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Purification of Dystrophin from Skeletal Muscle*

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Dystrophin was purified from rabbit skeletal muscle by alkaline dissociation of dystrophin-glycoprotein complex which was first prepared by derivatized lectin chromatography. Dystrophin-glycoprotein complex was isolated from digitonin-solubilized rabbit skeletal muscle membranes by a novel two-step method involving succinylated wheat germ agglutinin (sWGA) chromatography and DEAE-cellulose ion exchange chromatography. Proteins co-purifying with dystrophin were a protein triplet of M_r 59,000 and four glycoproteins of M_r 156,000, 50,000, 43,000, and 35,000, all previously identified as components of the dystrophin-glycoprotein complex. Alkaline treatment of sWGA/DEAE-purified dystrophin-glycoprotein complex resulted in complete dissociation of the dystrophin-glycoprotein complex. In order to separate dystrophin from its associated proteins, alkaline-dissociated dystrophin-glycoprotein complex was sedimented by sucrose gradient centrifugation. The residual glycoproteins which contaminated peak dystrophin-containing gradient fractions were then removed by WGA-Sepharose adsorption. The resulting protein appeared as a single band with an apparent M_r of 400,000 on overloaded Coomassie Blue-stained gels. The absence of WGA-peroxidase staining on nitrocellulose transfers of the pure protein indicated that the pure protein was devoid of contaminating glycoproteins. Antisera raised against the carboxyl terminus of human skeletal muscle dystrophin (which does not cross-react with the carboxyl terminus of the chromosome 6-encoded dystrophin-related protein) recognized the pure protein as did antisera specific for the amino terminus of human dystrophin. These data indicate that the protein isolated is indeed the intact, predominant skeletal muscle isoform product of the Duchenne muscular dystrophy gene.

Duchenne muscular dystrophy (DMD)[1] is caused by a defective gene found on the X-chromosome. Dystrophin, the large molecular weight protein product of the DMD gene (1), is localized to the sarcolemmal membrane of normal skeletal muscle (2–5) but is absent from the skeletal muscle of DMD humans (1, 2, 6), xmd dogs (7), and mdx mice (1, 5). The amino acid sequence of dystrophin, which has been deduced from the DMD gene cDNA sequence (8), suggests that dystrophin is a membrane-associated cytoskeletal protein involved in the anchoring of sarcolemmal proteins to the underlying cytoskeleton. Biochemical studies have demonstrated that dystrophin could be greatly enriched from detergent-solubilized skeletal muscle membranes using wheat germ agglutinin (WGA)-Sepharose (9–11) because it is tightly associated with several sarcolemmal glycoproteins (12, 13). These results indicate that the localization of dystrophin to the cytoplasmic face of the sarcolemma (2–5) results from a tight association of dystrophin with a multi-subunit complex of integral membrane glycoproteins. However, the component(s) of the dystrophin-glycoprotein complex which bind dystrophin directly have not been identified, thus requiring pure dystrophin for binding studies. In addition, nearly all of the structural information about dystrophin has been deduced from cDNA sequence (8).

Initially, the dystrophin-glycoprotein complex was isolated from digitonin-solubilized rabbit skeletal muscle membranes using WGA-Sepharose and DEAE-cellulose chromatography (9) and further purified by sucrose density gradient centrifugation in the presence of 0.1% digitonin (12). In the present study, we found that substituting succinylated WGA (sWGA)-agarose for WGA-Sepharose resulted in dystrophin-glycoprotein complex preparations of the same purity and yield as previously reported (12) while obviating the sucrose gradient step. Furthermore, we demonstrate that the purified dystrophin-glycoprotein complex is dissociated by alkaline treatment. We have combined the novel two-step purification of the dystrophin-glycoprotein complex using sWGA-agarose with alkaline dissociation of the complex to purify dystrophin to homogeneity. This protocol required only commercially available materials and yielded protein in sufficient quantity to afford the necessary biochemical, functional, and ultrastructural characterization of dystrophin.

MATERIALS AND METHODS

Purification of Dystrophin-Glycoprotein Complex—Heavy microsomes were prepared from rabbit skeletal muscle (14) and washed twice with 0.6 M KCl in 50 mM Tris-HCl, pH 7.4, 0.165 M sucrose, 0.1 mM PMSF, and 0.75 mM benzamidine to remove contractile proteins. 1.2–1.5 g of KCl-washed membranes were solubilized in 1.0% digitonin, 0.5 M NaCl, and protease inhibitors as previously described (9). The digitonin-solubilized membranes were circulated overnight on an 80-ml sWGA-agarose column (Vector Laboratories, Inc., Burlingame, CA), washed extensively with buffer A (0.1% digitonin, 50 mM Tris-HCl, pH 7.4, 0.75 mM benzamidine, 0.1 mM PMSF) containing 0.5 M NaCl (300 ml) followed by buffer A (300 ml), and then eluted with 300 ml of 0.3 M *N*-acetylglucosamine in buffer A. Eluted fractions (4 ml each) containing dystrophin were applied to a 3-ml DEAE-cellulose column and sequentially eluted with the following NaCl concentrations in buffer A: 0 mM (40 ml), 25 mM (40 ml), 35 mM (40 ml), 50 mM (150 ml), 75 mM (150 ml), 100 mM (200 ml),

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[1] The abbreviations used are: DMD, Duchenne muscular dystrophy; WGA, wheat germ agglutinin; sWGA, succinylated wheat germ agglutinin; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

110 mM (100 ml), 175 mM (40 ml), and 375 mM (40 ml). 4-ml fractions were collected from the 175 mM elution.

Purification of Dystrophin—Dystrophin-glycoprotein complex which eluted in the 175 mM NaCl wash (40 ml total volume) was concentrated to 0.6 ml in an Amicon stirred ultrafiltration cell (YM100 membrane, 25 p.s.i.), titrated to pH 11 with 1 M NaOH, and incubated for 1 h at 22 °C with mixing. 12.5-ml linear 5–20% (w/v) sucrose gradients containing 0.5 M NaCl in buffer A or buffer B (0.1% digitonin, 50 mM Tris, pH 11, 0.75 mM benzamidine, and 0.1 mM PMSF) were prepared using a BioComp Gradient Master density gradient former. The alkaline-treated dystrophin-glycoprotein complex was loaded onto a sucrose gradient containing 0.5 M NaCl in buffer B and overlaid with 0.1 ml of buffer B containing 0.5 M NaCl. Gradients were centrifuged at 4 °C in a Beckman VTi 65.1 vertical rotor for the indicated time at 200,000 × *g*. Twenty 0.6-ml fractions were collected from the top of the gradients using an Isco model 640 density gradient fractionator. After centrifugation and fractionation, gradient fractions were titrated back to pH 7.4 with 1 M HCl. Sucrose gradient fractions 16–18 from a 3-h sedimentation of alkaline-treated dystrophin-glycoprotein complex were incubated for 2 h with 0.5 ml of WGA-Sepharose (Pharmacia LKB, Uppsala, Sweden) which had been pre-equilibrated in buffer A containing 0.5 M NaCl and 0.5 M sucrose. The WGA-Sepharose was pelleted (500 × *g* for 10 s) and the supernatant treated three additional times by the same procedure with fresh 0.5-ml aliquots of WGA-Sepharose over a period of 14 h. The resulting supernatant, which contained pure dystrophin, was concentrated in a Centricon 100 (Amicon) and analyzed.

Polyclonal Antibodies—Polyclonal antisera against chemically synthesized peptides representing either the first 15 amino-terminal amino acids (MLWWEEVEDCYERED) or the last 10 carboxyl-terminal amino acids (PGKPMREDTM) of the predicted human skeletal muscle dystrophin sequence (8) were raised in New Zealand White rabbits (12) and affinity-purified against the synthetic peptides as described by Sharp and Campbell (15). Neither of these affinity-purified antisera cross-reacts with a synthetic peptide representing the carboxyl-terminal 12 amino acids (CCPNVPSRPQAM) of the chromosome 6 dystrophin-related protein (16).

Miscellaneous—Detergent-solubilized proteins were quantitated by a modified Lowry procedure (17) after protein precipitation with 5% trichloroacetic acid in the presence of 0.5 mg/ml sodium deoxycholate (18) using BSA as standard. Samples were analyzed by SDS-PAGE (19) (3–12% gradient gel) in the presence of 1% β-mercaptoethanol and stained with Coomassie Blue or transferred to nitrocellulose (20). Coomassie Blue-stained gels were analyzed densitometrically using a Hoefer GS 300 scanning densitometer and GS-360 data analysis software. M_r was calculated from a curve prepared using the Sigma MW-SDS-200 kit and pure skeletal muscle ryanodine receptor (21) as standards. Prestained M_r standards shown in the figures were purchased from Bethesda Research Laboratories. Nitrocellulose transfers were stained with 1 μg/ml peroxidase-conjugated WGA (Sigma) by the same method previously described using ^{125}I-labeled WGA (9) or stained with affinity-purified polyclonal antisera as previously described (22).

RESULTS AND DISCUSSION

As a result of preliminary experiments with a variety of lectins differing in sugar specificity, we found that sWGA-agarose could be useful in the purification of dystrophin-glycoprotein complex. It is apparent from Fig. 1 (*left*) that a high M_r (400,000) protein was a major component of the proteins eluted from the sWGA-agarose with 0.3 M *N*-acetylglucosamine. The identification of this protein as dystrophin was confirmed on immunoblots (not shown) stained with monoclonal and polyclonal anti-dystrophin antibodies (12, 13). Densitometric analysis of the fractions shown in Fig. 1 (*left*) indicated that dystrophin comprised 25% of the total protein compared with only 5% when WGA-Sepharose was used (9). A small (~10%) amount of dystrophin-glycoprotein complex was eluted from the sWGA-agarose column when washed with buffer A containing no NaCl (not shown). This material could be recovered after recirculation on a WGA-Sepharose column. However, the majority of dystrophin was specifically eluted only when 0.3 M *N*-acetylglucosamine was included in the wash (Fig. 1, *left*). The peak dystrophin-containing fractions eluted from the sWGA-agarose column (Fig. 1, *left*) were also enriched in proteins of M_r 297,000, 109,000, 101,000, 88,000, a 59,000 triplet, 50,000, a 43,000 doublet, 35,000, 30,000, and 25,000. The eluate from the sWGA-agarose column was devoid of sodium channel or dihydropyridine receptor which were major substituents of a similar elution profile from WGA-Sepharose (9). The M_r 109,000 protein was identified as sarcoplasmic reticulum Ca^{2+}-ATPase using monoclonal antibodies (22) specific for this protein (not shown). However, neither the M_r 109,000 nor 101,000 proteins cosedimented with the dystrophin-glycoprotein complex on sucrose gradients (not shown). We have previously observed

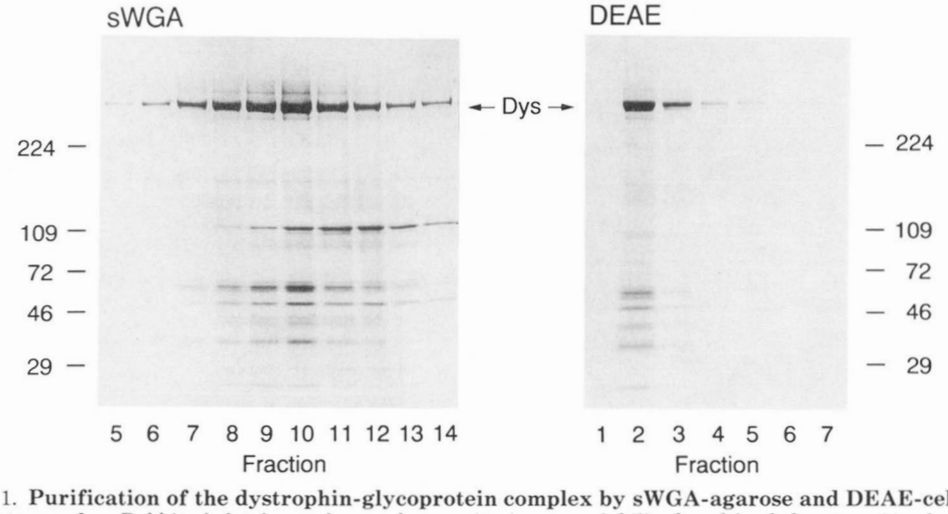

FIG. 1. **Purification of the dystrophin-glycoprotein complex by sWGA-agarose and DEAE-cellulose chromatography.** Rabbit skeletal muscle membranes (1 g) were solubilized and loaded onto a 80-ml sWGA-agarose column as described under "Materials and Methods." After extensive washing, the column was eluted with 0.3 M *N*-acetylglucosamine. 0.5 ml of fractions 5–14 collected from the *N*-acetylglucosamine elution were concentrated (Centricon 100) and analyzed on a Coomassie Blue-stained gel (*sWGA*). The dystrophin-containing fractions from the *N*-acetylglucosamine elution were then applied to a 3-ml DEAE-cellulose column and step-eluted with increasing NaCl concentrations as described under "Materials and Methods." 0.13 ml of fractions 1–7 of the 175 mM NaCl wash were analyzed on a Coomassie Blue-stained gel (*DEAE*). The molecular weight standards ($\times 10^{-3}$) are indicated on the *left* and *right*.

that ion exchange chromatography is useful in separating the sarcoplasmic reticulum Ca^{2+}-ATPase from the dystrophin-glycoprotein complex (9). When the dystrophin-enriched eluate from the sWGA-agarose column was applied to DEAE-cellulose and step-eluted with increasing NaCl concentrations, the 175 mM NaCl wash (Fig. 1, *right*) was enriched in dystrophin and proteins of M_r 88,000, a 59,000 triplet, 50,000, a 43,000 doublet, 35,000, and 25,000, all previously identified as components of the dystrophin-glycoprotein complex (11, 12). The M_r 156,000 dystrophin-associated glycoprotein is only faintly apparent in Fig. 1 (*right*) because it stains very poorly with Coomassie Blue (12), a phenomenon which has also been observed with the heavily glycosylated mucins (23) and several erythrocyte membrane glycoproteins (24). The copurification of the M_r 156,000 glycoprotein with dystrophin was confirmed on immunoblots stained with monoclonal antibody VIA4$_1$ (not shown), which is specific for the M_r 156,000 glycoprotein (12). WGA-peroxidase-stained nitrocellulose transfers of the DEAE-cellulose fractions eluting with 175 mM NaCl revealed the presence of several high M_r glycoproteins ranging from 300,000 to 500,000 (not shown) which also contaminated the dystrophin-glycoprotein complex prepared using WGA-Sepharose (12). These high M_r glycoproteins do not appear to be components of the dystrophin-glycoprotein complex as they do not strictly cosediment with dystrophin on sucrose density gradients (12) and are not immunoprecipitated by anti-dystrophin antibodies (not shown). The average yield from five dystrophin-glycoprotein complex preparations was 330 ± 36 μg when 1.5 g of skeletal muscle membranes was used as starting material.

The dystrophin-glycoprotein complex isolated using WGA-Sepharose as the lectin matrix was shown to sediment as a large 18 S complex on sucrose gradients (12). Likewise, the dystrophin-glycoprotein complex isolated using sWGA-agarose chromatography exhibited a sedimentation peak in fractions 10 and 11 of 5–20% sucrose gradients (Fig. 2, *top*), a gradient profile identical to that observed for complex obtained via WGA-Sepharose and run under identical conditions (12). Thus, it appears that the dystrophin-glycoprotein complex isolated using sWGA-agarose was of identical size, composition, and purity as the preparation using WGA-Sepharose without the need for a sucrose density gradient step.

We have reported that the dystrophin-glycoprotein complex could be disrupted by SDS or molar concentrations of KI (9), and recently, the purification of rabbit skeletal muscle dystrophin after SDS denaturation has been reported (25). However, SDS is essentially an irreversible denaturant and the removal of KI requires prolonged dialysis. Recently, a M_r 400,000 protein with homology to dystrophin was shown to be extracted from *Torpedo californica* electric organ membranes by alkaline treatment (26). Thus, the effectiveness of alkaline treatment in dissociating the dystrophin-glycoprotein complex was evaluated (Fig. 2). Purified dystrophin-glycoprotein complex (Fig. 1, *right*) was concentrated, titrated to pH 11 with 1 M NaOH, and then centrifuged through a 5–20% linear sucrose gradient containing 50 mM Tris, pH 11, for 90 min. When the gradient profile of the alkaline-treated dystrophin-glycoprotein complex (Fig. 2, *bottom*) is compared with that of untreated complex sedimented on a gradient containing 50 mM Tris-HCl, pH 7.4 (Fig. 2, *top*), it is apparent that the components of the alkaline-treated complex no longer cosediment and that all sediment as much smaller entities. A sucrose gradient profile identical to that shown in Fig. 2 (*bottom*) was observed when alkaline-treated dystrophin-glycoprotein complex was sedimented through a 5–20% sucrose gradient containing 50 mM Tris-HCl, pH 7.4 (not shown).

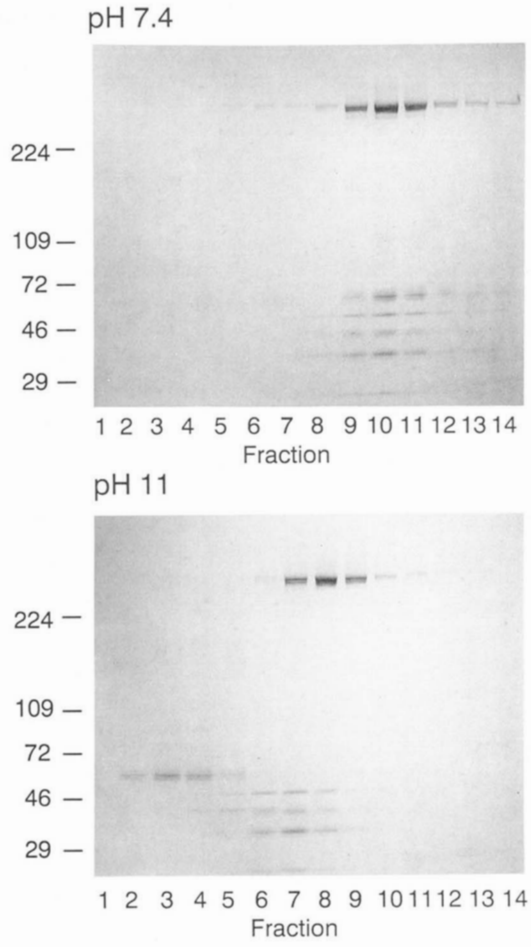

FIG. 2. **Effect of alkaline treatment on sedimentation of the dystrophin-glycoprotein complex through 5–20% linear sucrose gradients.** Coomassie Blue-stained gels of 0.3 ml of sucrose gradient fractions 1–14 of purified dystrophin-glycoprotein complex sedimented (90 min) through a 5–20% sucrose gradient containing 50 mM Tris-HCl, pH 7.4 (*pH 7.4*) or dystrophin-glycoprotein complex which had been alkaline-treated and centrifuged through a 5–20% sucrose gradient containing 50 mM Tris, pH 11 (*pH 11*). The positions of the molecular weight standards ($\times 10^{-3}$) are indicated on the *left*.

Alkaline incubations from 30 s to 1 h yielded similar results (not shown). These data indicate that the dystrophin-glycoprotein complex can be dissociated with alkaline treatment. In the absence of detergent, alkaline-treated dystrophin cosedimented with the 11 S standard catalase on either 5–20% sucrose gradients or 15–40% glycerol gradients (not shown) which is in good agreement with the sedimentation of tetrameric spectrin (27). This result indicates that alkaline-treated dystrophin sediments with a size consistent with a dystrophin dimer. This result also suggests that alkaline-treated dystrophin has not undergone extensive unfolding since it cosediments with spectrin, a protein with which it shares significant sequence and structural similarity. The M_r 59,000 dystrophin-associated protein sedimented near the top of the gradient and was completely separated from dystrophin (Fig. 2, *bottom*). The sedimentation peak of the M_r 156,000 dystrophin-associated glycoprotein was found in fraction 5 as detected with monoclonal antibody VIA4$_1$ (not shown). However, the M_r 50,000, 43,000, 35,000, and 25,000 dystrophin-associated proteins cosedimented, possibly as a complex smaller than dystrophin (Fig. 2, *bottom*).

Alkaline treatment followed by a 3-h sucrose gradient centrifugation time (Fig. 3, *top*) separated dystrophin from the

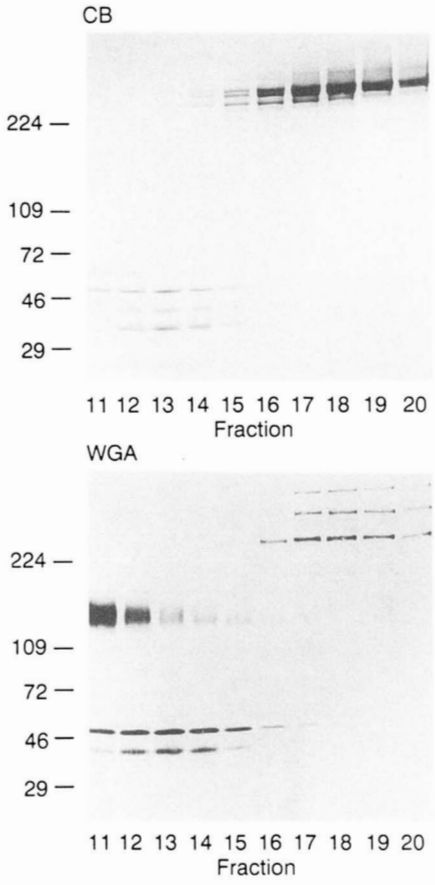

FIG. 3. **Separation of dystrophin from dystrophin-associated proteins.** Coomassie Blue-stained gel (*CB*) or peroxidase-conjugated WGA-stained nitrocellulose transfer (*WGA*) of 0.15 ml of sucrose gradient fractions 11–20 of dystrophin-glycoprotein complex which had been alkaline-treated and sedimented (3 h) through a 5–20% sucrose gradient containing 50 mM Tris, pH 11, is shown. The positions of the molecular weight standards ($\times 10^{-3}$) are shown on the *left*.

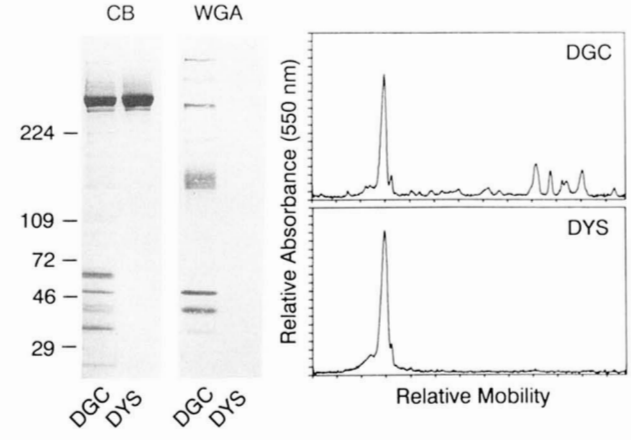

FIG. 4. **Comparison of pure dystrophin to dystrophin-glycoprotein complex.** Coomassie Blue-stained gel (*CB*) or WGA peroxidase-stained nitrocellulose blot (*WGA*) of 20 μg of dystrophin-glycoprotein complex (*DGC*) and 11 μg of pure dystrophin (*DYS*) is shown. The average of five densitometric scans of the Coomassie Blue-stained gel lanes are shown on the *right*. The positions of the molecular weight standards ($\times 10^{-3}$) are shown on the *left*.

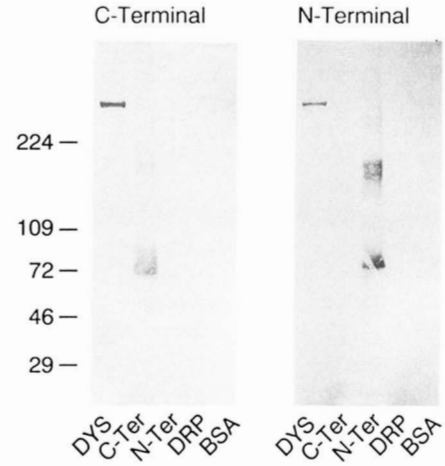

FIG. 5. **Immunoblot staining of pure dystrophin with human dystrophin sequence-specific polyclonal antisera.** Nitrocellulose transfers containing 1 μg of pure dystrophin (*DYS*), BSA-conjugated synthetic peptides representing the carboxyl terminus (*C-Ter*) and the amino terminus (*N-Ter*) of human dystrophin, the carboxyl terminus of the chromosome 6-encoded dystrophin-related protein (*DRP*), or unconjugated BSA (*BSA*) were stained with affinity-purified antisera raised against synthetic peptides representing the carboxyl or amino termini of human dystrophin as described under "Materials and Methods." The positions of the molecular weight standards ($\times 10^{-3}$) are shown on the *left*.

M_r 50,000, 43,000, and 35,000 glycoprotein multiplet of the dystrophin-glycoprotein complex. However, this procedure alone was not sufficent to purify dystrophin from the high M_r glycoproteins which contaminate dystrophin-glycoprotein complex preparations (12) and cosediment with uncomplexed dystrophin (Fig. 3, *bottom*). In order to completely purify dystrophin, the high M_r glycoproteins which contaminated peak dystrophin-containing gradient fractions (Fig. 3) were removed by WGA-Sepharose adsorption (Fig. 4). Ninety-eight percent of the protein in the WGA void appeared as a single high M_r band on overloaded Coomassie Blue-stained gels while the remaining 2% of protein composed a band of slightly lower M_r (Fig. 4). Although it is difficult to accurately determine the M_r of extremely large proteins by SDS-PAGE, the predominant band in the WGA void from six consecutive preparations was calculated to have an average M_r of 402,000 ± 6,800 which is in close agreement with the predicted M_r (8). The predominant component appears to be intact dystrophin as it is recognized by antisera specific for either the amino or carboxyl termini of human skeletal muscle dystrophin (Fig. 5). The minor, lower M_r component in the dystrophin preparation was stained only by the carboxyl-terminal specific antisera (Fig. 5) indicating that it is most likely a proteolytic fragment. Also shown in Fig. 5, the affinity-purified carboxyl-terminal antisera does not recognize the BSA-conjugated synthetic peptide corresponding to the carboxyl terminus of the chromosome 6-encoded dystrophin-related protein (16). In addition, affinity-purified antisera specific for the carboxyl terminus of the chromosome 6 dystrophin-related protein (16) do not stain pure dystrophin (not shown). Nitrocellulose transfers stained with peroxidase-conjugated WGA revealed no contaminating glycoproteins in the pure dystrophin (Fig. 4). The typical yield of pure dystrophin was 30 μg when 1.2–1.5 g of rabbit skeletal muscle membranes was used as starting material.

In this report, we have demonstrated the purification of dystrophin in sufficient quantity to allow careful biochemical, functional, and ultrastructural characterization. Based on previous estimates that dystrophin makes up only 0.002% of all muscle protein (1), this rapid method represents a 50,000-fold purification of dystrophin. All of the chromatography matrices used in this method are commercially available, making this method accessible to all laboratories interested

in studying the structure and function of dystrophin. The protein isolated is recognized by antisera specific for both the amino and carboxyl termini of the predominant skeletal muscle isoform (28) of human dystrophin (8). These data indicate that the protein isolated is the intact translation product of the Duchenne muscular dystrophy gene. Furthermore, the pure dystrophin is devoid of contaminating high M_r glycoproteins (Fig. 4). It is important to demonstrate this absence of the high M_r glycoproteins, which do not stain well with Coomassie Blue (Fig. 3), because their presence could confuse morphological studies (11, 29) of dystrophin preparations.

Two key developments greatly facilitated the purification of dystrophin. First, that the skeletal muscle voltage-dependent sodium channel (30) and the dihydropyridine receptor (31) are terminally sialated allowed the purification of the dystrophin-glycoprotein complex (Fig. 1) without the sucrose gradient step used previously (12). sWGA is negatively charged at physiological pH and does not appear to bind *N*-acetylneuraminic acid (32), in contrast to WGA, which is positively charged and binds both *N*-acetylneuraminic acid and *N*-acetylglucosamine residues.

The second development important in the purification of dystrophin was the observation that the dystrophin-glycoprotein complex can be dissociated by alkaline treatment (Fig. 3). It has long been known that the plasma membrane can be separated from the underlying cytoskeleton by simple alkaline treatment (33). Human erythrocyte band 4.2 purified by a method involving alkaline treatment (34) binds saturably to stripped red blood cells (34), pure ankyrin, and band 3 (35). These data suggest that another cytoskeletal protein with a membrane association as tight as that of dystrophin remains functional after alkaline treatment. That alkaline-treated dystrophin sediments with a size similar to spectrin, a protein with which dystrophin shares a high degree of homology, indicates that dystrophin has not undergone a gross deformation. Ultrastructural and additional biochemical studies are currently under way to determine whether the purified dystrophin exhibits properties characteristic of "native" dystrophin. In conclusion, the present work will immediately allow the direct study of dystrophin which is necessary to confirm structural predictions deduced from the cDNA sequence and address the possibility of post-translational modifications important to the physiological function of dystrophin.

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