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INHIBITION OF ACTIN-DYSTROPHIN INTERACTION BY INOSITIDE PHOSPHATE

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SUMMARY : Dystrophin, the protein absent from Duchenne dystrophy, is a member of the α-actinin protein family and located in the membrane cytoskeleton. It bridges a transmembrane glycoprotein complex with actin filaments. This work investigates the binding of dystrophin issued from *Torpedo marmorata* electric organ with actin in the presence of the phosphoinositide PIP_2 that regulates α-actinin and filamin binding with actin. The interaction was inhibited (80%) by PIP_2 and reached its minimum above 20 μM PIP_2, but the effect was abolished when PIP_2 was previously cleaved by phospholipase C. Using antibodies directed against the 27 kDa actin binding domain of α-actinin, a reliable carrier for actin binding sites ABS-1, ABS-2 and ABS-3 also involved in dystrophin and filamin, it was shown that PIP_2 affects the ABS-3 environment.

Dystrophin (1), the protein absent or mutated in Duchenne and Becker muscular dystrophies, is believed to form a flexible link between the actin cytoskeleton and extracellular matrix via a membrane-associated glycoprotein complex (2). The interaction between dystrophin and actin has been demonstrated by NMR studies using synthetic peptides of dystrophin (3,4) and recombinant fragments of dystrophin in conjonction with sedimentation assays (5-8). Partially purified dystrophin has provided similar evidence

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(7,9,10). The important problem of isolating pure dystrophin has considerably slowed down the biochemical study of dystrophin-actin interaction, which includes the study of related regulations (11-13). However, it has been possible to obtain (in the µg order) pure dystrophin using membranes from electric organ of *Torpedo marmorata*, rich in this protein, and high affinity antipeptide antibodies.

This study investigates the effect on purified dystrophin of phosphoinositide diphosphate (PIP_2), known to regulate the association of filamin (14) and α-actinin (15) to actin through direct interaction with the actin binding protein (15). The *in vitro* inhibition of actin interaction with dystrophin issued from *Torpedo* electric organ by PIP_2 is described.

MATERIALS AND METHODS

Protein purification :

Torpedo marmorata dystrophin was obtained as detailed by Lebart et al (16). In brief, it was prepared from AChR-rich membranes (17) after alkaline extraction (18). Dystrophin was isolated from extract in only one step by affinity chromatography using Sepharose 4B-insolubilized anti-dystrophin antibody, extensively washed in 0.5 M NaCl, eluted from immunoadsorbant with 0.1 M formic acid (pH = 2.6) and immediately neutralized with 2 M Tris. Rabbit skeletal muscle actin (19) and gizzard filamin (20) purifications have been previously described. Phospholipase C was obtained from Sigma. The 27 kDa actin-binding domain issued from thermolysin cleavage of gizzard α-actinin was purified as described (21). The C-terminal peptide of sequence 3549-3569 common to human, chicken and *Torpedo* dystrophins was synthesized (22). Protein biotinylation was performed (23) via lysine residues. Antibodies directed against sequence 108-134 (27mer) in actin-binding domain (ABD) of α-actinin (anti-27mer antibodies) have been previously described (22), and anti-dystrophin antibodies were induced (22) in rabbit using insolubilized dystrophin peptide of sequence 3549-3569.

Protein interactions :

Dystrophin-actin cosedimentation was performed in 2 mM $MgCl_2$, 150 mM NaCl, 10 mM Tris-HCl pH 7.5, at 20°C for 30 min at 30 p.s.i. in an Airfuge (Beckman Instruments). Proteins from the resulting supernatants and pellets were analyzed by immunoblotting (24) after SDS-PAGE 5-18% (25), using anti-dystrophin antibodies. Dystrophin-actin interaction was also monitored using enzyme-linked immunoassay as previously detailed (20,22). Phosphatidylinositol 4,5-biphosphate (PIP_2) from Sigma was

sonicated in 50 mM Tris-HCl, pH 7.5 for 15 min at 0°C just before incubation. In some experiments, PIP_2 (100 µM) was hydrolyzed (26) by phospholipase C (0.5 U/ml) for 1 h at 20°C in 4 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5. The binding of biotinylated actin or biotinylated anti-27mer antibodies was detected at 405 nm with alkaline phosphatase-labeled streptavidin (0.5 µg/ml).

RESULTS AND DISCUSSION

Dystrophin was purified from membrane extract by affinity chromatography using high affinity (K_d = 1 nM) anti-dystrophin antibodies. These antibodies are directed against a conserved sequence common to human, chicken and *Torpedo* dystrophins. They have allowed, after insolubilization, obtention of dystrophin solutions (Fig. 1) at concentrations of 0.5 to 1 µg/ml, as evaluated by colorimetry (27). The presence of membrane cytoskeleton

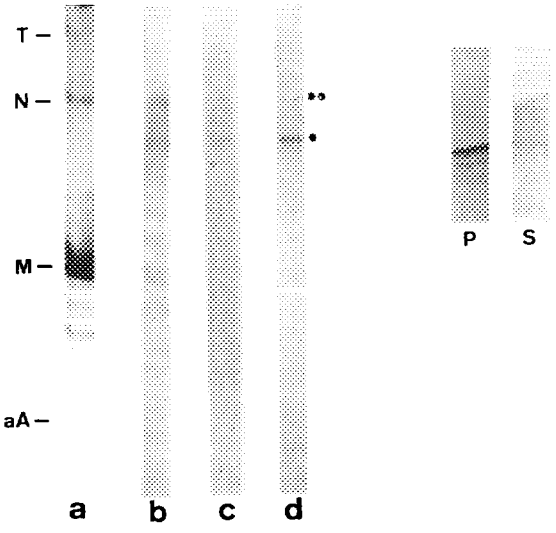

Fig. 1. Dystrophin purification.
Dystrophin purity was checked after silver staining of 2-12% gradient SDS-PAGE gel of the extract (b) and the eluted fraction resulting from affinity chromatography (c). A myofibrillar extract from rabbit muscle showing titin (T), nebulin (N), myosin heavy chain (M) and α-actinin (aA) of 3 $x10^6$ kDa, 8 $x10^5$ kDa, 2 $x10^5$ and 1 $x10^5$ kDa apparent M.W. was used as molecular weight marker after Coomassie blue staining (a). *Torpedo* dystrophin (*) copurified with some oligomer (**) was revealed by immunoblotting using anti-dystrophin antibody. Dystrophin (0.5 µg/ml) was also revealed by immunoblotting in pellet (p) and supernatant (s) after cosedimentation assay with rabbit F-actin (1 mg/ml).

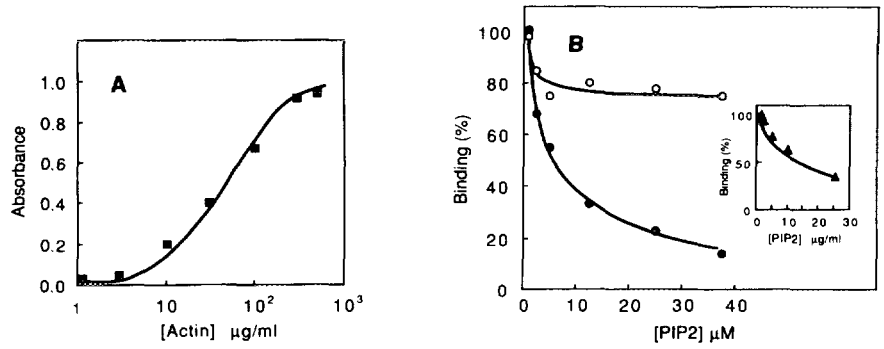

Fig. 2 . Dystrophin interaction with actin .
(A), Dystrophin binding to actin was carried out by direct ELISA. Dystrophin (0,3 µg/ml) was coated on plastic plates and biotinylated actin in 2 mM $MgCl_2$, 100 mM KCl, 0.2 mM ATP, 0.1 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5, was added at increasing concentrations (0-660 µg/ml). Interaction (■) was followed using alkaline phosphatase-labeled streptavidin and monitored at 405 nm using p-Nitrophenyl phosphate as substrate. (B), binding (%) of dystrophin and filamin (inset) to actin was obtained at a fixed concentration of actin (100 µg/ml) using PIP_2 (●,▲) and cleaved PIP_2 (○) added at increasing concentrations. The incubation solution was always supplemented by 0.5% gelatin and 3% gelatin hydrolysate.

associated proteins such as actin, α-actinin or filamin, was not detected after the affinity chromatography step, using immunoblotting and specific antibodies (not shown). Possible structural effects linked to drastic membrane alkaline extraction (pH 11) and acidic elution from affinity column (pH 2.6) on the dystrophin conformation have been previously analyzed using rotary-shadowed images (28,29). The authors reported a similar structure and the same capacity for self-association in oligomeric structures after these treatments. The ability of dystrophin to interact with actin could then be confirmed using a cosedimentation assay analyzed by immunoblotting (Fig. 1) which revealed the presence of dystrophin in the pellet, and a solid phase immunoassay (Fig. 2A) in which biotinylated actin bound coated dystrophin in a saturable manner.

The effect of exogenous PIP_2 on the actin binding ability of dystrophin was then analyzed. We have observed (Fig. 2B) that in the presence of PIP_2 concentrations increasing up to 50 µM, the interaction regularly decreased below 20% when PIP_2 concentration exceeded 20 µM. In identical experimental conditions (Fig. 2B, inset)

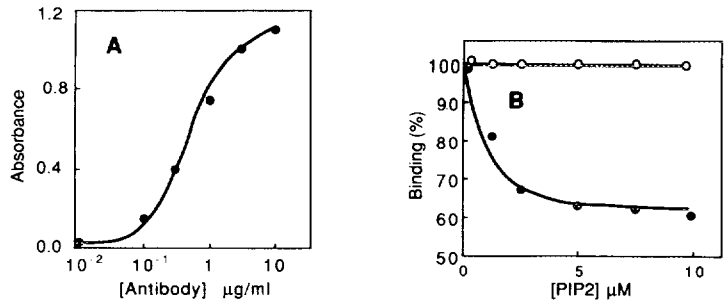

Fig. 3 . 27 kDa actin binding domain (ABD)-PIP_2 interaction.
(A), Reactivity of anti-27mer antibodies was checked (●) by direct ELISA on coated ABD (0.3 µg/ml) issued from α-actinin cleavage, in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5. Antibody binding was monitored at 405 nm using alkaline phosphatase labeled anti-rabbit IgG antibodies (1/2000). (B), Binding (%) of anti-27mer antibodies to coated ABD (0.3 µg/ml) was followed at increasing PIP_2 concentrations (●) using fixed antibody concentration (1 µg/ml). As a control, accessibility of coated (0.3 µg/ml) biotinylated ABD to alkaline phosphatase-labeled streptavidin was checked in the presence of increasing PIP_2 concentrations (○). Interaction conditions and monitoring are as described above.

actin-filamin binding was largely altered by PIP_2 used in similar concentrations. Using a different approach monitoring viscosity and sedimentation, Furahashi demonstrated that PIP_2 used at 10 µM abolished gelation and filamin binding in the presence of F-actin (14). In order to control the specificity of PIP_2 effect, we have also used phospholipase C-cleaved PIP_2 (26). Hydrolyzed PIP_2 was largely unable to affect dystrophin-actin binding (Fig. 2B).

In a previous study (22), we have characterized an antibody population directed against the α-actinin sequence 108-134 (27mer) which is conserved to 66% extent in filamin and human dystrophin (30,31). This sequence carries the actin binding site ABS-3 of sequence 127-134 contiguous to the ABS-2 site (sequence 150-176). This antibody reacts with a strong affinity (K_d = 3 nM) with the 27 kDa actin binding domain (ABD) issued from α-actinin thermolysin cleavage (Fig. 3A). It also crossreacts with the 70 kDa actin binding domain of filamin (22), but poorly with *Torpedo* dystrophin (not shown). ABD was purified so as to preserve the original protein folding (21). It is the smallest entity of reliable folding, carrying the three sequences (ABS-1, ABS-2 and ABS-3) known to participate in actin interface (20,32) with α-actinin (33), filamin (22) and dystrophin (4,7,31). In the presence of increasing PIP_2 concentrations, the reactivity of these antibodies with ABD

was decreased by 40% (Fig. 3B). Beyond 5 μM PIP_2 , we were not able to observe an additional effect on the binding of the antibody. The eventuality that large PIP_2 micelles (34) could cap the coated ABD, thus restricting access to the antibody, was tested using biotinylated-ABD and streptavidin as probe. We have observed that streptavidin retained the same binding ability whether or not PIP_2 was present in concentrations of up to 100 μM (Fig. 3B).

In conclusion, we have reported an *in vitro* effect of inositide phosphate PIP_2 on dystrophin-actin interaction which resulted in the inhibition of protein binding. Using the 27 kDa actin-binding domain derived from α-actinin cleavage as a native carrier for ABS sites, we have detected a PIP_2 effect near ABS-3. The large conservation of the actin binding sequences observed in dystrophin led us to conclude that a similar location of the phospholipid could severly affect dystrophin interaction with actin.

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