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PHOSPHORYLATION OF DYSTROPHIN: EFFECTS ON ACTIN BINDING

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SUMMARY. Dystrophin is phosphorylated by several protein kinases. In this work, we have studied the effects of dystrophin phosphorylation on the binding to actin. Purified dystrophin was phosphorylated *in vitro* by the catalytic subunit of cAMP-dependent protein kinase (PKA), casein kinase II (CK-II), and protein kinase *c* (PKC). The results demonstrate that phosphorylation of dystrophin by PKA phosphorylation caused a three fold increase in dystrophin binding to actin. In contrast, phosphorylation by CK-II or PKC inhibited the binding to actin. These results indicate that phosphorylation of dystrophin modulates its interaction with the actin cytoskeleton. It is suggested that phosphorylation may be one mechanism for regulating protein turnover in muscle membrane-skeleton.

Dystrophin, the product of the Duchenne/Becker muscular dystrophy gene, is an actin-binding protein of 427 kDa. It is expressed in striated muscle, smooth muscle, and in neurons where it is localized to the inner face of the plasma membrane.

Four domains have been identified in dystrophin from the predicted amino acid sequence (1). Through the C-terminal domain dystrophin associates with a transmembrane glycoprotein complex (2,3), while through the N-terminal domain it binds to actin (4-11). Therefore, it is generally accepted that dystrophin is a major linker between the muscle cytoskeleton and the sarcolemma. Absence or molecular abnormalities of dystrophin may impair the mechanical stability of the sarcolemma leading to muscle fiber degeneration.

We and other have recently shown that dystrophin is a substrate for a variety of protein kinases both *in vitro* and *in vivo* (12-15). Phosphorylation is one post-translational mechanism that modulates protein-protein interactions. This prompted us to study

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whether the binding of dystrophin to actin is modulated by its phosphorylation by cAMP-dependent protein kinase, casein kinase II, and protein kinase *c*.

MATERIALS AND METHODS

Preparation of muscle membrane fraction

Membranes enriched in sarcolemma were isolated from fast-twitch muscles of the rabbit. About 240 g of hind-leg and back muscles were homogenized with 900 ml of 0.3 M KCl, 20 mM sodium phosphate, 20 mM sodium pyrophosphate, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM EGTA, pH 7.0 with Osterizer (max. speed for 10 s). The homogenate was centrifuged at 14000 g for 20 min. The pellet was homogenized in the same buffer for 10 s and centrifuged as described above. The supernatant (Sp2) was kept. The pellet was then washed once with 5 mM Tris/HCl, pH 7.0 (900 ml) and homogenized in the same medium three times for 30 sec. The homogenate was centrifuged at 14000 g for 20 min. The final supernatant was combined with Sp2, filtered through three layers of cheese-cloth and centrifuged at 142000 g for 30 min. The resulting membrane pellet was resuspended in about 200 ml of 0.6 M KCl, 0.303 M sucrose, 50 mM Tris/HCl, pH 7.4, incubated for 30 min. at 0°C, and centrifuged at 142000 g for 35 min. The membranes were resuspended in 0.25 M sucrose, 10 mM histidine, pH 7.0, and stored at -80°C until used.

Purification of dystrophin

Membrane preparations (about 200 mg protein) were washed with 0.6 M KCl, 50 mM Tris/HCl, pH 7 and pelleted at 142000 g for 35 min. The pellet was resuspended in 50 mM Tris/HCl, pH 7.4, loaded on a layer of 7 ml of 0.303 M sucrose, 50 mM Tris/HCl, pH 7.4 and centrifuged at 1000 g for 10 min. for eliminating residual contractile proteins. The 0.303 M sucrose layer was collected and pelleted at 150000 g for 30 min. Dystrophin-glycoprotein complex was purified according to Ervasti et al., 1991 (2). Dystrophin was concentrated using Amicon and stored at -20°C in 50% glycerol. By electrophoresis, dystrophin preparations were found to contain the 59kDa subunit of the glycoprotein complex.

Purification of F-actin

F-actin was prepared from acetone powder from rabbit muscle as described by Pardee and Spudich (16). Before each experiment, F-actin was subjected to a single cycle of depolymerization/polymerization. F-actin was resuspended in 120 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 0.25 mM EGTA, 0.1 mM dithiothreitol (DTT), 20 mM Tris/HCl, pH 7.4, at a protein concentration of 4 mg/ml.

Phosphorylation by exogenous protein kinases

Phosphorylation by PKA and CK-II

Dystrophin (about 0.5 µg) in 100 µl of 20 mM Tris pH 7.4, 120 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 0.25 mM EGTA, 0.1 mM DTT, 5 mM ATP was incubated with either 5 mU of the purified catalytic subunit of PKA (Sigma, P-2645) or 0.4 mU of purified CK II (Boehringer Mannheim, #1500767), for 10 min. at room temperature. The reaction was stopped by cooling at 0°C for 10 min.

Phosphorylation by PKC

Dystrophin was preincubated for 5 min. at 30°C with 100 µl of phosphatidylserine/diacylglycerol (0.4/0.04 mg/ml), 2 mM $CaCl_2$, 3 µM 12-O-tetradecanoylphorbol 13-acetate (TPA). After the addition of 0.5 U PKC (purified from rat brain, Calbiochem) and 5 mM ATP dystrophin was incubated for 5 min. at 30°C and

then centrifuged in a Beckman Airfuge for 10 min. at 20 psi to sediment excess of phospholipid. The solution was then cooled at 0°C for 10 min.

Binding of non-phosphorylated and phosphorylated dystrophin to F-actin
F-actin (75 μg) was added to the tubes containing non phosphorylated or phosphorylated dystrophin and was incubated for 15 min. at 0°C with careful stirring and then for further 60 min. at 0°C without stirring. After centrifugation in a Beckman Airfuge (20 psi for 30 min.), the supernatant and the pellet were collected separately, solubilized in SDS-sample buffer, and loaded on a 5-10% polyacrylamide linear gel. SDS/polyacrylamide gel electrophoresis was carried out according to Laemmli (17). After electrophoresis gels were stained with silver (18).

Densitometric measurements
Densitometry of the stained gels was carried out in a BIO-RAD, Model GS-670, Imaging densitometer.

Protein concentration was measured according to Lowry et al. (19), using bovine serum albumin as standard.

RESULTS AND DISCUSSION

When purified dystrophin was incubated in the presence of F-actin and then centrifuged at high speed, a small amount of dystrophin was found in the pellet, in agreement with previous results (4,8,9). The fraction of total dystrophin sedimented by F-actin varied in different preparations. In some preparations up to 20% of total dystrophin added was sedimented by F-actin, while in other preparations no appreciable dystrophin could be sedimented under the same experimental conditions (FIG. 1). These results suggest that native dystrophin is able to bind to actin. However, the variability found among different batches of purified dystrophin in the binding to actin suggests that some modulatory mechanisms of this interaction may exist.

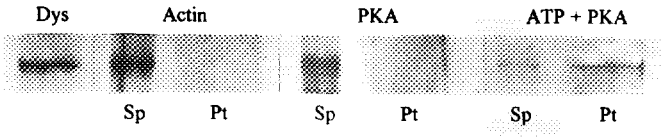

Fig. 1. ***Activation of dystrophin binding to actin after phosphorylation by PKA.*** Dystrophin phosphorylation and binding to F-actin were as described in Materials and Methods. SDS-gel electrophoresis of the supernatant and the pellet was carried out on 5-10% polyacrylamide gradient gel. After electrophoresis the gel was stained with silver. Only the region corresponding to dystrophin migration is shown. Key: Dys: 0.5 μg of purified dystrophin; Actin: 0.5 μg of purified dystrophin incubated with 75 μg of F-actin; PKA: 0.5 μg of purified dystrophin preincubated with 5mU of purified catalytic subunit of PKA; PKA+ATP: 0.5 μg of purified dystrophin preincubated with 5mU of purified catalytic subunit of PKA and 5 mM ATP. Sp: supernatant; Pt: pellet.

We and others have shown that dystrophin is phosphorylated both *in vitro* and *in vivo* by several protein kinases (12-15). We therefore decided to investigate whether phosphorylation of dystrophin by cAMP-dependent protein kinase (PKA) does affect its binding to F-actin. As shown in Fig. 1, preincubation of purified dystrophin with the catalytic subunit of PKA alone, did not affect the binding of dystrophin to actin. However, when ATP was included in the preincubation medium the amount of dystrophin in the pellet increased considerably. Using five different dystrophin preparations the amount of dystrophin sedimented after phosphorylation increased at least three times, from 15.4±10.8% to 52.7±7.6% of total dystrophin in the incubation medium ($p<0.02$). Computer search for consensus sites for phosphorylation by PKA showed seven potential sites. These sites are in the rod-like domain of dystrophin (serines 616, 1033, 2678 and 2793; threonines 1535, 1647 and 2621). No one site is located in the amino terminal domain of dystrophin were actin-binding sites have been demonstrated (4-11). Therefore, the modulation of actin binding by PKA cannot be ascribed to the phosphorylation of serine/threonine residues in the actin binding sites. On the other hand, it is conceivable that phosphorylation of the rod-like domain may either indirectly influence the conformation of the amino-terminal domain or affect the degree of aggregation of dystrophin. An increased aggregation of phosphorylated dystrophin should promote its sedimentation by high speed centrifugation. This possibility was ruled out because 100% of phosphorylated dystrophin was recovered in the supernatant fraction after centrifugation in the absence of F-actin (not shown). Effects on dystrophin-actin interaction by phosphorylation of actin can also be ruled out since in our experimental conditions incubation of dystrophin with actin was carried out at 0°C, conditions in which the added PKA should be inactive. Furthermore, it has been reported that phosphorylation of actin by PKA increases the oligomerization of F-actin (20). However, we did not find any change in actin sedimentation pattern in our experiments. In conclusion, our results demonstrate that phosphorylation of the rod-like domain of dystrophin by PKA modulates its actin-binding activity.

Dystrophin is phosphorylated by other protein kinases, such as CaM kinase, casein kinase II, and protein kinase *c*. Interestingly, sequence analysis has shown that potential phosphorylation sites by CK-II and PKC are located in or near the actin binding sites (threonine 134, and serine 136, respectively). This prompted us to investigate the effects

of dystrophin phosphorylation by these two kinases on actin binding activity. As shown in Fig. 2, neither kinase was able to increase the binding of dystrophin to actin. Interestingly, the results seem to indicate that phosphorylation of dystrophin by CK-II or PKC has an inhibitory effect on actin binding, as shown by the decreased percentage of dystrophin sedimented as compared to the control values, although the results are not statistically significant ($p<0.6$). However, the absence of statistical significance may be due to either the variability of control values among different dystrophin preparations or to the fact that under control conditions the amount of dystrophin sedimented by F-actin is low so that a decrease in amount of dystrophin sedimented after phosphorylation by CK-II can hardly be appreciated. We therefore investigated the effects of CK-II phosphorylation on dystrophin phosphorylated by PKA. Fig. 2 clearly shows that when dystrophin is phosphorylated by both PKA and CK-II there is a statistically significant decrease in actin-binding activity ($p<0.05$). As indicated above, a potential site of phosphorylation by CK-II is threonine 134 that is located in the actin-binding site 2 (6).

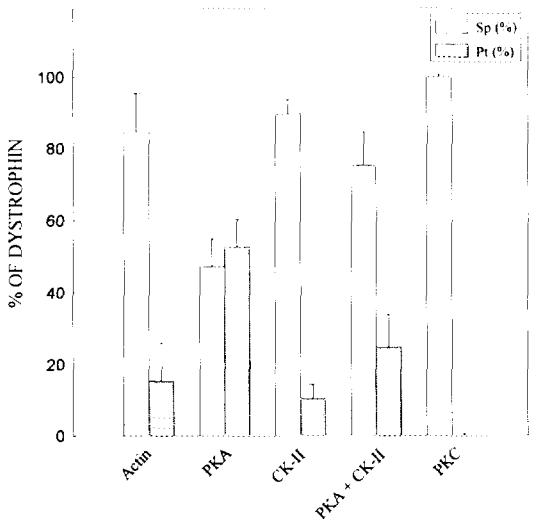

Fig. 2. **Opposite effects of phosphorylation by PKA or CK-II and PKC on the binding of dystrophin to actin.**
The histogram shows the percentage of dystrophin recovered in the supernatant (Sp) and in the pellet (Pt) after phosphorylation. Dystrophin phosphorylation, binding to F-actin, SDS-gel electrophoresis, and densitometry were as described in Materials and Methods and in Fig. 1. Key: Actin: 0.5 μg of purified dystrophin incubated with 75 μg of F-actin; PKA: dystrophin after phosphorylation by PKA; CK-II: dystrophin after phosphorylation by CK-II; PKA+CK-II: dystrophin after phosphorylation by PKA and CK-II; PKC: dystrophin after phosphorylation by PKC.

These results are suggestive for an inhibition of actin-binding activity of dystrophin by the incorporation of a phosphate group in the actin-binding site. Unfortunately, we do not have yet experimental evidence indicating that this site is indeed phosphorylated by CK-II under our experimental conditions.

In conclusion, our results indicate that phosphorylation of dystrophin by protein kinases has different modulatory effects on actin-binding activity.

Dystrophin is a component of muscle membrane cytoskeleton and it is thought to represent an important linker between the actin-cytoskeleton and the cell membrane, thus conferring the necessary mechanical stability to the sarcolemma (9). Regulation of cytoskeleton-membrane interactions is a basic cellular function that, in other cell systems, occurs during cell division, growth, or locomotion. Phosphorylation and de-phosphorylation of interacting proteins is one mechanism that is generally accepted to play an important role in these processes. Adult skeletal muscle do not divide or locomote. However, since the interactions of dystrophin with the other cytoskeletal proteins are of high affinity (10), mechanisms that modulate these interactions are needed, for example, for the replacement of individual proteins during the normal turnover of the membrane. We therefore would like to speculate that one physiological role of dystrophin phosphorylation in adult skeletal muscle is the modulation the cytoskeletal-membrane interactions during the normal turnover of the sarcolemma.

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