Actin–Dystrophin Interface†

Eric Fabbrizio, Armelle Bonet-Kerrache, Jean J. Leger, and Dominique Mornet*

Pathologie Moléculaire du Muscle, Faculté de Pharmacie, INSERM U.300, Avenue Charles Flahault, 34060 Montpellier Cédex 1, France

Received March 15, 1993; Revised Manuscript Received July 23, 1993®

ABSTRACT: Dystrophin, an elongated cytoskeletal molecule which is deficient in Duchenne muscular disease, contains an actin-binding domain in its N-terminal portion. We show that this part interacted with actin in the native molecule. By molecular biology techniques, four recombinant proteins were expressed in *Escherichia coli* using the pMAL vector which allowed us to obtain soluble proteins directly after purification. These constructions were tested for their ability to bind actin under various conditions, and their apparent dissociation constants were determined. The effects of other actin-binding proteins such as caldesmon and tropomyosin were analyzed in comparison to the actin-binding properties of these constructions. These results support the potential concept of a multiple actin-binding contact in the N-terminal region of dystrophin. Differences in the functional domains are discussed relative to similar α-actinin–actin-binding sites.

Dystrophin is a 427-kDa protein encoded by the Xp21 gene (Koenig et al., 1987) which is present in a wide range of tissues. Its absence causes Duchenne muscular dystrophy (Hoffman et al., 1988; Koenig et al., 1988). This protein consists of four main domains. The N-terminal domain was reported to have homology with α-actinin (Hammonds, 1987). The central domain is a rod-shaped elongated structure with repetitive sequences arranged in triple-helical segments (Koenig et al., 1990; Cross et al., 1990). This domain also contains four potential hinges that may give flexibility to the protein (Koenig et al., 1990) and be responsible for the previously described associative properties of the molecule (Sato et al., 1992; Fabbrizio et al., 1993). The third domain is a cysteine-rich region with sequence similarity to the C-terminal region of α-actinin (Noegel et al., 1987; Koenig et al., 1988). The fourth domain constitutes the C-terminal part of dystrophin and seems to be specific to "dystrophin family" proteins. Indeed, utrophin, a recently fully sequenced protein (Tinsley et al., 1992) related to dystrophin but encoded on an autosomal gene (Love et al., 1989), and DP71, a small dystrophin transcript product (Bar et al., 1991; Lederfein et al., 1992), both have very strong homology with the C-terminal part of dystrophin. This latter domain with part of the cysteine-rich region may be involved in membrane anchorage via the glycoprotein complex (Yoshida et al., 1991; Suzuki et al., 1992; Ervasti & Campbell, 1991; Ervasti et al., 1991; Ohlendieck et al., 1991). However, the low amount (about 0.002%) of dystrophin in skeletal muscle (Hoffman et al., 1988) has been a major limitation for carrying out detailed investigations on the hypothetical properties deduced from its sequence.

Investigations on the potential role and function of dystrophin have mainly involved comparisons of muscle fiber properties in normal and mdx mice, a choice model for dystrophin deficiency studies. This protein is thought to have an important role in maintaining membrane integrity since dystrophin-containing fibers were found to be less sensitive to hypoosmotic solutions than dystrophin-deficient fibers (Mencke et al., 1991). No important differences in the intrinsic mechanical properties of both types of fibers have been noted, but dystrophin could protect the sarcolemma from mechanical damage (Hutter et al., 1991). A possible role in calcium regulation has also been proposed. Dystrophin may act as a mechanical sensor on specific calcium channels which are inactivated by stretching (Franco et al., 1991). This could explain the specific rise in the calcium influx in dystrophin-deficient muscle (Turner et al., 1990). The ability of dystrophin to provide membrane stability was deduced by analogy with the erythrocyte spectin structure (Speicher et al., 1983). This stability could be enhanced by a meshwork of actin filaments, as occurs in actin–spectrin complex formation.

Molecular biology studies recently revealed two properties of dystrophin. First, two recombinant proteins were produced from the N-terminal part of dystrophin (about 240 residues) and found to bind actin in *in vitro* interactions or within COS cells (Way et al., 1992; Hemmings et al., 1992). Second, the same strategy was applied to the cysteine-rich regions of dystrophin which was shown to bind calcium in overlay experiments (Milner et al., 1992).

The N-terminal part of dystrophin thus seems to be an important functional domain. Previous NMR[1] experiments using dystrophin peptides from the N-terminal part revealed that this protein contains two potential actin-binding sites (Levine et al., 1990a, 1992). In this study, we focused on the actin–dystrophin interface. Two strategies were used: (i) a biochemical investigation on the natural protein extracted from rabbit skeletal muscle was carried out, and we found for the first time that actin interacted with a dystrophin-enriched preparation; (ii) the actin-binding region of dystrophin was

† This research was supported by grants from INSERM, CNRS, and the Association Française contre les Myopathies. E.F. received a fellowship from the AFM.

* To whom correspondence and reprint requests should be addressed.

® Abstract published in *Advance ACS Abstracts,* September 15, 1993.

[1] Abbreviations: ABS1, actin-binding site 1; ABS2, actin-binding site 2; CaD, caldesmon; TpM, tropomyosin; IPTP, isopropyl β-D-thiogalactopyranoside; IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; NMR, nuclear magnetic resonance; K_d, apparent dissociation constant; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; DMS, dimethyl suberimidate; MBS, 3-maleimidobenzoic acid *N*-hydroxysuccinimide; BS3, bis(sulfosuccinimidyl) suberate; DFNB, 1,5-difluoro-2,4-dinitrobenzene; sulfo-MBS, *m*-maleimidobenzoyl *N*-hydroxysulfosuccinimidate; DMA, dimethyl adipimidate; DEB, diepoxy-1,3-butadiene; pPDM, *N,N'-p*-phenylenedimaleimide; EEDQ, *N*-(ethoxycarbonyl)-2-dihydroquinoline; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

0006-2960/93/0432-10457$04.00/0

also dissected to determine the relative importance of both of the previously described actin-binding sites. We used an expression system to obtain soluble recombinant proteins expressing four different N-terminal parts of human dystrophin in *Escherichia coli*. Each construction was also characterized for its actin-binding properties and potential to disturb actin interactions with other actin-binding proteins such as caldesmon (CaD) or tropomyosin (TpM).

MATERIALS AND METHODS

Protein Preparation. (A) Dystrophin. Dystrophin from rabbit skeletal muscle was prepared as previously described (Fabbrizio et al., 1993).

(B) Actin. G-Actin from rabbit skeletal muscle was extracted from acetonic powder (Spudich & Watt, 1971) with slight modifications (Eisenberg & Kielly, 1974). Briefly, G-actin was polymerized for 2 h without stirring, adjusting the solution to a final concentration of 100 mM KCl and 2 mM $MgCl_2$. The KCl concentration was then increased to 0.8 M before ultracentrifugation at 40 000 rpm for 2 h. F-Actin was washed in buffer containing 2 mM Tris-HCl, pH 8.0, 10 mM KCl, 0.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 1 mM NaN_3. A second ultracentrifugation at 40 000 rpm for 1 h allowed us to obtain F-actin which was recuperated in the same buffer as above but without $MgCl_2$. All experiments presented in this work were performed using F-actin.

(C) Caldesmon and Tropomyosin. CaD and TpM were purified from fresh chicken gizzard (Bretscher, 1984) with a modified caldesmon preparation (Mornet et al., 1988).

Construction of Plasmids Encoding Different Fusion Proteins. The pEX construction with the N-terminal human dystrophin fragment (Lemaire et al., 1988), cloned by PCR, was digested with *Sma*I/*Hin*dIII enzymes and introduced in the pMAL vector (Ozyme) using an *Eco*RI linker. This construction was named A_0 and corresponded to residues 1–431. Other constructions were made. A_{03}, which contained residues 1–198, was obtained after A_0 fragment digestion with *Nsi*I/*Hin*dIII enzymes and a ligation step after Klenow fragment treatment. A_{01}, containing residues 1–68, was obtained after *Bam*HI/*Hin*dIII digestion of A_0 and a ligation step after Klenow fragment treatment. A_{02}, containing 382 residues from 49 to 431, was cloned after purification of the *Nar*I (Klenow)/*Hin*dIII insert, in a pMAL derivative form, named pIH902, at *Stu*I/*Hin*dIII sites. All constructions were electroporated in TB1 cells. The nonrecombinant protein was also produced and used as the negative control for functional tests. These constructions consist of a 42-kDa N-terminal part, corresponding to the maltose-binding protein, and a C-terminal part, corresponding to the dystrophin fragments mentioned above, except for the nonrecombinant protein whose C-terminal part contained a 9-kDa β-galactosidase fragment.

Expression of Recombinant Proteins and Purification. The different fusion proteins were expressed at 37 °C when the OD reached 0.6 by chemical induction with 0.3 mM IPTG for 2 h. Purification was performed as recommended by the manufacturer. Briefly, the induced culture was pelleted and resuspended in buffer I (10 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, pH 7.4) and sonicated three times for 2 min (three times more with A_{03}). The soluble extract was recuperated and loaded onto the amylose resin column preequilibrated in buffer I. Elution was carried out using 10 mM maltose in buffer I.

Cosedimentation Assays. Prior to sedimentation performed in an Airfuge (Beckman), F-actin was incubated with the different proteins for 5 min in buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM $CaCl_2$, and 1 mM NaN_3. The total volume of each sample was 150 μL. Samples were centrifuged to 30 psi for 20 min at room temperature with or without F-actin and analyzed as follows: the total mixture before cosedimentation, the pellet resuspended in the above described buffer, and the supernatant. All samples were then boiled in an equal amount of SDS loading buffer, analyzed on SDS–acrylamide gel, and visualized by Coomassie blue staining. The dystrophin-enriched preparations was mixed with F-actin in a 1/5 molar ratio. When CaD or TpM were mixed with F-actin, a 1/7 molar ratio was used. For each experiment, F-actin was always added last. Recombinant proteins were added with F-actin at different concentrations. Densitometric analysis of the gels was performed, and the apparent dissociation constants (K_d) of A_0, A_{01}, and A_{02} were deduced from the equation $(A)(P)/(PA) = K_d$, which was transformed to $(PA)/(A) = -(PA)/K_d + Pt/K_d$, where (PA) is the concentration of recombinant protein complexed with F-actin, (A) is the concentration of F-actin, and Pt is the maximum number of complexes that can be formed.

Limited Proteolysis. The A_0 protein was submitted to lysyl-C endoprotease (Boerhinger) treatment in the absence and presence of F-actin. Analysis was performed on SDS–PAGE after Coomassie blue staining.

Western Blot and Immunodetection. The Western blot technique was used after cosedimentation of the dystrophin-enriched preparation in the presence or absence of F-actin and performed as previously described (Pons et al., 1990).

Cross-Linking Experiments. Different chemical cross-linkers were used at concentrations of 1 and 10 mM. A_0 (300 μg/mL) was mixed with F-actin (1 mg/mL). After addition of the agent, the time course was followed for 45 min. The different cross-linkers used were EDC, DMS, MBS, BS3, DFNB, sulfo-MBS, DMA, DEB, pPDM, and EEDQ. They were all purchased from Sigma or Pierce. The EDC cross-link between CaD and F-actin was performed with a CaD/F-actin molar ratio of 1/7, as previously described (Harricane et al., 1992).

RESULTS

Binding of Native Dystrophin Molecules with Actin. Standard 0.5 mg/mL concentrations of dystrophin-enriched preparations were analyzed after Airfuge centrifugation in the absence or presence of a 4–5 molar excess of F-actin, which is considered to be the best molar ratio for dystrophin/actin interactions (Way et al., 1992). The Coomassie blue stained gel shown in Figure 1 revealed that native dystrophin interacted with F-actin. Comparison of F-actin-cosedimented proteins with the remaining proteins in the supernatant showed that dystrophin was specifically pelleted by actin (Figure 1B). This was confirmed when an anti-dystrophin antibody (H'5A3) was used whose epitope was previously located in the C-terminal part of dystrophin. This interaction appeared to be selective since dystrophin was present in the supernatant when no actin was added. We also noted a protein band of about 80 kDa which copurified with dystrophin, and it is currently being analyzed.

Purification of the Recombinant Protein. The N-terminal binding site of dystrophin was studied using different constructions expressed in *E. coli*. As shown in Figure 2, the recombinant proteins, named A_0 (first 431 residues) and A_{03} (first 198 residues), encompassed both actin-binding sites ABS1 and ABS2, and the others, named A_{01} (first 68 residues) and A_{02} (382 residues), contained ABS1 and ABS2, respectively. Purification of A_0 is given as an example in Figure 3,

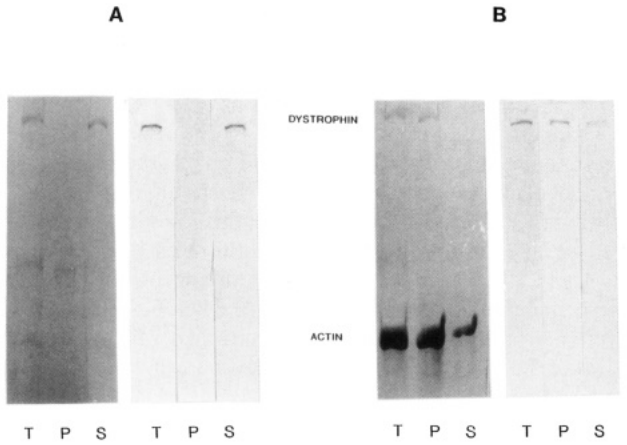

FIGURE 1: Cosedimentation with native dystrophin. Forty micrograms of the dystrophin-enriched preparation was cosedimented alone (A) or mixed with 80 μg of F-actin (B), as described under Materials and Methods. Samples from total (T), pellet (P), and supernatant (S) were analyzed on SDS 3–9% gradient PAGE and visualized after Coomassie blue staining (left panels of A and B) or after electroblotting on nitrocellulose membrane (right panels of A and B). Anti-dystrophin monoclonal antibody H'5A3 (dilution 1/3), raised against the C-terminal part of dystrophin, and a goat anti-mouse second antibody coupled to alkaline phosphatase (dilution 1/5000) revealed the 400-kDa dystrophin band.

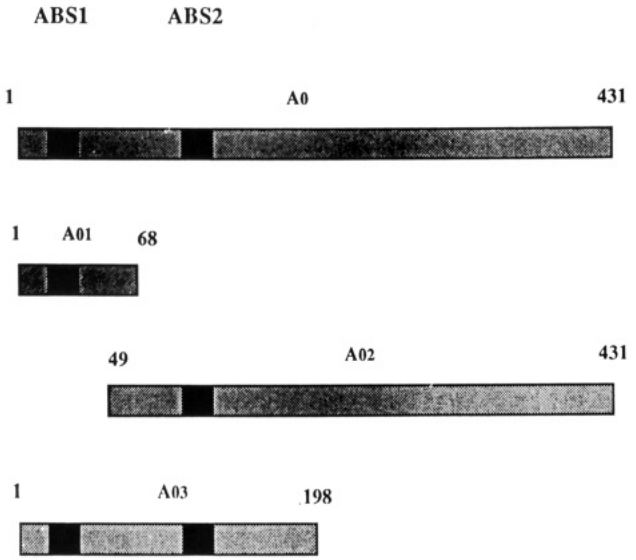

FIGURE 2: Representation of the different constructions. The different dystrophin fragments A_0, A_{01}, A_{02}, and A_{03} were constructed as described under Materials and Methods. Actin-binding sites ABS1 and ABS2 correspond to residues 86–117 and 350–375 on the dystrophin amino acid sequence and are indicated by solid boxes for each construction. A_0 and A_{03} contained both actin-binding sites ABS1 and ABS2. A_{01} and A_{02} contained actin-binding sites ABS1 and ABS2, respectively.

where the recombinant protein is shown to be soluble in the bacterial extract (Figure 3, lane 3 versus 4). Purification through the amylose column allowed recovery of significant amounts of protein (about 1.5–2 mg/150-mL culture). A_{03} was less soluble than the other proteins. Nevertheless, all of these proteins were used directly to determine their binding properties toward F-actin.

Properties of N-Terminal Recombinant Dystrophin. Cosedimentation experiments were performed with each of the described recombinant proteins, and the results are presented in Figure 4. Note that A_{03} sedimented alone under high-speed centrifugation, contrary to the other recombinant proteins, and was thus not useful for our study (Figure 4A).

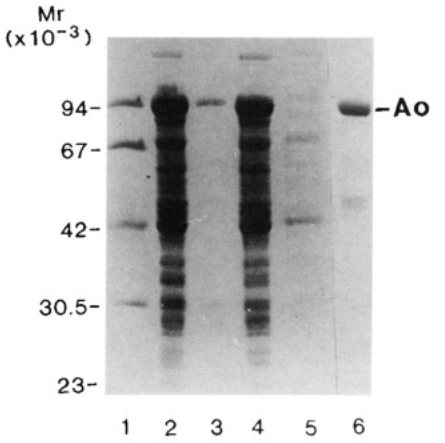

FIGURE 3: Purification of the A_0 dystrophin recombinant protein. A_0 was expressed in *E. coli* after chemical induction with 0.3 mM IPTG. Purification was performed on an amylose resin column. The main steps during A_0 purification were analyzed by 10% SDS–PAGE and revealed by Coomassie blue staining. The total induced bacterial extract and the nonsoluble and the soluble fractions of the bacterial extract are shown in lanes 2, 3, and 4, respectively. The soluble fractions were recuperated after centrifugation at 9000*g* for 30 min of the total sonicated bacterial extract. This fraction was diluted 5-fold with column buffer before being loaded onto the amylose column. The sample in lane 5 corresponded to flow-through of the diluted soluble fraction. After extensive washing with column buffer, A_0 was eluted with 10 mM maltose in column buffer and corresponds to the sample in lane 6. The standard molecular weights are shown in lane 1.

A_0, A_{01}, and A_{02} proteins cosedimented with F-actin in a specific manner (Figure 4B). The nonrecombinant protein was used as the negative control did not cosediment with F-actin, confirming the specificity of the observed interaction (not shown). Similar experiments using various ratios of A_0, A_{01}, and A_{02} to actin were analyzed by densitometric measurements of the gels as described under Materials and Methods. We thus estimated an apparent K_d of 5.7, 1.25, and 4 μM for A_0, A_{01}, and A_{02}, respectively.

A limited proteolysis experiment was performed to investigate the F-actin/dystrophin interaction. A_0 was submitted to lysyl-C endoprotease treatment in the absence and presence of F-actin. The results in Figure 5 show disappearance of the 94-kDaA_0 protein band when no F-actin was added (Figure 5A), while it remained quite intact in the presence of F-actin over the time course (Figure 5B). This recombinant protein was thus clearly protected by F-actin, confirming that it was a functional protein.

Effect of Dystrophin Recombinant Protein on Two Actin-Binding Proteins: Caldesmon and Tropomyosin. This F-actin–dystrophin interface was investigated using CaD, another actin-binding protein (Figure 6). A specific effect was observed when CaD was added. A_0 and A_{01} displaced CaD in the supernatant under conditions in which this protein usually cosediments with actin (Figure 6B,C). The A_{02} protein, contrary to A_0 and A_{01} proteins, did not as fully influence CaD behavior since it did not prevent CaD from sedimenting with F-actin under the same conditions (Figure 6D). Control experiments without recombinant proteins confirmed that this behavior was specific (Figure 6A).

When TpM was used in the presence of A_0 or A_{01} (Figure 7), the F-actin/TpM interaction was not affected by dystrophin recombinant proteins, contrary to the F-actin/CaD interaction (Figure 7B). When CaD and TpM were added in the presence of A_0 or A_{01} recombinant proteins, a significant amount of CaD was found in the pellet (Figure 7C), confirming the role of TpM in reinforcing the F-actin/CaD interaction. Control

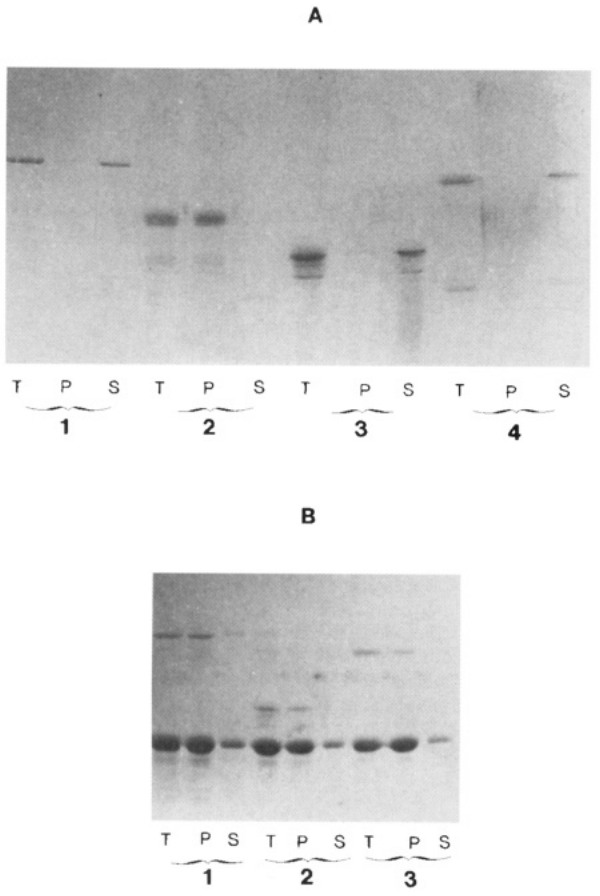

FIGURE 4: Cosedimentation of the different recombinant proteins. Recombinant proteins (A_0, 20 μg; A_{01}, 10 μg; and A_{02}, 20 μg) were ultracentrifuged under the conditions described under Materials and Methods without F-actin (A) or with 40 μg of F-actin (B). Total (T), pellet (P), and supernatant (S) samples were analyzed on a 10% SDS–PAGE and visualized after Coomassie blue staining. Samples from A_0 in panel 1, A_{03} in panel 2, A_{01} in panel 3, and A_{02} in panel 4 are shown in (A). Samples from A_0 in panel 1, A_{01} in panel 2, and A_{02} in panel 3 are shown in (B).

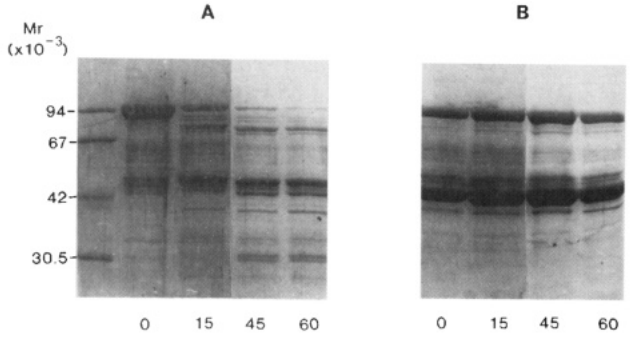

FIGURE 5: Limited proteolysis of the A_0 protein. Three-tenth milligram of A_0 was mixed with 50 mM Tris-HCl, pH 7.5, in the absence (A) or presence (B) of 1 mg of F-actin. Then 15 units of lysyl-C-endoprotease was added. The time course was followed for 1 h and is indicated in minutes. The reaction was stopped by the addition of loading buffer and 5 min of boiling. Samples were analyzed on a 10% SDS–PAGE and visualized after Coomassie blue staining.

cosedimentation with CaD and TpM in the presence of F-actin is also presented (Figure 7A).

Cross-Linking Experiments. All cross-linkers described under Materials and Methods were used in their optimal conditions, but our attempts to show an actin–dystrophin union were unsuccessful. Oligomerization of the A_0 protein or actin was not always initiated. In another set of experiments, we used EDC to covalently link F-actin and CaD. The reaction was stopped after the addition of β-mercaptoethanol for each selected condensation time, and the new covalent products were identified as previously described (Harricane et al., 1992). This modified F-actin solution was used to completely cosediment 20 μg of A_0 (Figure 8). The results show that actin molecules involved in the different cross-link products did not affect the cosedimentation pattern of A_0 since A_0 was always fully able to cosediment with this F-actin solution and remove the un-cross-linked CaD in the supernatant (Figure 8, lanes P1–3 and S1–3). This result is in line with the experiment reported above (Figure 6) when F-actin and CaD were not treated with the EDC agent. Moreover, it demonstrates that two different interfaces were involved in each interaction.

DISCUSSION

The present study demonstrates for the first time that native dystrophin in a dystrophin-enriched preparation is able to cosediment with F-actin. This is strongly in favor of an F-actin interaction *in vivo*, as also suggested by experiments in COS cells on the expression of the N-terminal part of dystrophin fused to the C-terminal part of α-actinin (Hemmings et al., 1992). However, we cannot exclude the possible intervention of another component present in this preparation that might have enhanced the F-actin/dystrophin interaction. Recent reports suggest that talin could be a potential partner of such an interaction (Senter et al., 1993). It was shown that two N-terminal dystrophin peptides (residues 17–26 and 128–156) that bind actin have their own counterparts on actin, corresponding to residues 86–117 and 350–375, respectively, and they were consequently named dystrophin–actin-binding sites 1 (ABS1) and 2 (ABS2), respectively (Levine et al., 1990a, 1992). The N-terminal actin-binding region of dystrophin was dissected to assess the relative importance of these sites. Using molecular biotechnology, recombinant proteins encompassing the dystrophin–actin-binding site were produced as follows: A_0 and A_{03} contained ABS1 and ABS2. A_{01} contained only ABS1, while A_{02} was restricted to ABS2. All recombinant proteins were soluble in the concentration range investigated here, except that A_{03} sedimented alone in cosedimentation assays. These aggregative properties were also observed with dystrophin constructs such as that corresponding to the first 233 residues (Way et al., 1992). This is in line with problems encountered by others in testing similar sequence constructions in cosedimentation experiments without addition of Triton X-100 (Hemmings et al., 1992). The results presented here compared to previous experiments using dystrophin recombinant proteins are unique in that the A_{01} and A_{02} recombinant proteins, both only containing one previously defined actin binding site (Levine et al., 1992), were capable of cosedimenting with F-actin. This means that A_{01} and A_{02} each contained a functional actin-binding site. We found a low dissociation constant for A_{01}, and this result is in partial disagreement with the suggestion from α-actinin studies that ABS1 is not very important in the F-actin–dystrophin interface. Indeed, α-actinin has homology with dystrophin in this actin-binding region, and deletion of residues KTFT, also present at the dystrophin ABS1 site, or mutations at this site do not drastically modify α-actinin binding to F-actin (Kuhlman et al., 1992). The apparent K_d for an N-terminal dystrophin fragment (1–256) construct was estimated to be 44 μM (Way et al., 1992). We found an apparent K_d of 5.7 μM for A_0 containing both actin-binding sites, as was also the case in Way's construct. It is interesting to note that the K_d for A_0 was higher than those corresponding to each separate actin-binding site. This might suggest an

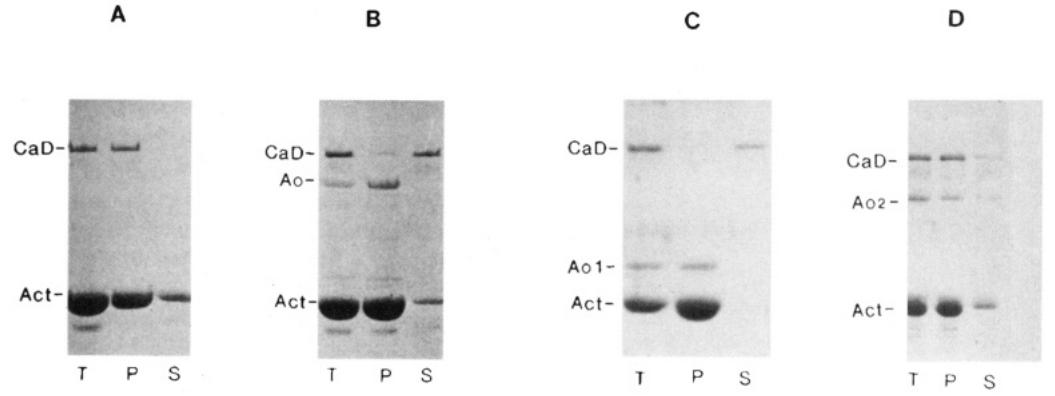

FIGURE 6: Effect of A_0, A_{01}, and A_{02} on the CaD/F-actin interaction. The cosedimentation conditions were as described under Materials and Methods. Forty micrograms of actin, always added at the end of the mixture preparation, was used for each experiment. CaD was used at a CaD/F-actin molar ratio of 1/7. CaD cosedimentation with F-actin is shown (A) alone, (B) in the presence of A_0 (20 μg), (C) in the presence of A_{01} (10 μg), and (D) in the presence of A_{02} (20 μg). Total (T), pellet (P), and supernatant (S) samples were analyzed on a 10% SDS–PAGE and visualized after Coomassie blue staining.

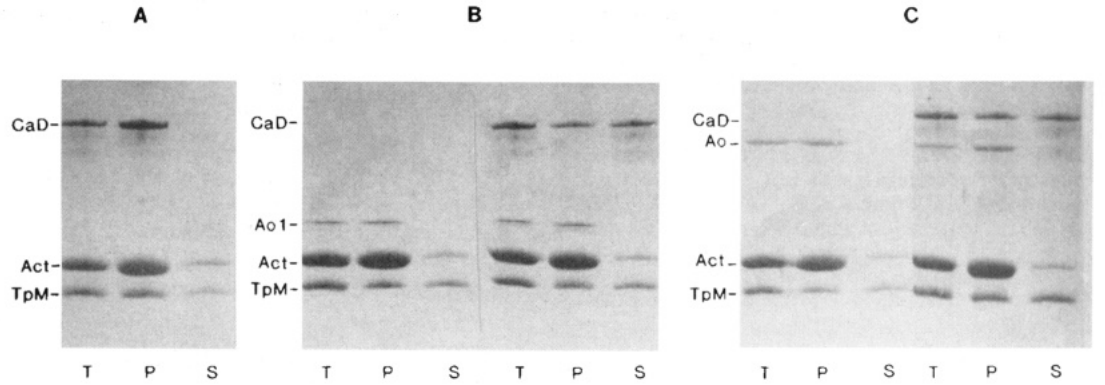

FIGURE 7: Effect of A_0 and A_{01} on the TpM/F-actin interaction. The cosedimentation conditions were as described in Figure 6. TpM was added at a TpM/F-actin molar ratio of 1/7. TpM cosedimentations are shown (A) in the presence of CaD, (B left) in the presence of A_{01}, (B right) in the presence of A_{01} and CaD, (C, left) in the presence of A_0, and (C, right) in the presence of A_0 and CaD. Total (T), pellet (P), and supernatant (S) samples were analyzed on a 10% SDS–PAGE and visualized after Coomassie blue staining.

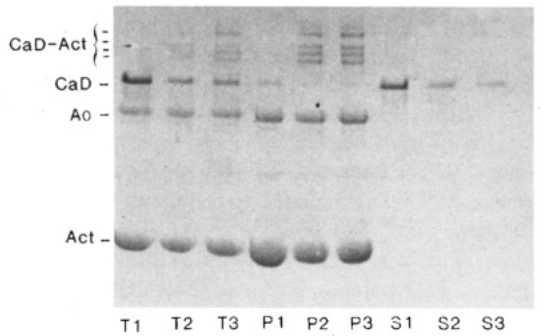

FIGURE 8: Cosedimentation of the CaD/F-actin cross-linked products in the presence of A_0. The cross-link reaction between F-actin and CaD was performed with 10 mM EDC under the conditions described under Materials and Methods. The reaction was stopped by the addition of β-mercaptoethanol for each condensation time, and the cross-link mixture products were cosedimented in the presence of A_0 as described in Figure 4. Total (T), pellet (P), and supernatant (S) samples corresponding to each condensation time (0, 3, and 6 min) were analyzed on a 10% SDS–PAGE and visualized after Coomassie blue staining and are indicated as 1, 2, and 3, respectively. CaD–Act indicates the different cross-link products that appeared during the time course.

inhibitory effect of the ABS2 site on the ABS1 site due to the spatial proximity of both sites. Nonetheless these results could be modified *in vivo* since, on one hand, dystrophin may be capable of forming dimers and dimerization modifies K_d values and, on the other, dystrophin is anchored to the membrane via the glycoprotein complex. It is not yet known whether these two actin-binding sites are located closely together in the tertiary sequence of dystrophin. Crystallization of the N-terminal part of dystrophin could provide some information on the real structures involved in these interactions. The only certainty is that actin sites are located in the same subdomain on the outer face but not on the same side of the protein, as deduced from the recently reported actin crystal structure (Kabsch et al., 1990). Since each construction containing one actin-binding site can bind actin, each actin-binding site might link a different actin monomer belonging (or not) to the same filament.

It is conceivable that *in vivo* specific proteins and/or other actin-binding proteins may serve as modulators of this interaction, depending on the state of the cell. In cosedimentation assays, A_0 or A_{01} inhibited CaD, another actin-binding protein, from binding actin (Figure 6). CaD was shown to bind actin segments 1–7 and 20–41 (Levine et al., 1990b). The close proximity with the dystrophin ABS1 site may explain our results. When A_0 or A_{01} bound actin, steric hindrance may have affected the adjacent caldesmon site on actin, thus explaining the presence of caldesmon in the supernatant. However, it is also possible that once binding of dystrophin recombinant protein occurred, an actin conformational change was promoted that buried the actin–caldesmon site and prevented its association with the twisted actin filaments. Experiments with 3-fold increased concentrations of CaD did not disturb the A_0/actin interaction, and CaD still could not be substantially pelleted with F-actin (not shown), suggesting that the observed inhibition was not competitive; thus two different actin interfaces were involved.

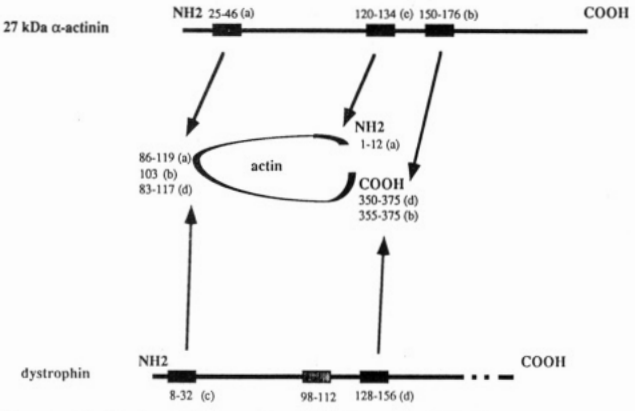

FIGURE 9: Deduced maps of α-actinin–actin-binding sites on actin compared with dystrophin. Dystrophin, actin, and the 27-kDa α-actinin fragment are represented on this scheme. Previously identified actin-binding sites of α-actinin and dystrophin are indicated in solid black boxes. Residues 25–46 on α-actinin (a; Mimura et al., 1987) or 8–32 on dystrophin (c; Levine et al., 1990a) interact with 86–119 actin peptide (a; Mimura et al., 1987) or near residues 103 (b; Lebart et al., 1990) and 83–117 actin peptide (d; Levine et al., 1992). Residues 150–176 on α-actinin (b; Lebart et al., 1990) or 128–156 on dystrophin (d; Levine et al., 1992) interact with the last 20 amino acids of actin (b; Lebart et al., 1990) and actin residues 350–375 (d; Levine et al., 1992). The 27-kDa N-terminal fragment of α-actinin covalently cross-linked with EDC agent to actin at N-terminal residues 1–12 (a; Mimura et al., 1987). This implies that the recently determined third α-actinin–actin-binding site located between residues 120–134 (e; Kuhlman et al., 1992) binds actin N-terminal residues, opposite the corresponding part on dystrophin (residues 98–112) as indicated with a grey box since EDC cannot cross-link A_0 with actin.

This result was confirmed in cross-linking experiments of CaD with actin cosedimented in the presence of A_0. It is very interesting that caldesmon also needs the actin C-terminal part to make a binary complex (Crosbie et al., 1991). This is a region involved in the F-actin–dystrophin interface since it partially corresponds to the ABS2 site. However, no effect on CaD was observed with the A_{02} protein. In addition, IAEDANS labeling at C_{374} did not induce any changes in the binding of A_0 to F-actin (not shown). These data suggest involvement of more ABS1 in this phenomenon, indicating its importance within the dystrophin–actin-binding site. The other set of experiments using TpM (another actin-binding protein) instead of CaD showed no effect (Figure 7), confirming our finding on CaD-specific behavior in the presence of A_0. It should now be determined whether similar events, whose functional significance requires clarification, occur *in vivo*.

Further evidence of the F-actin/dystrophin interaction was pointed out in a cross-linking experiment. By analogy with previous reports, the present experiments were performed in the best conditions to covalently link actin and different actin-binding proteins. In fact, different actin-binding proteins have a preferred attachment site in the 12 N-terminal actin residues which are abundant in acidic amino acids (Vandeckerkhoeve et al., 1978) and suitable for EDC cross-linking experiments (Mimura et al., 1987; Sutoh et al., 1986; Bartegi et al., 1990). But here EDC was not efficient in promoting actin–dystrophin complex stabilization. Nevertheless, cosedimentation of actin and CaD EDC cross-link products in the presence of A_0 (Figure 8) provided further insight into certain results summarized here in Figure 9. The dystrophin part (residues 98–112), which is homologous to the α-actinin region, did not covalently link to F-actin with any of the cross-linkers used, even with EDC. This means that dystrophin, which contains at least two known actin-binding sites, does not functionally behave in the same way as α-actinin, which contains three reported actin-binding sites (Figure 9). It could also explain why we found a significantly important ABS1 site in dystrophin, although this is not the case in α-actinin (Hemmings et al., 1992).

Actin is a protein that is present in every eukaryotic cell studied to date. It is involved in various forms of cellular motility and in the dynamics of the cytoplasmic matrix. In agreement with recent reports (Way et al., 1992; Hemmings et al., 1992; Levine et al., 1990a), our results clearly show that dystrophin belongs to the actin-binding protein family since it contains functional actin-binding sites. The fact that multiple actin-binding sites coexist in dystrophin molecules suggests that different actin monomers may be linked to one dystrophin. Other natural and mutated dystrophin constructs should now be analyzed since this is the best strategy to produce significant amounts of the actin-binding segment of dystrophin. The location of dystrophin within the cell is possibly essential for this binding. Since the C-terminal part of dystrophin is anchored in the membrane via the glycoprotein complex, there must be a correlation between the dystrophin location and its F-actin interaction.

REFERENCES

Bar, S., Barnea, E., Levy, Z., Neuman, S., Yaffe, D., & Nudel, U. (1990) *Biochem. J. 272*, 557–560.

Bartegi, A., Fattoum, A., & Kassab, R. (1990) *J. Biol. Chem. 265*, 2231–2237.

Bretscher, A. (1984) *J. Biochem. 259*, 12873–12880.

Crosbie, R. H., Chalovich, J. M., & Reisler, E. (1992) *Biochem. Biophys. Res. Commun. 184*, 239–245.

Cross, R. A., Murray, S., & Kendrick-Jones, J. (1990) *FEBS Lett. 262*, 87–92.

Eisenberg, E., & Kielly, W. W. (1974) *J. Biol. Chem. 249*, 4742–4748.

Ervasti, J. M., & Campbell, K. P. (1991) *Cell 66*, 1121–1131.

Ervasti, J. M., Kahl, S. D., & Campbell, K. P. (1991) *J. Biol. Chem. 266*, 9161–9165.

Fabbrizio, E., Harricane, M. C., Pons, F., Leger, J., & Mornet, D. (1993) *Biol. Cell. 76*, 167–174.

Franco, A., Jr., & Lansman, J. B. (1990) *Nature 344*, 670–673.

Harricane, M. C., Fabbrizio, E., Arpin, C., & Mornet, D. (1992) *Biochem. J. 287*, 633–637.

Hammonds, R. G. (1987) *Cell 51*, 1.

Hemmings, L., Kuhlman, P. A., & Critchley, D. R. (1992) *J. Cell Biol. 116*, 1369–1380.

Hoffman, E. P., Fischbeck, K. H., Brown, R. H., Johnson, M., Medori, R., Loike, J. A., Harris, J. B., Waterston, R., Brooke, M., Sprecht, L., Kupsky, W., Chamberlain, J., Caskey, C. T., Shapiro, F., & Kunkel, L. M. (1988) *N. Engl. J. Med. 318*, 1363–1368.

Hutter, O. F., Burton, F. L., & Bovell, D. L. (1991) *J. Muscle Res. Cell Motil. 12*, 585–589.

Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., & Holmes, K. C. (1990) *Nature 347*, 37–44.

Koenig, M., & Kunkel, L. M. (1990) *J. Biol. Chem. 265*, 4560–4566.

Koenig, M., Hoffman, E. P., Bertelson, C. J., Monaco, A. P., Feener, C. A., & Kunkel, L. M. (1987) *Cell 50*, 509–529.

Koenig, M., Monaco, A. P., & Kunkel, L. M. (1988) *Cell 53*, 219–228.

Kuhlman, P. A., Hemmings, L., & Critchley, D. R. (1992) *FEBS Lett. 304*, 201–206.

Lebart, M. C., Mejean, C., Boyer, M., Roustan, C., & Benyamin, Y. (1990) *Biochem. Biophys. Res. Commun. 173*, 120–126.

Lederfein, D., Levy, Z., Augier, N., Mornet, D., Morris, G., Fuchs, O., Yaffe, D., & Nudel, U. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5346–5350.
Lemaire, C., Heilig, R., & Mandel, J. L. (1988) *EMBO J. 7*, 4157–4162.
Levine, B. A., Moir, A. J. G., Patchell, V. B., & Perry, S. V. (1990a) *FEBS Lett. 263*, 159–162.
Levine, B. A., Moir, A. J. G., Audemard, E., Mornet, D., Patchell, V. B., & Perry, S. V. (1990b) *Eur. J. Biochem. 193*, 687–696.
Levine, B. A., Moir, A. J. G., Patchell, V. B., & Perry, S. V. (1992) *FEBS Lett. 298*, 44–48.
Love, D. R., Hill, O. F., Dickson, G., Spurr, N. K., Byth, B. C., Marsden, R. F., Walsh, F. S., Edwards, Y. H., & Davies, K. E. (1989) *Nature 339*, 55–58.
Mencke, A., & Jockusch, H. (1991) *Nature 349*, 69–71.
Milner, R. E., Busaan, J., & Michalak, M. (1992) *Biochem. J. 288*, 1037–1044.
Mimura, N., & Asano, A. (1987) *J. Biol. Chem. 262*, 4717–4723.
Mornet, D., Harricane, M. C., & Audemard, E. (1988) *Biochem. Biophys. Res. Commun. 155*, 808–815.
Noegel, A. A., Witke, W., & Schleicher, M. (1987) *FEBS Lett. 221*, 391–396.
Ohlendieck, K., Ervasti, J. M., Snook, J. B., & Campbell, K. P. (1991) *J. Cell Biol. 112*, 135–148.
Pons, F., Augier, N., Heilig, R., Léger, J., Mornet, D., & Léger, J. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7851–7855.
Sato, O., Nonomura, Y., Kimura, S., & Murayama, K. (1992) *J. Biochem. 112*, 631–633.
Senter, L., Luise, M., Presotto, C. P., Betto, R., Teresi, A., Ceoldo, S., & Salviati, G. (1993) *Biochem. Biophys. Res. Commun. 192*, 899–904.
Speicher, D. W., David, G., Yurchenko, P. D., & Marchesi, V. T. (1983) *J. Biol. Chem. 258*, 14931–14947.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4871–4886.
Suzuki, A., Yoshida, M., Yamamoto, H., & Ozawa, E. (1992) *FEBS Lett. 308*, 154–160.
Sutoh, K., & Hatano, S. (1986) *Biochemistry 25*, 435–440.
Tinsley, J. M., Blake, D. J., Roche, A., Fairbrother, U., Riss, J., Byth, B. C., Knight, A. E., Kendrick-Jones, G. K., Love, D. R., Edwards, Y. H., & Davies, K. E. (1992) *Nature 360*, 591–593.
Turner, P. R., Fong, P., Denetclaw, W. F., & Steinhardt, R. A. (1991) *J. Cell Biol. 115*, 1701–1712.
Vandeckerkhoeve, J., & Weber, K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1106–1110.
Way, M., Pope, B., Cross, R. A., Kendrick-Jones, J., & Weeds, A. G. (1992) *FEBS Lett. 301*, 243–245.
Yoshida, M, & Ozawa, E. (1990) *J. Biochem. 108*, 748–752.