Interaction of proteolytic fragments of calmodulin with caldesmon and calponin

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Interaction of five tryptic fragments of calmodulin with caldesmon and calponin was analysed by native gel electrophoresis. In the presence of Ca^{2+} intact calmodulin interacts with caldesmon and calponin with apparent K_d values equal to 0.23 and 1.3 μM respectively. The interaction was abolished in the absence of Ca^{2+} . Two large tryptic fragments of calmodulin obtained in the presence of Ca^{2+} (TR1C, residues 1–77, and TR2C, residues 78–148) interact with caldesmon with apparent K_d values of 11.9 and 4.6 μM . Affinity of TR2C to calponin (K_d 3.8 μM) was comparable with that of native calmodulin and was much higher than the corresponding value for TR1C (K_d 41 μM). The short

C-terminal tryptic peptide of calmodulin obtained in the presence of EGTA (TR3E, residues 107–148) interacts with caldesmon and calponin with K_d values of 23.9 and 12.1 μM , whereas the large N-terminal peptide TR1E (residues 1–106) interacts with both caldesmon and calponin with a very low affinity (K_d 60 μM). Thus although both N- and C-terminal domains of calmodulin are involved in the interaction with caldesmon and calponin, the C-terminal part of calmodulin (residues 78–148) is of special importance and has the highest contribution for caldesmon and calponin binding.

INTRODUCTION

Calmodulin is a ubiquitous eukaryotic protein playing a major role in the Ca^{2+} -dependent regulation of a wide variety of cellular events [1,2]. Significant progress has been achieved in analysing the mechanism of calmodulin action and determination of the structure of calmodulin–target protein complexes [3–6]. However, only proteins having high affinity to calmodulin were analysed in detail.

Caldesmon and calponin belong to the group of non-muscle and smooth-muscle proteins involved in the regulation of muscle contraction and cytoskeleton functioning [7–9]. Both caldesmon and calponin interact with a number of Ca-binding proteins, such as calmodulin, troponin C, S-100 protein and caltropin [10,11]. Three putative calmodulin-binding sites are located in the C-terminal part of caldesmon [12,13] and the dissociation constants for caldesmon–calmodulin complex ranges from 0.1 to 1 μM [7,14]. The calmodulin-binding site (or sites) is located in the N-terminal half of calponin [8,15] and it has been shown that one or two molecules of calmodulin bind to calponin with K_d values varying from 0.1 to 3 μM [11,16]. This means that the affinity of calmodulin to both caldesmon and calponin is much lower than that to myosin light chain kinase (MLCK) or cyclic nucleotide phosphodiesterase (PDE) where dissociation constants are in the low nanomolar range. The structural basis for the interaction of calmodulin with low- and high-affinity calmodulin-binding proteins might be different, therefore it was worthwhile to analyse calmodulin binding to caldesmon and calponin in more detail. In the present study we have investigated the interaction of native calmodulin and its five tryptic peptides with caldesmon and calponin in order to localize target protein binding sites of calmodulin.

MATERIALS AND METHODS

Protein preparations

Caldesmon and calponin were isolated from duck gizzard by

previously described methods [17,18]. Bovine brain calmodulin was purified according to the Gopalakrishna and Anderson method [19]. The purity of all proteins was checked by SDS/PAGE on 10–12 % gels by the method of Laemmli [20]. Protein concentration was determined by UV absorbance with the following absorption coefficient values: caldesmon, $\epsilon_{280}^{0.1\%}$ 0.33 [21]; calponin, $\epsilon_{280}^{0.1\%}$ 0.74 [22]; calmodulin, $\epsilon_{278}^{0.1\%}$ 0.2 [19].

Trypsinolysis of calmodulin and purification of calmodulin peptides

Calmodulin (1.5–2 mg/ml) in 20 mM NH_4HCO_3 was subjected to trypsinolysis either in the presence of 2 mM EGTA or in the presence of 1 mM CaCl_2 . In the first case the weight ratio of calmodulin to *N*-tosyl-L-phenylalanylchloromethane (TPCK)-treated trypsin was equal to 300:1–500:1 and incubation lasted for 7–10 min at 22 °C. In the second case the weight ratio calmodulin:trypsin was equal to 30:1–50:1 and incubation lasted for 40–60 min at 22 °C. Reaction was stopped by addition of PMSF to a final concentration of 0.1 mM and protein composition was analysed by native 15% PAGE in 25 mM Tris/80 mM glycine, pH 8.6 [23]. Purification of calmodulin tryptic peptides obtained in the presence of CaCl_2 (TRC peptides) or in the presence of EGTA (TRE peptides) was achieved by hydrophobic chromatography on phenyl-Sepharose as described by Brzeska et al. [24]. In the presence of 2 mM CaCl_2 peptide TR2C (residues 78–148) was not adsorbed on phenyl-Sepharose, whereas peptide TR1C (residues 1–77) was tightly bound to the matrix and was eluted by 2 mM EGTA.

Isocratic elution was used for purification of TRE peptides. The short C-terminal peptide TR3E (residues 107–148) does not interact with phenyl-Sepharose in the presence of 2 mM CaCl_2 and was eluted in the flowthrough. Under conditions used the largest N-terminal peptide TR1E (residues 1–106) weakly interacts with phenyl-Sepharose and was eluted after TR3E. The most tightly bound peptide TR2E (residues 1–90) appeared at the end of the elution profile. Fractions obtained after hydro-

Abbreviations used: MLCK, myosin light chain kinase; PDE, cyclic nucleotide phosphodiesterase; VU-60, mutant calmodulin containing an E31A point mutation.

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Figure 1 Dependence of the peak area on the quantity of intact calmodulin (1) and its tryptic peptides TR1C (2) and TR2C (3) loaded on the gel

phobic chromatography were analysed by native gel electrophoresis (either in the presence or in the absence of 6 M urea). The fractions containing homogeneous peptides were collected and subjected to repeated freeze-drying. Concentration of calmodulin peptides was determined by the method of Spector [25] using calmodulin as a standard.

Interaction of caldesmon and calponin with calmodulin and its peptides

The method of native gel electrophoresis was used for analysing interaction of caldesmon and calponin with calmodulin peptides. Calmodulin (or calmodulin peptides) (8–15 µM) in 10 mM glycine/30 mM Tris (pH 8.6), containing 10% (v/v) glycerol and 1% (v/v) mercaptoethanol was mixed with increasing quantities of caldesmon or calponin (molar ratio target protein/calmodulin varied from 0.15 to 7). The incubation mixture contained either 1 mM CaCl₂ or 2 mM EGTA. After incubation for 10–15 min at room temperature the samples (30–35 µl) were subjected to native gel electrophoresis which was also run either in the presence of 1 mM CaCl₂ or in the presence of 2 mM EGTA. Gels were stained with Coomassie Blue R-250 and scanned on an LKB Ultrascan XL densitometer. Each track was scanned two or three times at different positions and average areas of the peaks were calculated. If the shape of the band was irregular the number of scans collected was increased. As a rule the difference between peak areas determined by this way does not exceed 10–15%. All experiments were performed under conditions when the area under the calmodulin (or its peptides) peak was linearly dependent on the quantity of the protein (or peptide) loaded on the gel (Figure 1).

If calmodulin (or calmodulin peptides) interacts with the target protein then addition of increasing quantities of calponin or caldesmon will result in decrease of the intensity of the band of free (non-bound) calmodulin. Assuming that calmodulin (M) forms an equimolar complex with a target protein (T) the following equation can be used:

$$K = (T - M + m)m / (M - m)$$

where K is the apparent dissociation constant, T is the total concentration of target protein, M is the total concentration of calmodulin (or its peptides) and m is the concentration of free (unbound) calmodulin (or its peptides). Solving this square

equation against m and plotting m [which is proportional to the area of the peak of free (unbound) calmodulin] against T (total target protein concentration) we can determine the apparent dissociation constant.

It is supposed that under certain conditions 2 mol of calmodulin are bound per mol of calponin [11,15]. Therefore in the case of calponin the experimental data were fitted to a cubic equation describing the reaction $T + M \rightleftharpoons TM + M \rightleftharpoons TM_2$ [11]. However, the quality of fitting in this case was not better than that obtained by using the single-site model and therefore we used this simple model for describing our experimental results.

RESULTS

Interaction of calmodulin with target proteins measured by native gel electrophoresis

The method of native gel electrophoresis was successively used for analysing interaction of a number of Ca-binding proteins with caldesmon and components of troponin complex [10,23,26]. Under the conditions used isolated caldesmon has a rather low electrophoretic mobility and mainly remains on the top of the gel (Figure 2). On the other hand isolated calmodulin has high electrophoretic mobility and migrates as a sharp band in the lower part of the gel (Figure 2). When a fixed amount of calmodulin was titrated by caldesmon the intensity of the band of isolated calmodulin was decreased and the band becomes smeared (Figure 2). This fact can be explained by calmodulin–caldesmon complex formation. If this explanation is correct then in the absence of Ca²⁺ when calmodulin is unable to interact with caldesmon we should not observe any changes in the gel pattern. Indeed when calmodulin was titrated by caldesmon in the presence of EGTA and electrophoresis was performed in the absence of Ca²⁺ we did not observe any changes in the intensity of the band of isolated calmodulin even at a molar ratio of caldesmon/calmodulin equal to 2 (Figure 3). This means that native gel electrophoresis can be used for estimation of calmodulin binding to different target proteins.

We analysed the ability of this method to measure the interaction of calmodulin with caldesmon and calponin. The apparent dissociation constants of calmodulin–caldesmon and calmodulin–calponin complexes determined by native gel electrophoresis were equal to 0.23 and 1.3 µM respectively (Table 1). These values are close to the upper limits of the corresponding constants described in the literature [7,11,14,16]. In order to check the sensitivity of this method we compared the interaction of caldesmon with native calmodulin and its mutant (VU-60)

Figure 2 Interaction of intact wild-type calmodulin with caldesmon

Native gel electrophoresis of isolated calmodulin (lane 1), of a mixture of calmodulin with caldesmon (lanes 2–11) and of isolated caldesmon (lane 12). Calmodulin (12.8 µM; 6.9 µg on each track) was titrated by caldesmon in the presence of 1 mM CaCl₂. Molar ratio caldesmon/calmodulin was equal to 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.1 and 1.2 for lanes 2–11 respectively. The concentration of caldesmon in the samples loaded on lanes 11 and 12 was equal to 15.4 µM. Positions of free calmodulin (CaM) are marked by arrows.

Figure 3 Interaction of calmodulin with caldesmon in the presence of 2 mM EGTA (1) or in the presence of 1 mM CaCl_2 (2)

12.8 μM calmodulin (6.9 μg on each track) was titrated by caldesmon and subjected to the gel electrophoresis. Peak area of free calmodulin (in relative units) was plotted against total concentration of caldesmon added. Data were fitted to the following equation: Peak area = $A \times [\sqrt{\{(T-M+K)/2\}^2 + M \times K} - (T-M+K)/2]$, where symbols are defined as follows: T , total caldesmon concentration; M , total calmodulin concentration; K , apparent dissociation constant, and A , coefficient of proportionality. Solid line represents best fit curve with K_d 0.29 μM . Error bars indicate standard deviation.

containing a point mutation E31A. The first Ca-binding site of VU-60 is defective and this mutant has a significantly reduced affinity for caldesmon [27]. Addition of excess caldesmon results in complete disappearance of the band of free wild-type calmodulin (Figure 4). At the same time even in the presence of a 1.5-fold excess of caldesmon significant quantities of VU-60 mutant remained free (Figure 4). The apparent K_d for the VU-60 mutant was 4.7 μM and was more than 16 times larger than the corresponding constant for the wild-type calmodulin. These results correlate well with our earlier published results [27] and indicate that the method of native gel electrophoresis is sensitive and can be applied for analyses of calmodulin–target protein interaction.

Interaction of calmodulin peptides with caldesmon

Limited proteolysis of calmodulin in the presence of EGTA results in production of three peptides, TR1E (residues 1–106), TR2E (residues 1–90) and TR3E (residues 107–148) [24,28,29]. Addition of increasing quantities of caldesmon was accompanied by a significant decrease in the intensity of the TR3E band and only a marginal decrease and smearing of the TR1E band. These results mean that the C-terminal fragment of calmodulin interacts with caldesmon with higher affinity than its larger N-terminal counterpart. Indeed in experiments performed on isolated TRE peptides we found that the apparent K_d for TR1E was equal to 63 μM , whereas the corresponding value for TR3E was equal to 24 μM (Figure 5A, Table 1). It is worthwhile mentioning that the affinity of a shorter N-terminal peptide TR2E (residues 1–90) to caldesmon was comparable with that of the C-terminal peptide and was equal to 26 μM (Table 1). This may indicate that residues 91–106 of TR1E peptide somehow interfere with proper interaction of this peptide with caldesmon.

Limited trypsinolysis of calmodulin in the presence of Ca^{2+}

Table 1 Apparent dissociation constants (in μM) of intact calmodulin and its fragments to caldesmon and calponin

The results are mean $\pm$ S.D. with a number in parentheses representing the number of independent experiments each of which contains no less than six data points.

Calmodulin or its peptides	Caldesmon	Calponin
Intact calmodulin (residues 1–148)	0.23 ± 0.1 (9)	1.3 ± 0.4 (6)
TR1C (1–77)	11.9 ± 3.2 (3)	41.4 ± 6.7 (3)
TR2C (78–148)	4.6 ± 2.0 (3)	3.8 ± 1.3 (4)
TR1E (1–106)	63.4 ± 24.4 (4)	298.0 ± 138.0 (3)
TR2E (1–90)	25.2 ± 5.9 (4)	118.0 ± 50.0 (3)
TR3E (107–148)	23.9 ± 4.6 (4)	12.1 ± 3.7 (3)

Figure 4 Titration of wild-type calmodulin (1) and calmodulin mutant VU-60 (12.8 μM , 6.9 μg on each track) (2) by caldesmon

Fraction of calmodulin remained unbound is plotted against total caldesmon concentration. Solid lines represent best fit curves with K_d equal to 0.29 μM for wild-type calmodulin and 4.7 μM for VU-60 mutant.

results in production of two main peptides, TR1C (residues 1–77) and TR2C (residues 78–148) [24,28–30]. The C-terminal TR2C peptide interacts with caldesmon with a K_d of about 5 μM , whereas the N-terminal TR1C peptide has lower affinity, having a K_d of about 12 μM (Figure 5B, Table 1). These results correlate with the results obtained on TRE peptides and indicate that a large C-terminal TR2C peptide harbours the centre of high affinity to caldesmon. This centre is not completely destroyed even in a shorter TR3E peptide (residues 107–148), although this peptide interacts with caldesmon with lower affinity than TR2C (residues 78–148) peptide. There is the second caldesmon-binding site located in the N-terminal part of calmodulin. This centre seems to be located in the peptide TR1C (residues 1–77). It is worthwhile to mention that the larger N-terminal peptides (TR1E or TR2E) have lower affinity to caldesmon than TR1C. This could be due to the difference in the overall structure of these peptides or to the interfering effect of residues 78–106 when they are included in the N-terminal fragments of calmodulin.

Interaction of calmodulin and its peptides with calponin

The pI value of calponin is equal to 9.9 [8], therefore at pH 8.6 calponin has a very low electrophoretic mobility and practically

Figure 5 Interaction of calmodulin peptides with caldesmon

(A) Titration of isolated TR1E (1) (5.3 μ M, 2 μ g on each track) and TR3E (2) (5.3 μ M, 0.8 μ g on each track) peptides of calmodulin by caldesmon. (B) Titration of isolated TR1C (1) (5.6 μ M, 1.5 μ g on each track) and TR2C (2) (6.8 μ M, 1.7 μ g on each track) peptides of calmodulin by caldesmon. Fraction of calmodulin peptides remaining unbound is plotted against total caldesmon concentration. Solid lines represent best fit curves.

does not enter the gel (Figure 6). Titration of calmodulin by calponin is accompanied by a significant decrease in the intensity of the band of free calmodulin and appearance of new bands with intermediate mobility located in the upper part of the gel and probably corresponding to the calmodulin–calponin complexes formed (Figure 6). As already mentioned the apparent K_d value for calmodulin–calponin complex, as measured by decrease in the intensity of free calmodulin band, was equal to 1.3 μ M. Titration of a mixture of TRC peptides of calmodulin by calponin results in a significant decrease in the intensity of TR2C and only a marginal decrease in the intensity of the TR1C band. Similar results were obtained when isolated TRC peptides of calmodulin were mixed with calponin (Figure 7A). The apparent K_d for TR1C was about 40 μ M, whereas the corresponding constant for TR2C was equal to 4 μ M and was only slightly larger than the K_d value for intact calmodulin (Table 1). This means that the C-terminal fragment of calmodulin (residues 78–148) contains the calponin-binding site.

Figure 6 Interaction of calmodulin with calponin

Calmodulin (10.5 μ M, 5.7 μ g on each track) was mixed with calponin in the presence of 1 mM CaCl_2 and subjected to native gel electrophoresis. Molar ratio calponin/calmodulin was equal to 0, 0.18, 0.35, 0.53, 0.71, 0.88, 1.06, 1.23, 1.41, 1.59, 1.76 for tracks 1–11 respectively. Concentration of calponin in the samples loaded on tracks 11 and 12 was equal to 18.5 μ M.

We also analysed the interaction of TRE peptides of calmodulin with calponin. The short C-terminal TR3E fragment (residues 107–148) has a rather high affinity to calponin ($K_d = 12 \mu$ M), whereas the large N-terminal fragments (TR1E and TR2E) interact with calponin with a very low affinity (Figure 7B, Table 1). The data obtained indicate that the high-affinity calponin-binding site is located in the C-terminal part of calmodulin (residues 78–148). Even a shorter C-terminal fragment (residues 107–148) has high affinity to calponin. The second much weaker calponin-binding site is located in the N-terminal part of calmodulin (residues 1–77).

DISCUSSION

Limited trypsinolysis of calmodulin in the presence of Ca^{2+} results mainly in the cleaving of the peptide bond at Lys-77 [31], although peptide bonds of Arg-74 and Lys-75 are partially susceptible to proteolysis [28]. When calmodulin is cleaved by trypsin in the presence of EGTA peptide bonds of Arg-106, Arg-90 and to a smaller extent of Lys-77 were split by enzyme [28,31]. The resulting mixture of TRC or TRE peptides can be completely separated by HPLC [28,29] performed in the presence of acetonitrile, i.e. under rather harsh conditions. A much milder procedure for peptide purification was introduced by Brzeska et al. [24] and is based on hydrophobic chromatography on phenyl-Sepharose. Using this procedure we were unable to separate peptides differing by 3–4 residues (e.g. fragments 1–74, 1–75 and 1–77 or 75–148, 76–148 and 78–148). At the same time in the course of this type of purification the peptides are not exposed to organic solvents and the probability of their denaturation is reduced. This fact seems to be especially important when the interaction of peptides with low-affinity calmodulin-binding proteins is to be analysed.

Calmodulin fragments are much less effective than intact calmodulin in binding and activating a number of enzymes. For example none of the fragments activates calcineurin or PDE [28], although at very high concentrations TR2C peptide binds to PDE and activates phosphorylase kinase and erythrocyte Ca-ATPase [28,29]. In the case of caldesmon we also found that two parts of calmodulin obtained in the presence of CaCl_2 (TR1C and TR2C peptides) interact with the target protein with affinity which was at least 20 times lower than that of the native calmodulin (Table 1), although both halves of calmodulin bind to caldesmon. This can be interpreted as an indication of the presence of two caldesmon-binding sites in the calmodulin structure. This conclusion agrees with our results obtained on

Figure 7 Interaction of calmodulin peptides with calponin

(A) Titration of TR1C (1) (6.8 μ M, 1.8 μ g on each track) and TR2C (2) (6.8 μ M, 1.7 μ g on each track) peptides of calmodulin by calponin. B. Titration of TR1E (1), TR2E (2) and TR3E (3) peptides of calmodulin (5 μ M, less than 1.8 μ g of peptide on each track) by calponin. Fractions of peptides remaining unbound is plotted against total calponin concentration. Solid lines represent best fit curves.

calmodulin mutants [27]. Mutation of residues 118–120 located in the C-terminal part of calmodulin strongly affects its interaction with caldesmon. A very strong effect on caldesmon binding was observed when the first or the second N-terminal Ca-binding sites were modified by point mutations ([27] and this paper). The caldesmon-binding site located in the C-terminal part of calmodulin has higher affinity than the second site located in the N-terminal part of molecule (compare data for TR1C and TR2C). Since affinity of TR2C was about four times higher than that of TR3E we may suppose that residues 78–107 play important roles in high-affinity binding of caldesmon to calmodulin. The low-affinity caldesmon-binding site seems to be restricted by residues 1–77 of calmodulin. Any residues added to this N-terminal fragment of calmodulin impair interaction with caldesmon (Table 1). This could be due to the fact that the structure of TR1E (and probably TR2E) is different from the corresponding parts of calmodulin in the intact molecule [30].

Intact calmodulin interacts with calponin with low affinity [8,11,15,16] and calmodulin fragments bind calponin with even

lower affinity (Table 1). However, TR2C peptide of calmodulin interacts with calponin with a K_d which was only three times higher than the corresponding value for intact calmodulin. This means that the main calponin-binding site is located in the C-terminal half of calmodulin and is restricted by residues 78–148. It is worthwhile mentioning that even a shorter peptide TR3E (residues 107–148) interacts with calponin with a rather high affinity. At the same time all fragments derived from the N-terminal part of calmodulin interact only weakly with calponin. Thus the contribution of the N-terminal part of calmodulin is less pronounced in calponin than in caldesmon binding.

Two structural models of the interaction of Ca-binding and target proteins have been postulated in the literature. Calmodulin-binding sites of a number of target proteins have a structure of amphiphilic α -helix. Peptide analogous to the calmodulin-binding sites of MLCK and calmodulin-dependent protein kinase II, forming an amphiphilic α -helix, is located in a tunnel formed by a bent calmodulin molecule [4–6]. A similar type of interaction was postulated for the peptide containing the first calmodulin-binding site (site A, residues 651–667) of caldesmon and calmodulin [32]. Another type of interaction was postulated for troponin C (which is similar to calmodulin) and troponin I. In binary troponin C–troponin I complex troponin C has an extended conformation similar to that found in crystallized isolated troponin C (or calmodulin) [33,34]. In this case a large portion of the mainly α -helical central part of troponin I wraps around the central helix of troponin C in an antiparallel manner [26,34]. Recently, published work of Mabuchi et al. [35] indicates that the mode of interaction of intact caldesmon and of its fragments with calmodulin may be different and that intact caldesmon interacts with an extended molecule of calmodulin. If this suggestion is correct, then we may suppose that the structure of the calmodulin–caldesmon complex is similar to that of the troponin C–troponin I complex. In this case the C-terminal domain of calmodulin tightly interacts with site A (residues 651–667) of caldesmon and the N-terminal domain of calmodulin interacts with the second calmodulin-binding site of caldesmon (site B, residues 744–752) which is involved in the regulation of actomyosin ATPase activity [12,13]. If the same type of suggestions are applicable to calmodulin–calponin complex, then the low effectivity of calmodulin in reversion of calponin-induced inhibition of actomyosin ATPase activity can be explained by the low affinity of the N-terminal domain of calmodulin to calponin. We plan to check these suggestions by analysing the interaction of caldesmon (or calponin) fragments with different parts of the calmodulin molecules.

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