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N-terminal domain of dystrophin

Armelle Bonet-Kerrache*, Eric Fabbrizio, Dominique Mornet

INSERM U 300, Faculté de Pharmacie, Avenue Charles Flahault, 34060 Montpellier, France

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Abstract Contro-versial experiments have been published on calmodulin binding of dystrophin. In this study, we used recombinant proteins and the techniques of affinity chromatography and ELISA to show that the N-terminal part of dystrophin binds calmodulin specifically in a calcium-dependent manner. The calcium-dependent interaction of calmodulin and dystrophin does not directly regulate binding of actin to dystrophin, but may regulate dystrophin interactions with other associated proteins.

Key words: Duchenne muscular dystrophy; Dystrophin; Calmodulin; Actin; Recombinant protein

1. Introduction

Dystrophin is a 400 kDa elongated protein coded by a gene located on Xp21 [1]. It is a low quantity protein representing 0.002% of total muscular cell proteins [2], but is essential for the membrane integrity of the cell. Its absence causes Duchenne muscular dystrophy (DMD), a progressive lethal disease [3]. Immunocytochemical studies have shown that it is localized on the cell membrane where it binds an integral membrane complex of glycoproteins that link the extracellular domain via the laminin-M receptor [4,5]. The role of dystrophin in this DMD dysfunction has yet to be elucidated, but two hypotheses are commonly put forth: the first suggests that dystrophin has a role in stabilizing the sarcolemmal membrane and the second a role in regulating the intracellular calcium concentration [6,7].

There is now evidence which suggests that an absence of dystrophin may upset calcium levels. An elevated level of intracellular calcium has been reported in DMD muscle cells [8,9], leading to an increased protein degradation rate in the cell [8,10]. An increase in the parvalbumin concentration was noted in muscle tissue from mdx mice [11], and an increased calmodulin (CaM) level was observed in dystrophic hamsters and in DMD muscles [12,13].

CaM is a small heat-stable calcium-binding protein found in most eucaryotic cells. CaM is implicated in many cellular processes such as regulation of cell motility, cyclic nucleotide levels or calcium fluxes [14,15]. The binding of CaM to dystrophin has already been investigated but the results are controversial. Madhavan et al. proposed two consensus sequences, one located in the N-terminal part of dystrophin and the other in the C-terminal part of the molecule [16]. To partially address this point, we investigated possible CaM binding to the N-terminal part of dystrophin using a soluble recombinant protein designed in our laboratory, rather than the full length protein. Moreover, we investigated its possible regulation of dystrophin–actin interactions.

*Corresponding author. Fax: (33) 67 04 21 40.

Abbreviations: ABS, actin binding site; ATP, adenosine triphosphate; BSA, bovine serum albumin; CaM, calmodulin; DMD, Duchenne muscular dystrophy; DTT, dithiothreitol; EGTA, ethyleneglycolether tetraacetic acid; ELISA, enzyme linked immunosorbent assay; MBP, maltose binding protein; NR, nonrecombinant protein; PAGE-SDS, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

2. Experimental

2.1. Protein preparations

Proteins were purified by previously described methods: bovine brain calmodulin [17], rabbit skeletal actin [18] with slight modifications [19], recombinant proteins of human dystrophin, named A0 (first 431 residues), A01 (first 68 residues), A02 (residues 49 to 431) and nonrecombinant protein (NR) [20]. These constructions consist of a 42 kDa N-terminal part, corresponding to the maltose binding protein (MBP), and a C-terminal part, corresponding to the dystrophin fragments mentioned above, except for the NR whose C-terminal part contained a 9 kDa β-galactosidase fragment.

2.2. Calmodulin–Sepharose column

Proteins were dialysed against a 20 mM imidazole, pH = 7.0, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM DTT, 2 mM $CaCl_2$ buffer (A), loaded on a 1 × 10 cm column of calmodulin-Sepharose-4B (Pharmacia, Uppsala Sweden), washed several times in the same buffer then with an imidazole salt buffer (B) and eluted with the above imidazole buffer (C) containing 5 mM EGTA.

2.3. Immunological techniques

ELISA was carried out by the method of Engvall [21]. Proteins (F-actin and calmodulin) were coated at 200 μg/ml and 10 μg/ml, respectively, on microtitre plate wells (Nunc) and incubated overnight at 4°C. After washing with PBS-Tween, wells were saturated with 1% BSA-PBS. Fusion proteins were incubated for 1 h at 37°C in decreasing concentrations. For actin-coated plates, CaM was applied at 1 mg/ml in 5 mM $CaCl_2$ or EGTA for 1 h at 37°C. The first antibody used, 4E8, is a monoclonal antibody raised against MBP protein which was produced and characterized in the laboratory. The second antibody is an alkaline phosphatase conjugated anti-mouse antibody diluted as recommended by the manufacturer (Jackson Immuno Reseach).

2.4. Actin-binding

Skeletal muscle actin (20 μM) and N-terminal recombinant dystrophin (5 μM) were mixed at 25°C in 20 mM Tris-HCl, pH = 7.5, 2 mM $MgCl_2$, 1 mM DTT, 1 mM ATP with CaM (10 μM) $CaCl_2$ or EGTA (2 mM). Samples (0.1 ml) were then centrifuged at 30 PSI in a Beckman airfuge, 30 min at 20°C. Pellets and supernatants were subjected to SDS-PAGE.

2.5. Other procedures

Protein concentrations were determined by spectrophotometric measurements in a Kontron Uvikon 930 using the following extinction coefficients: CaM $\varepsilon^{1\%}_{277\,nm} = 1.9$ [22], actin $\varepsilon^{1\%}_{280\,nm} = 11$ [23], except for recombinant proteins whose concentrations were measured by the Bradford Assay using BSA for calibration [24].

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3. Results

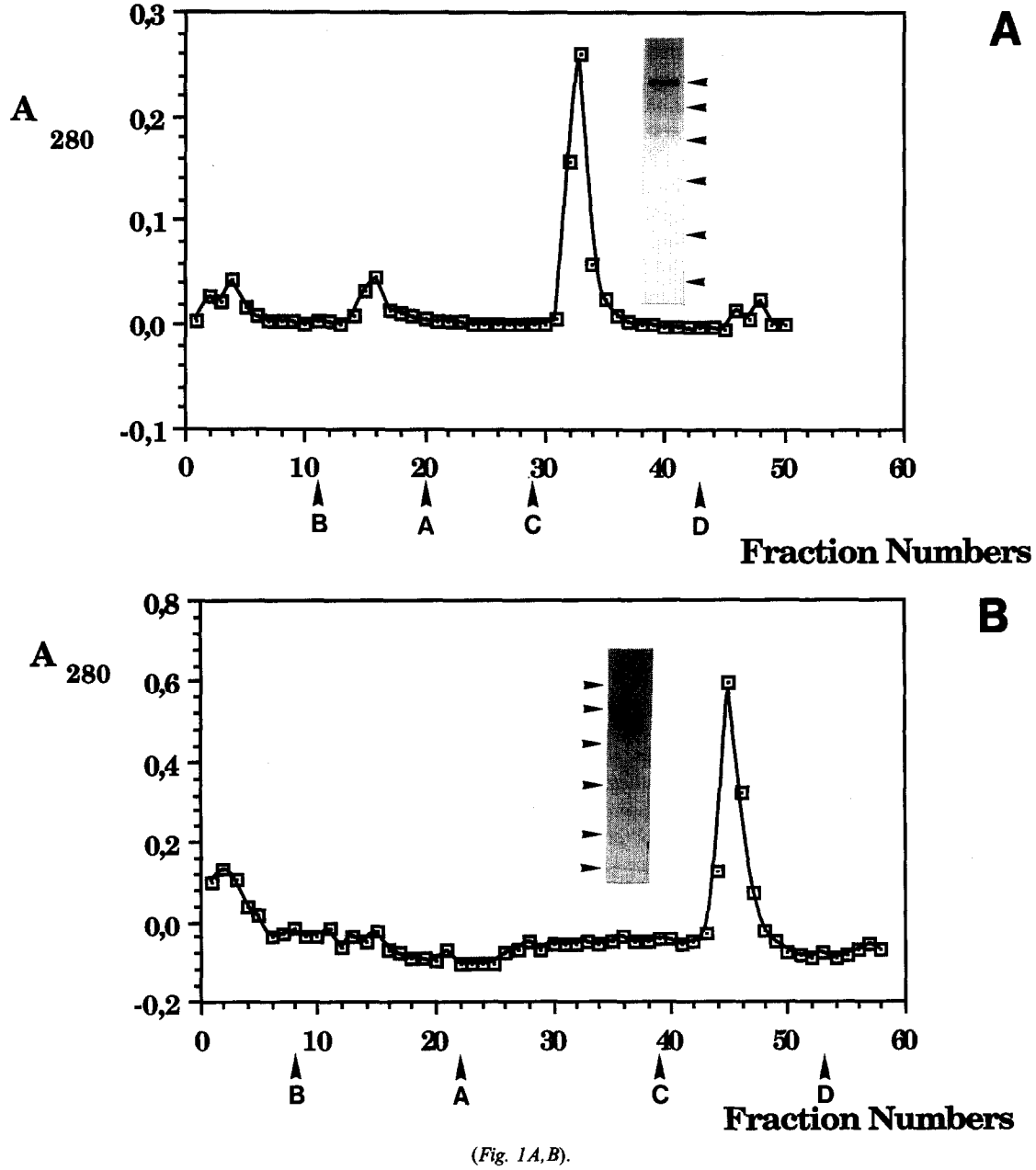
3.1. Calmodulin binding to the N-terminal part of dystrophin

NR and the three fragments A0(1–431), A01(1–68), A02(49–431) were applied to a calmodulin-Sepharose column under high and low ionic strength conditions (Fig. 1). Each fraction, absorbing at 280 nm, was identified on Coomassie blue stained gel. A0 specifically bound the CaM-Sepharose column since elution only occurred in the presence of EGTA (buffer C), and not with ionic strength buffer (buffer B) (Fig. 1A). We obtained the same profile with A01 and A02 (Fig. 1B,C). On the other hand, NR did not bind the affinity resin at all (Fig. 1D), showing that binding of A0, A01 and A02 was specific to the dystrophin part of the recombinant protein.

Secondly, the interaction of recombinant proteins with coated CaM was investigated by a direct ELISA technique and the presence of dystrophin fused proteins was detected by anti-MBP antibody. Each recombinant protein was incubated at concentrations ranging from 0.001 mg/ml to 0.75 mg/ml in the presence or absence of calcium (Fig. 2). The results clearly showed binding of A0 to CaM in the presence of calcium, but not in its absence. A01 and A02 bound coated CaM in the same manner (data not shown).

3.2. Calcium-calmodulin induced modulation of the dystrophin–actin interface

Since the N-terminal part of dystrophin is known to bind actin [20,25], we investigated how this dystrophin–actin interaction might be disturbed by the presence of the calcium–calmodulin complex. In cosedimentation assays, actin, recombinant proteins and CaM, in presence or absence of calcium, were mixed together in a molar ratio of actin/recombinant protein/CaM = 4/1/2. A0 cosedimented with actin (Fig. 3, lanes 4 and 5) but A0 was not disturbed by the presence of calcium–CaM,



(Fig. 1A,B).

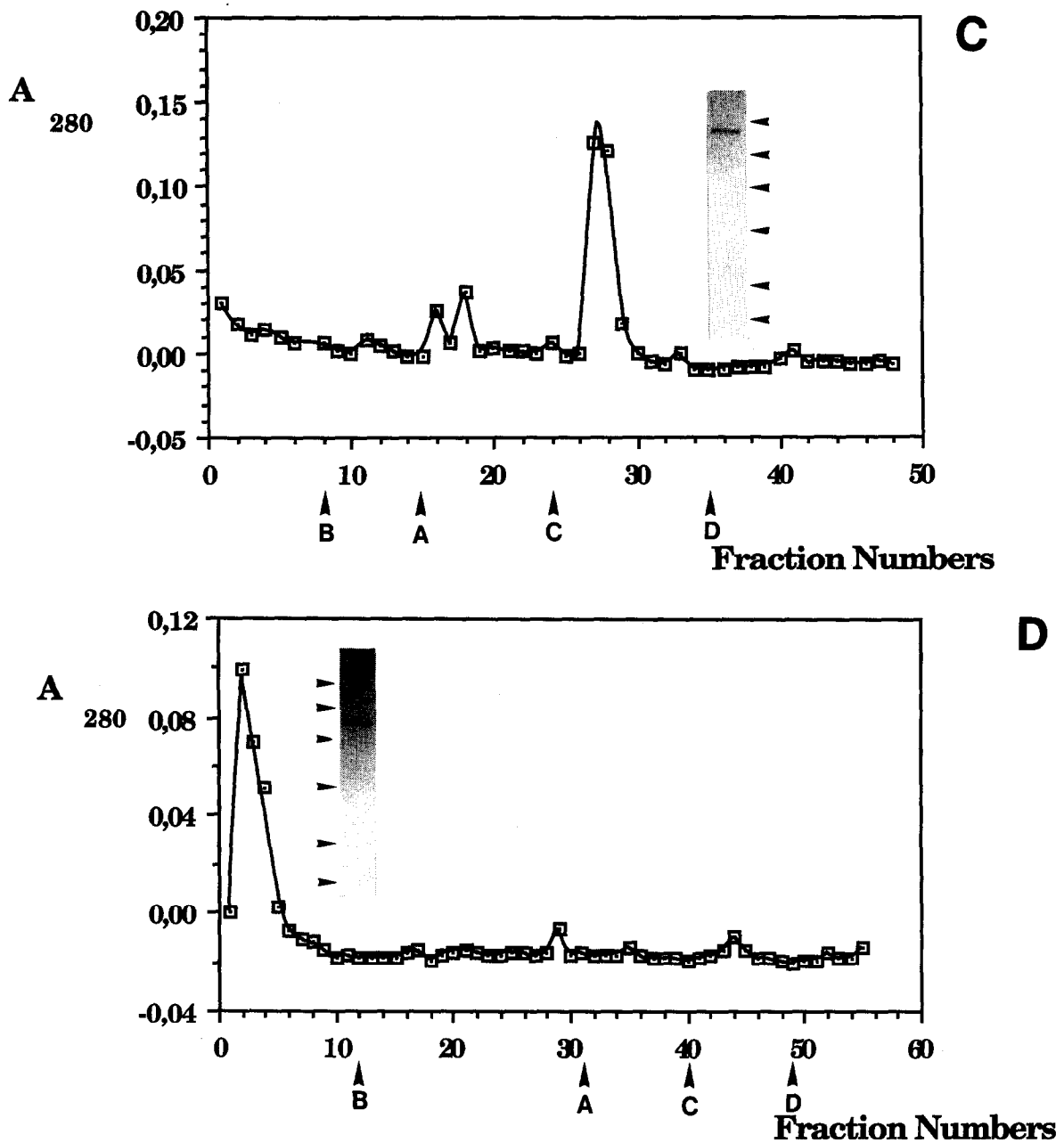


Fig. 1. Binding of dystrophin recombinant proteins A0 (A), A01 (B), A02 (C) and NR (D) to immobilized calmodulin. 1 to 1.5 mg of A0, A01, A02 or NR was dialyzed against buffer A (see section 2) and applied on a 1 × 10 cm CaM-sepharose-4B column. The column was washed and buffer A + 0.3 M KCl (buffer B) was applied. Bound protein was eluted with buffer C (5 mM EGTA) and buffer D (5 mM EGTA + 0.3 M KCl). Buffer was added at the point indicated by the arrow. The inset shows 12.5% SDS-PAGE analysis of fractions 33, 45, 27 or 2 corresponding to the A0, A01, A02 material removed by EGTA (A, B or C) or unbound material (D). The positions of M_r markers (as Fig. 3) are shown.

nor was CaM cosedimented with A0 and actin (Fig. 3, lane 6 and 7). The difference in the motility of CaM in lanes 6s and 7s was due to the binding of calcium to the molecule. In the same way, using the ELISA technique, binding of recombinant proteins on coated actin was not disturbed by calcium–CaM (Fig. 4).

4. Discussion

Madhavan et al. [16] revealed the presence of two putative CaM-binding domains on dystrophin, one located in the N-terminal part of dystrophin at position 18–42 and the other in the C-terminal part at position 3,374–3,398. We thus used recombinant proteins from the N-terminal part of human dystrophin, which were already used in our laboratory for their actin-binding properties [20], to investigate binding of the N-terminal part of dystrophin with calcium–CaM.

We show that binding of A0 (1–431), A01 (1–68) and A02 (49–431) to CaM is not only specific to the dystrophin part of the molecules (NR did not bind), but is also calcium-dependent. Binding of dystrophin to CaM has already been shown using an overlay technique, but this result was refuted by other au-

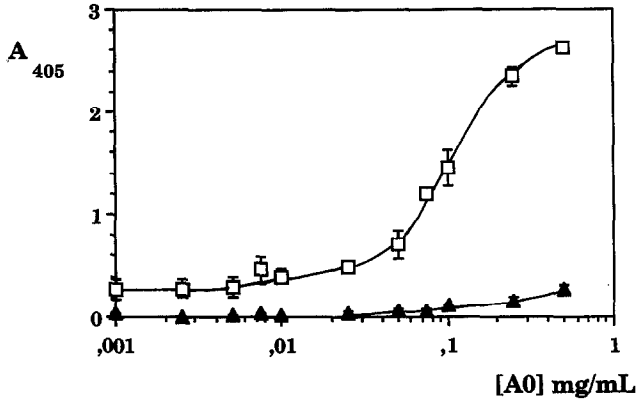


Fig. 2. Interaction of dystrophin recombinant protein A0 with CaM. In direct ELISA experiments, interaction of increased A0 concentrations (0.001 to 0.75 mg/ml) with coated CaM in the presence of $CaCl_2$ (□) or EGTA (▲) was followed at 405 nm with monoclonal anti-MBP antibody ($n = 3$).

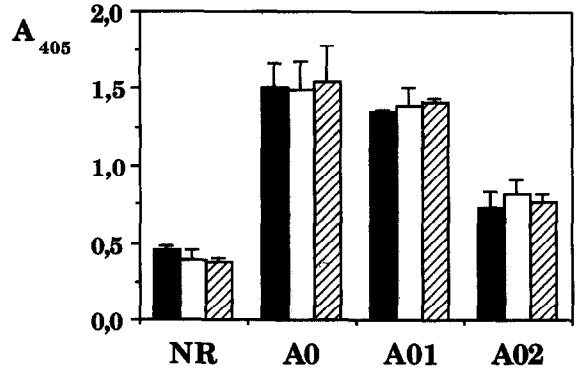


Fig. 4. Modulation of the dystrophin N-terminal recombinant protein–actin interface with calcium–calmodulin. A direct ELISA experiment was carried out as described in section 2. F-actin was coated at 200 μg/ml, NR, A0, A01 and A02 were incubated for 1 h at 0.3 mg/ml (■), wells were washed and CaM was also incubated for 1 h at 1 mg/ml with 5 mM of $CaCl_2$ (□) or EGTA (▨) ($n = 3$).

thors using the same technique [16,26]. We used CaM-sepharose and ELISA techniques and found that the N-terminal part of dystrophin unquestionably possesses a CaM-binding site. This is relevant in view of the recent paper of Madhavan and Jarrett [27] which showed CaM-activated phosphorylation of dystrophin in the C-terminal part of the molecule but not in the N-terminal part. CaM-binding to the N-terminal part of dystrophin may allow phosphorylation of the C-terminal part of the same, or adjacent, dystrophin molecule in view of the latest model of Matsumura and Campbell [28].

We also show here that the function of this binding is not for regulation of the dystrophin–actin interface, since cosedimentation and ELISA experiments did not show any modulation. Recombinant proteins seemed to more strongly bind actin than CaM, and we did not see any ternary complex in cosedimentation analyses.

An important question in the light of these results is where the CaM binding site is located (Fig. 5). The putative site is situated between residues 18 to 42 which overlap with the actin-binding site 1 (ABS1) corresponding to residues 17 to 28 [29]. However, A02 also binds CaM, so are there one or two sites in A0? If there is a single interaction site, it must be in the overlap region between A01 and A02 and so between residues 49 and 68 (Fig. 5a). However, this region is not an optimal sequence for CaM interactions [30]. If there are two CaM binding sites, there should be one in the A01 sequence and another in the A02 sequence (Fig. 5b). Since in our cosedimentation conditions we found no ternary complex (actin-recombinant protein–CaM) these sites should overlap with ABS1 and ABS2.

Ervasti and Campbell [26] showed no binding of a dystrophin–glycoprotein complex with calcium–CaM using the CaM-Sepharose affinity precipitation technique. This suggests that CaM binding may have a modulatory effect on the interface of dystrophin with one of the DAG (dystrophin associated

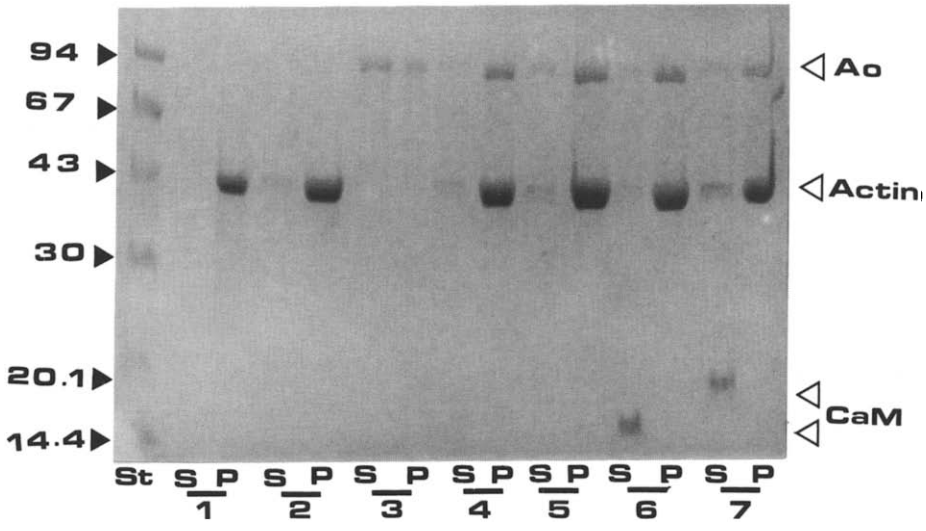


Fig. 3. Binding of dystrophin recombinant protein A0 to actin and its modulation by calcium–calmodulin. A0-actin and CaM were cosedimented at 200,000 × g for 30 min in a Beckman airfuge. Supernatants (S) and pellets (P) were analysed by SDS-PAGE. St: standards (M_r in kDa); lanes 1 and 2: actin alone (20 μM) with (1) or without calcium (2); lane 3: A0 alone (5 μM); lanes 4 and 5: actin + A0 with (4) or without (5) calcium; lanes 6 and 7: actin + A0 + CaM (10 mM) with (6) or without (7) calcium.

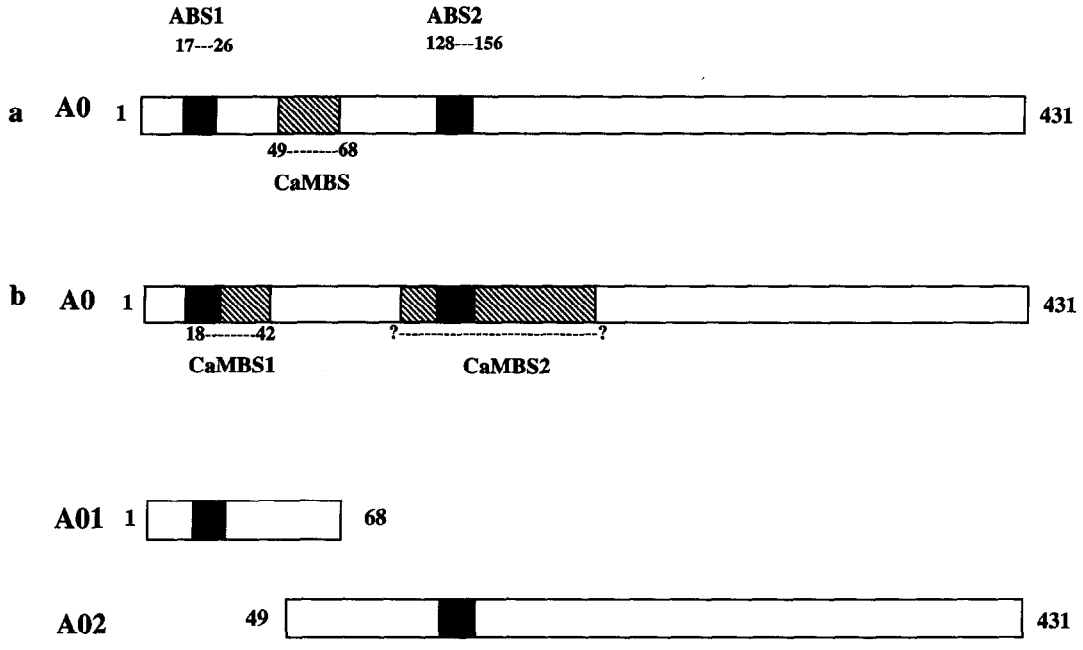


Fig. 5. Hypothetical localization of calmodulin binding sites on dystrophin N-terminal recombinant proteins. (a) Calmodulin binding site (CaMBS) is unique in the overlap region between A01 and A02; (b) two CaMBS: CaMBS1 corresponds to the putative CaMBS described by [16]. A0 (first 431 residues), A01 (first 68 residues) and A02 (residues 49 to 431). Boxes in black represent actin binding sites (ABS), shaded boxes represent CaMBS.

glycoproteins) or DAP (dystrophin associated proteins), and more particularly with 43 kDa (β-dystroglycan) or 59 kDa (syntrophin-I) proteins, as mediated by phosphorylation of the C-terminal of dystrophin.

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