Complexes between 15 kDa caldesmon fragment and actin investigated by immuno-electron microscopy

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The regulatory system of smooth muscle thin filaments is thought to involve a major calcium-calmodulin and actin binding protein: caldesmon. A dissective approach was used to isolate a 35 kDa C-terminal fragment of the molecule and to produce antibodies reacting against both the intact and the 15 kDa N-terminal end of this parental fragment. While this purified 15 kDa caldesmon fragment demonstrates a weak actin association, we observed that it cross-links actin filaments into loose bundles. These structures were labelled with a selective antibody and showed regular periodic striation with repeats of approximately 40 nm. This work brings additional information to previous reports using an actin and calmodulin binding 25 kDa C-terminal fragment of the caldesmon molecule [(1989) J. Biol. Chem. 264, 2869–2875]. We demonstrate that a purified fragment corresponding to a sequence smaller than 96 amino acids, which contains no cystein residue, is able to interact with actin at a single site which is not the calmodulin modulated.

Immuno-electron microscopy; Actin; Caldesmon

1. INTRODUCTION

Caldesmon, found recently in smooth [1], corresponds to a major protein involved in control of the actomyosin interaction. Further characterization showed that this protein interacts with actin and calmodulin and also with tropomyosin and myosin. The myosin binding site is located in the N-terminal part of the molecule [2], the actin and calmodulin binding sites [3] and the tropomyosin binding region [4] are confined to its C-terminal end. This has been carefully reported, with the help of the C-DNA deduced amino-acid sequence, in a tentative map of the major binding domains of caldesmon [5]. Antibodies have already been used as specific antagonists to investigate the caldesmon role in the thin filament regulation [6,7]. In this study, we observed by electron microscopy the complexes actin-caldesmon and actin-15 kDa fragment of caldesmon. We used specific antibody to selectively label the induced actin filament aggregation. The results presented here enabled us to propose an explanation for the bundling properties of caldesmon on actin filaments as being an induced conformational change rather than an oxidation process of the molecule.

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2. MATERIALS AND METHODS

2.1. *Protein preparations*

Turkey gizzard smooth muscle caldesmon and specific fragments were obtained from fresh material following the previously described procedure [8]. Skeletal F-actin was obtained according to Eisenberg and Kielly [9]. Extinction coefficients were 280 nm, 11.0 for F-actin [10] and caldesmon or purified caldesmon fragment concentrations were determined by Lowry assay using bovine serum albumin as standard [11].

2.2. *Immunization procedure*

Two rabbits were immunized using multiple injections (2 mg/injection) of 35 kDa caldesmon fragment emulsified in Freund's complete adjuvant and standard immunization protocols. The specificity of our antibodies was assessed by Western immunoblotting.

2.3. *Electron microscopic conditions*

Electron microscopy was performed using negative staining as previously described [11]. Two different procedures were used for antibody labelling: (i) The caldesmon and antibody complex was obtained by preincubation for 10 min (0.3 mM CaD and diluted antiserum 1/250), then mixed with F-actin at a caldesmon/actin molar ratio of 1/7. (ii) antibody was added to the actin-caldesmon complex in the same dilution and incubated 15 min before electron microscopy processing. The same procedures were followed when a 15 kDa caldesmon fragment was used instead of native caldesmon.

3. RESULTS

As shown in Fig. 1, the immunoblot of the protein bands is strongly positive for each isolated species. Thus we have produced an antibody that selectively recognizes native caldesmon by its C-terminal end, either as 35 kDa or as 15 kDa segments. The antiserum

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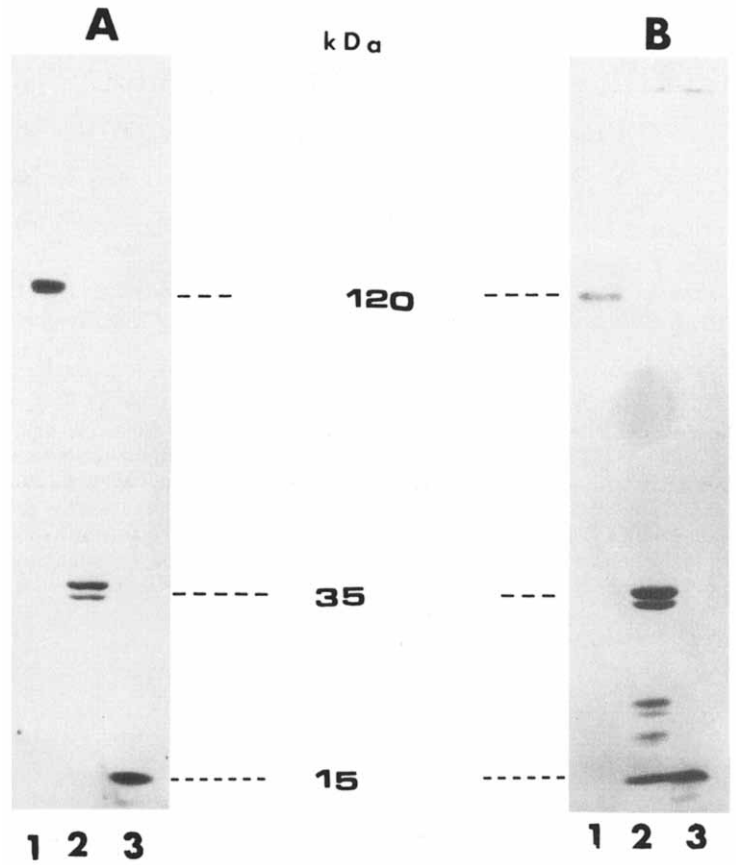

Fig. 1. Immunoblot of SDS analysis of purified native caldesmon and caldesmon fragments. Proteins purified samples are: lane 1, native caldesmon; lane 2, 35 kDa C-terminal thrombic fragment; lane 3, 15 kDa submaxillaris-fragment. (A) Coomassie Blue staining. (B) Corresponding immunoblot shown by 35 kDa antiserum diluted 1000 times. Note: The pattern in lane 2 shows some breakdown products.

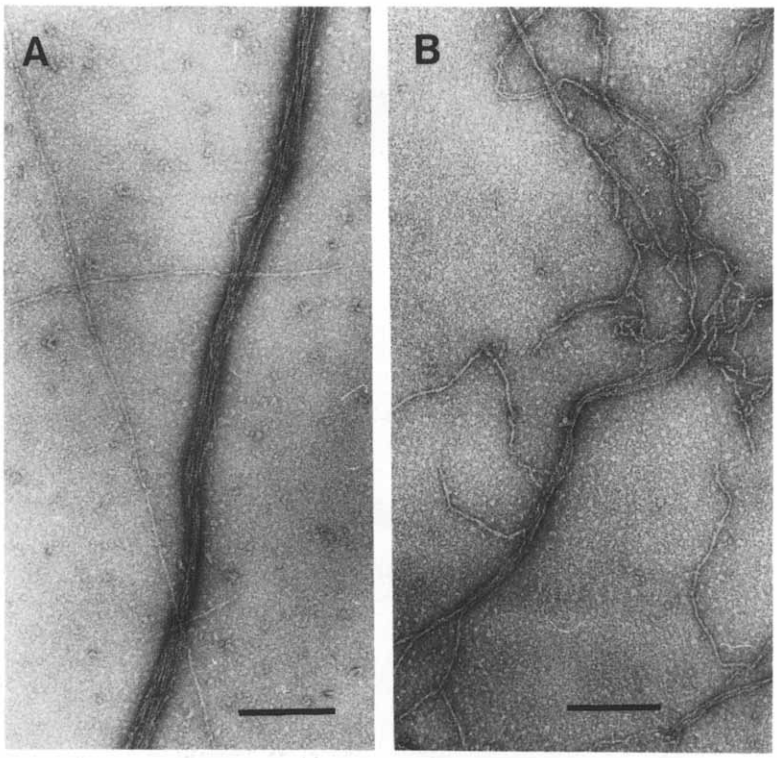

Fig. 2. Ultrastructural study: F-actin was incubated for 10 min with native caldesmon (A) or with 15 kDa fragment (B). Bar: 0.25 μm.

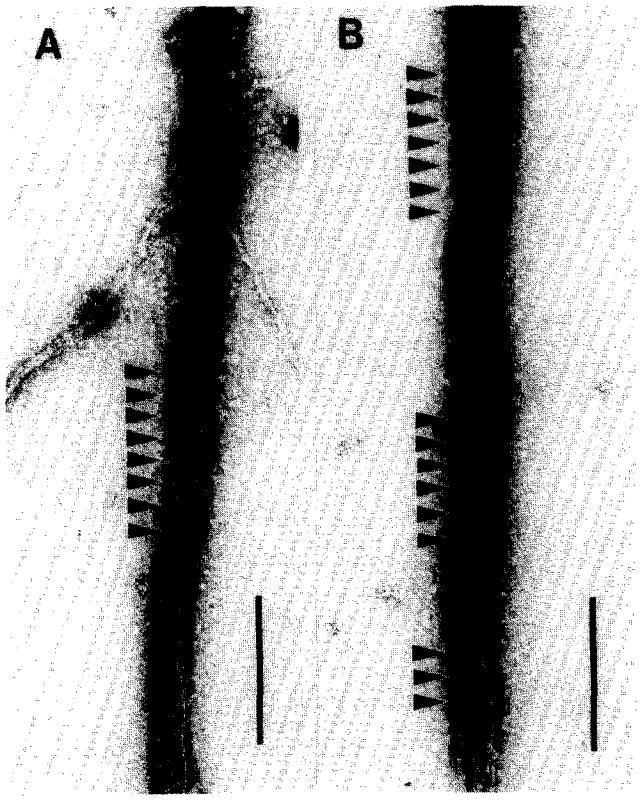

Fig. 3. Electron micrographs of the complex of actin +15 kDa caldesmon + antibody. Alternative striation is indicated by black arrows and are clearly visible in A and B. Note: Loose bundles increased upon longer incubation times but are always different and less packed than those obtained with native caldesmon. Bar: 0.25 μm.

did not recognize actin or the 90 kDa N-terminal part of caldesmon.

We compared in the same 7/1 actin/caldesmon ratio for each experiment, electron microscopic images (Fig. 2) of actin mixed with native caldesmon (A), 15 kDa fragments (B).

When native caldesmon was mixed with F-actin, large bundles were induced and a few free F-actin filaments were present. Longitudinal associations of twisted F-actin were tightened in these structures, so filaments could not be counted. (Similar structures were obtained with 35 kDa fragment [11].)

On the contrary, observed negative staining of the 15 kDa caldesmon fragment and actin association gave rise to large bundles with networks of weak twisted filaments. These structures formed an irregular wide net, as shown in Fig. 2B, in which lateral associations of loosely packed actin filaments were easily counted.

These structures were then observed, with the help of 35 kDa antibody, at higher magnification allowing us to localize caldesmon or caldesmon fragments in the actin induced bundles.

Caldesmon is located (Fig. 3) along the actin filaments with a periodicity of 37 nm. However, the images obtained with 15 kDa fragment enabled us to show on looser, less packed and straight bundles, clearer cross-striation patterns of these structures. The two experimental procedures used for the complex formation induced similar structures, indicating that this selective antibody does not prevent or weaken the actin-caldesmon interface.

4. DISCUSSION

In this investigation, attempts were made to determine whether caldesmon oxidation is needed to assemble F-actin filaments into bundles. To accomplish this, turkey gizzard caldesmon was proteolytically cleaved. We have specifically produced a 15 kDa fragment which recognized actin, and we have controlled by sequencing that no cysteine residue was present in its amino acid sequence.

We demonstrated that the 15 kDa fragment contains an actin binding region inducing weak links between actin filaments, rather than compact aggregations of tight bundles.

Additionally, we observed a striation within the actin bundles with a periodicity of 37 to 40 nm when labelled by the specific 35 kDa antibody, in complete agreement with recent results [13–14]. In comparison with chymotryptic 40 kDa fragment, the thrombic 35 kDa C-terminal domain is shortened by 32 residues in its N-terminal extremity [5], thus we can conclude that this shortening does not alter the antigenicity of the produced antibody. Riseman et al. [14] used a 25 kDa C-terminal end of the parental 40 kDa chymotryptic fragment and they were surprised to observe that it contrasted with the reduced intact molecule, cross-links of F-actin into tightly ordered bundles. Here we demonstrate that a fragment as small as our 15 kDa fragment, the N-terminal end of the parental 35 kDa thrombic fragment (see Fig. 3), is also able to produce loosely ordered bundles which thickened upon incubation. This result shows the presence of two kinds of actin binding sites within caldesmon. We propose that this peptide of 96 amino acids (without cysteine residue) must induce a conformational change in the actin filaments, increasing their ability to aggregate. However, this interaction could not be dependent on calmodulin switching, since this fragment does not contain any calmodulin binding site [8].

We conclude that caldesmon bundle formation involves different interaction sites with actin, at least two or more according to the last report of Wang et al. [15]. This effect on actin assembly and the possible existence of weak and strong forms of actin-caldesmon complex is in agreement with recent proposals of Galazkiewicz et al. [16]. This is presently investigated with the regulatory function of caldesmon being carried out on the acto-myosin complex.

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