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DYSTROPHIN, THE PROTEIN THAT PROMOTES MEMBRANE RESISTANCE

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SUMMARY : Deficiency of dystrophin, a 427-kDa subsarcolemma membrane protein, is responsible for Duchenne muscular dystrophy. The function of this protein is not clear but its subcellular distribution suggests that it is an important link between the cytoskeleton and the extracellular matrix, thus maintaining membrane integrity. The N-terminus of dystrophin was shown to bind actin *in vivo* and *in vitro* via two major actin binding sites. The role of dystrophin/actin interactions has been investigated and the results presented here demonstrate for the first time that the N-terminal part of dystrophin is able (i) to interact with G-actin monomers, and (ii) to slowly promote G->F actin transformation. This conversion was shown to be stimulated the presence of calmodulin in a calcium dependent manner. This is evidence that dystrophin is an anchor protein for actin involved in the control of membrane cell shape deformation and developing a calmodulin-calcium induced F-actin network, thus stiffening the myotube membrane cytoskeleton.

The mechanical force generated by the acto-myosin complex in muscle cells leads to marked variations in cell shape. This process affects different parameters of the cell membrane, including its resistance. Dystrophin, a giant cytoskeletal protein that is absent in Duchenne muscular pathology (1), links the extracellular matrix to cytoplasmic F-actin via a membrane glycoprotein complex (2). Its presence in muscle is actually thought to preserve muscle cell membrane integrity by yet unknown mechanisms while it seems important for stretch inactivated calcium channel activity (3).

To perform experiments on dystrophin, purification of the native protein was unable to provide enough pure material for experiments on dystrophin, because of the low amounts of this molecule in muscle (about 0.002% of the total muscle proteins (1)), but also because pure dystrophin is difficult to isolate without its associated proteins (4, 5). The strategy has thus been to express recombinant proteins corresponding to the N-terminal part of the molecule in *E. coli*. Various dystrophin constructs have been previously produced and their respective F-actin binding properties were studied (6-10) according to the number of actin-binding sites (ABS) present in their sequence. The recent renumbering of ABSs proposed by Winder and Kendrick-Jones refered to three neigbouring ABSs which are present in the N-terminal extremity of dystrophin (11). The study presented here focussed on such recombinant fragment (called Ao) containing these three ABSs. Under standard conditions, it was reported that the N-terminal pGEX construct was not able to interact with G-actin (8). However, when rather similar

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constructs produced in pMAL vectors were used immediately after purification through an amylose column in newly defined conditions, specific G-actin binding was observed. Titration by the ELISA technique provided direct evidence of the dystrophin-G-actin association. The second approach ensured this interaction and also showed that this process is time dependent, leading progressively to a G- into F-actin conversion. Dystrophin-induced F-actin formation was confirmed using a cosedimentation technique. This approach hinders any native actin modification, e.g. pyrene incorporation in residue 374 of actin to monitor such a polymerization process (12). Dystrophin performances in converting G to F- actin were thus investigated in the presence of the calmodulin-calcium (CaM-Ca^{2+}) complex, which was recently shown to bind the N-terminus of dystrophin (13). This latter experiment affords information on the respective roles of these three proteins during F-actin network formation.

MATERIAL AND METHODS

Protein preparations: Actin was purified from rabbit skeletal muscle as previously described (9) F-actin was stored at 4°C in buffer A (2 mM Tris HCl, pH 8.0, 10 mM KCl, 0.1 mM $CaCl_2$ and 1 mM NaN_3). G-actin was obtained as follows: F-actin was diluted to 2 mg/mL in buffer B (2 mM HEPES pH 7.0, 0.1 mM ADP, 0.1 mM $CaCl_2$ and 1 mM NaN_3). After centrifugation at 200000 g for 1 h, the pellet was resuspended in buffer B and sonicated with a Branson model sonifier three times for 2 min. After centrifugation as described above, G-actin was collected in the supernatant, titrated by spectrophotomtric measurement in a Kontron Uvikon 930 using actin ε 1% at 280nm = 1.1 and stored at 4°C at 1.5 mg/mL.

Construction of plasmids encoding the Ao recombinant protein fused to the C-terminal part of the maltose binding protein (MBP) and its purification are described elsewhere (9). Concentration of the fusion protein Ao was measured by the Bradford assay using BSA for calibration (13). CaM was purified as in (13).

Cosedimentation assays: Cosedimentation of G-actin with dystrophin recombinant proteins was performed in an Airfuge (Beckman). Buffer was 2 mM Tris-HCl pH 7.5. Ten µM of G-actin was added to 2.5 µM of Ao recombinant protein and the mixture was incubated for 0 to 60 min before centrifugation to 28 psi for 15 min. Comparative experiments were performed in presence and absence of 3 µM CaM. Control experiments, in the presence of EGTA, clearly demonstrated the specificity of CaM-Ca^{2+} on dystrophin which increased the G to F-actin conversion.

Immunological techniques: Binding of dystrophin recombinant proteins to G-actin was carried out using a solid phase immunoassay (ELISA) as in (13).

Affinity chromatography: The CaM Sepharose 4B column was purchased from Pharmacia (Uppsala Sweden). Dystrophin recombinant protein solutions 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, loaded on a 1x10 cm CaM affinity column. Ao (2 µM) specifically bound to this CaM-Sepharose column since, after washing several times in the same buffer then with an imidazole salt buffer, elution only occured in the presence of 5 mM EGTA buffer (C) and not with ionic strength buffer (D), (buffer C in presence of 0.3 M KCl). On such an immobilized CaM-dystrophin recombinant protein complex, a solution of G-actin (10 µM) was applied and the excess G-actin was eluted during the washing step. Bound proteins were only eluted with EGTA buffer (C). G-actin or these different buffers were added at the point indicated with arrows. No other proteins were eluted at high ionic strength (buffer D).

RESULTS

Specific binding of the N-terminal part of dystrophin to G-actin

Analysis of the G-actin / dystrophin interaction by ELISA methods gave the titration curve shown in Fig. 1. It was characterized in our conditions, by differences in the curves

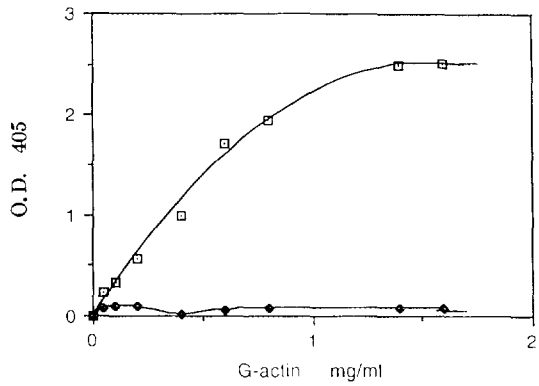

Figure 1. ELISA titration of the complex between G-actin and the dystrophin N-terminal recombinant protein.
The ELISA titration of the binding of dystrophin recombinant protein Ao to G-actin is presented (❑) with the non-recombinant protein NR (◆) included as control. Absorbance was measured at 405 nm

according to the presence of dystrophin recombinant protein Ao, (which contains all three ABSs), comparatively to the non-recombinant protein (NR) while G-actin or Ao alone, remained in the supernatant.

The Ao protein was found to be specifically taken up when a G-actin affinity column (made in the laboratory using a sulfo link coupling gel provided by Pierce) was used. Analysis of the resin by SDS polyacrylamide slab gel electrophoresis showed the presence of actin and Ao, with both proteins retained on the column even after extensive washing (results not shown). This point will be illustrated later in the results obtained when using another affinity column.

Dystrophin induces G->F actin transformation

In light of the dystrophin-G-actin interaction, Ao was assayed with G-actin to observe a dystrophin-induced actin polymerization process. G->F actin conversion can be easily analyzed at different times by cosedimentation assays. After ultracentrifugation, there was a time-dependent increase in F-actin quantities, while the remaining G-actin was detected in the supernatant (Fig. 2A). Note that in our conditions the non-recombinant protein (NR) as well as CaM did not induce any polymerization with G-actin.

The CaM·Ca^{2+} complex was recently demonstrated to specifically bind the dystrophin N-terminus (13), but was unable to modulate F-actin-dystrophin interactions, contrary to the situation concerning F-actin-utrophin interactions (14). The influence of CaM on the actin polymerization process was studied. As shown comparatively in Fig. 2A and 2B, F-actin formation was significantly increased during the first steps of this process. This dystrophin-induced phenomenon was not temperature dependent and not observed when Ca^{2+} was omitted (data not shown).

Interestingly, CaM never cosedimented with the F-actin-dystrophin complex, as previously observed when using purified F-actin (13-14). However, a dystrophin-CaM-G-actin ternary complex was revealed in the presence of Ca^{2+} when the CaM affinity column was used. This affinity chromatography column was particularly efficient for retaining dystrophin. After washing the CaM-affinity column, a G-actin solution was applied onto the column (Fig.3A, first arrow) and, once the G-actin excess was eliminated, the remaining proteins were specifically

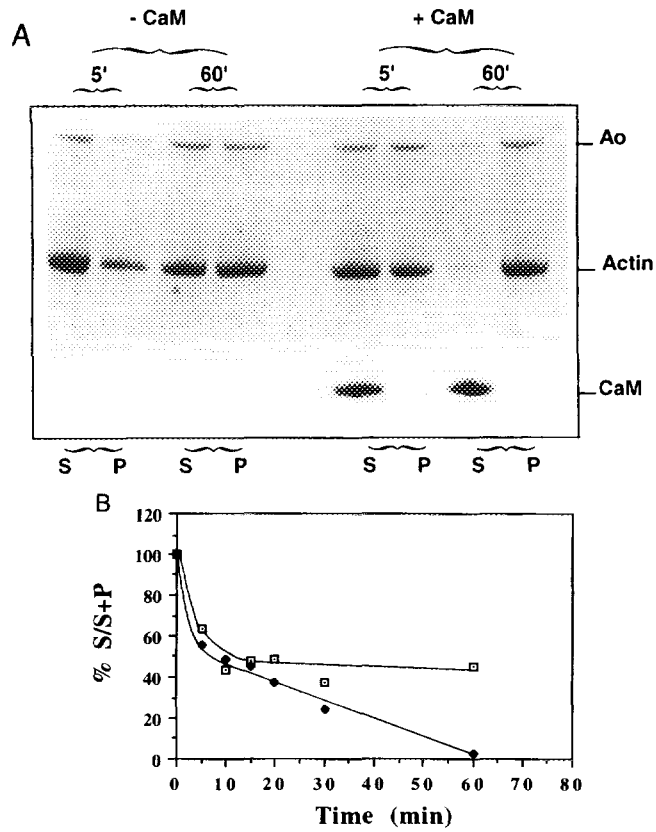

Figure 2. Transformation of G into F-actin in the presence of Ao.

In part (A) as well as for each following experiments, the supernatant (S) and the pellet (P) resuspended in 100 µL of the above buffer were boiled in an equal amount of SDS loading buffer, analyzed on 10% SDS polyacrylamide gel and visualized after Coomassie blue staining. The mixture of G-actin and Ao in the presence or absence of CaM-Ca^{2+} was analyzed after 5 and 60 min of incubation and comparative analysis of the various supernatant and pellet fractions allowed us to determine the performance of G to F-actin conversion according to the proteins present in the mixtures. Part (B) presents the scanning curves corresponding to the results of cosedimentation experiments between Ao and actin in the presence (❑) and absence (◆) of CaM. Variation in the ratio S/S+P (supernatant/supernatant+pellet) are presented over the time course (t from 0 to 60 min), showing the time dependence of the G to F-actin conversion as well as its stimulation by CaM. In similar conditions the addition of 150 mM KCl to the G-actin solution induced polymerization of F-actin in a CaM-Ca^{2+} independent manner.

eluted by EGTA (Fig. 3A, arrows C and D). Analysis of the elution profile (Fig. 3B) demonstrated the combined presence of actin and dystrophin confirming the G-actin affinity column results described above.

DISCUSSION

Many studies previously demonstrated that dystrophin was able to associate with the γ–actin filaments. However, a recent work suggests that the N-terminal dystrophin recombinant protein likely interacts in skeletal muscle cells with α– and ß–actin filaments, which emanate from M and Z lines and are colocalized with dystrophin in costameres, rather than with cytoskeletal F-actin (15). Consequently, it was important to investigate whether one property of

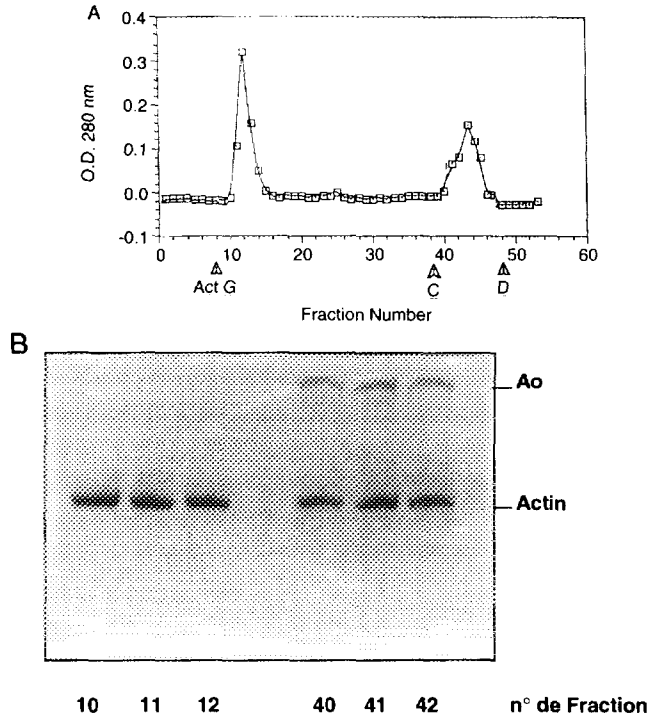

Figure 3. Ao and G-actin binding to the CaM column.
Part (A) shows the elution patterns of the CaM-Sepharose-4B column: first arrow corresponds to the step where G-actin solution was applied onto the column and arrows C and D refer to elution buffers as described in materials and methods. The part (B) shows the 12.5% SDS-PAGE analysis of fractions 11 to 14, and 40 to 43, corresponding to the G-actin unbound material or Ao-G-actin material removed by EGTA. The positions of Mr markers have confirmed the identity of the proteins Ao and actin by their respective migration in accordance with their calculated molecular weight (high molecular weight markers from Bio-Rad Laboratories).

the dystrophin N-terminal part, containing all three ABSs previously described in this dystrophin domain, is responsible for a G->F actin conversion. On the basis of the results presented here, we conclude that dystrophin can interact with actin monomers and induce a polymerization process which can be activated in a CaM-Ca^{2+} dependent manner. We utilized the dystrophin-membrane anchorage model (16) and derived a new concept for understanding the structure-function of dystrophin. First dystrophin-actin linkage could be a starting point for the formation of a modulable CaM-Ca^{2+} dependent F-actin-network. Many severing proteins are able to depolymerize F-actin in muscle and non-muscle cells such as gelsolin, an ubiquitous protein (17,18).

-Why does dystrophin bind to F-actin?- We can assume that the F-actin network linked to dystrophin may only be built when Ca^{2+} is released in the muscle cells. Ca^{2+} release leads to various Ca^{2+}-complex formations, such as troponin-Ca^{2+} and CaM-Ca^{2+}. The muscle cell membrane that already possesses the spectrin-ankyrin-F-actin-talin-vinculin network (19,20) may suddenly require membrane resistance reinforcement to respond to muscle cell membrane modifications. The dystrophin-F-actin network is thus formed. At the end of a contraction, such an induced secondary F-actin network may break down by the action of a severing protein. This leaves dystrophin linked to few actin monomers which are then able to furnish actin nuclei in a

CaM-Ca^{2+} dependent manner, for the anchorage of a novel dystrophin-F-actin network in response to new cell events. This complex formation could additionaly be regulated by effects of dystrophin phosphorylation (21). The fact that overexpression of Dp71 helps to recover normal membrane glycoprotein complex levels in dystrophin-deficient muscles, but fails to fight against membrane degradation, sheds further light on the importance of the differential protein information contained in dystrophin (22, 23). One missing function of Dp71 in comparison to dystrophin is the actin binding properties of the latter molecule. This property highlighted a very important function of dystrophin in the present study. In addition, the fact that the dystrophin-F-actin interaction is not modulated by the CaM-Ca^{2+} complex while utrophin-F-actin complex is, indicates that, at least with respect to the above function of dystrophin, it can never be replaced by utrophin. Generation of natural occurring mutants in the dystrophin N-terminus which confer severe clinical disorders may help to determine the importance of these zones in polymerization (24, 25).

The mechanical function of dystrophin was recently measured in muscle cell by measurement of the local stiffness of the membrane (26). The other point is that an absence of dystrophin leads to Ca^{2+} leaks (27). The absence of dystrophin is related to decreased muscle performance (28-31) and an unability to maintain steady contraction in mdx mice as compared to normal mouse muscles, indicating muscle fatigue (32), and deformability of the muscle membrane leading to weakened resistance (26). These overall results indicate that during the contraction process dystrophin increases membrane resistance by providing supplementary modulable anchorage of F-actin to reinforce such network linked to the membrane.

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