Dystrophin is phosphorylated by endogenous protein kinases

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Dystrophin, the protein coded by the gene missing in Duchenne muscular dystrophy, is assumed to be a component of the membrane cytoskeleton of skeletal muscle. Like other cytoskeletal proteins in different cell types, dystrophin bound to sarcolemma membranes was found to be phosphorylated by endogenous protein kinases. The phosphorylation of dystrophin was activated by cyclic AMP, cyclic GMP, calcium and calmodulin, and was inhibited by cyclic AMP-dependent protein kinase peptide inhibitor, mastoparan and heparin. These results suggest that membrane-bound dystrophin is a substrate of endogenous cyclic AMP- and cyclic GMP-dependent protein kinases, calcium/calmodulin-dependent kinase and casein kinase II. The possibility that dystrophin could be phosphorylated by protein kinase C is suggested by the inhibition of phosphorylation by staurosporin. On the other hand dystrophin seems not to be

a substrate for protein tyrosine kinases, as shown by the lack of reaction of phosphorylated dystrophin with a monoclonal anti-phosphotyrosine antibody. Sequence analysis indicates that dystrophin contains seven potential phosphorylation sites for cyclic AMP- and cyclic GMP-dependent protein kinases (all localized in the central rod domain of the molecule) as well as several sites for protein kinase C and casein kinase II. Interestingly, potential sites of phosphorylation by protein kinase C and casein kinase II are located in the proximity of the actin-binding site. These results suggest, by analogy with what has been demonstrated in the case of other cytoskeletal proteins, that the phosphorylation of dystrophin by endogenous protein kinases may modulate both self assembly and interaction of dystrophin with other cytoskeletal proteins *in vivo*.

INTRODUCTION

Dystrophin, the protein coded by the Duchenne muscular dystrophy gene, is a 125 nm-long asymmetrical protein (Pons et al., 1990) of molecular mass 427 kDa. It is expressed mainly in striated muscles, skeletal and cardiac, where it is localized to the cytoplasmic face of the sarcolemma (Arahata et al., 1988; Bonilla et al., 1988; Watkins et al., 1988; Zubrzycka-Gaarn et al., 1988; Cullen et al., 1991; Wakayama and Shibuya, 1991), and to the junctional transverse tubule membrane (Knudson et al., 1988; Salviati et al., 1989; Bornemann and Schmallbruch, 1991; Yarom et al., 1992). In the sarcolemma, dystrophin has been shown to be associated with a glycoprotein complex (Ervasti and Campbell, 1991). Different isoforms of dystrophin are generated in smooth muscle and neurons by differential splicing at the N- or the C-terminal respectively (Feener et al., 1989; Barnea et al., 1990). A 71 kDa isoform of dystrophin is expressed in liver and other non-muscle tissues (Lederfein et al., 1992; Rapaport et al., 1992). Four domains have been identified based on the amino-acid sequence deduced from the cDNA sequence (Koenig et al., 1988). Because three domains have high similarity, with corresponding domains of α -actinin and spectrin, it is currently believed that dystrophin is a component of the cytoskeleton underlying muscle plasma membrane, i.e. the membranoskeleton.

Many cytoskeletal proteins, such as spectrin, ankyrin, adducin, band 4.1 and band 4.9, are phosphorylated by several protein kinases (see Bennet, 1990, for review). Thus, it was of interest to examine whether dystrophin also is a substrate of protein kinases. Our results show that dystrophin in isolated sarcolemma and triads is phosphorylated by endogenous cyclic AMP-dependent, cyclic GMP-dependent protein kinase, Ca^{2+} /calmodulin-depen-

dent kinase (CaM kinase), casein kinase II, and PKC activities. On the other hand, dystrophin is not phosphorylated by endogenous protein tyrosine kinases. (Part of this work has been presented at the symposium 'Current Status of Research on the Xp21 Myopathies', Rome, 1992.)

MATERIALS AND METHODS

Anti-phosphotyrosine monoclonal antibody (6G9) was purchased from Gibco Brl, Gaithersburg, MD, U.S.A.). Cyclic AMP-dependent protein kinase peptide inhibitor, mastoparan, staurosporin, heparin, and wheat-germ-agglutinin-Sepharose were obtained from Sigma. [γ - ^{32}P]ATP was obtained from Amersham. All other chemicals used were reagent grade. Anti-[dystrophin (60 kDa peptide)] polyclonal antibody was a gift from Dr. E. P. Hoffman, Pittsburgh, PA, U.S.A.

Isolation of sarcolemma and triads

Membrane preparations were isolated from fast-twitch muscles of the rabbit. Highly purified membranes from plasmalemma were obtained with a method that has been modified from those of Jones (1988) and Ohlendieck et al. (1991). About 220–240 g of rabbit hind-leg and back muscles were homogenized with 750 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. The muscle was homogenized with Polytron PT-20 (one cycle of 5 s duration at setting 5). The homogenate was centrifuged at 14000 g_{max} for 20 min (Sorvall GSA rotor, 10000 rev./min). The pellet was extracted again with the buffer described above and then washed twice with 5 mM Tris/HCl, pH 7.0 (125 ml/tube). The final pellet was resuspended in 5 mM Tris/HCl, pH 7.0, and homogenized with Polytron PT-20 (three

Abbreviations used: CaM kinase, Ca^{2+} /calmodulin-dependent kinase; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; PMA, phorbol 12-myristate 13-acetate; NP40, Nonidet P-40; anti-D60, anti-dystrophin antibody.

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cycles of 30 s duration at setting 5). The homogenate was centrifuged at 14000 g_{max} for 20 min. The supernatant was filtered through three layers of gauze and centrifuged at 43 500 g_{max} for 30 min. The pellet was hand-homogenized using a glass homogenizer in 30 ml of distilled water and mixed with 30 ml of 2 M sucrose/0.6 M KCl/0.1 M sodium pyrophosphate/0.2 M Tris/HCl, pH 7.0. Samples (10 ml) of the membrane fraction were then loaded into a centrifuge tube (Beckman rotor 60Ti), after which 7 ml of 0.6 M sucrose and 7 ml of 0.25 M sucrose were layered on top. After centrifugation at 50000 rev./min for 65 min the membrane fraction banding at the 0.25–0.6 M sucrose interface was collected. After diluting with 3–4 vol. of distilled water the sarcolemmal membranes were pelleted by centrifugation at 40000 rev./min for 30 min (Beckman 60Ti rotor) and resuspended in 0.25 M sucrose/10 mM histidine, at pH 7.0. The first homogenization buffer contained the following protease inhibitors: 0.23 mM phenylmethanesulphonyl fluoride (PMSF), 0.83 mM benzamidine, 1 mM iodoacetamide, 1.1 mM leupeptin, 0.7 mM pepstatin, and 76.8 nM aprotinin. All other buffers contained PMSF, iodoacetamide, and benzamidine.

Triads were purified using the method of Mitchell et al. (1983). Briefly, 180 g of rabbit back and hind-leg muscles were homogenized in 900 ml of 10 % (w/v) sucrose/0.5 mM EDTA/20 mM sodium pyrophosphate/20 mM sodium phosphate/1 mM $MgCl_2$, pH 7.1, in a Waring blender. The homogenate was centrifuged at 9000 rev./min (Sorvall, GSA rotor) for 15 min and the resulting supernatant was filtered through three layers of cheesecloth and centrifuged at 11000 rev./min (Sorvall, GSA rotor). The pellet was resuspended in about 90 ml of 10 % (w/v) sucrose/20 mM sodium pyrophosphate/20 mM sodium phosphate/5 mM Hepes, pH 7.1, and was layered on top of a sucrose gradient of the following composition: 18 ml of 28–50 % sucrose continuous gradient, 12 ml of 25 % (w/v) and 4 ml of 14 % (w/v) sucrose. All sucrose solutions contained 20 mM sodium pyrophosphate/20 mM sodium phosphate/5 mM Hepes, pH 7.1. After centrifugation at 27000 rev./min (Beckman, SW28 rotor) for 90 min a dense membrane band at about 30 % (w/v) sucrose was collected, diluted 1:3 with 20 mM sodium pyrophosphate/20 mM sodium phosphate/5 mM Hepes, pH 7.1, and centrifuged at 31000 rev./min (Beckman, rotor 60Ti) for 60 min. The pellet was resuspended in 18 ml of 10 % (w/v) sucrose/20 mM sodium pyrophosphate/20 mM sodium phosphate/5 mM Hepes, pH 7.1, and was layered on top of a discontinuous sucrose gradient consisting of the following sucrose layers: 0.5 ml of 45 %, 4.5 ml of 36 %, 6 ml of 34 %, 6 ml of 32 %, 6 ml of 28 %, 6 ml of 25 %, and 4 ml of 15 % sucrose. After centrifugation at 27000 rev./min for 90 min (Beckman, SW28 rotor) membranes banding at the 32–34 % (w/v) sucrose interface were collected, diluted with 5 mM Hepes, pH 7.1, and centrifuged at 150000 g_{max} for 45 min. Purified triads were resuspended in 10 % (w/v) sucrose/5 mM Hepes, pH 7.1, and frozen in liquid N_2 .

Phosphorylation of membrane proteins

Basal conditions for membrane protein phosphorylation

Sarcolemmal membrane proteins were phosphorylated by incubating at 30 °C for 5 min in 0.1 ml of the following medium: 2.5 mM Mg_2SO_4 /2.5 mM EGTA/1 mM dithiothreitol (DTT)/20 mM NaF/50 μM [γ - ^{32}P]ATP/20 mM histidine, pH 7.0.

Cyclic AMP-dependent protein kinase phosphorylation

This was carried out by incubating at 30 °C for 5 min in 0.1 ml of the following medium: 2.5 mM Mg_2SO_4 /2.5 mM

EGTA/1 mM DTT/20 mM NaF/50 μM [γ - ^{32}P]ATP/2.5 μM cyclic AMP/20 mM histidine, pH 7.0.

Cyclic GMP-dependent protein kinase phosphorylation

The activity was measured by incubating at room temperature for 15 min in 0.1 ml of the following medium: 8 mM $MgCl_2$ /10 mM EGTA/20 mM NaF/50 μM [γ - ^{32}P]ATP/100 μM cyclic GMP/50 mM Pipes/Tris, pH 6.8.

CaM protein kinase phosphorylation

This was performed by incubating at 30 °C for 5 min in 0.1 ml of the following medium: 2.5 mM Mg_2SO_4 /1 mM DTT/20 mM NaF/50 μM [γ - ^{32}P]ATP/10 μM $CaCl_2$ /2 μM calmodulin/20 mM histidine, pH 7.0.

Protein kinase C phosphorylation

This was carried out as described by Yuan and Sen (1986). After pre-incubation at 4 °C for 1 h in 50 ml of 0.625 mM EGTA/0.075 % Triton X-100/20 mM Tris/HCl, pH 7.5, membranes were incubated for 3 min at 30 °C in 1.7 mM $MgCl_2$ /0.17 mM EGTA/1.7 mM DTT/3.3 μM sodium orthovanadate/1.5 μM [γ - ^{32}P]ATP/0.3 mg/ml phosphatidylserine/0.03 mg/ml diolein/2 μM phorbol 12-myristate 13-acetate (PMA)/2 mM $CaCl_2$ /20 mM Tris/HCl, pH 7.5.

Protein tyrosine kinase phosphorylation

The activity was assayed by incubating at 30 °C for 5 min in 0.1 ml of the following medium: 10 mM $MgCl_2$ /25 mg of BSA/0.15 % Nonidet P-40 (NP40)/70 nM sodium orthovanadate/60 μM [γ - ^{32}P]ATP/30 mM Hepes, pH 7.4. The reaction was stopped by the addition of 30 ml of SDS solution [2.3 % (w/v) SDS/10 % (w/v) glycerol/5 % (w/v) 2-mercaptoethanol/62.5 mM Tris/HCl, pH 6.8].

SDS/PAGE and Western blotting were performed as previously described (Salviati et al., 1989). Autoradiography of blotted paper was done with Kodak XO Mat at –80 °C for 24 to 72 h. After autoradiography the blot was stained with anti-dystrophin antibody (anti-D60; Hoffman et al., 1987). Densitometry of the autoradiographic film was performed using a GS-300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.).

Immunoprecipitation of phosphorylated dystrophin

Sarcolemmal membrane protein, after phosphorylation with [γ - ^{32}P]ATP, was solubilized with 1 % (w/v) Triton X-100. After centrifugation to remove insoluble material, the supernatant was incubated overnight at 4 °C with anti-dystrophin antibody [1:100 with 0.15 M NaCl/0.05 % NaN_3 /0.1 M sodium phosphate buffer, pH 7.2 (PBS)]. The immunocomplex was then sedimented by incubation with 10 mg of Protein A for 30 min and centrifugation at 15000 g for 5 min. The pellet was washed once with PBS and solubilized with SDS solution. After SDS/PAGE the gel was dried and exposed to X-ray film at –80 °C for 24–72 h.

Sequence analysis

Sequence analysis was performed on dystrophin as reported by Knudson et al. (1988) (Swiss Protein Sequence Database no. M18026).

RESULTS

Standard procedures for the purification of membrane fractions for skeletal muscle sarcolemma include several extraction steps of the myofibrillar pellet to remove sarcoplasmic reticulum membranes before plasma membranes are liberated by extensive homogenization from the myofibrils. During these steps, dystrophin is usually degraded by endogenous proteases, even in the presence of several protease inhibitors. Thus, in the sarcolemmal membrane preparations purified in this way dystrophin accounts for less than 0.05% of total membrane protein (Salviati et al., 1989), i.e. two orders of magnitude lower than that reported recently by Ohlendieck and Campbell (1991). Preliminary experiments have shown that the addition of a calcium-chelating agent (EGTA) to the homogenizing medium is very effective in preventing degradation of dystrophin during the extraction steps. Thus, membranes purified from rabbit sarcolemma in the presence of EGTA (see the Materials and methods section) are highly enriched in a high-molecular-mass protein of about 420 kDa (Figure 1, lane c). In immunoblot, this protein was shown to be stained by an anti-dystrophin antibody (Figure 1, lane e). Densitometry of the electrophoretic gels stained with Coomassie Blue showed that dystrophin accounted for at least 1–2% of total protein in the membrane fraction.

Phosphorylation of sarcolemma-associated dystrophin

Sarcolemma membranes were incubated with [γ - 32 P]ATP for 5 min under basal conditions and in the presence of EGTA.

Figure 1 Protein and phosphorylation patterns produced by endogenous protein kinases of purified sarcolemma membrane preparations from rabbit skeletal muscle

Sarcolemma membrane preparations were purified from rabbit back and leg muscles in the absence (lanes b and d) or in the presence (lanes c, e, f, g and h) of 5 mM EGTA in all buffers. Sarcolemma membrane preparations were incubated with 50 mM [γ - 32 P]ATP at 30 °C for 5 min under basal conditions (see the Materials and methods section). After stopping the reaction with the addition of 30 ml of SDS solution (see the Materials and methods section), membrane proteins were separated by SDS/PAGE on 5–10% (w/v) polyacrylamide linear gradient gels and transferred to nitrocellulose. The filter was stained with Ponceau Red, destained and exposed to an X-ray film at –80 °C for 24–48 h. The nitrocellulose filter was subsequently stained with anti-dystrophin antibody. Lanes a–c: SDS/polyacrylamide gels stained with Coomassie Blue. Lanes d, e and h: Western blots stained with anti-dystrophin antibody. Lane f: Western blot stained with Ponceau Red. Lanes g and i: autoradiography. Lane contents: a, molecular-mass markers; b and d, sarcolemma membranes prepared in the absence of EGTA; lanes c, e, f, g and h, sarcolemma membranes prepared in the presence of 1 mM EGTA; i, sarcolemma membrane proteins immunoprecipitated by anti-dystrophin antibody after incubation with [γ - 32 P]ATP and solubilization with Triton X-100.

After incubation, membrane proteins were separated by SDS/PAGE and transferred to a nitrocellulose filter. The phosphorylated proteins were identified by autoradiography. As shown in Figure 1 (lane g), several proteins were found to be phosphorylated under these conditions by endogenous protein kinase(s). Among these were proteins in the high-molecular-mass range. To ascertain whether dystrophin was phosphorylated, the same piece of nitrocellulose used for autoradiography was subsequently stained with the anti-dystrophin antibody. As shown in Figure 1 (lane h), the high-molecular-mass protein that was phosphorylated was also stained by the anti-dystrophin antibody. Since electrophoretic co-migration does not allow a conclusive identification of the protein, we investigated whether we could immunoprecipitate the phosphorylated dystrophin extracted from sarcolemma membrane by detergent solubilization. Figure 1 (lane i) shows that our specific anti-dystrophin antibody was capable of purifying 32 P-labelled dystrophin. These results indicate that the high-molecular-mass 32 P-labelled band in Figure 1 (lane g) is, in fact, dystrophin and not another high-molecular-mass protein such as the ryanodine receptor, and that dystrophin bound to the sarcolemma is phosphorylated by endogenous protein kinases.

Two other phosphorylated proteins were immunoprecipitated by the antibody. These proteins, in the range of 40–60 kDa, could be either proteolytic products of dystrophin or protein components of the dystrophin complex that were solubilized by the detergent (Campbell and Kahl, 1989).

In order to identify the specific protein kinases that phosphorylate dystrophin, we first incubated sarcolemma membranes under conditions that specifically activated or inhibited endogenous cyclic AMP- and cyclic GMP-dependent protein kinases. As shown in Figure 2, the addition of either cyclic AMP (lane b) or cyclic GMP (lane d) stimulated the incorporation of 32 P into dystrophin (6- and 8-fold respectively). Cyclic AMP-stimulated phosphorylation of dystrophin was almost completely inhibited by an oligopeptide which is a specific inhibitor of cyclic AMP-dependent protein kinase (Cheng et al., 1986; results not shown).

In similar experiments the phosphorylation of dystrophin was almost doubled by the addition of 2 μ M calmodulin and 10 μ M calcium (Figure 2, lane f). The addition of 10 μ M mastoparan, a specific inhibitor of CaM kinase (Malencik and Anderson, 1983), completely inhibited dystrophin phosphorylation under these conditions (Figure 2, lane g). These results indicate that dystrophin associated with sarcolemma membranes is a substrate for endogenous cyclic AMP-dependent, cyclic GMP-dependent, and CaM kinases.

In another set of experiments we tested the effects of other protein kinase inhibitors on their abilities to inhibit the phosphorylation of dystrophin. As shown in Figure 2, heparin, an inhibitor of casein kinase II (Tuazon and Traugh, 1991; Figure 2, lane i), and staurosporin, an inhibitor of protein kinase C (Tamaoki et al., 1986; Figure 2, lane j), both inhibited dystrophin phosphorylation by about 80%. These results suggest that dystrophin is phosphorylated also by endogenous casein kinase II and protein kinase C. This conclusion is supported by the findings that dystrophin solubilized from sarcolemma membranes by alkaline extraction (Chang et al., 1989) was phosphorylated by exogenous protein kinase C purified from bovine brain (A. Donella-Deana and G. Salviati, unpublished work).

Dystrophin is not a substrate for endogenous protein tyrosine kinases, since a monoclonal anti-phosphotyrosine antibody did not react with dystrophin after phosphorylation (results not shown).

Figure 2 Phosphorylation of dystrophin by several endogenous protein kinase activities

Sarcolemma membrane protein (100 µg) was phosphorylated under assay conditions as described in the Materials and methods section. SDS/PAGE, Western blotting and autoradiography were carried out as described in the legend to Figure 1. Only the region containing high-molecular-mass proteins is shown. Upper lane: autoradiography; lower lane: immunostaining with anti-dystrophin antibody. Lanes a and b: cyclic AMP-dependent protein kinase activity (lane a, no cyclic AMP; b, +2.5 µM cyclic AMP); lanes c and d: cyclic GMP-dependent protein kinase activity (lane c, no cyclic GMP; lane d, +100 µM cyclic GMP); lanes e-g: CaM kinase activity (lane e, no additions, lane f, +10 µM Ca²⁺ and 2 µM calmodulin; lane g, +10 µM Ca²⁺, 2 µM calmodulin and 10 µM mastoparan). Lanes h-j, casein kinase and protein kinase C activities (lane h, basal conditions; lane i, +1 mg/ml heparin; lane j, +100 nM staurosporin).

Figure 3 Dystrophin bound to triads is phosphorylated by endogenous protein kinases

Triads were prepared according to Mitchell et al. (1983). Samples containing 100 µg of triad protein were used. All experimental conditions were as described in the legend to Figure 2. Lane a, molecular-mass markers stained with Ponceau Red; lane b, protein pattern after staining with Ponceau Red; lane c, autoradiography; lane d, anti-dystrophin staining (◀, indicates dystrophin band); lanes e-g, cyclic AMP-dependent protein kinase activity (lane e, no additions; lane f, +2.5 µM cyclic AMP; lane g, +2.5 µM cyclic AMP and 10 µM cyclic AMP-dependent protein kinase peptide inhibitor). Lanes e'-g', CaM kinase activity (lane e', no additions; lane f', +10 µM Ca²⁺ and 2 µM calmodulin; lane g', +Ca²⁺-calmodulin and 10 µM mastoparan). Arrows indicate the position of dystrophin.

Phosphorylation of triad-associated dystrophin

We have previously shown that dystrophin has dual localization in skeletal muscle (Salviati et al., 1989), the sarcolemma and the junctional T-tubules. It has also been demonstrated that junctional T-tubules contain cyclic AMP-dependent protein kinase (Salvatori et al., 1990). We therefore carried out a series of experiments to study whether dystrophin associated with triads was also phosphorylated *in vitro* by endogenous protein kinases.

As shown in Figure 3, lane c, dystrophin bound to triads was phosphorylated under basal conditions. The phosphorylation was activated by cyclic AMP (+140%; Figure 3, lane f), Ca²⁺ and calmodulin (+400%; Figure 3, lane f') and was inhibited by the cyclic AMP-dependent protein kinase peptide inhibitor (Figure 3, lane g) and mastoparan (Figure 3, lane g').

DISCUSSION

In this paper we present evidence that dystrophin, the product of the gene which is missing in Duchenne muscular dystrophy, is phosphorylated *in vitro* by endogenous cyclic AMP- and cyclic GMP-dependent protein kinases, CaM kinase, protein kinase C, and casein kinase II. Sequence analysis of dystrophin (Koenig et al., 1988) has identified seven consensus sequences specific for cyclic AMP- and cyclic GMP-dependent protein kinases. The potential phosphorylatable serines were at positions 616, 1033, 2678 and 2793, whereas threonines were at positions 1535, 1647 and 2621. All these potential sites are in the central, rod portion of the molecule. At the present time, we do not have experimental evidence as to whether dystrophin is phosphorylated at one site only or at multiple sites, as has been shown for β-spectrin (Harris and Lux, 1980). Our results show that dystrophin can be phosphorylated also by CaM kinase, casein kinase II, and protein kinase C, since the incorporation of ³²P into the protein is increased by activators of these kinases and decreased or abolished by mastoparan, staurosporin, and heparin (i.e. inhibitors of CaM kinase, protein kinase C, and casein kinase II respectively). By searching consensus sequences of serine and threonine for protein kinase C and casein kinase II phosphorylation, we were unable to identify such sites since too many (over 60) potential sites are present in the molecule. It is, however, of interest that potential sites of phosphorylation by protein kinase C (serines 136 and 147) and casein kinase II (threonine 134) are located in the actin-binding site 2 (Levine et al., 1990; Way et al., 1992). On the other hand, many potential sites for casein kinase II phosphorylation are in the C-terminal domain.

Our results show that dystrophin is not phosphorylated by endogenous tyrosine kinases, since an anti-phosphotyrosine antibody did not react with dystrophin after phosphorylation, while other proteins of the sarcolemma membrane preparation were indeed phosphorylated under the same conditions. This result is in contrast with sequence analysis, which shows a potential site for tyrosine kinase at tyrosine 3215 in the cysteine-rich domain.

Our results indicate that dystrophin is phosphorylated *in vitro*, thus raising the question of the biological significance of this post-translational event. It is important to stress that in our experiments dystrophin is phosphorylated by endogenous protein kinases, suggesting that such an event may also occur *in vivo*. The question is then whether the phosphorylation of dystrophin affects the biochemical and physiological properties of the protein. There are several possible ways by which phosphorylation affects the biochemical properties of dystrophin. First, modulation of self assembly: it has been proposed that dystrophin forms a dimer with antiparallel orientation *in vivo* (Hoffman and Kunkel, 1989). Phosphorylation of serine-threonine residues in the rod portion could modulate the protein-protein interaction. Secondly, modulation of actin interaction: there are potential phosphorylation sites in the second actin-binding site. The addition of a phosphate to this site may modify the affinity of the site for actin. Thirdly, modulation of the interaction with other cytoskeletal and membrane proteins: the phosphorylation of residues in the C-terminus may affect the interaction with the

glycoprotein complex. Phosphorylation of other membrane skeleton proteins has two main effects. In the case of proteins such as erythrocyte proteins 4.1 and 4.9 and ankyrin, phosphorylation decreases the affinity for the interaction with target proteins [see Bennet (1990) and references therein]. On the other hand, phosphorylation of spectrin does not seem to affect self-association (Ungewickell and Gratzer, 1978) or the association with ankyrin (Lu and Tao, 1986) or with actin (Brenner and Korn, 1979). At the present time, it is impossible to speculate as to the full consequences of dystrophin phosphorylation, since a physiological function of dystrophin has not been elucidated. However, our finding that this important protein is phosphorylated by endogenous protein kinases suggests that dystrophin's function is subjected to potentially significant regulatory influences.

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REFERENCES

- Arahata, K., Ishiura, S., Ishiguro, T., Tsukahara, T., Suhara, Y., Eguchi, C., Ishihara, T., Nonaka, I., Ozawa, E. and Sugita, H. (1988) *Nature (London)* **333**, 861–863
- Barnea, E., Zuk, D., Simantov, R., Nudel, U. and Yaffe, D. (1990) *Neuron* **5**, 881–888
- Bennet, V. (1990) *Physiol. Rev.* **70**, 1029–1065
- Bonilla, E., Samitt, C. E., Miranda, A. F., Hays, A. P., Salviati, G., DiMauro, S., Kunkel, L. M., Hoffman, E. P. and Rowland, L. P. (1988) *Cell* **54**, 447–452
- Bornemann, A. and Schmalbruch, H. (1991) *Muscle Nerve* **14**, 1177–1181
- Brenner, S. L. and Korn, E. D. (1979) *J. Biol. Chem.* **254**, 8620–8627
- Campbell, K. P. and Kahl, S. D. (1989) *Nature (London)* **338**, 259–262
- Chang, H. W., Bock, E. and Bonilla, E. (1989) *J. Biol. Chem.* **264**, 20831–20834
- Cheng, H. C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten, S. M. and Walsh, D. A. (1986) *J. Biol. Chem.* **261**, 989–992
- Cullen, M. J., Walsh, J., Nicholson, L. V. B., Harris, J. B., Zubrzycka-Gaarn, E. E., Ray, P. N. and Worton, R. G. (1991) *Neuromuscular Disorders* **1**, 113–119
- Ervasti, J. M. and Campbell, K. P. (1991) *Cell* **66**, 1121–1131
- Feener, C. A., Koenig, M. and Kunkel, L. M. (1989) *Nature (London)* **338**, 509–511
- Harris, H. W. and Lux, S. E. (1980) *J. Biol. Chem.* **255**, 11512–11520
- Hoffman, E. P. and Kunkel, L. M. (1989) *Neuron* **2**, 1019–1029
- Hoffman, E. P., Brown, R. H., Jr. and Kunkel, L. M. (1987) *Cell* **51**, 919–928
- Jones, R. L. (1988) *Methods Enzymol.* **157**, 85–91
- Knudson, C. M., Hoffman, E. P., Kahl, S. D., Kunkel, L. M. and Campbell, K. P. (1988) *J. Biol. Chem.* **263**, 8480–8484
- Koenig, M., Monaco, A. P. and Kunkel, L. M. (1988) *Cell* **53**, 219–228
- Lederfein, D., Levy, Z., Augier, N., Mornet, D., Morris, G., Fuchs, O., Yaffe, D. and Nudel, U. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5346–5350
- Levine, B. A., Moir, A. J. G., Patchell, V. B. and Perry, S. V. (1990) *FEBS Lett.* **263**, 159–162
- Lu, P. W. and Tao, M. (1986) *Biochem. Biophys. Res. Commun.* **139**, 855–860
- Malencik, D. A. and Anderson, S. R. (1983) *Biochem. Biophys. Res. Commun.* **114**, 50–56
- Mitchell, R. D., Palade, P. and Fleischer, S. (1983) *J. Cell Biol.* **96**, 1008–1016
- Ohlendieck, K. and Campbell, K. P. (1991) *FEBS Lett.* **283**, 230–234
- Ohlendieck, K., Ervasti, J. M., Snook, J. B. and Campbell, K. P. (1991) *J. Cell Biol.* **112**, 135–148
- Pons, F., Augier, N., Heilig, R., Leger, J., Mornet, D. and Leger, J. J. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7851–7855
- Rapaport, D., Lederfein, D., Dendunnen, J. T., Grootsholten, P. M., Vanommen, G. J. B., Fuchs, O., Nudel, U. and Yaffe, D. (1992) *Differentiation* **49**, 187–193
- Salvatori, S., Damiani, E., Barhanin, J., Furlan, S., Salviati, G. and Margreth, A. (1990) *Biochem. J.* **267**, 679–687
- Salviati, G., Betto, R., Ceoldo, S., Biasia, E., Bonilla, E., Miranda, A. F. and DiMauro, S. (1989) *Biochem. J.* **258**, 837–841
- Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* **135**, 397–402
- Tuazon, P. T. and Traugh, J. A. (1991) in *Advances in Second Messenger and Phosphoprotein Research* (Greengard, P. and Robinson, G. A., eds.), vol. 23, pp. 123–164, Raven Press, New York
- Ungewickell, E. and Gratzer, W. (1978) *Eur. J. Biochem.* **88**, 379–385
- Wakayama, Y. and Shibuya, S. (1991) *Acta Neuropathol.* **82**, 178–184
- Watkins, S. C., Hoffman, E. P., Slayter, H. S. and Kunkel, L. M. (1988) *Nature (London)* **333**, 863–866
- Way, M., Pope, B., Cross, R. A., Kendrick-Jones, J. and Weeds, A. G. (1992) *FEBS Lett.* **301**, 243–245
- Yarom, R., Morris, G. E., Froede, R. and Schaper, J. (1992) *Experientia* **48**, 614–616
- Yuan, S. and Sen, A. K. (1986) *Biochim. Biophys. Acta* **886**, 152–161
- Zubrzycka-Gaarn, E. E., Bullman, D. E., Karpati, G., Burghes, A. H., Belfall, B., Klamut, H. J., Talbot, J., Hodges, R. S., Ray, P. N. and Worton, R. G. (1988) *Nature (London)* **333**, 466–469