of avian caldesmon) has been modeled with a synthetic peptide analogue, as described above, which mimics both the affinity and the Trp fluorescence changes observed with caldesmon [30], and forms an α-helix when bound to calmodulin [10]. However, the ability to regulate actin-activated myosin ATPase activity, a property of caldesmon and larger caldesmon fragments [8,9] is not found in this peptide, even though it can co-sediment with actin [30]. A peptide analogue of the second site (residues 675–695 of avian caldesmon) also has a Trp which undergoes spectral changes when bound to CaM [9]. Sequences overlapping the carboxyl-terminal end of site 2 also bind to F-actin and inhibit actomyosin ATPase activity [8,9]. These interactions could be like the allosteric control that CaM exhibits in binding to auto-inhibited protein kinases such as MLCK

[24] and CaM-dependent protein kinase II [31]. In these systems, the CaM binding and auto-inhibitory sequences are in adjacent or overlapping linear segments. If the calmodulin–caldesmon complex shares some features of the kinase complexes, it may be possible to invoke an allosteric control of an actin binding site by interaction with calmodulin at a proximal site. If this comparison is correct, then we may suggest that, while caldesmon could interact with calmodulin using elements of both sites, it might also allosterically modify the actin binding properties of one site by interaction at another. This hypothesis can be experimentally verified by investigating the interaction of caldesmon fragments or synthetic peptides containing only one calmodulin-binding site with selected calmodulin mutants.

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