Mapping of contact sites in the caldesmon–calmodulin complex

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The interaction of intact calmodulin and its four tryptic peptides with deletion mutants of caldesmon was analysed by native gel electrophoresis, fluorescence spectroscopy and zero-length cross-linking. Deletion mutants H2 (containing calmodulin-binding sites A and B) and H9 (containing sites B and B') interacted with intact calmodulin to form complexes whose stoichiometries varied from 2:1 to 1:1. The N-terminal peptides of calmodulin (TR1C, residues 1–77, and TR2E, residues 1–90) bound H2 with higher affinity than H9. At the same time H2 was less effective than H9 in binding to the C-terminal peptides of calmodulin TR2C (residues 78–148) and TR3E (residues 107–148). The N-terminal peptides of calmodulin (TR1C and TR2E) could be

cross-linked to intact caldesmon and its deletion mutants H2 and H9. The similarity in the primary structures of sites A and B' of caldesmon and our measurements of the affinities of H2 and H9 to calmodulin and its peptides strongly indicate an orientation of the protein complex where sites A and B' interact with the N-terminal domain of calmodulin, whereas site B interacts with the C-terminal domain of calmodulin. The spatial organization of contact sites in the caldesmon–calmodulin complex agrees with the earlier proposed two-dimensional model of interaction of the two proteins [Huber, El-Mezgueldi, Grabarek, Slatter, Levine and Marston (1996) *Biochem. J.* **316**, 413–420].

INTRODUCTION

Caldesmon is a multifunctional actin-binding protein involved in the regulation of smooth-muscle contraction and non-muscle cell motility [1,2]. Caldesmon action is regulated by a Ca^{2+} -binding protein whose nature is not well determined. Under conditions *in vitro* the inhibitory activity of caldesmon on the actomyosin ATPase is reversed by a number of EF-hand proteins such as caltropin, troponin C, S-100 and calmodulin [1–5]. Therefore calmodulin is one of the candidates for the role of a regulator of caldesmon functioning. Much effort has been directed at localizing calmodulin-binding sites of caldesmon. It is well accepted that the C-terminal domain of caldesmon contains two [6] or three [7,8] high-affinity calmodulin-binding sites. Caldesmon-binding sites of calmodulin are not so well determined. It is supposed that calmodulin interacts with caldesmon in an extended conformation [9] and that both N- and C-terminal globular domains as well as the central α -helix of calmodulin are involved in caldesmon binding [10]. The data obtained on proteolytic fragments of calmodulin [11] and calmodulin mutants [12] agree with this suggestion and indicate that multiple sites of calmodulin interact with caldesmon. However, the mutual arrangement of calmodulin and caldesmon contact sites is unknown. In the present study we analysed the interaction of intact caldesmon and its deletion mutants with calmodulin and its proteolytic fragments to obtain information about the spatial organization of the caldesmon–calmodulin complex.

MATERIALS AND METHODS

Protein preparation

Intact caldesmon and calmodulin were isolated from duck gizzard and bovine brain by methods described earlier [13,14]. Caldesmon

mutants 606C, H2 and H9 were obtained by bacterial expression in the pMW172 plasmid/BL21(DE3) cell system described by Redwood and Marston [15] and Huber et al. [16]. Tryptic peptides of calmodulin were obtained as described earlier [11] and purified either by hydrophobic chromatography on phenyl-Sepharose [17] or by ion-exchange chromatography on Q-Sepharose with the modified method of Tsalkova and Privalov [18]. The concentration of intact caldesmon and calmodulin was determined spectrophotometrically taking $A_{1\text{ cm}, 280}^{1\%}$ as 0.33 for caldesmon [19] and 0.19 for calmodulin [20]. The concentration of deletion mutants of caldesmon was determined by using fluorescamine [21,22]. In this case caldesmon or its deletion mutants dissolved in 40 mM sodium borate, pH 9.0, containing 1% (w/v) SDS were mixed with fluorescamine (0.3 mg/ml in acetone). The final concentration of fluorescamine was 0.06 mg/ml and the content of amino groups varied between 2 and 20 nmol per 1 ml sample. The fluorescence was excited at 390 nm and measured at 484 nm. Knowing the amino acid composition from which we calculated the total quantity of amino groups, we estimated the peptide concentration. The concentration of calmodulin peptides was determined by the dye-binding method of Spector [23] with calmodulin as standard. The homogeneity of proteins used was checked by SDS/PAGE [24].

Protein–protein interaction

Interaction of caldesmon and its mutants with calmodulin and its proteolytic fragments was measured by three independent methods.

Native gel electrophoresis was run on 15% polyacrylamide gel in 80 mM Tris/glycine, pH 8.6, in the presence of 1 mM CaCl_2 or 2 mM EGTA. Samples containing fixed quantities of calmodulin or its tryptic peptides were titrated with increasing quantities of

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deletion fragments of caldesmon. The interaction of calmodulin with caldesmon fragments is accompanied by a decrease in the intensity of the band of free calmodulin and an increase in the quantity of the band corresponding to the calmodulin–caldesmon fragment complex. Gels stained by Coomassie R-250 were scanned on an LKB Ultrascan XL densitometer and the peak area was plotted against the ratio [caldesmon fragment]/[calmodulin] [7] or against the total concentration of caldesmon fragment [11]. From the first graph we determined the stoichiometries of the complexes formed. Knowing the total concentration of calmodulin and the stoichiometries of the complexes formed, we determined the total concentration of caldesmon-binding sites. This value was used to estimate apparent dissociation constants of caldesmon fragments from intact calmodulin by using a non-linear binding equation [10,11]. A similar approach was used when tryptic peptides of calmodulin were titrated with caldesmon fragments.

The caldesmon–calmodulin interaction was also determined by using fluorescence spectroscopy. A fixed quantity of caldesmon (or its deletion mutants) was titrated with calmodulin. Interaction of caldesmon (or its Trp-containing fragments) with calmodulin is accompanied by an increase in Trp fluorescence [7,10,25]; therefore, by measuring the dependence of Trp fluorescence on the total concentration of calmodulin added, one can determine the apparent binding constant of caldesmon (or its fragments) to calmodulin [10]. Titration was performed in a 10 mM Tris/HCl buffer, pH 7.4, containing 100 mM KCl, 0.1 mM CaCl_2 and 4 mM 2-mercaptoethanol. The fluorescence was excited at 290 nm and measured at 322 nm. Fluorescence intensity was plotted against the ratio [calmodulin]/[caldesmon fragment] and the stoichiometry of the complex formed was determined from the intercept of the line drawn through the initial points of titration and the line drawn at the maximal level of fluorescence. Knowing the stoichiometry of the complex and the total concentration of caldesmon fragment we determined the total number of calmodulin-binding sites and by using an earlier published non-linear equation [11] we also determined the apparent dissociation constant of the two proteins.

The two-step procedure introduced by Grabarek and Gergely [26] was used for zero-length protein cross-linking. Carboxy groups of calmodulin or its proteolytic fragments (approx. 12 μM) in 25 mM Mes, pH 6.0, containing 0–40 mM NaCl were activated by the addition of 5 mM *N*-hydroxysuccinimide and 3 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide. The mixture was incubated for 15 min at 20 °C and the reaction was stopped by the addition of 2-mercaptoethanol to a final concentration of 20 mM. Caldesmon or caldesmon fragments were added to the mixture and the incubation was continued for 45 min at 25 °C. The molar ratio of calmodulin fragments to intact caldesmon was equal to 1:4.5. The molar ratio of calmodulin peptides to caldesmon fragments was in the range 5:1 to 9:1. Cross-linking was performed both in the presence and in the absence of CaCl_2 . For this purpose 0.2 mM CaCl_2 or 8 mM EGTA were added before mixing activated calmodulin with caldesmon. Aliquots of cross-linked samples were analysed by gradient (8–22 %) PAGE in the presence of SDS [24].

RESULTS

Interaction of calmodulin with caldesmon fragments

We analysed the interaction of three deletion mutants of caldesmon with calmodulin. The caldesmon fragment, H2, contains two calmodulin-binding sites (A and B); the fragment, H9, also contains two calmodulin-binding sites (B and B'); and the

Figure 1 Titration of intact calmodulin with H2 (A) and H9 (B)

(A) Calmodulin (15.4 μM) was titrated with increasing quantities of H2. The molar ratio of H2 to calmodulin was 0, 0.38, 0.63, 0.76, 0.89, 1.02, 1.14, 1.27, 1.41 and 1.52 for lanes 1–10 respectively. Isolated H2 (23.4 μM) was loaded on lane 11. (B) Calmodulin (15.4 μM) was titrated with increasing quantities of H9. The molar ratio of H9:calmodulin was 0, 0.27, 0.40, 0.54, 0.68, 0.81, 0.94, 1.08, 1.21, 1.35 and 1.49 for lanes 1–11 respectively. Isolated H9 (22.9 μM) was loaded on lane 12. The position of isolated calmodulin (CM) and the calmodulin–caldesmon fragment complex is indicated at the left.

deletion mutant, 606C, contains three calmodulin-binding sites (A, B and B') [2,7]. Comparing the interaction of different caldesmon fragments with calmodulin we hoped to get information on the spatial organization of the caldesmon–calmodulin complex.

Calmodulin was titrated with increasing quantities of the caldesmon fragments H2 or H9 and the composition of incubation mixture was analysed by native gel electrophoresis run either in the absence (2 mM EGTA) or in the presence of CaCl_2 (1 mM). Under the conditions used the free caldesmon fragments H2 and H9 do not enter the gel. Calmodulin does not interact with caldesmon in the absence of Ca^{2+} . This was also confirmed for the interaction of calmodulin with caldesmon fragments in that the intensity of the band of free calmodulin was not changed after titration with caldesmon fragments H2 and H9. Titration of calmodulin with H2 or H9 performed in the presence of Ca^{2+} was accompanied by a significant decrease in the intensity of the band of free calmodulin and the appearance of an additional band with electrophoretic mobility lower than that of free calmodulin (Figure 1). This means that under the conditions used, calmodulin interacts with H2 and H9 and forms a complex with lower electrophoretic mobility. We plotted the peak area of free calmodulin against the ratio [caldesmon fragment]/[calmodulin] (Figure 2) and found that the intercept of the line drawn through the titration points and the abscissa was close to 2 for both H2 and H9. Similar results were obtained when the peak area of the complex calmodulin–caldesmon fragment was plotted against the molar ratio [caldesmon fragment]/[calmodulin] (Figure 2). This means that under the conditions used, saturation was achieved when 2 mol of H2 or H9 were bound per mol of calmodulin. Knowing the stoichiometry of the complex formed and measuring the dependence of the peak area of isolated calmodulin against the total concentration of added caldesmon fragments, we determined the apparent dissociation constants of the complex formed. All these calculations were performed

Figure 2 Determination of the stoichiometry of the complex of calmodulin with caldesmon fragments measured by native gel electrophoresis

A fixed quantity of intact calmodulin ($15.4 \mu\text{M}$) was titrated with the caldesmon fragment H2 (▲, ●) or H9 (▼, ■). The peak area (as a percentage) of free calmodulin (▲, ▼) or the complex of calmodulin with caldesmon fragments (●, ■) is plotted against the molar ratio of caldesmon fragments to calmodulin.

assuming that H2 and H9 form complexes of the same (2:1) stoichiometry with calmodulin, that the binding of 2 mol of peptides to 1 mol of calmodulin is independent and that two sites of calmodulin interact with the peptides with the same affinity. This assumption is simplistic but a more complex model suggesting interdependence of the binding sites or different affinities of the two binding sites cannot be adequately verified from the limited number of experimental points obtained by the method of native gel electrophoresis. The average dissociation constants determined were 0.24 ± 0.07 S.D. μM for H2 and 5.9 ± 3.4 S.D. μM for H9. An apparent dissociation constant was also determined from the dependence of the peak area of the complex formed on the concentration of caldesmon fragment added. In this case the dissociation constants were 0.54 ± 0.08 S.D. μM for H2 and 2.09 ± 0.27 S.D. μM for H9. Thus both approaches give comparable results.

A theoretical analysis of the titration curves indicates that titrations performed at an initial calmodulin concentration (P_0) significantly higher than the apparent dissociation constant (K_d) provide an exact determination of the stoichiometry of the complex formed [27]. At the same time these conditions do not allow very precise determinations of K_d values, although computer fitting gives reliable values of K_d [27]. Thus the results presented indicate that both H2 and H9 can form 2:1 complexes with calmodulin, and H2 interacts with calmodulin with higher affinity than H9. These results agree with those of Zhuang et al. [28] who have shown that peptides containing site A (in our case the H2 fragment) possess a higher affinity for calmodulin than do peptides containing site B (in our case the H9 fragment). It is worth mentioning that at saturation the stoichiometry of the complex between caldesmon deletion fragments and calmodulin was equal to 2:1 and was different from that of the intact caldesmon–calmodulin complex, which has a stoichiometry of 1:1 [11]. Previously this unusual stoichiometry was observed by fluorescent titration of H2 by calmodulin [7]; therefore we reinvestigated the interaction of caldesmon fragments with calmodulin by fluorescence spectroscopy.

Figure 3 Binding of calmodulin to caldesmon fragments measured by Trp fluorescence

H2 ($4 \mu\text{M}$) (curve 1), H9 ($4 \mu\text{M}$) (curve 2) or 606C ($1.5 \mu\text{M}$) (curve 3) were titrated with calmodulin; the fluorescence enhancement at 322 nm (F/F_0) is plotted against the molar ratio of calmodulin to caldesmon fragments. The lines indicate the fluorescence if the affinity were infinite and linearly related to binding. Binding of all fragments can be accounted for on the basis of 2 mol of caldesmon fragments to 1 mol of calmodulin.

Titration of the caldesmon fragments 606C, H2 and H9 with calmodulin results in a 1.5–2.5-fold increase in the Trp fluorescence intensity (Figure 3). The experimental points were fitted by a non-linear equation [10,27] on the assumption that the binding of the first and second molecules of peptide each induce the same increase in the fluorescence and that these effects are additive. Fitting the experimental points by this equation provides a determination of the maximal value of fluorescence (horizontal lines on Figure 3). The stoichiometry of the complex peptide to calmodulin thus determined varied from 1.75 to 2.2. This means that at saturation 1 mol of calmodulin can bind 2 mol of caldesmon fragments. The apparent average K_d values, determined on the assumption that at saturation calmodulin independently binds 2 mol of peptides, were 91 ± 64 , 295 ± 110 and 886 ± 350 nM for 606C, H2 and H9 respectively. Thus the largest caldesmon fragment 606C possesses the highest affinity for calmodulin, whereas the smallest C-terminal fragment of caldesmon, H9, has the lowest affinity for calmodulin.

Fluorescent titration of caldesmon or its fragments by calmodulin is accompanied by two processes: an increase in the fluorescence intensity at 320–322 nm and a blue shift in the fluorescence maximum [7,10,25]. These two processes can be separated by following only the blue shift of the Trp fluorescence spectrum maximum or by measuring the intensity of the fluorescence maximum at each concentration of calmodulin [12]. Unfortunately, the blue shift in the fluorescence maximum is not very large and this complicates the precise measurement of its dependence on calmodulin concentration as well as the determination of the parameters of the interaction. In our case we measured only the fluorescence at 322 nm. Although this is an integral parameter depending on two above mentioned processes it clearly reflects the calmodulin–caldesmon interaction.

The reported values of the stoichiometry of the peptide–calmodulin complex varies from 1:1 for H9 [7] and for two synthetic peptides containing a single calmodulin-binding site [28] to 2:1 for H2 [7]. Our results indicate that under the

Figure 4 Zero-length cross-linking of calmodulin and the caldesmon fragments H2 and H9

Calmodulin (lane 2) was activated by 1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide and *N*-hydroxysuccinimide (lane 1) and mixed with the caldesmon fragments H2 (lanes 3–5) or H9 (lanes 6–8). Cross-linking was performed in the presence of either 0.2 mM CaCl_2 (lanes 3 and 6) or 8 mM EGTA (lanes 5 and 8). H2 and H9 alone were loaded on lanes 4 and 7 respectively. Cross-linking was performed in the presence of 40 mM NaCl at a molar ratio of calmodulin to caldesmon fragments of 1:5.5.

conditions used both H2 and H9 can form 2:1 complexes with calmodulin. These results agree with our results obtained by means of native gel electrophoresis. Thus according to the results of fluorescent titration and native gel electrophoresis, H2 and H9 can form 2:1 complexes with calmodulin at saturation.

We continued our investigation of the interaction of caldesmon fragments with calmodulin by means of zero-length cross-linking. When calmodulin was activated by soluble carbodi-imide, its protein band on SDS/PAGE became smeared and a diffuse band with a lower apparent molecular mass appeared on the gel (Figure 4, lanes 1 and 2). This is due to partial intramolecular cross-linking of calmodulin and modification of its carboxy groups. When activated calmodulin was mixed with H2 or H9 in the presence of EGTA no cross-linking occurred and we observed only protein bands corresponding to free caldesmon fragments and activated calmodulin (Figure 4, lanes 5 and 8). In the presence of Ca^{2+} both H2 and H9 were cross-linked to calmodulin, and complexes with apparent molecular masses of 25–28 kDa appeared on the gel (Figure 4, lanes 3 and 6). Under identical conditions H2 was more effectively cross-linked to calmodulin than was H9 (Figure 4, lanes 3 and 6). This corresponds to a lower affinity of H9 for calmodulin as shown by native gel electrophoresis and fluorescence spectroscopy. The apparent molecular mass of the cross-linked complex was close to that expected for a 1:1 complex of calmodulin with caldesmon fragments. Even at a rather large excess of caldesmon fragments over calmodulin we were unable to detect complexes with higher molecular masses. This means that under the conditions of cross-linking only 1 mol of caldesmon fragment was covalently bound per mol of calmodulin. Our failure to detect 2:1 caldesmon fragment–calmodulin complexes could be due to different reasons. It is possible that only one (out of two) peptide-binding sites of calmodulin has activated carboxy groups properly oriented for cross-linking with caldesmon fragments or else the cross-linking of the first mole of fragment prevents the covalent binding of the second mole of fragment. Moreover the cross-linking experiments were performed under conditions (ionic strength and pH value) significantly different from those of the fluorescence measurements or native gel electrophoresis. This also might affect the stoichiometry of the complex formed.

Figure 5 Cross-linking of calmodulin and its tryptic peptides to caldesmon

Caldesmon alone (lanes 1 and 1') or its mixture with intact calmodulin (lanes 2 and 3) or calmodulin peptides TR1C (residues 1–77) (lanes 4 and 5), TR2C (residues 78–148) (lanes 6 and 7), TR2E (residues 1–90) (lanes 8 and 9) and TR3E (residues 107–148) (lanes 10 and 11) after zero-length cross-linking were subjected to 8–22% gradient PAGE. Cross-linking was performed in the presence of either 0.2 mM CaCl_2 (lanes with even numbers) or 8 mM EGTA (lanes with odd numbers). The incubation mixture contained 40 mM NaCl.

The results presented indicate that caldesmon fragments can interact with calmodulin to form complexes with variable stoichiometries. At low concentration caldesmon fragments form a 1:1 complex with calmodulin. In this case only one (out of two) caldesmon-binding sites of calmodulin interacts with the caldesmon fragment. The same stoichiometry is achieved if the caldesmon fragment interacts simultaneously with two different sites on one molecule of calmodulin. At high concentration the caldesmon fragments can form 2:1 complexes with calmodulin. In this case each of two peptide-binding sites of calmodulin is occupied by a separate molecule of caldesmon fragment interacting with calmodulin through two different sites.

Interaction of caldesmon with tryptic peptides of calmodulin

When calmodulin with activated carboxy groups was mixed with caldesmon in the presence of Ca^{2+} ions we observed the formation of a cross-linked complex with a molecular mass close to the sum of the apparent molecular masses of calmodulin and caldesmon (Figure 5). This cross-linking was specific and was not observed in the absence of Ca^{2+} ions. Similar experiments were performed with tryptic fragments of calmodulin. The N-terminal peptides of calmodulin TR1C (residues 1–77) and TR2E (residues 1–90) were Ca^{2+} -dependently cross-linked to intact caldesmon, whereas the C-terminal peptides TR2C (residues 78–148) and TR3E (residues 107–148) did not form covalent complexes with intact caldesmon either in the presence or in the absence of Ca^{2+} ions (Figure 5). These results mean that certain carboxy groups in the N-terminal domain of calmodulin are located in close proximity to Lys residues of caldesmon and provide zero-length cross-linking. This conclusion agrees with our results indicating the involvement of the N-terminal domain of calmodulin in caldesmon binding [11]. Earlier, using native gel electrophoresis, we showed that the C-terminal peptides of calmodulin (TR2C and TR3E) also interact with caldesmon and their affinity for caldesmon is similar to or even higher than that of the N-terminal peptides of calmodulin [11]. At the same time we were unable to cross-link either TR2C or TR3E to caldesmon. This means that the orientation of carboxy groups is unfavourable for cross-linking the C-terminal peptides of calmodulin to caldesmon. Thus, although calmodulin contains at least two caldesmon-binding sites located in the N- and C-terminal parts, only one site located in the N-terminal part can be revealed by zero-length cross-linking. Therefore even if two molecules of H2 or H9 interact with a molecule of calmodulin, only one molecule of peptide bound to the N-terminal part of calmodulin will be

Figure 6 Cross-linking of tryptic peptides of calmodulin to deletion mutants of caldesmon

Cross-linking of H2 and H9 was performed at low ionic strength (25 mM Mes), whereas 606C was cross-linked to calmodulin peptides at intermediate ionic strength (25 mM Mes containing 40 mM NaCl). The reaction was performed in the presence of either 0.2 mM CaCl_2 (**A**) or 8 mM EGTA (**B**). Caldesmon fragments alone (lane 1) or the cross-linked mixture of the corresponding caldesmon fragments with TR1C (residues 1–77) (lane 2), TR2C (residues 78–148) (lane 3), TR2E (residues 1–90) (lane 4) or TR3E (residues 107–148) (lane 5) were subjected to 8–22% gradient SDS/PAGE.

cross-linked. This explains our results on the cross-linking of H2 and H9 to calmodulin (Figure 4).

Interaction of caldesmon fragments with calmodulin peptides

Continuing our investigation, we analysed the interaction of deletion mutants of caldesmon with proteolytic fragments of calmodulin. We found that at low ionic strength (25 mM Mes, pH 6.0) H2 and H9 can be Ca^{2+} -dependently cross-linked to the calmodulin peptides TR1C and TR2E (Figure 6). We failed to detect cross-link formation between caldesmon fragments and the C-terminal peptides of calmodulin (TR2C or TR3E) (Figure 6). The fragment 606C has the highest affinity for calmodulin (see the results of fluorescence spectroscopy). Therefore at low ionic strength (25 mM Mes) 606C was cross-linked to TR1C or TR2E independently of the presence of Ca^{2+} ions in the incubation mixture. When the cross-linking was performed at intermediate ionic strength (25 mM Mes containing 40 mM NaCl) complex formation was dependent on the presence of Ca^{2+} ions (Figure 6). The results presented indicate that the N-terminal peptides of calmodulin (TR1C and TR2E) can be cross-linked both to the intact caldesmon and to its three deletion mutants. As expected, cross-linking revealed complexes with an apparent molecular mass close to the sum of the molecular masses of the caldesmon fragment and the calmodulin peptides. This means that under the conditions used, calmodulin peptides form 1:1 complexes with caldesmon fragments. It is worth mentioning that both H2 (containing calmodulin-binding sites A and B) and H9 (containing calmodulin-binding sites B and B') were cross-linked to the N-terminal peptides of calmodulin. Because both caldesmon fragments contain site B we can suppose that this site interacts with the N-terminal part of calmodulin. Alternatively it must be suggested that the N-terminal peptides of calmodulin interact

Figure 7 Titration of the calmodulin peptide TR2E (residues 1–90) (2.3 μM) with the caldesmon fragments H9 (curve 1) and H2 (curve 2)

The fraction of TR2E remaining unbound is plotted against the total concentration of caldesmon fragment added. Solid lines represent best-fit curves with K_d values of 280 and 770 μM for H2 and H9 respectively.

Table 1 Apparent dissociation constants (in μM) of calmodulin peptides TR1C and TR2E for deletion mutants of caldesmon

The results represent means $\pm$ S. D. with the number of independent experiments in parentheses, each of which contains not less than six data points.

Calmodulin peptide	H2	H9
TR1C (residues 1–77)	1527 $\pm$ 100 (2)	6050 $\pm$ 1000 (2)
TR2E (residues 1–90)	280 $\pm$ 26 (2)	770 $\pm$ 30 (2)

with site A of H2 and site B' of H9. This is possible only if sites A and B' are more similar to each other than to site B. Indeed Wang et al. [29] detected similarity between sites A and B'. To determine which site or sites of caldesmon interact with the N-terminal part of calmodulin we analysed the interaction of caldesmon fragments with calmodulin peptides by means of native gel electrophoresis.

Titration of the N-terminal peptides of calmodulin (TR1C or TR2E) by caldesmon fragments is accompanied by a decrease in the intensity of the band of free (unbound) calmodulin peptide (Figure 7). This means that the N-terminal peptides of calmodulin interact with the caldesmon fragments H2 and H9. The apparent dissociation constant of the N-terminal calmodulin peptides for H2 was one-third to one-quarter of the corresponding value for H9 (Table 1), and the larger N-terminal peptide of calmodulin (TR2E) interacted with caldesmon fragments with higher affinity than the shorter (TR1C) peptide of calmodulin.

Titration of the C-terminal peptides of calmodulin (TR2C and TR3E) with H2 or H9 was not accompanied by a decrease in the intensity of free (unbound) peptide. Instead we observed a smearing of the band of the free calmodulin peptide, which was due to the formation of an additional band located close to the band of the free calmodulin peptide; this led to an increase in the

Figure 8 Titration of the C-terminal peptides of calmodulin TR2C (residues 78–148) and TR3E (residues 107–148) with caldesmon fragments

(A) Titration of TR2C (4.6 μM) with H9. The molar ratio of H9 to TR2C was 0, 11.2, 18.0, 24.8, 31.5, 38.3, 45.1 and 51.8 for lanes 1–8 respectively. (B) Titration of TR3E (4.15 μM) with H2. The molar ratio of H2 to TR3E was 0, 11.2, 18.0, 24.8, 31.5, 38.3, 45.1 and 51.8 for lanes 1–8 respectively. (C) Increase in the intensity of the overall calmodulin peptide band TR2C (●, ■) and TR3E (▼, ▲) after titration with caldesmon fragments H9 (●, ▼) and H2 (■, ▲). Solid lines represent best-fit curves with K_d values of 360 and 320 μM for the complexes TR2C–H9 and TR3E–H9 respectively.

integral intensity of the band. We concluded that this additional band represented the formation of a complex between calmodulin peptides and caldesmon fragments. Owing to the very close electrophoretic mobilities of these bands we were unable to scan either band separately (Figures 8A and 8B). We plotted the integral intensity of two badly separated bands against the total concentration of caldesmon fragments added (Figure 8C). The H9 fragment of caldesmon induced 1.9-fold and 3-fold increases in the integral intensities of the bands of TR2C and TR3E respectively, and the apparent dissociation constants determined from these results were 360 and 320 μM respectively. Caldesmon fragment H2 induced no more than a 20% increase in the intensity of the integral band of both TR2C or TR3E; we were unable to determine the apparent dissociation constant.

Summing up, we conclude that the caldesmon fragment H2 has a higher affinity for the N-terminal peptides of calmodulin and interacts very weakly with the C-terminal peptides of calmodulin. In contrast, the caldesmon fragment H9 has a lower

affinity for the N-terminal peptides of calmodulin and interacts with the C-terminal peptides of calmodulin.

DISCUSSION

Calmodulin has a dumb-bell-like shape, each sphere of which contains two Ca^{2+} -binding sites [30]. The N- and C-terminal domains of calmodulin have different affinities for Ca^{2+} ions and differ in amino acid compositions. Therefore these two domains of calmodulin might interact with two distinct sites of caldesmon. The caldesmon fragment H2 contains two calmodulin-binding sites, sites A and B [7]. The C-terminal fragment of caldesmon, H9, also contains two calmodulin-binding sites (B and B') [7]. Wang et al. [29] demonstrated that site A is more similar to site B' and that both sites A and B' are different from site B. Therefore we can suppose that sites A and B' of caldesmon will interact with one domain of calmodulin and site B of caldesmon with a different domain. There are two possibilities: either sites A and B' interact with the N-terminal and site B with the C-terminal domain of calmodulin (Figure 9A) or sites A and B' interact with the C-terminal and site B with the N-terminal domain of calmodulin (Figure 9B). If this suggestion is correct then 1 mol of calmodulin can bind 1 or 2 mol of H2 or H9 depending on the ratio of calmodulin to caldesmon fragment. A similar explanation is acceptable for the 606C caldesmon mutant. In intact caldesmon, the rotational freedom of the different parts of the molecule is restricted and therefore the polypeptide chain is bent in such a manner that sites A and B' interact with one and site B with another domain of calmodulin. We tried to determine the spatial organization of the calmodulin–caldesmon complex.

To understand which sites are involved in the interaction with the N- and C-terminal domains of calmodulin we analysed the interaction of deletion mutants of caldesmon with tryptic peptides of calmodulin. Fragment H2 has a higher affinity than fragment H9 for the N-terminal peptides of calmodulin (Figure 7). If H2 and H9 interact with the N-terminal peptides of calmodulin through site B, which is common to them, then the affinities of H2 and H9 should be the same. Because H2 has a higher affinity than H9 for the N-terminal peptides of calmodulin we can suppose that these fragments interact with calmodulin peptides through different sites. The similarity of site A to site B' [29] makes it likely that these sites interact with the same domain of calmodulin. Site A has higher affinity than site B' for calmodulin [7,28]. This explains the higher affinity of H2 than that of H9 both for intact calmodulin and for the N-terminal calmodulin peptides. Such a spatial organization agrees with the model shown in Figure 9(A).

This suggestion agrees with our results obtained with the C-terminal peptides of calmodulin. Indeed the results of native gel electrophoresis indicate that H9 binds to the C-terminal peptides of calmodulin more strongly than H2 (Figure 8). This can be explained by the fact that H9 contains not only the complete sequence of site B but also additional amino acids downstream from site B. This will preserve an intact structure of site B and promote the interaction of H9 with the C-terminal peptides of calmodulin.

In conclusion our results indicate an organization of the caldesmon–calmodulin complex where (1) site A of caldesmon interacts with the N-terminal domain of calmodulin, (2) the polypeptide chain of caldesmon parallels that of calmodulin permitting interaction of site B with the C-terminal domain of calmodulin and (3) the polypeptide chain of caldesmon returns, running antiparallel to the polypeptide chain of calmodulin and providing interaction of site B' with the N-terminal domain of calmodulin. This model (see Figure 9) agrees with our earlier

Figure 9 Hypothetical model of the interaction of caldesmon fragments H2 and H9 with calmodulin

(A) Formation of 1:1 or 2:1 peptide–calmodulin complexes where sites A and B' interact with the N-terminal domain and site B with the C-terminal domain of calmodulin. (B) Formation of 1:1 or 2:1 peptide–calmodulin complexes where sites A and B' interact with the C-terminal domain and site B with the N-terminal domain of calmodulin. (C) Hypothetical two-dimensional model of calmodulin–caldesmon interaction [12].

published results [2,12] and proposes, in agreement with Mornet et al. [31], that the peptide chain of domain 4 of caldesmon folds into a comparatively compact structure forming multiple contacts with calmodulin.

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