Involvement of caldesmon at the actin–myosin interface

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Addition of myosin subfragment 1 (S-1) to the actin–caldesmon binary complex, which forms bundles of actin filaments resulted in the formation of actin/caldesmon-decorated filaments [Harricane, Bonet-Kerrache, Cavadore & Mornet (1991) *Eur. J. Biochem.* **196**, 219–224]. The present data provide further evidence that caldesmon and S-1 compete for a common actin-binding region and demonstrate that a change occurs in the actin–myosin interface induced by caldesmon. S-1 digested by trypsin, which has an actin affinity 100-fold weaker than that of native S-1, was efficiently removed from actin by caldesmon, but not completely dissociated. This particular ternary complex was stabilized by chemical cross-linking with carbodi-imide, which does not have any spacer arm, and revealed contact interfaces between the different protein components. Cross-linking experiments showed that the presence of caldesmon had no effect on stabilization of actin–(20 kDa domain), whereas the actin–(50 kDa domain) covalent association was significantly decreased, to the point of being virtually abolished.

INTRODUCTION

In smooth muscle, regulation of actomyosin ATPase is mediated through the interaction of proteins associated with the thin filament [1–3]. Tropomyosin, a component of the thin filament, is involved in this process, along with caldesmon, which is a specific component of the F-actin filament in smooth muscle cells [4,5]. Myosin inhibition of actin-activated ATP hydrolysis promoted by caldesmon is removed by Ca^{2+} /calmodulin binding to caldesmon [6,7]. Conformational changes occur in both caldesmon and tropomyosin when bound to the actin filament, and interactions between them amplify the caldesmon-induced inhibition of actin-dependent myosin ATPase activity [8]. However, the exact molecular mechanisms responsible for the inhibition of ATPase activity are not yet known.

The N-terminal region of the caldesmon molecule contains a binding site for the myosin subfragment 2 (S-2) region [9–11], but the caldesmon region responsible for inhibition of myosin/actin-activated ATPase activity involves the C-terminus, which contains actin- and calmodulin-binding sites [12–14].

It has also been reported that the difference between skeletal muscle heavy meromyosin (HMM) and myosin subfragment 1 (S-1) is due only to the presence of the S-2 region in HMM [15], and not to the associated light-chain composition. We previously analysed the effect of skeletal S-1 on the actin–caldesmon complex to obtain more detail on the interaction of both caldesmon and the head portion of myosin with the actin filament. The use of S-1 instead of the HMM molecule, which contains both the S-1 and the S-2 regions, allows differentiation of ‘non-productive’ myosin head binding from the binding causing actin-activated ATP hydrolysis [16]. Using the full-length caldesmon molecule, we have now further characterized the ternary complex (caldesmon–S-1–actin) in the rigour state by electron microscopy. The actin filament was decorated by native S-1 in the presence of caldesmon as demonstrated by gold particle labelling, which detected caldesmon-specific antibodies [17].

In this study we utilized trypsin-split S-1 (i.e. S-1, digested by trypsin), which has an actin affinity 100-fold weaker than that of native S-1, and investigated its interaction with actin in the presence of caldesmon. Because of the lower actin affinity of this

S-1 derivative [18], we used chemical cross-linking experiments to demonstrate that caldesmon alters the actomyosin interface. We also studied the stability of the actin–caldesmon–(trypsin-split S-1) ternary complex in the presence of either calmodulin/ Ca^{2+} or tropomyosin.

The results of these experiments confirm the competitive binding between caldesmon and S-1 for the same actin-binding region [19]. These data also provide new accurate details on the existence of an actin–caldesmon–S-1 ternary complex.

EXPERIMENTAL

Protein preparations

Caldesmon was prepared from turkey gizzard muscle according to the procedure of Bretscher [20]. Rabbit skeletal actin was obtained as described by Eisenberg & Kielly [21]. Rabbit skeletal native S-1 and trypsin-split S-1 were obtained as previously described [22]. Chicken gizzard tropomyosin was obtained according to Bretscher [20]. Bovine brain calmodulin was purchased from Sigma. The concentrations of S-1, F-actin and tropomyosin were estimated spectrophotometrically using values for $A_{1\text{cm},280}^{1\%}$ of 7.5, 11.0 and 3.3 respectively. The caldesmon and calmodulin concentrations were measured using values for $A_{1\text{cm},278}^{1\%}$ of 3.3 and 2.0 respectively. Protein band absorbance was measured with a high-resolution gel densitometer (Hoefer Scientific Instruments, GS300) at 580 nm. The molecular masses of proteins were estimated by comparing their electrophoretic mobilities with those of the following protein markers: myosin heavy chain (200 kDa), phosphorylase B (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa).

SDS/polyacrylamide gel electrophoresis

PAGE was performed with 3–15% gradient slab gels containing 0.8% *NN'*-methylene bisacrylamide and 0.1% SDS according to Laemmli [23]. Proteins were either directly viewed under u.v. light and/or stained with Coomassie Blue R-250 and diffusion-destained.

Abbreviations used: EDC, 1-ethyl-[3-dimethylamino]propyl]carbodi-imide; S-1, myosin subfragment 1; S-2, myosin subfragment 2; HMM, heavy meromyosin.

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Fig. 1. Comparison of the effects of EDC treatment of the native proteins caldesmon, actin and trypsin-split S-1

Time courses of the first 9 min of cross-linked product formation are shown. (a) Caldesmon dimer formation (240 kDa), (b) actin trimer formation (120 kDa), (c) covalent association of the 50 kDa and 20 kDa domains of trypsin-split S-1 (LC, light chains). Lanes T contain molecular mass markers.

Carbodi-imide modification

Native or fluorescently labelled protein molecules associated in binary and ternary complexes were incubated with 2 mM-1-ethyl-[3-(3-dimethylamino)propyl]carbodi-imide (EDC) (Serva) for 3, 6, 9 or 12 min at room temperature in 50 mM-Mes buffer, pH 6.5. The reactions were stopped by the addition of 0.1 M-2 β -mercaptoethanol and aliquots were separated by SDS/PAGE as previously described [24].

Binding assays

The binding of trypsin-split S-1 domains (27, 50 and 20 kDa) to actin in the presence and absence of caldesmon was measured by sedimenting the different complexes in a Beckman ultracentrifuge (170 000 *g*, 30 min) and determining the free trypsin-split S-1 by SDS/PAGE analysis as previously described [25]. According to the method of Chalovich *et al.* [4], caldesmon was added to actin, stirred for several minutes prior to the addition of trypsin-split S-1 at a caldesmon/actin ratio of 1:7. The solution was centrifuged and the fraction of trypsin-split S-1 was estimated by densitometric measurements of the 50 kDa heavy chain band present on SDS electrophoretic gels.

RESULTS

In establishing the contact zones resulting from complex formation between different proteins, the EDC-promoted cross-linkings were particularly useful for many actin-binding proteins. These cross-linking experiments included both proteins used in this study, as reported separately for caldesmon [26] and trypsin-split S-1 [27].

The EDC-induced covalent associations in all protein mixtures were controlled under similar conditions. This study was carried out in three steps because of the possible protein-protein association of caldesmon, which is able to form dimers, of F-actin, which results from the polymerization of G-actin molecules, or of the interdomain interaction in trypsin-split S-1. We first analysed the effects of EDC treatment on each protein alone, then on the actin-caldesmon and actin-trypsin-split-S-1 binary complexes, and finally on the actin-caldesmon-trypsin-split-S-1 ternary complex in the presence and absence of tropomyosin and/or calmodulin.

EDC treatment of each native protein

Each protein was submitted to EDC (2 mM) treatment for

Fig. 2. Covalent association of two binary complexes upon EDC treatment

(a) Actin-caldesmon (Act-CaD) cross-linking increased with time during the first 12 min. (b) Actin-trypsin-split-S-1 association linked by EDC gave two major products of 60 kDa [actin-20 kDa domain] and 90 kDa [actin-50 kDa domain]. LC, light chains.

12 min. The Coomassie Blue patterns obtained during the time course of EDC treatment are shown in Fig. 1. With caldesmon a major new species appeared with a molecular mass of 240 kDa, as a result of dimerization of the molecule. Other oligomers were also stabilized by the chemical cross-linker and there was a high tendency for caldesmon to self-associate (Fig. 1a). Under these conditions, treatment of F-actin with EDC resulted in the formation of only small quantities of stabilized 120 kDa product, which corresponded to actin trimer formation, while very little dimer formation occurred (Fig. 1b). After EDC treatment, the trypsin-split S-1, which contains an association of 27, 50 and 20 kDa domains, produced only a small amount of a protein band around 70 kDa (Fig. 1c). This was identified as being a covalent association of 50 kDa and 20 kDa fragments by fluorescent labelling of the 20 kDa SH-1 cysteine group (as previously described by Harricane *et al.* [17]).

EDC treatment of binary mixtures

The actin-caldesmon complex at a 7:1 molar ratio was submitted to EDC treatment. Four main cross-linked products (Fig. 2a) appeared rapidly, and were determined by fluorescent incorporation of either actin or caldesmon to be a covalent

Fig. 3. Covalent associations produced by EDC treatment of the ternary actin-caldesmon-trypsin-split-S-1 ternary complex

(a) Lane T, molecular mass markers; lane A, covalent products formed during EDC treatment for 6 min of actin-caldesmon complex; lane B, covalent products formed during EDC treatment for 6 min of actin-trypsin-split-S-1; lane C, caldesmon association after 6 min of EDC treatment. The time course of the ternary complex over 12 min of treatment with EDC is shown (lanes 0, 3, 6, 9 and 12); mainly the actin-(20 kDa domains) of S-1 was stabilized. LC, light chains; CaD-act, caldesmon-actin. (b) Comparative densitometric measurements at 580 nm actin-(20 kDa domain) (○, ●) and actin-(50 kDa domain) (□, ■) cross-linked products in the absence (○, □) and in the presence (●, ■) of caldesmon during EDC treatment.

association of actin and caldesmon at a 1:1 ratio by the method of Bartegi *et al.* [26]. Treatment of the other actin and trypsin-split S-1 binary complex with EDC resulted in the stabilization of two actin cross-linking products, one with the 50 kDa fragment (corresponding to the 90 kDa protein band) and another with the 20 kDa fragment (corresponding to the 60 kDa protein band), as previously demonstrated by Kassab *et al.* [28]. These results are presented in Fig. 2(b).

EDC treatment of the ternary mixtures

The actin-caldesmon-trypsin-split-S-1 complex was obtained at a 7:1:1:7 molar ratio and then submitted to EDC treatment. The products were identified by electrophoresis analysis to be similar to products obtained from previously reported binary complexes, as shown in lane A (EDC-treated actin-caldesmon), lane B (EDC-treated actin-trypsin-split-S-1) and lane C (EDC-treated caldesmon) of Fig. 3(a).

The time course of the cross-linking process (Fig. 3a) shows that caldesmon influenced the covalent attachment of trypsin-split S-1 to actin, but also that trypsin-split S-1 decreased the actin-caldesmon association for all cross-linked products except the 240 kDa species. Since fluorescence in both actin and caldesmon was observed with this product (results not shown), it was identified as being an actin-caldesmon covalent association. Nevertheless, caldesmon dimer formation seemed to occur concomitantly during EDC treatment.

However, the main observation was the significant decrease in the formation of the 90 kDa protein band while the 60 kDa protein band appeared normally. After 9 and 12 min, some products migrating as a 90 kDa band were slightly visible. The Coomassie Blue-stained gel was densitometrically analysed, as shown in Fig. 3(b). Formation of the actin-(20 kDa domain) band was only slightly affected by the presence of caldesmon. In contrast, the actin-(50 kDa domain) covalent union was strongly affected by the presence of caldesmon. This effect of caldesmon was completely abolished when tropomyosin was added to the actin-caldesmon-trypsin-split-S-1 ternary complex. The presence of tropomyosin enhanced the caldesmon-actin covalent union, whereas the addition of calmodulin decreased the caldesmon-actin covalent union with a concomitant increase in actin-(50 kDa domain) product formation (results not shown).

The actin-binding properties of trypsin-split S-1 in the presence and absence of caldesmon were estimated using airfuge centrifugation with increasing actin concentrations. Densitometric measurements of the trypsin-split S-1 remaining in the supernatant indicated about a 30-fold decrease in the actin affinity relative to that of native S-1 in the absence of caldesmon ($K_a = 2.0 \pm 1.1 \mu\text{M}^{-1}$; mean $\pm$ S.D.; $n = 4$). This affinity was significantly decreased by about 7-fold in the presence of caldesmon ($K_a = 0.3 \pm 0.9 \mu\text{M}^{-1}$).

DISCUSSION

The results in this study confirm and provide new data on the competition between caldesmon and S-1 for one common actin-binding region. Each protein alone interacts with actin at two dependent binding sites at least [19,28]. Thus both proteins can still be associated on the same actin filament, forming a ternary complex in which binding of one partner to actin weakens the binding of the other partner.

In previous studies [17] we revealed the presence of an actin-caldesmon-S-1 ternary complex. The use of trypsin-split S-1 instead of chymotryptic S-1 confirms the existence of weakly and strongly associated forms of actin-caldesmon complex [16] which contain at least two different interaction sites between actin and caldesmon [29].

The use of chemical cross-linking experiments showed that S-1 decreased the association of actin-caldesmon for all cross-linked products, as shown in Fig. 3(a). However, there was not complete exclusion of caldesmon from actin, as shown by the small amount of caldesmon-actin covalent complexes remaining stabilized during EDC modification of the ternary complex. On the other hand, Fig. 3(a) clearly indicates that caldesmon influenced the S-1 covalent attachment of S-1 to actin. Indeed, the actin-(50 kDa domain) covalent union was strongly affected by the presence of caldesmon, while actin-(20 kDa domain) binding was only slightly altered (Fig. 3b). Thus the ternary complex exists as covalent species of F-actin-caldesmon-S-1, in which S-1 is linked by its 20 kDa domain but not by its 50 kDa domain. A schematic drawing of the cross-linking experiments showing that the covalent linkage induced by EDC differed for the ternary complex is shown in Fig. 4. This result is in accordance with that obtained on dibromobimane modification of S-1, which revealed

Fig. 4. Schematic representation of the different actin complexes with caldesmon and trypsin-split S-1

(a) Binary complex of actin and caldesmon. (b) Binary complex of actin and trypsin-split S-1; there is strong interface between actin and the 50 kDa and 20 kDa domains. (c) Ternary complex of actin-caldesmon-trypsin-split-S-1. The interface between actin and trypsin-split-1 is weakened by the presence of caldesmon. Only the 20 kDa domain is linked to actin, as revealed by EDC treatment.

a decrease in the affinity for actin [30], but in that particular case it was shown to result in an internal covalent cross-linking which excluded 20 kDa domain from the actin interface [30]. This covalent link induced by dibromobimane was assumed to stabilize a conformation change, a process which may enable caldesmon to have a regulatory effect on smooth muscles.

The C-terminal part of actin, close to the N-terminus and involved in the caldesmon-actin interface, could be of importance as suggested by Makuch *et al.* [31]. There is evidence from chemical cross-linking, n.m.r. and immunological studies that caldesmon interacts with the N-terminal segment of actin [19,26,32]. However, the negatively charged residues 1-7 of actin appear to be involved in its binding to caldesmon and to S-1 in the presence of nucleotide [26,28]. This N-terminus of smooth muscle actin, which differs in four of the seven N-terminal amino acids from the skeletal muscle actin sequence, participated to a lesser degree than expected in the caldesmon-actin interface. Our results further suggest that the regulatory function of caldesmon would be to alter binding of the 50 kDa domain of S-1 to the N-terminal part of actin, but to leave the association with the 20 kDa domain of S-1 intact (see Fig. 4c). This is of particular interest considering the recent evidence [33] that Fab-(1-7)-peptide is efficient in inhibiting the actin-S-1 association in the presence of analogous nucleotides.

Studies using a mosaic multiple-binding model for ternary complex formation [34,35] are in agreement with our present results demonstrating that S-1 binds actin with a different orientation in the presence of caldesmon. These recent studies by other authors showed that both proteins, S-1 and caldesmon, can be either associated on the same filament and/or in competition

for one common actin-binding site, but under different conditions.

Our present results provide evidence that the 50 kDa domain of S-1 is removed from the actin interface in the presence of caldesmon. Indeed, S-1 ATPase activity was previously shown to be dependent on the integrity of this central domain [36,37]. Therefore the N-terminal region of actin, which is not accessible to the 50 kDa domain of S-1 in the presence of caldesmon, is suggested to be responsible for inhibition of the ATPase activity of actomyosin. The nature of this inhibitory effect must be investigated further to define the specific spatial disposition of caldesmon on actin which induces conformational change in actin and promotes inhibition at the S-1-actin binding interface.

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