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Association of dystrophin and an integral membrane glycoprotein

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DUCHENNE muscular dystrophy (DMD) is caused by a defective gene found on the X-chromosome[1]. Dystrophin is encoded by the DMD gene and represents about 0.002% of total muscle protein[2]. Immunochemical studies have shown that dystrophin is localized to the sarcolemma in normal muscle but is absent in muscle from DMD patients[3-7]. Many features of the predicted primary structure of dystrophin are shared with membrane cytoskeletal proteins[8], but the precise function of dystrophin in muscle is unknown. Here we report the first isolation of dystrophin from digitonin-solubilized skeletal muscle membranes using wheat germ agglutinin (WGA)-Sepharose. We find that dystrophin is not a glycoprotein but binds to WGA-Sepharose because of its tight association with a WGA-binding glycoprotein. The association of dystrophin with this glycoprotein is disrupted by agents that dissociate cytoskeletal proteins from membranes. We conclude that dystrophin is linked to an integral membrane glycoprotein in the sarcolemma. Our results indicate that the function of dystrophin could be to link; this glycoprotein to the underlying cytoskeleton and thus help either to preserve membrane stability or to keep the glycoprotein non-uniformly distributed in the sarcolemma.

The specific binding of dystrophin to wheat germ agglutinin (WGA)-Sepharose is shown in Fig. 1 (*a-d*). Dystrophin was solubilized from rabbit skeletal muscle membranes with 0.5 M NaCl and 1% digitonin (Fig. 1*a* and *b*, lane 1). The solubilized membranes were first passed through an antibody affinity column to remove the ryanodine receptor[9] (relative molecular mass, M_r, 450,000). This step is important because of the similar apparent mobilities on SDS-polyacrylamide gels of dystrophin and a major proteolytic fragment of the ryanodine receptor[9]. The flow-through of the ryanodine-receptor affinity column was then applied to a WGA-Sepharose 4B column. Immunoblot-analysis of solubilized membranes and of solubilized membranes that had been repeatedly passed through a WGA-Sepharose column showed that dystrophin was completely removed from solubilized membranes by WGA-Sepharose (Fig. 1*b*, lane 2). Coomassie-blue staining of both samples run on SDS-polyacrylamide gels (Fig. 1*a*) indicated that there had been no change in the protein components because most of the proteins in the rabbit skeletal muscle membrane preparation are not WGA-binding glycoproteins. The protein adsorbed to WGA-Sepharose was also analysed by SDS-PAGE and immunoblotting (Fig. 1*c* and *d*). In the absence of *N*-acetyl-D-glucosamine (NAG), the α_1 and α_2 subunits of the dihydropyridine (DHP) receptor and a protein of M_r 400K were the main proteins bound to WGA-Sepharose (Fig. 1*c*, lane 1). The high-molecular weight protein

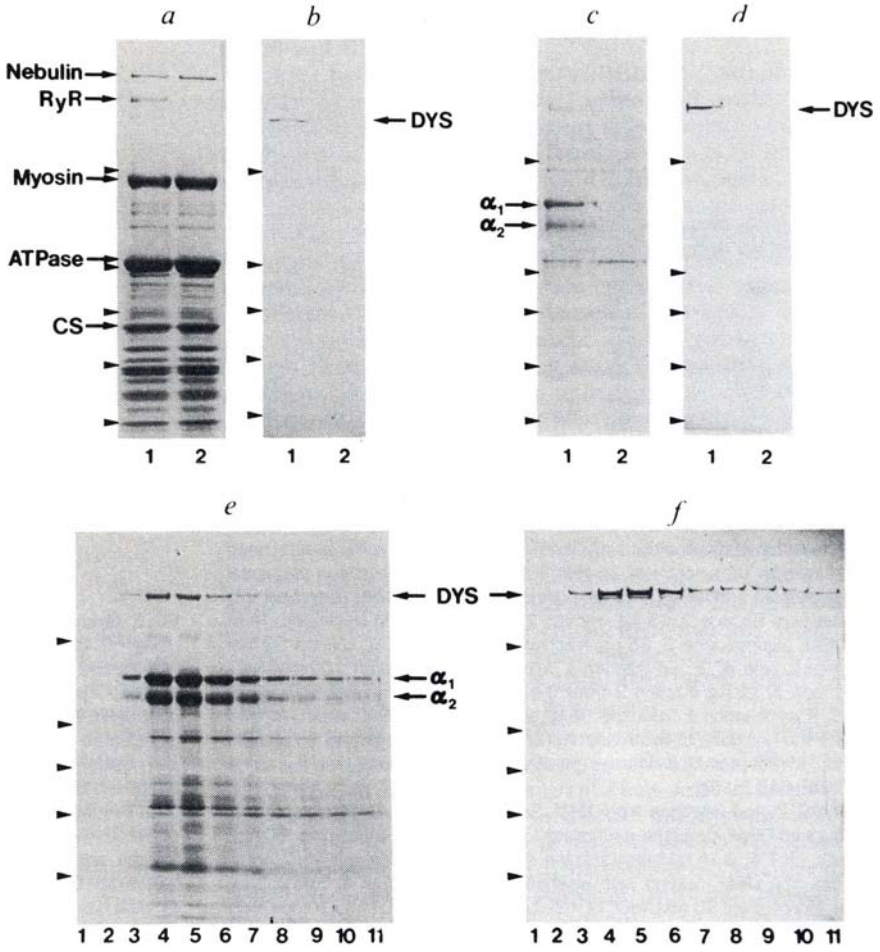

FIG. 1 Specific binding of dystrophin to WGA-Sepharose (*a-d*) and elution with *N*-acetyl-D-glucosamine (*e-f*). Coomassie blue-stained gels (*a, c* and *e*) and immunoblots of identical gels stained with sheep polyclonal anti-dystrophin antibodies (*b, d* and *f*) are shown. *a* and *b*, Lane 1, digitonin-solubilized rabbit skeletal-muscle membranes (75 μg); lane 2, column flow-through from four independent WGA-Sepharose columns (75 μg). Arrows indicate the positions of nebulin, ~700K; ryanodine receptor/Ca^{2+}-release channel, ~450K (RyR); myosin, 205K; (Ca^{2+}+Mg^{2+})ATPase, 105K (ATPase); and calsequestrin, 63K (CS). *c* and *d,* WGA-Sepharose beads (50 μl) incubated with solubilized membranes in the absence (lane 1) or presence (lane 2) of *N*-acetyl-D-glucosamine. *e* and *f,* Lanes 1-11 are 100 μl aliquots of NAG-eluted WGA-Sepharose column fractions. Arrows indicate the positions of dystrophin (DYS) in panels *b, d, e* and *f* and the α_1 and α_2 subunits of the DHP receptor in panels *c* and *e*. For all panels, arrowheads indicate the positions of the molecular weight standards: from top to bottom their M_r values ($\times 10^{-3}$) are 208, 100, 68, 43 and 25.

METHODS. For *a* and *b,* rabbit skeletal muscle membranes[9] (800 mg) were solubilized with a solution of 1% digitonin and 0.5 M NaCl containing protease inhibitors at a protein concentration of 4 mg ml^{-1}, as previously described[9], then passaged through XA7-Sepharose for removal of ryanodine receptor[9] and through four different columns of WGA-Sepharose (15ml each). For *c* and *d,* membranes (2 mg) were solubilized and passed through XA7-Sepharose as already described, then applied to 50 μl WGA-Sepharose in the absence or presence of 0.3 M NAG. After incubation the WGA-Sepharose beads were washed extensively and subjected to SDS-PAGE. For *e* and *f,* digitonin-solubilized membranes (800 mg) were applied to XA7-Sepharose and circulated overnight on 15 ml WGA-Sepharose. After extensive washing, the WGA-Sepharose column was eluted with five column volumes of 0.3 M NAG and 2.5 ml fractions were collected. Yields of 3 to 5 mg from 800 mg solubilized membranes were typical. In all experiments, WGA-Sepharose buffers contained 50 mM Tris-HCl, pH 7.4, 0.75 mM benzamidine and 0.1 mM phenylmethylsulphonyl fluoride. Samples were fractionated on 3-12% gradient SDS-polyacrylamide gels[17] and either stained with Coomassie blue or transferred to nitrocellulose paper[18] and stained with either sheep polyclonal anti-dystrophin antibodies[19] or anti-α_1 and anti-α_2 antibodies[20,21] for identification of the α_1 and α_2 subunits of the DHP receptor (not shown). Gel lanes were scanned using a Hoefer GS 300 scanning densitometer and analysed using GS-360 data analysis software. Protein concentrations were determined as previously described[22].

was identified as dystrophin by immunoblotting (Fig. 1*d*, lane 1). The specificity of the absorption of dystrophin to WGA-Sepharose was tested by repeating the experiment in the presence of NAG. Only myosin ($M_r \approx 205K$) and the ($Ca^{2+}+Mg^{2+}$) ATPase ($M_r \approx 105K$) bound nonspecifically to WGA-Sepharose in the presence of NAG (Fig. 1*c*, lane 2). Our results therefore demonstrate that dystrophin specifically binds to WGA-Sepharose following solubilization in digitonin. The specific elution of dystrophin from WGA-Sepharose by NAG is shown in Fig. 1*e* and *f*; the yield of NAG-eluted material is ~5 mg from 800 mg starting membranes. The peak protein fractions that are eluted with NAG are enriched in dystrophin and the DHP receptor, as shown by Coomassie-blue staining and immunoblotting of SDS-polyacrylamide gels. Dystrophin makes up about 5% of the total NAG-eluted protein as estimated from integration of densitometer scans of the gels.

The fact that dystrophin could be isolated using WGA-Sepharose following detergent solubilization of skeletal muscle membranes indicated to us that dystrophin might be associated with the DHP receptor, or that it could be a glycoprotein. To investigate the possible association of dystrophin with the DHP receptor, we combined and fractionated NAG-eluted fractions from WGA-Sepharose using DEAE-cellulose. Elution of the DEAE-cellulose column with a linear gradient of NaCl indicated that most of the DHP receptor eluted in a broad pattern from 50-110mM NaCl, whereas dystrophin eluted at 110-300 mM NaCl. Consequently we developed a step gradient of NaCl to separate the DHP receptor from dystrophin more effectively (Fig. 2). The first four fractions are enriched in DHP receptor and do not contain dystrophin (as determined by Coomassie blue-stained gels and immunoblots; Fig. 2*a* and *b*, lanes 2-5). The final fraction is enriched in dystrophin (corresponding to 30-40% of the protein by densitometry) and is essentially depleted of DHP receptor (Fig. 2*a* and *b*, lane 6). Proteins co-eluting with dystrophin migrated on Coomassie blue-stained gels at positions corresponding to M_rs of 325K, 250K, 225K, 172K, 162K, 143K, 57K, 47K, 39K and 31K; the mobility of dystrophin indicated that its M_r was ~360K. Immunoblotting after each step in the preparation showed that dystrophin was isolated intact and not degraded. Thus the decrease in apparent M_r of isolated dystrophin when compared with the value of 427K predicted from the complementary DNA sequence[8] may result from proteolytic processing after synthesis or simply from the difficulty in measuring high molecular weight proteins accurately on SDS gels.

To investigate whether dystrophin is a glycoprotein, we stained protein blots of DEAE-isolated dystrophin and purified DHP receptor with ^{125}I-labelled lectins. ^{125}I-labelled WGA specifically stained the α_2 subunit of the DHP receptor (Fig. 3, lane 1) but did not stain dystrophin (Fig. 3, lane 2). Concanavalin A (con A) labelled with ^{125}I was also used to stain blots and together with results of endoglycosidase H digests confirmed that dystrophin is not a glycoprotein (data not shown). So we conclude that dystrophin must be retained by WGA-Sepharose as a result of an association with a glyco-mediator which survives solubilization of the membranes. The isolated dystrophin also binds specifically to con A-Sepharose (not shown). As glycoproteins are the only molecules that will specifically bind both to WGA-Sepharose and con A-Sepharose, the mediator must be a glycoprotein. We also show (Fig. 3) that the dystrophin

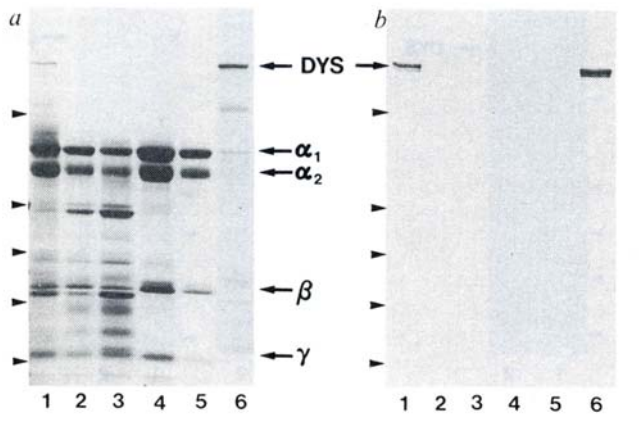

FIG. 2 Isolation of dystrophin using DEAE-cellulose. A Coomassie blue-stained gel *(a)* and an immunoblot of an identical gel stained with sheep polyclonal anti-dystrophin antibodies *(b)* are shown. Lane 1,10 μg NAG-eluted sample (Fig. 1*e*, lane 5); lane 2, 13 μg fraction 1 from the 50 mM NaCl wash from the DEAE column; lane 3, 15 μg fraction 2 from the DEAE-column 50 mM NaCl wash; lane 4, 25 μg fraction 1 from the DEAE-column 100 mM NaCl wash; lane 5,11 μg fraction 2 from the DEAE-column 100 mM NaCl wash; lane 6, 8 μg fraction 1 from the DEAE-column 250 mM NaCl wash. Arrows indicate the positions of dystrophin (DYS) and the α_1, α_2, β and γ subunits of the DHP receptor. Molecular weight standards (arrowheads) are the same as those used for Fig. 1.

METHODS. Eluted fractions from WGA-Sepharose (Fig. 1*e*) were applied to 2 ml packed DEAE-Cellulose equilibrated in buffer A (0.1% digitonin, 50 mM Tris-HCl, pH 7.4, 0.75 mM benzamidine and 0.1 mM phenylmethylsulphonyl fluoride). The DEAE-column was washed with 10ml buffer A, followed by buffer A containing 50 mM NaCl (50 ml), 100 mM NaCl (200 ml), 110 mM NaCl (100ml) and 250 mM NaCl (25ml). Fractions (2.5ml) were collected for all washes. Analysis of the 110 mM NaCl wash indicated a protein pattern similar to that in lanes 4 and 5, but much less intense (not shown). Washing of the DEAE-column with NaCl concentrations between 110 mM NaCl and 250 mM revealed a protein pattern similar to that in lane 6 and did not preferentially remove any protein (not shown). Approximately 300 μg, isolated dystrophin (lane 6) was obtained from 700 mg solubilized membranes. SDS-PAGE, immunoblot analysis and densitometer scanning are described in the legend to Fig. 1.

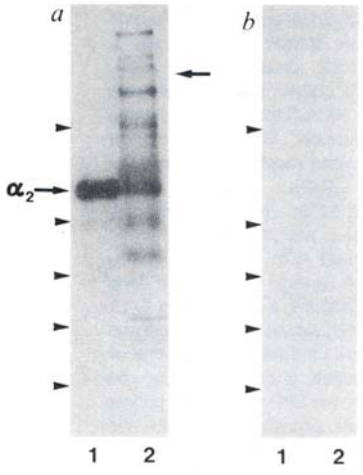

FIG. 3 Glycoprotein analysis of purified DHP receptor and isolated dystrophin using ^{125}I-labelled WGA. Autoradiographs of immunoblots incubated with ^{125}I-labelled WGA in the absence *(a)* or presence *(b)* of 0.3 M NAG are shown. Lane 1, 7 μg purified DHP receptor (Fig. 2, lane 4); lane 2, 7 μg isolated dystrophin (Fig. 2, lane 6). The position of the α_2 subunit of the DHP receptor is indicated by the arrow (α_2). The arrow on the right in panel a indicates the position of dystrophin determined by staining the blot with sheep polyclonal anti-dystrophin antibodies (after staining with ^{125}I-labelled WGA) and then overlaying the autoradiograph on the blot. Note that dystrophin does not stain with ^{125}I-labelled WGA. Molecular weight standards (arrowheads) are the same as those used for Fig. 1.

METHODS. DHP receptor (Fig. 2, lane 4) or isolated dystrophin (Fig. 2, lane 6) were separated on 3-12% SDS-polyacrylamide gels and transferred to nitrocellulose paper. Blots were blocked in PBS-Tween (50 mM NaH_2PO_4, 0.9% NaCl, 0.05% Tween-20) and incubated for 1 h at 25 °C with 250,000 c.p.m. ^{125}I-labelled WGA per ml PBS-Tween either with or without 0.3 M NAG. Transfers were washed extensively with PBS-Tween then with PBS, then dried and exposed to Kodak-XAR film using intensifying screens (Dupont). Exposures were for three days at 25 °C. No additional bands appeared after longer exposure. Identical results were obtained using ^{125}I-labelled con A (not shown). SDS-PAGE and immunoblot analysis are described in the legend to Fig. 1.

preparation contains several glycoproteins ranging from 40K to 500K, but no glycolipids. Therefore the mediator of the interaction between dystrophin and the lectin columns must be a glycoprotein containing both WGA and con A binding sites. Also, as the detergent concentration necessary to solubilize dystrophin is almost identical to that required to solubilize the ryanodine and DHP receptors (both integral membrane proteins), then the mediator must also be an integral membrane glycoprotein.

To investigate this association of dystrophin with the integral membrane glycoprotein, we attempted to dissociate the dystrophin-glycoprotein complex with treatments known to disrupt the interaction between cytoskeletal proteins and membranes[10-12], and then monitored the binding of dystrophin to WGA-Sepharose. We used NAG-eluted dystrophin because we needed large amounts of protein for Coomassie-blue staining and because it contained the DHP receptor, which is a complex

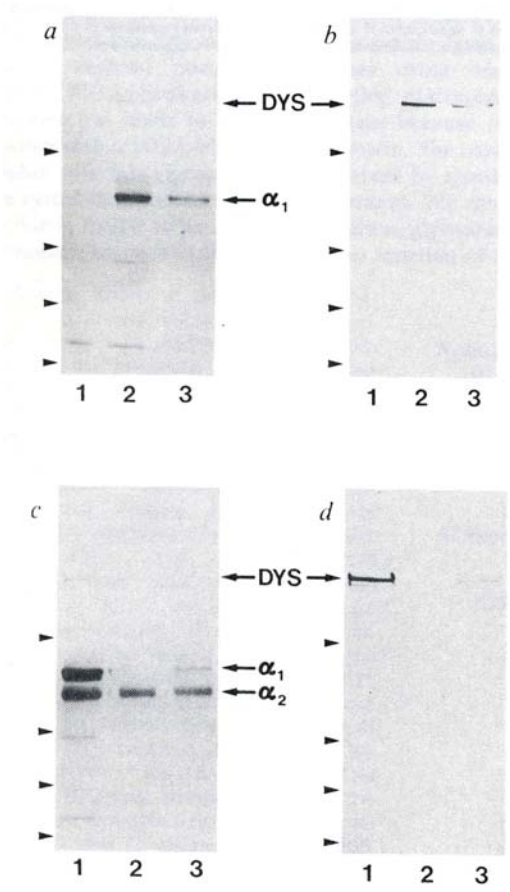

FIG. 4 Dissociation of dystrophin and membrane-associated glycoproteins using SDS and Kl. Shown are Coomassie Blue-stained gels (*a* and *c*) and immunoblots of identical gels stained with sheep polyclonal anti-dystrophin antibodies (*b* and *d*). Lane 1, no treatment; lane 2, 1% SDS treatment; lane 3, 2 M Kl treatment. In a and b, supernatants (100 μl) from the WGA-Sepharose beads are analysed. In *c* and *d*, NAG-eluates (100 μl) from the WGA-Sepharose beads used in *a* and *b* are analysed. The positions of dystrophin (DYS) and the α_1 and α_2 (*c* and *d* only) subunits of the DHP receptor are indicated by arrows. Molecular weight standards (arrowheads) are the same as those used for Fig. 1.

METHODS. 100 μl (20 μg) NAG-eluted sample from WGA-Sepharose (Fig. 1e, lane 5) was treated for 30 min at 25 °C either alone or with 1% SDS or 2 M Kl. Samples were dialysed overnight in 50 mM Tris-HCl, pH 7.4,0.5 M NaCl, 0.75 mM benzamidine and 0.1 mM phenylmethylsulphonyl fluoride-containing buffer only for the no-treatment sample, 1% SDS for the SDS-treated sample, or 0.5 M Kl for the Kl-treated sample, and then applied to 50 μl WGA-Sepharose prewashed either with buffer only, 0.1% SDS or 0.5 M Kl. The samples were incubated for 2 h at 25 °C with agitation, then pelleted. Following extensive washing with the appropriate buffer (buffer only, 0.1% SDS or 0.5 M Kl), the WGA-Sepharose beads were eluted with 0.3 M NAG. SDS-PAGE and immunoblot analysis are described in the legend to Fig. 1.

of several polypeptides with a major glycoprotein[13]. Figure 4 shows that NAG-eluted dystrophin rebinds completely to WGA-Sepharose after removal of NAG (lane 1). Thus, dystrophin retains its association with the WGA-binding glycoprotein after isolation and dialysis to remove NAG. When the NAG-eluted sample is treated with 1% SDS, followed by dilution to 0.1% SDS and removal of NAG by dialysis, dystrophin no longer binds to WGA-Sepharose (Fig. 4*a* and *b*, lane 2), even though the α_2 subunit of the DHP receptor (a WGA-binding glycoprotein) still binds to WGA-Sepharose (Fig. 4*c* and *d*, lane 2). Thus, SDS is able to dissociate the dystrophin-glycoprotein complex and the free dystrophin does not bind to WGA-Sepharose.

Membrane cytoskeletal protein interactions are known to be sensitive to molar concentrations of potassium iodide[10,11]. We tested the effect of Kl on the interaction of dystrophin with the WGA-binding glycoprotein by treating the NAG-eluted sample with 2 M Kl. After dilution of Kl to 0.5 M and removal of NAG by dialysis, the sample was applied to WGA-Sepharose. In Fig. 4, lane 3 it is shown that Kl dissociates the dystrophin-glycoprotein complex and that Kl-treated dystrophin no longer binds to WGA-Sepharose, even though the α_2 subunit of the DHP receptor still binds to WGA-Sepharose. Similar results were obtained with con A-Sepharose (not shown). ^{125}I-labelled WGA staining of the material in the void volume from the WGA-Sepharose beads and of the NAG-eluted material (not shown) indicates that all WGA-binding glycoproteins bound to WGA-Sepharose after treatment with SDS or Kl. So, dystrophin binds to WGA-Sepharose as a result of its tight association with a WGA-binding glycoprotein and this association is sensitive to SDS and Kl.

Here we have reported the first isolation of dystrophin from skeletal muscle membranes: as dystrophin is present in very low concentrations in muscle, a procedure representing a 17,000-fold purification of dystrophin from whole muscle is an important advance. Previously dystrophin could only be studied indirectly, for example by using immunological probes for the protein. We have also demonstrated that dystrophin is tightly associated with an integral membrane glycoprotein and that this association is disrupted by agents that dissociate cytoskeletal proteins from membranes. The similarities between dystrophin and the cytoskeletal proteins α-actinin and spectrin[8] further indicate that the function of dystrophin could be to link an integral membrane glycoprotein to the underlying cytoskeleton. This association of dystrophin with a WGA-binding glycoprotein has been found in membranes from rabbit and rat cardiac muscle and skeletal muscle from guinea pig, dog, mouse and human (results not shown). We conclude that the localization of dystrophin to the cytoplasmic face of the sarcolemma results from a tight association with an integral sarcolemma membrane glycoprotein and we propose that this glycoprotein serves as a 'dystrophin receptor' which links dystrophin to the sarcolemma membrane. Although we have not yet identified this 'receptor', the results shown in Fig. 3 indicate that it could be one of the glycoproteins migrating with M_r 480K, 400K, 362K, 300K, 226K, 205K, 133K, 100K, 75K, 62K, 47K or 37K.

The dystrophin-glycoprotein complex may have an important structural and/or functional role in muscle: it could contribute to stabilization of the membrane or of a non-uniform distribution of a membrane glycoprotein, or it could link dystrophin to the extracellular matrix. We believe that the low concentration of dystrophin itself favours a role in maintaining the heterogeneous membrane arrangement of a scarce glycoprotein, possibly an ion channel or cell-surface receptor. The association of dystrophin with a sarcolemma glycoprotein is comparable to the association of ankyrin with red-cell or brain membranes[10-12] in that molar concentrations of KI can disrupt the complex. Recently ankyrin has been shown to be associated with the (Na^+K^+ATPase in kidney[14] and with the Na^+ channel in the brain[15]; these associations with ankyrin were proposed to be responsible for the non-uniform distribution of these proteins

in epithelial cells or neuronal cells respectively. Dystrophin may have a similar role in muscle and could be responsible for a non-uniform distribution of a membrane glycoprotein in the sarcolemma membrane.

The absence of dystrophin in DMD muscle may result in the displacement or dysfunction of the membrane glycoprotein that is normally associated with dystrophin. The lack of the dystrophin-glycoprotein complex may weaken sarcolemma membrane integrity and this may be critical for fast muscle fibres, which appear to be the first site of degeneration[16]. It remains to identify the 'dystrophin receptor' in the sarcolemma membrane and to determine the role of dystrophin in the function of the membrane glycoprotein. □

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