Dystrophin-Glycoprotein Complex Is Highly Enriched in Isolated Skeletal Muscle Sarcolemma

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Abstract. mAbs specific for protein components of the surface membrane of rabbit skeletal muscle have been used as markers in the isolation and characterization of skeletal muscle sarcolemma membranes. Highly purified sarcolemma membranes from rabbit skeletal muscle were isolated from a crude surface membrane preparation by wheat germ agglutination. Immunoblot analysis of subcellular fractions from skeletal muscle revealed that dystrophin and its associated glycoproteins of 156 and 50 kD are greatly enriched in purified sarcolemma vesicles. The purified sarcolemma was also enriched in novel sarcolemma markers (SL45, SL/TS230) and Na^+/K^+-ATPase, whereas t-tubule markers (α_1 and α_2 subunits of dihydropyridine receptor, TS28) and sarcoplasmic reticulum markers (Ca^{2+}-ATPase, ryanodine receptor) were greatly diminished in this preparation.

Analysis of isolated sarcolemma by SDS-PAGE and densitometric scanning demonstrated that dystrophin made up 2% of the total protein in the rabbit sarcolemma preparation. Therefore, our results demonstrate that although dystrophin is a minor muscle protein it is a major constituent of the sarcolemma membrane in skeletal muscle. Thus the absence of dystrophin in Duchenne muscular dystrophy may result in a major disruption of the cytoskeletal network underlying the sarcolemma in dystrophic muscle.

DYSTROPHIN, the protein product of the human Duchenne muscular dystrophy gene (DMD)[1] (18), is localized to the sarcolemma membrane in normal skeletal muscle (2, 5, 49, 51) but is absent from skeletal muscle of DMD humans (2, 18, 19), mdx mice (18, 49) and xmd dogs (13). Dystrophin represents ~0.002% of the total muscle protein (18) and its predicted primary structure suggests that it shares many features with membrane cytoskeletal proteins such as α-actinin and spectrin (29). Dystrophin can be isolated from skeletal muscle membranes using WGA chromatography because of its tight association with WGA-binding glycoproteins (7). Recently, we have shown that dystrophin exists in a large oligomeric complex which contains four glycoproteins of apparent M_r 156,000, 50,000, 43,000 and 35,000 and a protein triplet at 59,000 (15). One of the glycoproteins in the dystrophin-glycoprotein complex has been found to be greatly reduced in muscle from mdx mice and DMD patients. Therefore, the absence of dystrophin leads to the loss of at least one component of the dystrophin-glycoprotein complex and this could initiate the molecular pathogenesis of muscular dystrophy (15).

Since it is apparent that the degenerative processes leading to DMD are associated with the surface membrane of skeletal muscle, it is important to be able to study the structure of isolated sarcolemma to identify the normal protein composition of the sarcolemma membrane from skeletal muscle and to determine the relative abundance of dystrophin to other sarcolemma proteins. For the biochemical characterization of surface membrane components, which are involved in the pathology of muscle diseases, sarcolemma vesicles have to be prepared in a sufficient yield with a high degree of purity. A variety of procedures have been used to isolate skeletal muscle fractions enriched in sarcolemma, most involving density gradient centrifugation (3, 32, 40, 41). However, due to the low abundance of sarcolemma to other membranes in skeletal muscle, in particular sarcoplasmic reticulum, density gradient centrifugation has not produced a sarcolemma membrane preparation that gives a substantially different SDS-PAGE profile in comparison to purified sarcoplasmic reticulum.

Recently, Charuk et al. (12) successfully used wheat germ agglutination procedure after density gradient centrifugation for the subfractionation of cardiac sarcolemma. WGA is a homodimer that cross-links terminally linked *N*-acetyl-D-glucosamine and/or sialic acid (4), thus, lectin agglutination should aggregate specifically sealed right-side-out sarcolemma vesicles because the carbohydrate chains of membrane glycoproteins are extracellular (12).

Here we report the isolation of highly purified sarcolemma vesicles from rabbit skeletal muscle using sucrose density

1. *Abbreviations used in this paper*: DMD, Duchenne muscular dystrophy; DYS, dystrophin; NAG, *N*-acetyl-D-glucosamine; SL, sarcolemma; TS, transverse tubule system.

step gradient centrifugation and wheat germ agglutination. Our approach was to characterize the isolated sarcolemma vesicles by immunological analysis using mAbs. Novel proteins are introduced as specific markers for the sarcolemma, the sarcolemma and transverse tubule membrane, or the transverse tubule system of rabbit skeletal muscle, using immunoblot and immunofluorescence localization techniques.

Our findings demonstrate that the tightly associated dystrophin–glycoprotein complex (15) is restricted to the cell periphery of skeletal muscle cells and that dystrophin is a major protein constituent of the rabbit sarcolemma membrane, i.e., ~2% of the total skeletal muscle sarcolemma protein.

Materials and Methods

Isolation of Rabbit Skeletal Muscle Membranes

New Zealand white rabbits (5 lb) were euthanized by ketamine injection, and all subsequent steps were carried out at 0–4°C. Approximately 450 g of back and hind leg white skeletal muscles was excised, ground in a meat grinder and weighed. Tissue was homogenized in a Waring blender (Waring Products Div., New Hartford, CT) for three times 30 s in 7.5 vol of buffer A (20 mM sodium pyrophosphate, 20 mM sodium phosphate monohydrate, 1 mM $MgCl_2$, 0.303 M sucrose, 0.5 mM EDTA, pH 7.0) in the presence of protease inhibitors: aprotinin (76.8 nM), leupeptin (1.1 uM), pepstatin A (0.7 μM), benzamidine (0.83 mM), iodoacetamide (1 mM), and PMSF (0.23 mM). All other buffers contained the last three protease inhibitors at the above concentration. The homogenate was centrifuged in a JA-10 rotor (Beckman Instruments, Fullerton, CA) for 15 min at 14,000 *g* and the supernatant filtered through six layers of cheesecloth. The pellets were resuspended in buffer A (70% of the original volume) and rehomogenized and centrifuged as described before. The supernatants were combined and centrifuged in a JA-14 rotor (Beckman Instruments) for 30 min at 30,000 *g* and the resulting supernatant and microsomal pellets were used to isolate light microsomes and microsomes, respectively. Microsomal pellets were resuspended and incubated for 30 min in 400 ml of 0.6 M KCl, 0.303 M sucrose, 50 mM Tris-HCl, pH 7.4 and subsequently centrifuged in a Beckman 45Ti rotor (Beckman Instruments) for 30 min at 142,000 *g* to obtain KCl-washed microsomes. The final microsomal preparation was resuspended in buffer B (0.303 M sucrose, 20 mM Tris-maleate, pH 7.0).

Light microsomes were obtained from the supernatant of the JA-14 rotor spin, after adding solid KCl to a final concentration of 0.6 M to the suspension, by centrifuging it in a 45Ti rotor for 35 min at 142,000 *g*. The pellets were resuspended in 400 ml of buffer B and again treated with KCl and centrifuged as described above. The final pellets of light microsomes were resuspended in 30 ml of buffer B containing 0.6 M KCl and 5-ml aliquots were loaded onto 35 ml of 0.878 M sucrose, 0.6 M KCl, 20 mM Tris-maleate, pH 7.0 in ultraclear centrifuge tubes and centrifuged in an SW28 swinging bucket rotor (Beckman Instruments) at 112,000 *g* for 17 h. The crude surface membrane fraction at the 0.303 M/0.878 M sucrose interface and the pellets were collected, diluted with 0.6 M KCl, 20 mM Tris-maleate, pH 7.0 and centrifuged in a 45Ti rotor for 30 min at 142,000 *g*. All membrane preparations were resuspended in buffer B and stored frozen at −135°C. Protein concentration was determined as described (35) using BSA as standard. Approximately 30 mg of crude surface membrane was typically obtained from 450 g of skeletal muscle.

Triads and t-system vesicles were isolated according to Sharp et al. (43) and Rosemblatt et al. (36), respectively. Light sarcoplasmic reticulum vesicles were prepared as described previously (6).

Isolation of Purified Sarcolemma by Wheat Germ Agglutination

To further purify the crude surface membrane preparation, vesicles were treated with WGA in principle as described by Charuk et al. (12). Crude surface membrane vesicles were resuspended at a protein concentration of 1 mg/ml in buffer C (50 mM sodium phosphate, pH 7.4, 0.160 M NaCl) and gently mixed with an equal volume of 1 mg/ml wheat germ lectin (Sigma Chemical Co., St. Louis, MO) in buffer C. Total volume of this mixture was 60 ml and after a 10-min incubation on ice the solution was pelleted in a centrifuge (Eppendorf model 5402) for 90 s at 14,000 *g*. The lectin-agglutinated vesicles were resuspended in buffer D (20 mM Tris-HCl, pH 7.4, 0.303 M sucrose) and centrifuged as described above. This procedure was repeated and the resuspended pellets were then deagglutinated by incubation for 20 min in 18 ml of 0.2 M *N*-acetyl-D-glucosamine in buffer D. The deagglutinated suspension was centrifuged in an Eppendorf centrifuge for 90 s at 14,000 *g* and the supernatant pelleted in a TL100.3 rotor (Beckman Instruments) for 20 min at 150,000 *g*, resuspended in buffer D and centrifuged for 20 min at 150,000 *g* as described above. Subsequently the pellets were resuspended in 9 ml of buffer C and treated for a second time with lectin and finally deagglutinated as described above. The final preparation was designated purified sarcolemma. The nonagglutinated vesicles were centrifuged for 20 min at 150,000 *g* and the pellets consisted mostly of sarcoplasmic reticulum and transverse tubule vesicles. Starting with 30 mg crude surface membranes, the yield of purified sarcolemma and nonagglutinated vesicles was ~4 and 18 mg, respectively.

Monoclonal and Polyclonal Antibodies

mAbs were prepared against antigens associated with skeletal muscle sarcolemma, sarcolemma and transverse tubules, or transverse tubules and used for immunoblotting and immunofluorescence analysis. Production and characterization of mAbs against membrane marker proteins was carried out by immunizing mice with various rabbit skeletal muscle membrane preparations as described previously (9, 25, 26). An immunodot assay was used for the initial screening of the hybridoma supernatants, followed by immunoblot and immunofluorescence analysis to further characterize the specificity of the new mAbs.

New mAbs XVB9 and $IXCl_2$ are directed against a 45-kD sarcolemma protein and a 230-kD sarcolemma/transverse tubule protein, respectively.

For the characterization of previously established mAbs used in this study see the following publications: mAbs IID8 and IIH11 against the slow- and fast-twitch Ca^{2+}-ATPase of sarcoplasmic reticulum (25), mAbs IIF7 and IIID5 against the α_1-subunit of the 1,4-dihydropyridine receptor (26), mAb $IXE11_2$ against a 28-kD transverse tubule protein (27), mAb XA7 against the ryanodine receptor (9, 22), mAb $IVD3_1$ against the 50-kD glycoprotein of sarcolemma (27), mAb $VIA4_1$ against the 156 kDa glycoprotein of sarcolemma (15), mAb XIXC2 against dystrophin (15), mAb McB2 against the α_2-subunit of the Na^+/K^+-ATPase (Dr. K. J. Sweadner, Dept. of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA [48]). mAb MF20 against myosin was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa and mAb 5C5 against actin from Sigma Chemical Co. mAbs against marker proteins used in this study are summarized in Table I.

Polyclonal antisera against the α_2-subunit of the dihydropyridine receptor (42) and against the COOH-terminal decapeptide of dystrophin (15) were raised in guinea pig and rabbit, respectively. The decapeptide representing the COOH-terminal of human skeletal muscle dystrophin (Pro-Gly-Lys-Pro-Met-Arg-Glu-Asp-Thr-Met) (29) was chemically synthesized on a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) by the Alberta Peptide Institute, University of Alberta, Canada, using methods previously described (45). The COOH-terminal sequence of dystrophin is almost completely different from the COOH-terminal sequence of a dystrophin-related protein (31). The photoprobe, *p*-benzoylbenzoic acid, was coupled directly to the protected peptide resin during synthesis and the resulting photoprobe peptide was purified using reverse-phase HPLC. The purified peptide was photochemically coupled to BSA as described (34) and the peptide to protein ratio was 17:1.

Gel Electrophoresis and Densitometric Analysis

SDS-PAGE was performed according to Laemmli (30) in 3–12% polyacrylamide gels. Protein bands were visualized by Coomassie brilliant blue staining and also analyzed by Stains-all staining for Ca^{2+}-binding proteins (8). Densitometric scanning of Coomassie brilliant blue–stained SDS-PAGE gels was carried out on a computing densitometer (model 300A; Molecular Dynamics) and also on a densitometer (model GS 300; Hoefer Scientific Instruments, San Francisco, CA).

Immunoblotting and Lectin Staining

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes by the method of Towbin et al. (46). Prestained molecular weight standards were from Bethesda Research Laboratories (Gaithersburg, MD) and the apparent molecular masses were as follows: myosin, 224 kD; phosphorylase b, 109 kD; albumin, 72 kD; ovalbumin, 46 kD; and carbonic anhydrase, 29 kD. Nitrocellulose transfers were blocked in Blotto (50 mM so-

Table I. Subcellular Distribution of Membrane Marker Proteins in Skeletal Muscle

Specificity	M_r	Ab	Immunoblotting (isolated membranes)				Immunofluorescence (in situ)			Reference
			LSR	*TR*	*TS*	*SL*	SR	TS	SL	
	kD									
Sarcolemma										
Dystrophin	427	XIXC2	−	−	+	+++	−	−	+	7, 15
SL 156	156	$VIA4_1$	−	−	+	+++	−	−	+	15
SL 50	50	$IVD3_1$	−	−	+	+++	−	−	+	15, 27
SL 45	45	XVB9	−	−	+	+++	−	−	+	*
Transverse tubules and sarcolemma										
SL/TS 230	230	$IXC1_2$	−	+	+	+	−	+	+	*
Na^+/K^+-ATPase	112	McB2	−	+	+	+		(ND)		48
Transverse tubules										
DHPR subunits										
α_1	170	IIID5	−	+	++	−	−	+	−	26
α_1	170	IIF7	−	+	++	−	−	+	−	26
α_2	175	GP5SA	−	+	++	−		(ND)		43
β	52	$VD2_1$	−	+	++	−	−	+	−	26
TS28	28	$IXE11_2$	−	+	++	−	−	+	−	27
Junctional sarcoplasmic reticulum										
Ryanodine receptor	565	XA7	−	++	−	−		(ND)		9, 22
Free sarcoplasmic reticulum										
Fast Ca^{2+}-ATPase	110	IIH11	+++	++	++	+	++	−	−	25
Slow Ca^{2+}-ATPase	110	IID8	+++	++	++	+	++	−	−	25

* This study.
Abbreviations: DHPR, dihydropyridine receptor; LSR, light sarcoplasmic reticulum; TR, triads; TS, transverse tubule system; SL, sarcolemma.

dium phosphate, pH 7.4, 150 mM sodium chloride, 5% nonfat dry milk) and subsequently incubated overnight with primary antibody (hybridoma supernatant or 1:1,000 dilution of a concentrated antibody). Immunoblots were then washed with Blotto and incubated for 1 h with peroxidase-conjugated secondary antibody (Boehringer-Mannheim Biochemicals, Indianapolis, IN) at a dilution of 1:1,000. After washing the nitrocellulose blots with Blotto they were developed in 20 mM Tris-Cl, pH 7.5, 200 mM NaCl using 4-chloro-1-naphthol as substrate (27).

Immunoblots were also stained with ^{125}I-WGA agglutinin (ICN Radiochemicals, Irvine, CA) in the presence or absence of 0.3 M *N*-acetyl-D-glucosamine as described previously (7).

Immunofluorescence Microscopy

Immunofluorescence staining of transverse and longitudinal cryosections (8 μm) from rabbit skeletal muscle (gastrocnemius) was carried out as described previously (15). Cryosections were preincubated for 20 min with 5% normal goat antiserum in PBS (50 mM sodium phosphate, pH 7.4, 0.9% NaCl), followed by a 1-h incubation at 37°C with the primary antibody (hybridoma supernatants or 1:1,000 dilution of a concentrated antibody). After washing in PBS the sections were further incubated for 30 min at 37°C in PBS with a 1:50 dilution of affinity-purified FITC-labeled goat $F(ab')_2$ anti-mouse IgG (Boehringer-Mannheim) and subsequently examined in a Zeiss Axioplan fluorescence microscope.

Immunofluorescence labeling of biopsy samples of normal control and patients with Duchenne muscular dystrophy (obtained from the Department of Neuropathology, University of Iowa) were carried out with the cryosections of the control and the dystrophic muscle sample placed on the same microscope slide and the samples were therefore treated in an identical manner during all incubation and washing steps.

Histochemical Staining

Serial transverse cryosections of unfixed rabbit gastrocnemius muscle were stained for myosin ATPase after alkaline preincubations (pH 10.4) as described by Guth and Samaha (17). This method specifically labels the myosin ATPase of only fast-twitch skeletal muscle fibers.

Results

mAbs to Skeletal Muscle Membrane Proteins

mAbs against antigens that are closely associated with different muscle membrane systems were used to characterize fractions in the purification of sarcolemma vesicles from rabbit skeletal muscle. After initial screening with immunodot assays, positive cell cultures were assayed for antibodies able to recognize antigens in skeletal muscle membrane preparations by immunoblotting. New mAbs XVB9 and $IXC1_2$ label protein bands in KCl-washed microsomes of 45 and 230 kD, respectively (see Fig. 7). Immunoblot analysis of the purified dystrophin-glycoprotein complex (15) revealed that both proteins are not integral components of this complex (results not shown). mAbs that exhibited a strong labeling in immunoblotting, were tested using indirect immunofluorescence microscopy on skeletal muscle cryosections. There are principally three different categories of surface membrane antigens, those which are restricted to the sarcolemma, or the transverse tubules, or those that are localized in both membrane systems. While the new mAb XVB9 is directed against a 45-kD protein that is restricted to the sarcolemma, the new mAb $IXC1_2$ against 230-kD protein is found in both the sarcolemma and the transverse tubule (Fig. 1).

Other mAbs to skeletal muscle membrane proteins were previously characterized and are specific for the slow- and fast-twitch Ca^{2+}-ATPase of sarcoplasmic reticulum (25), the dihydropyridine receptor and TS28 protein of transverse tubules (26, 27), the ryanodine receptor of triads (9, 22), com-

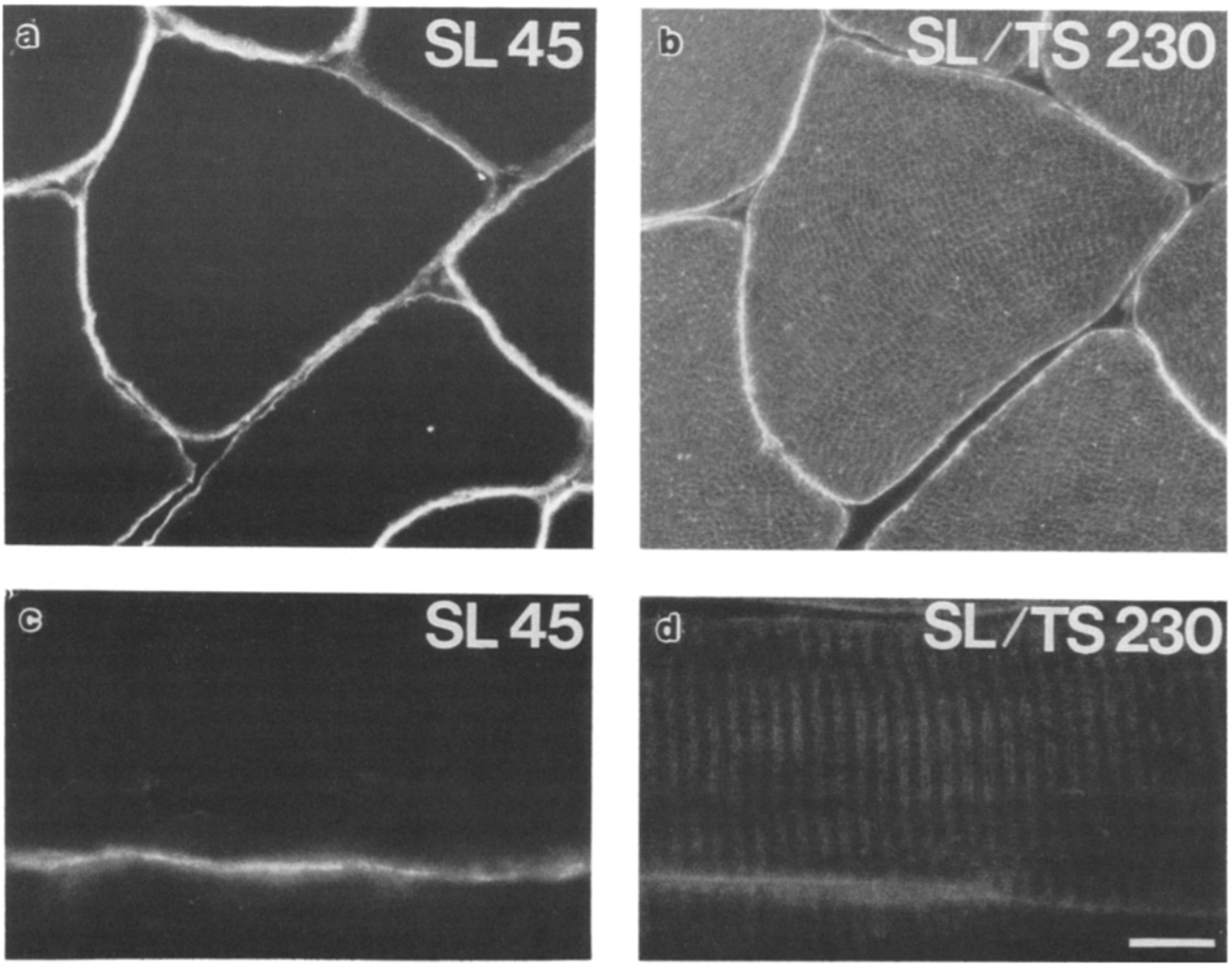


Figure 1. Immunofluorescence labeling of rabbit skeletal muscle with novel mAbs against SL45 and SL/TS 230. Cryosections from rabbit gastrocnemius muscle were prepared and labeled by the indirect immunofluorescence technique as described in Materials and Methods. Transverse (*a* and *b*) and longitudinal (*c* and *d*) cryosections from rabbit skeletal muscle, were labeled with mAb XVB9 against SL45 (*a* and *c*), mAb $IXC1_2$ against SL/TS 230 (*b* and *d*). Immunofluorescence labeling for SL45 was restricted to the sarcolemma, whereas mAb $IXC1_2$ to SL/TS 230 exhibited in addition to labeling of the cell periphery also an internal polygonal staining pattern in transverse sections and specific labeling of the A-I junction region in longitudinal cryosections. Bar, 5 μm.

ponents of the dystrophin–glycoprotein complex (15) and the Na^+/K^+-ATPase (48). Thus, these mAbs could then be used for further immunoblot staining to establish the purity and distribution pattern of specific antigens in the various skeletal muscle membrane systems. Table I summarizes the mAbs against marker proteins used in this investigation.

Immunofluorescence Localization of Surface Membrane Components

In immunofluorescence microscopy mAb XVB9 against a 45-kD protein exclusively labeled the cell periphery in transverse and longitudinal cryosections of rabbit skeletal muscle (Fig. 1, *a* and *c*). Staining was absent from the interior regions of skeletal muscle fibers, suggesting that the 45-kD protein is only associated with the sarcolemma. Examination of Fig. 1 *b* reveals that the immunofluorescence labeling of mAb $IXC1_2$ against a 230-kD protein is not confined to the sarcolemma region, but also extends to a polygonal internal staining pattern. These results are confirmed by longitudinal cryosections, which localize the 230-kD protein to the A-I junction and sarcolemma region (Fig. 1 *d*).

The localization pattern of the two new mAbs was compared with established marker proteins of rabbit skeletal muscle. The cell periphery was exclusively stained with mAbs XVB9 against the 45-kD protein, $IVD3_1$ against the dystrophin-associated 50-kD glycoprotein and XIXC2 against dystrophin (Fig. 2), demonstrating the specific association of these antigens with the muscle fiber plasma membrane or the intracellular cytoskeleton subjacent to the plasma membrane. All three antigens exhibited a uniform distribution throughout the sarcolemma in transversely and longitudinally cut cryosections (Fig. 2, *a–f*). The absence of any internal immunofluorescence staining establishes these antigens as convenient sarcolemma markers. mAb $VIA4_1$ against the dystrophin-associated 156-kD glycoprotein also labels the cell periphery, but only very weakly (15). The antibody recog-

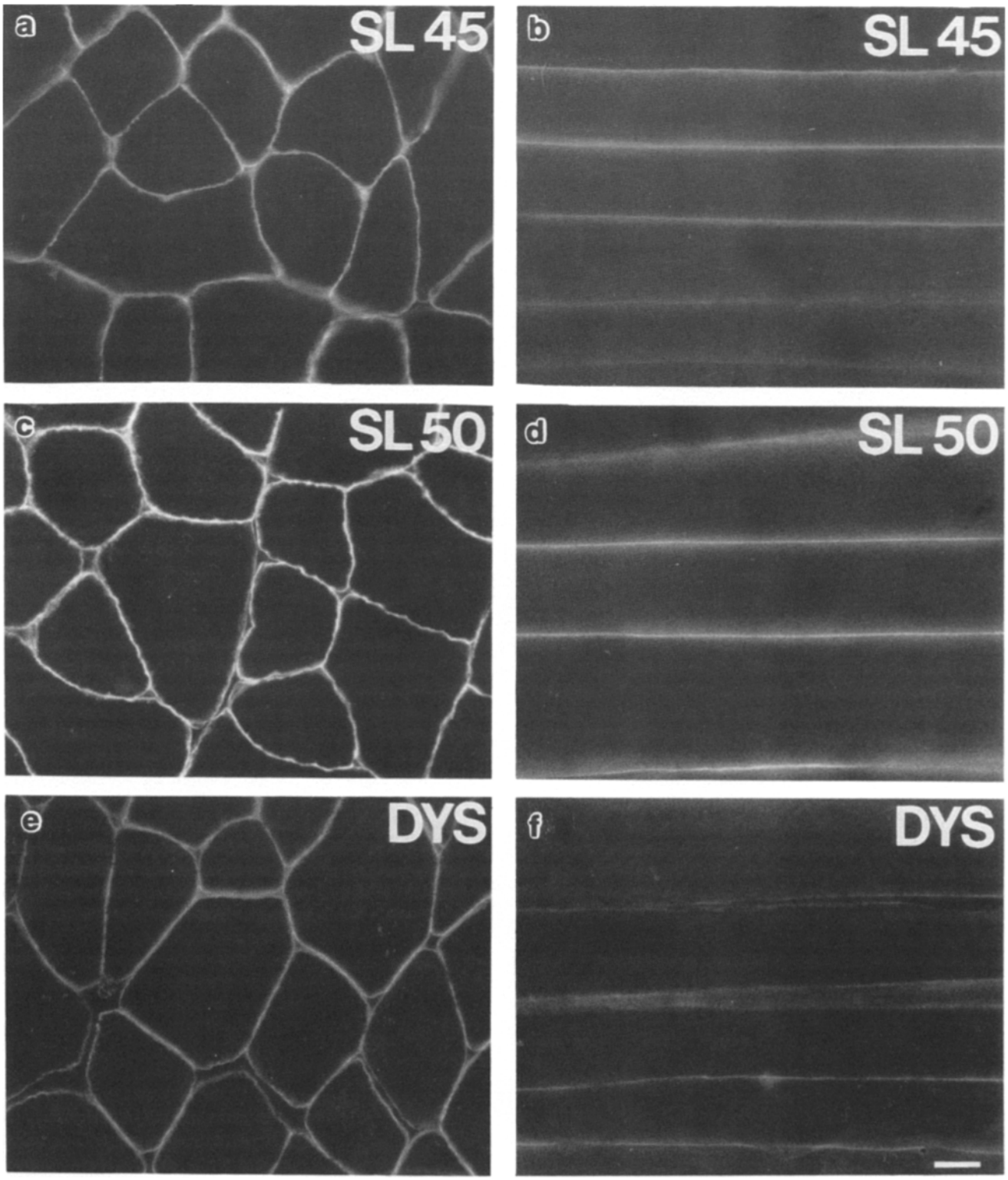


Figure 2. Distribution of SL45, SL50, and dystrophin in rabbit skeletal muscle. Immunofluorescence labeling of transverse (*a*, *c*, and *e*) and longitudinal (*b*, *d*, and *f*) cryosections from rabbit skeletal muscle, stained with mAb XVB9 against SL45 (*a* and *b*) mAb $IVD3_1$ against SL50 (*c* and *d*) and mAb XIXC2 against dystrophin (*e* and *f*). All three mAbs specifically labeled the cell periphery, whereas specific staining was not observed in internal regions of muscle fibers. Bar, 5 μm.

nizes the denatured protein in a much stronger manner and shows strong labeling of the 156-kD glycoprotein band in immunoblots (see Figs. 7, 9, and 10).

Fig. 3 compares the immunofluorescence labeling of mAb $IXC1_2$ against the 230-kD protein with that of mAb IIID5 against the α_1-subunit of the dihydropyridine receptor, a transverse tubule marker (26). Both proteins exhibit a polygonal staining pattern of the cell interior (Fig. 3, *a* and *c*),

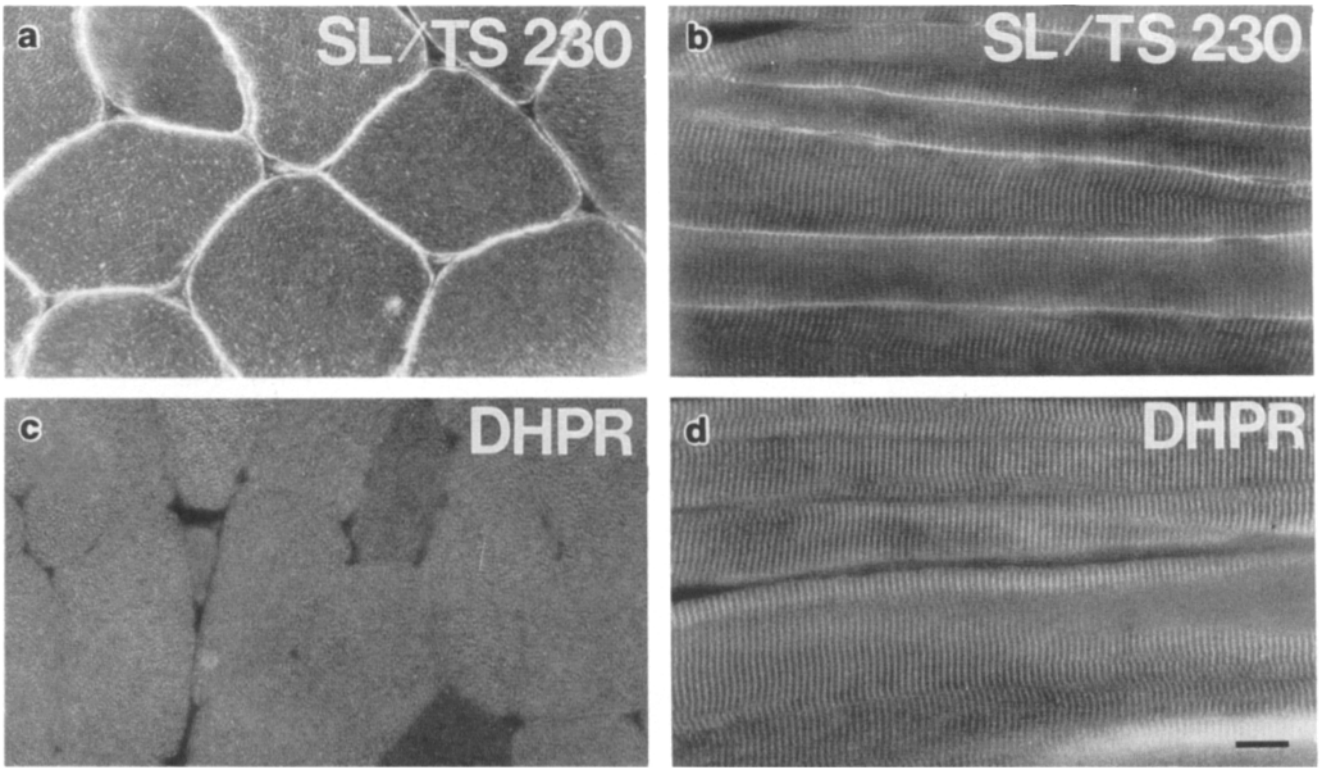


Figure 3. Distribution of SL/TS 230 and dihydropyridine receptor in rabbit skeletal muscle. Immunofluorescence labeling of transverse (*a* and *c*) and longitudinal (*b* and *d*) cryosections from rabbit skeletal muscle, stained with mAb $IXC1_2$ against SL/TS 230 (*a* and *b*) and mAb IIID5 against the α_1-subunit of dihydropyridine receptor (DHPR) (*c* and *d*). Both mAbs exhibited in transverse cryosections a polyclonal staining pattern throughout the cytoplasm of the myofibers and labeled the A-I junction region in longitudinal sections. In addition, mAb $IXC1_2$ also specifically labeled the cell periphery. Bar, 5 μm.

characteristic for their localization in transverse tubules. On the other hand, transverse and longitudinal cryosections of Fig. 3, *a* and *b* demonstrate that the staining pattern of mAb $IXC1_2$ extends to the sarcolemma region, whereas mAb IIID5 exclusively labels internal regions of the muscle fibers (Fig. 3, *c* and *d*).

Since muscles contain various fiber types, it is important to be aware of the fiber type specificity of mAbs used as markers for sarcolemma isolation. We have investigated the fiber type distribution of the different marker proteins used in this study using serial transverse sections of gastrocnemius muscle stained with each mAb. Fig. 4 *a* demonstrates histochemically the distribution of type I (slow-twitch) and type II (fast-twitch) fibers in serial transverse cryosections. Alkaline-stable myosin ATPase is a specific histochemical marker for fast-twitch type II fibers. The immunofluorescence staining pattern of mAb IIH11 and IID8 against the fast- and slow-twitch Ca^{2+}-ATPase, respectively, confirms the distribution pattern of the alkaline stable myosin ATPase (Fig. 4, *b* and *d*). mAb IIID5 to the dihydropyridine receptor strongly labeled fiber type II and less intensively fiber type I (Fig. 4 *c*), which reflects the different abundance of transverse tubules in fast and slow muscle fibers (14). After establishing the fiber type distribution of the serial transverse sections used in this investigation, the immunofluorescence staining pattern of novel marker proteins and components of the dystrophin glycoprotein complex were analyzed. All three mAbs against sarcolemma markers, XVB9, XIXC2, and $IVD3_1$, illustrated an equal distribution between fast and slow fibers (Fig. 4, *f–h*). Therefore, the components of the dystrophin–glycoprotein complex seem to be equally distributed between fibers of type I and II. mAb $IXC1_2$, which specifically stains both the cell periphery and the cell interior, shows an identical immunofluorescence intensity of the sarcolemma in both fiber types (Fig. 4 *e*). On the other hand, slow fibers exhibited much less internal polygonal labeling than fast fibers. This polygonal staining pattern is similar to that of IIID5, which is consistent with the fact that the 230-kD protein is closely associated with the transverse tubules and therefore more abundant in the interior of fast fibers than slow fibers.

Recent work in the field of DMD research has indicated the existence of dystrophin-related proteins that have sequence homology with dystrophin but are encoded by a different gene and therefore are likely to be present in DMD muscle (20, 23, 31). To be certain that we are identifying dystrophin and not dystrophin-related proteins, it is important to confirm the specificity of the antibodies used in our study by immunofluorescence experiments with normal and DMD muscle. This ensures that these antibodies are not cross-reactive with dystrophin-related proteins, spectrin or alpha-actinin (21, 33). Dystrophin-related proteins are most likely not affected in dystrophic muscle and would therefore be detectable in DMD muscle biopsies with cross-reactive antibodies. To establish the specificity of mAb XIXC2 against dystrophin and the polyclonal rabbit antisera against the COOH-terminal decapeptide of dystrophin, cryosections from normal and dystrophic skeletal muscle were tested with

these antibodies. Fig. 5 demonstrates that the cell periphery of normal human muscle cells is strongly labeled by both antibodies against dystrophin, whereas no immunofluorescence staining is observed in biopsy samples from four different DMD patients. In immunoblot analysis the polyclonal rabbit antisera against the COOH-terminal decapeptide, which strongly reacts with normal mouse muscle membranes, exhibited no staining of mdx muscle membranes (15) and mAb XIXC2 strongly labeled a 427-kD protein in normal human muscle extracts but not in biopsy samples from DMD patients (results not shown). These results establish mAb XIXC2 and the polyclonal antisera against the COOH-terminal decapeptide of dystrophin as excellent probes for dystrophin.

Isolation of Crude Surface Membranes from Rabbit Skeletal Muscle

The initial purification steps of isolating skeletal muscle sarcolemma involved the preparation of KCl-washed light microsomes and crude surface membranes. These procedures are outlined in the flow chart of Fig. 6. Sucrose step gradient centrifugation produced a fraction enriched in surface membrane components, as illustrated by the immunoblots of Fig. 7. The relative amounts of surface membrane markers (SL45, SL/TS 230) and components of the dystrophin–glycoprotein complex (dystrophin, SL156, SL50) is increased in crude surface membrane vesicles, as compared with KCl-washed and light microsomes. These results and the relatively high yield of 30 mg crude surface membrane vesicles obtained from 450 g of starting material establish the crude surface membrane fraction from rabbit skeletal muscle as a convenient source of sarcolemma vesicles for further purification steps.

Wheat Germ Agglutination of Sarcolemma Vesicles

Purified sarcolemma vesicles were prepared by a wheat germ agglutination procedure, as outlined in the flow chart of Fig. 8. Aggregation of sarcolemma vesicles by WGA should selectively isolate sealed right-side out vesicles, because of the extracellular position of the carbohydrate chains of membrane glycoproteins. Fig. 9 illustrates the protein composition of the sarcolemma vesicle fraction and the nonagglutinated vesicles, as analyzed by SDS-PAGE, lectin staining, and immunoblotting. The Coomassie blue–stained protein band pattern of the different fractions revealed that the protein composition of the sarcolemma membrane vesicles differed from the crude surface membranes and the nonagglutinated vesicles, especially with respect to the greatly diminished protein band of apparent molecular mass 110 kD and a more intense protein band pattern in the range between 20 and 60 kD (Fig. 9, *Coomassie*). Staining of an identical gel with the cationic carbocyanine dye "Stains-all" showed a greatly diminished staining intensity of Ca^{2+}-binding protein bands of 63 and 110 kD in the sarcolemma membrane fraction, as compared with the starting material and the nonagglutinated vesicles (results not shown). The red stained band of 110 kD and the blue stained band of 63 kD correspond to the Ca^{2+}-ATPase and calsequestrin, both components of the sarcoplasmic reticulum, which appear to be greatly reduced in the sarcolemma membrane fraction. Labeling with ^{125}I-WGA illustrates the enrichment of WGA-positive glycoproteins in the sarcolemma vesicle fraction compared with the starting material of crude surface membranes (Fig. 9, *WGA*). Besides the several glycoproteins in the range between 80 and 170 kD, two dominating WGA-positive glycoproteins of apparent 30 and 460 kD appear to be highly enriched in the sarcolemma vesicle fraction. On the other hand, the non-agglutinated vesicle fraction contained only one major WGA-binding protein of apparent 150 kD. Identical control blots were not labeled with ^{125}I-WGA in the presence of 0.3 M *N*-acetyl-D-glucosamine which demonstrates the specificity of the WGA binding pattern (results not shown).

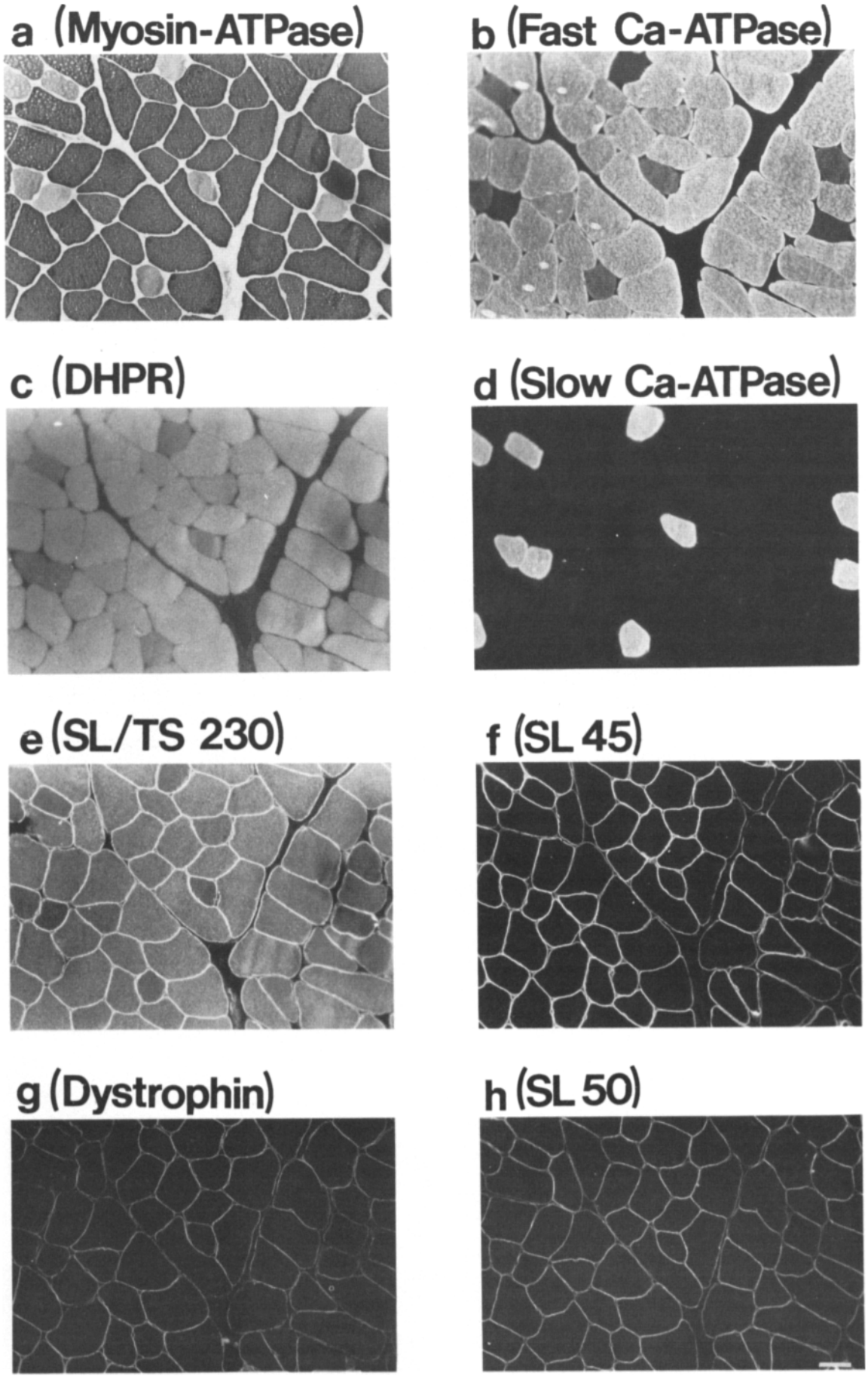
Transverse tubule markers, the α_2-subunit of the dihydropyridine receptor and TS28 protein, appear to be restricted to the nonagglutinated vesicle fraction (Fig. 9, α_2 *DHPR*, *TS28*). These results indicate that the crude surface membrane preparation contains transverse tubule vesicles with a predominant inside-out orientation, which are not agglutinated by WGA and therefore stay in the supernatant. As expected on the basis of these findings, mAb $IXC1_2$ against the SL/TS230 stains both, the nonagglutinated vesicles and the sarcolemma membrane fraction (Fig. 9, *SL/TS 230*). The 230-kD protein seems to be associated with the predominantly right-side-out sarcolemma vesicles and the inside-out transverse tubule vesicles, as already illustrated by the polygonal immunofluorescence pattern of the cell interior and as well staining of the cell periphery (Figs. 1 and 3). Labeling with mAb McB2 against the Na^+/K^+-ATPase revealed that this antigen seems to be also associated with both, the nonagglutinated vesicles and the sarcolemma membrane vesicle fraction (Fig. 9, *Na/K-ATPase*).

mAb XVB9, which appears in immunofluorescence microscopy to be a very good sarcolemma marker (Figs. 1 and 2), labels, in agreement with this result, almost exclusively the sarcolemma vesicle fraction (Fig. 9, *SL45*). Fig. 9 also illustrates that dystrophin and its associated glycoproteins of 156 and 50 kD are almost uniquely associated with the sarcolemma fraction using mAbs XIXC2, $VIA4_1$ and $IVD3_1$ as markers.

Subcellular Localization of Dystrophin Glycoprotein Complex in Isolated Sarcolemma Vesicles

mAbs against previously established sarcolemma and transverse tubule markers and the two novel markers that were introduced in this study were used to characterize the degree of purity of subcellular fractions from rabbit skeletal muscle. Membrane vesicles from light sarcoplasmic reticulum, triads and transverse tubules were isolated by established procedures and sarcolemma vesicles were isolated by wheat germ agglutination as described in Materials and Methods. Membrane fractions were carefully prepared in the presence of a protease inhibitor cocktail and separated on 3–12% SDS-PAGE gel to characterize the range of low and high apparent molecular mass marker proteins of interest in immunoblotting experiments.

The Ca^{2+}-ATPase of apparent 110 kD is a well-defined marker of the sarcoplasmic reticulum and mAb IIH11 against this antigen strongly labeled sarcoplasmic reticulum, triads, and transverse tubules, but only weakly sarcolemma vesicles (Fig. 10, Ca-ATPase). This Ca^{2+}-ATPase seems to be the most frequent contaminant of skeletal muscle membrane



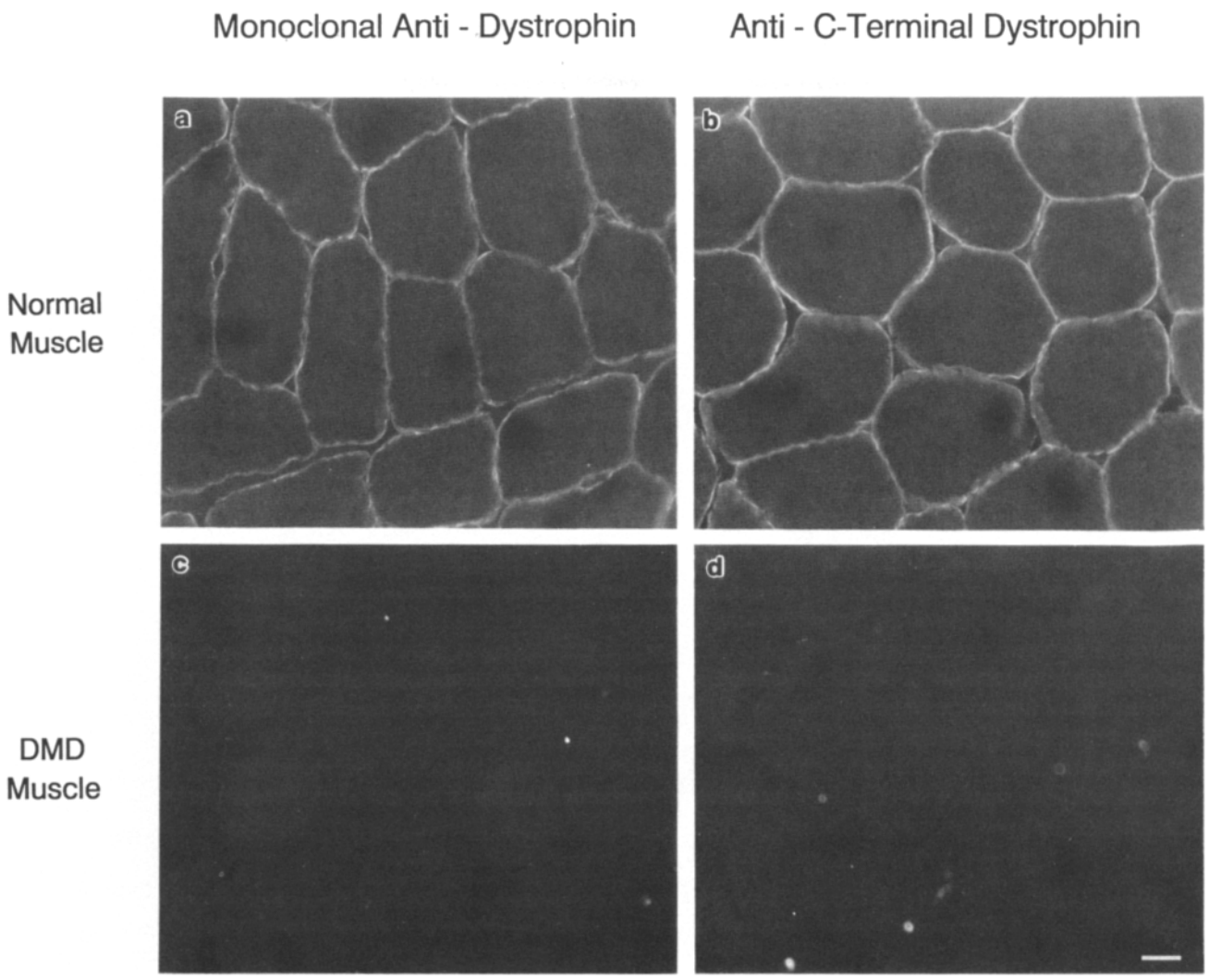


Figure 5. Immunofluorescence labeling of normal and dystrophic human muscle with monoclonal and polyclonal antibodies to dystrophin. Shown are transverse cryosections of normal human skeletal muscle (*a* and *b*) and dystrophic skeletal muscle from a DMD patient (*c* and *d*) labeled with mAb XIXC2 against dystrophin (*a* and *c*) and polyclonal rabbit antisera against the COOH-terminal decapeptide of dystrophin (*b* and *d*). Both antibodies localize dystrophin to the cell periphery of normal human muscle cells and exhibit no labeling of human dystrophic muscle. Bar, 5 μm.

preparations, possibly due to its high abundance. The WGA-agglutinated sarcolemma fraction exhibits, compared with transverse tubule preparations, only small amounts of contaminating components derived from the sarcoplasmic reticulum. To quantitate the amount of contaminating sarcoplasmic reticulum in the sarcolemma fraction, we compared immunoblot staining intensity of various amounts of sarcoplasmic reticulum and triads with sarcolemma. Densitometric scanning of autoradiographs of ^{125}I-secondary antibody labeled anti-Ca^{2+}-ATPase and anti-calsequestrin indicated that the content of sarcoplasmic reticulum in the sarcolemma enriched fraction was ~8% (results not shown).

Staining with mAb XA7 revealed that the ryanodine receptor Ca^{2+}-release channel is restricted to the triad fraction

Figure 4. Distribution of skeletal muscle membrane proteins in type I (slow) and type II (fast) myofiber of rabbit gastrocnemius. Serial transverse cryosections of rabbit gastrocnemius were histochemically stained for alkaline-stable myosin ATPase, a marker for type II (*fast*) myofibers (*a*) and immunofluorescently labeled with mAb IIH11 against fast Ca^{2+}-ATPase (*b*), mAb IIID5 against dihydropyridine receptor (*DHPR*) (*c*), mAb IID8 against slow Ca^{2+} ATPase (*d*), mAb $IXC1_2$ against SL/TS 230 (*e*), mAb XVB9 against SL45 (*f*), mAb XIXC2 against dystrophin (*g*), and mAb $IVD3_1$ against SL50 (*h*). The fibre type distribution of slow and fast Ca^{2+}-ATPase corresponds to the histochemical staining pattern of alkaline-stable myosin ATPase. The internal staining pattern of mAb IIID5 and $IXC1_2$ were both more intense in fast than in slow myofibers, which agrees with the higher abundance of transverse tubules in fast fibers compared with slow fibers. The intensity of labeling of the cell periphery of type I (slow) fibers with mAbs $IXC1_2$, XVB9, XIXC2 and $IVD3_1$ was indistinguishable from that of type II (*fast*) fibers. Bar, 20 μm.

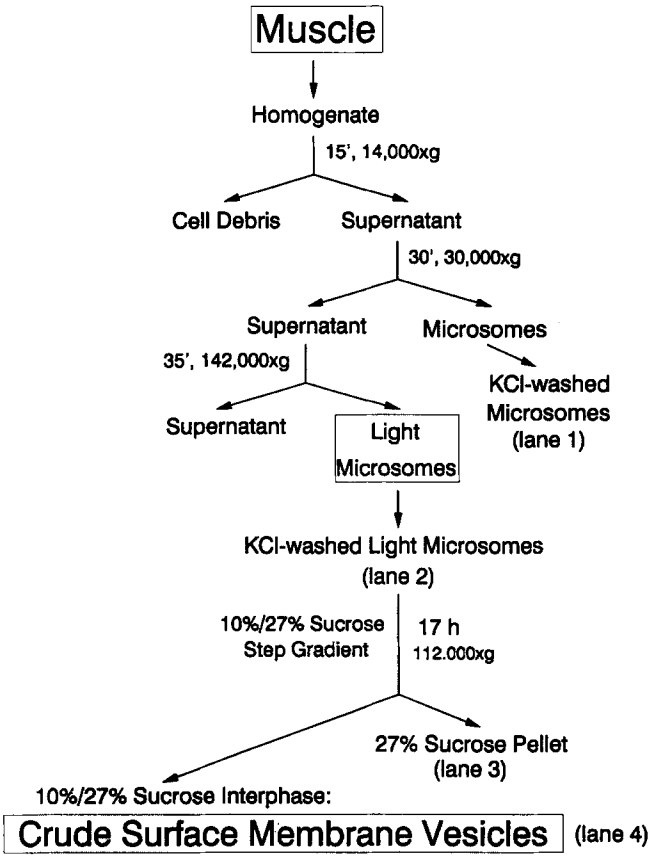


Figure 6. Flow chart of procedure to isolate crude surface membrane vesicles. KCl-washed light microsomes were prepared from rabbit skeletal muscle homogenate by differential centrifugation and subsequently crude surface membrane vesicles isolated by sucrose step gradient centrifugation. Next to the isolated fractions is the lane number in parentheses that will appear in the immunoblot analysis of Fig. 7.

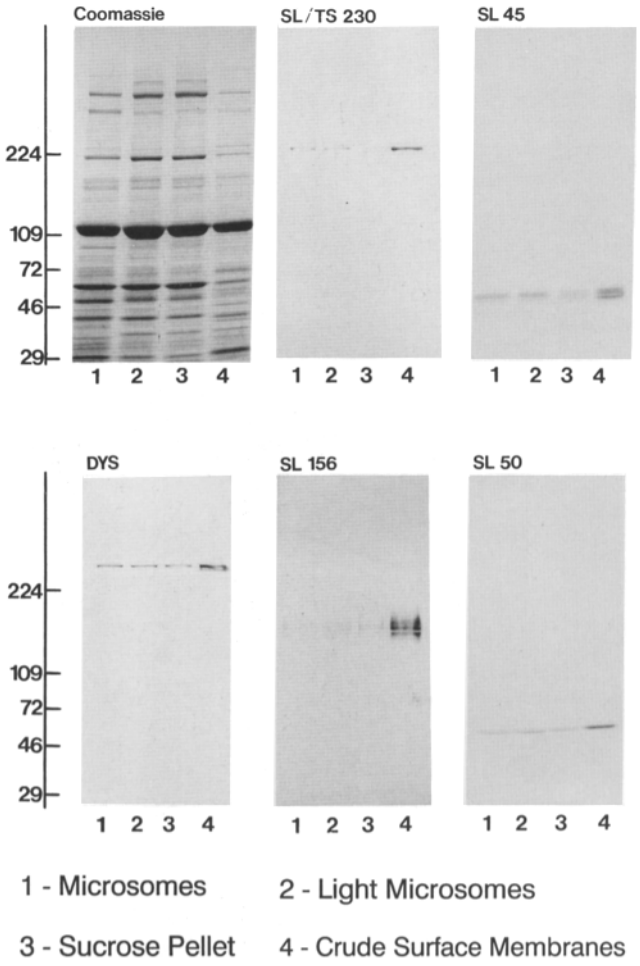


Figure 7. Immunoblot analysis of crude surface membrane preparation. Muscle membranes were prepared as described in Materials and Methods and separated by SDS-PAGE followed by immunoblotting with mAbs. Shown are a Coomassie blue-stained gel and identical immunoblots labeled with mAb $IXC1_2$ against SL/TS 230, mAb XVB9 against SL45, mAb XIXC2 against dystrophin, mAb $VIA4_1$ against SL156, and mAb $IVD3_1$ against SL50. Lanes *1–4* consist of KCl-washed microsomes, light microsomes, pellet from the sucrose-density step gradient and crude surface membranes from rabbit skeletal muscle (50 μg protein/lane). The molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

(Fig. 10, *RyR*). mAb IIID5 to the α_1-subunit of the dihydropyridine receptor labeled strongly the transverse tubule fraction and also triads, but not the purified sarcolemma vesicles, indicating a good removal of t-system from sarcolemma in the WGA agglutination procedure. Furthermore, purified sarcolemma was stained with sarcolemma marker mAb XVB9, and mAb $IXC1_2$ labeled, in accordance with its dual immunofluorescence localization pattern, sarcolemma, transverse tubule, and triad vesicles (Fig. 10, *SL/TS230*). mAb McB2 to the Na^+/K^+-ATPase, which is generally considered a surface membrane marker, exhibited labeling of triads, transverse tubules and purified sarcolemma (Fig. 10, *Na/K-ATPase*). Immunofluorescence labeling of skeletal muscle cryosections did not show strong enough labeling to clarify the localization of this antigen.

The initial screening of the four subcellular fractions from rabbit skeletal muscle established the high degree of purity of the sarcolemma preparation used in this study. The preparation is essentially free of triads and transverse tubules and contains only small amounts of sarcoplasmic reticulum vesicles, much less than for example the transverse tubule preparation. The yield of crude and purified sarcolemma vesicles from 450 g of skeletal muscle was ~30 and 4 mg protein, respectively. This makes the WGA agglutination procedure described in this investigation a good method to isolate purified sarcolemma vesicles in a sufficiently high yield to characterize the components of the dystrophin–glycoprotein complex in plasma membrane vesicles.

Identical immunoblots, which were used for the initial screening of the above characterized subcellular fractions of rabbit skeletal muscle, were labeled with mAbs XIXC2, $VIA4_1$ and $IVD3_1$ against the dystrophin–glycoprotein complex (Fig. 10). Strong staining of the protein bands for dystrophin, the 156-kD glycoprotein and 50-kD glycoprotein were found in the purified sarcolemma fraction and some weak labeling in the transverse tubule fraction. These results agree with the immunofluorescence labeling of the cell periphery with the same mAbs (Fig. 2) and indicate that the components of the dystrophin–glycoprotein complex are

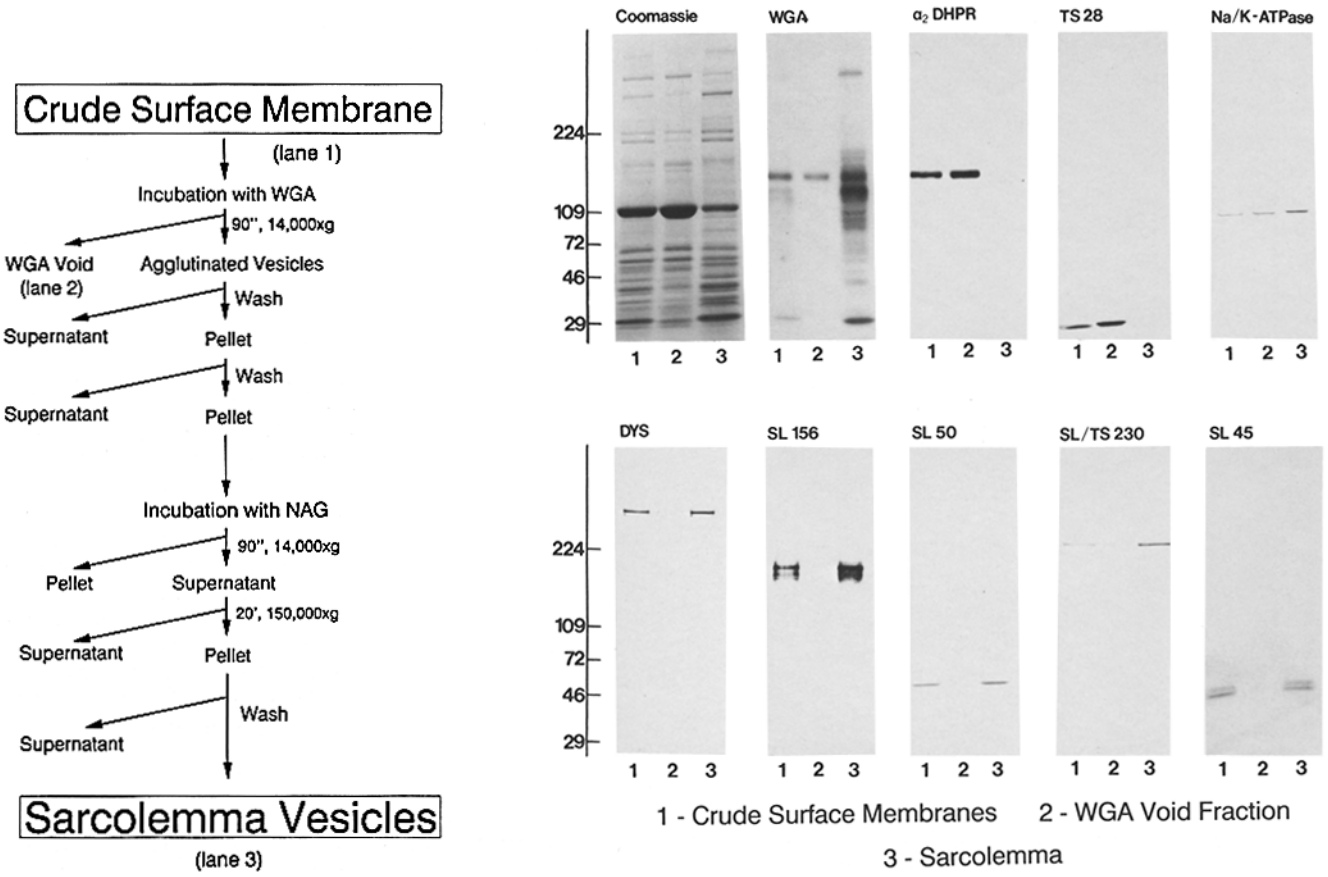


Figure 8. Flow chart of wheat germ agglutination procedure to isolate purified sarcolemma vesicles. Crude surface membranes were incubated with WGA and subsequently the agglutinated sarcolemma vesicles separated from the WGA void fraction, washed and finally deagglutinated by incubation with *N*-acetyl-D-glucosamine. Next to the isolated fractions is the lane number in parentheses that will appear in the immunoblot analysis of Fig. 9.

Figure 9. Immunoblot analysis of purified sarcolemma preparation. Shown are a Coomassie blue–stained gel and identical immunoblots labeled with ^{125}I-WGA, polyclonal guinea pig antisera against the α_2-subunit of dihydropyridine receptor (*DHPR*), mAb IXE11$_2$ against TS28, mAb McB2 against Na^+/K^+-ATPase, mAb XIXC2 against dystrophin, mAb VIA4$_1$ against SL156, mAb IVD3$_1$ against SL50, mAb IXC1$_2$ against SL/TS 230, and mAb XVB9 against SL45. Lanes *1–3* consist of crude surface membrane, WGA void fraction and purified sarcolemma from rabbit skeletal muscle (50 μg protein/lane). Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

highly enriched in the purified sarcolemma fraction and are probably absent or present only in very low abundance in the transverse tubule system.

Dystrophin Is a Major Component of Isolated Sarcolemma Membranes

To estimate the amount of dystrophin in the skeletal muscle plasma membrane we analyzed purified sarcolemma by densitometric scanning of Coomassie blue–stained gels. Fig. 11, *a* and *b* illustrates the Coomassie blue–stained protein band pattern of purified rabbit skeletal muscle sarcolemma separated by 3–12% SDS-PAGE, and the corresponding immunoblot staining of the dystrophin protein band of apparent molecular mass 427 kD using mAb XIXC2. This mAb was established to be highly specific for dystrophin by immunofluorescence analysis of normal control and DMD muscle samples, as shown in Fig. 5. Peak integration of densitometric scans of isolated rabbit skeletal muscle sarcolemma (Fig. 11 *c*) revealed that the protein band of apparent 427 kD accounts for 2.1 ± 0.7 (n = 8) % of the total protein of this membrane system. To account for possible variations in Coomassie blue staining intensity relative to the amount of total protein separated by SDS-PAGE, gels with different amounts of sarcolemmal protein were analyzed by densitometric scanning and the values averaged. Immunoadsorption experiments with XIXC2 immunoaffinity beads quantitatively remove the 427-kD band from digitonin-solubilized sarcolemma (results not shown). Therefore, the sarcolemmal protein band of apparent 427 kD is exclusively dystrophin. Our work is the first to demonstrate that dystrophin is a discrete sarcolemmal protein band in Coomassie blue–stained gels and is therefore a major constituent of the skeletal muscle plasma membrane.

The immunoblot analysis of WGA-agglutinated sarcolemma vesicles from rabbit skeletal muscle revealed that the dominant protein band of apparent 110-kD contains partly the Ca^{2+}-ATPase of sarcoplasmic reticulum and the Na^+/K^+-ATPase. The 210-kD band is myosin according to immunoblot staining, using mAb MF20 (results not shown) and the 230-kD band corresponds to the SL/TS 230 protein newly introduced in this study as a sarcolemma/transverse tubule marker. The dense protein pattern of lower apparent

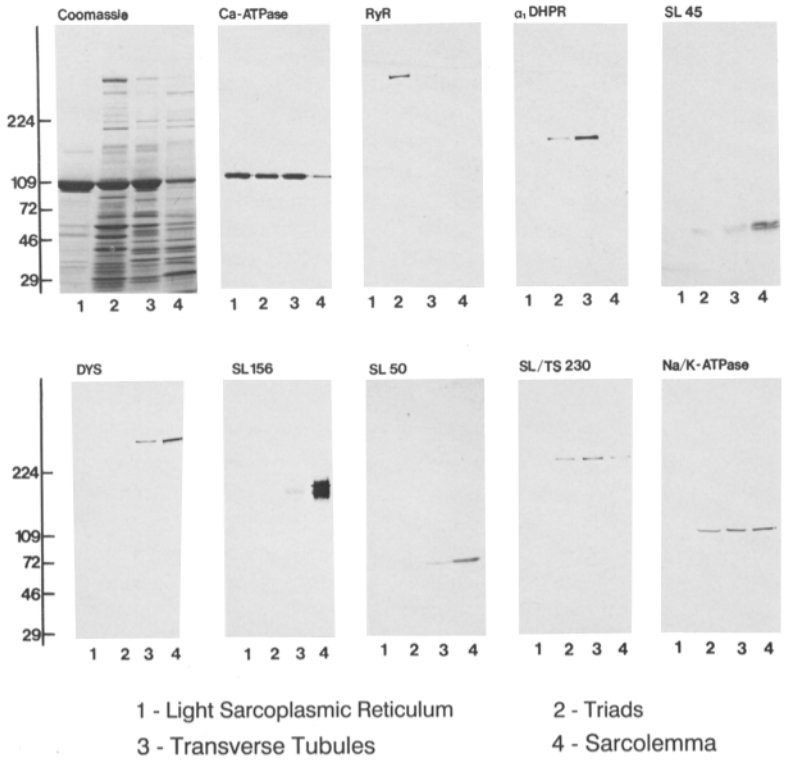


Figure 10. Immunoblot analysis of subcellular fractions from rabbit skeletal muscle. Shown is a Coomassie blue-stained gel and identical blots labeled with mAb IIH11 against Ca^{2+}-ATPase, mAb XA7 against ryanodine receptor (*RyR*), mAb IIF7 against the α_1-subunit of dihydropyridine receptor (*DHPR*), mAb XVB9 against SL45, mAb XIXC2 against dystrophin, mAb $VIA4_1$ against SL156, mAb $IVD3_1$ against SL50, mAb $IXCl_2$ against SL/TS 230, and mAb McB2 against Na^+/K^+-ATPase. Lanes *1–4* consist of light sarcoplasmic reticulum, triads, transverse tubules and sarcolemma from rabbit skeletal muscle (50 μg protein/lane). Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

molecular mass bands does not allow a perfect match of immunoblot labeling with mAb XVB9 against SL45 and $IVD3_1$ against SL50 and Coomassie blue–stained protein bands. Actin of 42 kD is also present in the purified sarcolemma, as detected by immunoblotting with mAb 5C5 (results not shown).

Discussion

This study describes the isolation and characterization of sarcolemma membranes from rabbit skeletal muscle using mAbs as markers. mAbs against skeletal muscle membrane proteins were first characterized by immunofluorescence labeling of muscle cryosections and then used in the characterization of purified sarcolemma. The mAbs used in this study label antigens with a very specific localization in skeletal muscle cryosections. mAb XVB9 recognizes a sarcolemma-specific protein of apparent 45 kD and mAb $IXCl_2$ labels a protein of apparent 230 kD, which is localized in the sarcolemma and the transverse tubules. Furthermore, the components of the dystrophin–glycoprotein complex are excellent sarcolemma markers as demonstrated by restricted sarcolemmal immunofluorescence labeling of their respective mAbs. Other marker proteins of various muscle membranes used in this investigation were characterized previously. Whereas the α_1 and α_2-subunit of the dihydropyridine receptor and TS28 protein are localized in the transverse tubules system (26, 27) the ryanodine receptor seems to be associated with triads (22) and the Ca^{2+}-ATPase is restricted to the sarcoplasmic reticulum (24). We used this strategy to estimate the purity of our sarcolemma preparation because it is advantageous to conventional marker enzyme assays. Most estimations of the purity of plasma membrane preparations rely on the use of marker enzyme activity (3, 40, 41). However, most surface membrane marker enzymes used are not uniquely associated with the sarcolemma, which causes problems in the interpretation of marker enzyme activities in subcellular fractionation studies. Both Na^+/K^+-ATPase and 5′-nucleotidase, which are widely used as plasma membrane markers, are not restricted to the cell periphery in skeletal muscle (37).

Most skeletal muscle sarcolemma preparations employ sucrose density gradient centrifugation and plasma membrane vesicles appear to be associated with light membrane fractions (3, 32, 40, 41). However, our immunoblot analysis indicates that crude sarcolemma preparations, isolated by density gradients, are strongly contaminated with components of the sarcoplasmic reticulum (results not shown). Therefore, a specific method for the isolation of sarcolemma is needed that relies on the structure and not density of the sarcolemma membrane. A wheat germ agglutination procedure was recently successfully used by Charuk et al. (12) in the subfractionation of cardiac sarcolemma and was applied in this study in a modified version to circumvent the above-mentioned problems in the purification of sarcolemma vesicles from rabbit skeletal muscle. The WGA-agglutinated vesicle fraction appeared to be highly enriched in sarcolemma markers and contained only small amounts of sarcoplasmic reticulum contaminations, and was essentially free of transverse tubules vesicles. Chang et al. (11) used ^{125}I-WGA as a specific plasma membrane marker in fractionation studies of rat tissue and found that WGA was a very suitable label of cell surface receptors. The enrichment of a 30-kD protein band in purified sarcolemma vesicles agrees with the SDS-PAGE analysis of skeletal muscle plasma membrane vesicles, isolated by the method of Seiler and Fleischer (40).

Immunoblot analysis of subcellular fractions from rabbit skeletal muscle demonstrate that components of the dystro-

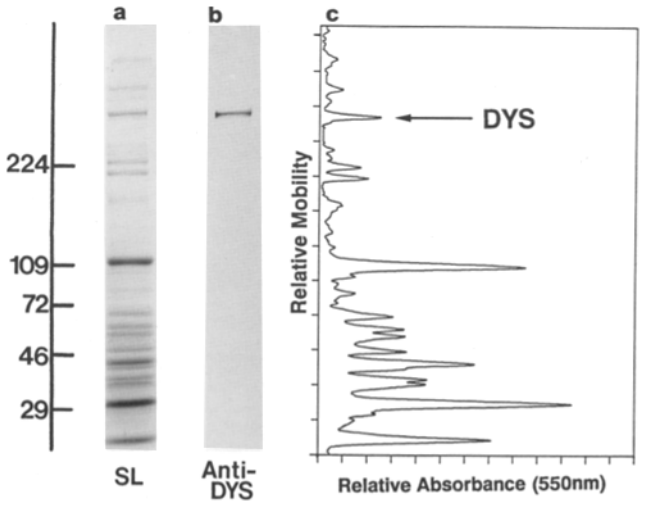


Figure 11. Densitometric scanning of SDS-PAGE of purified rabbit skeletal muscle sarcolemma. Shown is a Coomassie blue–stained gel of purified skeletal muscle sarcolemma (50 μg protein) (*SL*) (*a*), an identical immunoblot stained with mAb XIXC2 against dystrophin (*Anti-DYS*), (*b*) and a corresponding densitometric scan of the protein band pattern (*c*). The dystrophin band of apparent 427 kD (*arrow*) made up 2% of total rabbit sarcolemma protein and is therefore a major component of the purified sarcolemma fraction. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

phin–glycoprotein complex are highly enriched in sarcolemma vesicles. mAb XIXC2, $VIA4_1$ and $IVD3_1$ against dystrophin and its closely associated glycoproteins of apparent 156 and 50-kD, strongly labeled the purified sarcolemma fraction and also stained exclusively the cell periphery of transversely and longitudinally cut muscle cryosections. The 50-kD glycoprotein was already previously identified as a very convenient sarcolemma marker (27). The finding, that dystrophin is associated with the muscle surface membrane, agrees with cell fractionation studies of Salviati et al. (38). Here we report for the first time in subcellular fractionation studies that two dystrophin-associated glycoproteins are restricted to membrane vesicles which originated from the cell periphery.

Dystrophin is an extremely minor component of total skeletal muscle protein (18). However, our present immunoblot analysis and immunoadsorption data indicate that the 427-kD protein band of dystrophin accounts for two percent of the protein from isolated sarcolemma vesicles. This high abundance of dystrophin in the membrane cytoskeleton of skeletal muscle cells is comparable to that of the major membrane cytoskeleton component spectrin in other cell types (10). An elaborate plasma membrane skeleton is found in erythrocytes and spectrin appears to be the major component of this membrane skeleton (44). The predicted amino acid sequence of dystrophin indicates a long spectrin-like central rod domain of this molecule, which suggests an association of dystrophin with the subsarcolemmal cytoskeleton (29). Possibly dystrophin is a major component of the subsarcolemmal cytoskeletal network, which can be an important factor for the flexibility and integrity of the surface membrane during muscle contraction (28).

Our former finding of a substantial reduction of the SL156 glycoprotein from muscle of mdx mice and DMD patients (15) and the relatively high concentration of dystrophin in skeletal muscle sarcolemma reported in this study is analogous to results in erythrocytes of patients afflicted with hereditary elliptocytosis (1). Possibly in both diseases, the absence of a major cytoskeletal component can lead to the disruption of the cytoskeletal network causing fatal destabilization of the plasma membrane and associated proteins. Elevated intracellular Ca^{2+} levels and corresponding higher net degradation of muscle proteins in mdx mice (47) may be explained by the loss of a dystrophin-associated protein in the sarcolemma, which is involved in Ca^{2+} homeostasis. Dystrophin and its associated glycoproteins may be important factors in linking the cytoskeleton to the extracellular matrix, preserving plasma membrane stability, or maintaining a nonuniform distribution of low abundance glycoproteins, possibly ion channels or cell surface receptors.

Fast-twitch skeletal muscle fibers are affected earlier in muscle from DMD patients than slow-twitch fibers (50). Immunofluorescence localization studies of Schafer and Stockdale (39) identified sarcolemma-associated antigens with different distribution in fast and slow skeletal muscle fibers. Variability of staining intensity among fibers was also found for a sarcolemmal Na^+/K^+-ATPase in chicken muscle (16). Therefore, we have examined the fiber-type distribution of the dystrophin–glycoprotein complex. The three components of the dystrophin–glycoprotein complex investigated in this study are equally distributed between fast- and slow-twitch fibers, as illustrated by immunofluorescence labeling of serial, transverse cryosections of mixed fiber composition with mAbs. It remains to be determined why the fiber type plays a role in the early steps of abnormal muscle protein degradation and fiber necrosis in dystrophic muscle.

We conclude that the components of the dystrophin–glycoprotein complex are highly enriched in the skeletal muscle sarcolemma and that dystrophin, which is a minor component of total muscle protein, is a major constituent of the sarcolemma. Thus, the absence of dystrophin in DMD may lead to instability of the sarcolemma membrane due to its major role in the sarcolemma cytoskeleton. This destruction of sarcolemmal integrity may be the initial step in the molecular pathogenesis of muscular dystrophy.

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