Dystrophin-Glycoprotein Complex and Laminin Colocalize to the Sarcolemma and Transverse Tubules of Cardiac Muscle

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The expression and subcellular distribution of the dystrophin-glycoprotein complex and laminin were examined in cardiac muscle by immunoblot and immunofluorescence analysis of rabbit and sheep papillary muscle. The five dystrophin-associated proteins (DAPs), 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG, were identified in rabbit ventricular muscle and found to codistribute with dystrophin in both papillary myofibers and Purkinje fibers. The DAPs and dystrophin codistributed not only in the free surface sarcolemma but also in interior regions of the myofibers where T tubules are present. Neither the DAPs nor dystrophin were detected in intercalated discs, a specialized region of cardiac sarcolemma where neighboring myocardial cells are physically joined by cell–cell junctions. Similarly, in bundles of Purkinje fibers, which lack T tubules, DAPs and dystrophin were also found to codistribute at the free surface sarcolemma but were not detected either in the region of surface sarcolemma closely apposed to a neighboring Purkinje fiber or in interior regions of these myofibers. Comparison between the distribution of the dystrophin-glycoprotein complex and laminin showed that laminin codistributes with the components of this complex in both papillary myofibers and Purkinje fibers. These results are consistent with previous findings demonstrating that the extracellularly exposed 156-DAG (dystroglycan) of the skeletal muscle dystrophin-glycoprotein complex binds laminin, a component of the basement membrane. Although we demonstrate that DAPs, dystrophin, and laminin colocalize to the sarcolemma in rabbit and sheep papillary myofibers as they do in skeletal myofibers, the most striking difference between the subcellular distribution of these proteins in cardiac and skeletal muscle is that the dystrophin-glycoprotein complex and laminin also localize to regions of the fibers where T tubules are distributed in cardiac but not in skeletal muscle. These results imply that the protein composition and thus possibly some functions of T tubules in cardiac muscle are distinct from those of skeletal muscle. (*Circulation Research* 1993;72:349–360)

KEY WORDS • **dystrophin-glycoprotein complex • cardiac muscle • sarcolemma • transverse tubules • immunocytochemical labeling**

Dystrophin, the protein encoded by the Duchenne muscular dystrophy (DMD) gene, is most prominently expressed in skeletal and cardiac muscle.[1] Biochemical and immunochemical studies have shown that dystrophin isolated from rabbit skeletal muscle membranes is a component of a large oligomeric complex containing four associated glycoproteins (DAGs) of apparent M_r of 156,000, 50,000, 43,000, and 35,000, a protein triplet of 59,000, and a protein of 25,000.[2–6] Immunocytochemical studies have shown that dystrophin[2,6–11] and the dystrophin-associated proteins (DAPs) of 156 kd (156-DAG), 50 kd (50-DAG), 43 kd (43-DAG), and 35 kd (35-DAG) as well as the 59-kd protein triplet (59-DAP) are restricted to the cell periphery and apparently absent from transverse (T) tubules of skeletal muscle fibers.[2,5,6,12] Consistent with these results, it has been demonstrated that dystrophin[5,6,13] and its tightly associated proteins are highly enriched in purified skeletal muscle sarcolemma (SL).[5,6]

A study of the membrane organization of the dystrophin-glycoprotein complex[5] demonstrated that the intracellular dystrophin and 59-DAP are tightly linked to the extracellular 156-DAG (dystroglycan) via an oligomeric transmembrane complex composed of 50-DAG, 43-DAG, 35-DAG, and 25-DAG. Since dystrophin has some sequence homology with the actin binding protein α-actinin,[14] these previous studies have supported the idea that dystrophin via its associated proteins bridge the actin filament containing cytoskeleton to external components of the sarcolemma.[5] In support of this hypothesis, it has recently been demonstrated that 156-DAG (dystroglycan) in skeletal muscle binds laminin, a component of the basal lamina,[15] and that the

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A.O.J. is a recipient of a grant-in-aid from the Heart and Stroke Foundation of Ontario, Canada. K.P.C. is an Investigator of the Howard Hughes Medical Institute and a recipient of National Institutes of Health grant HL-14388 and a grant from the Muscular Dystrophy Association. R.K. is a recipient of a Research Traineeship Award from the Canadian Heart and Stroke Foundation. J.M.E. is the Carl M. Pearson Fellow of the Muscular Dystrophy Association.

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Received December 24, 1991; accepted October 20, 1992.

TABLE 1. Specificity of Primary Antibodies

Antigen		Antibody		Tissue cryosection specificity/feasibility			
Name	Species	Name	Type	Rabbit	Sheep	Dog	References
156-DAG	Rabbit	DAG156	Sheep AP	+	NF	...	15
59-DAP	Rabbit	DAP59	Sheep AP	+	NF	ND	23
50-DAG	Rabbit	DAG50	Sheep AP	+	NF	ND	23
		$IVD3_1$	Mouse MAb	+	+	+	2, 12
43-DAG	Rabbit	DAG43	Sheep AP	+	NF	ND	23
35-DAG	Rabbit	DAG35	Sheep AP	+	NF	ND	23
Dystrophin	Human (skeletal, C-terminus)	...	Rabbit AP	NF	+	+	2
Laminin	Mouse (EHS sarcoma)	...	Rabbit serum	NF	+	ND	27

DAG, dystrophin-associated glycoprotein; AP, affinity-purified antibody; +, feasible; NF, not feasible; DAP, dystrophin-associated protein; ND, not determined; MAb, monoclonal antibody; EHS, Engelbreth-Holm-Swarm.

NH_2-terminal region of dystrophin is both structurally and functionally homologous to the NH_2-terminal region of α-actinin.[16]

Since DMD affects the performance of skeletal muscle fibers quite severely, major efforts have focused on understanding the structure and function of dystrophin as well as its interaction with the DAPs in skeletal muscle. Nonetheless, patients with DMD as well as female carriers of the DMD gene have a much higher chance of developing cardiomyopathy than normal individuals, suggesting that the lack of dystrophin in cardiac muscle cells may also impair the function of the heart in these individuals.[17]

At present, our knowledge about the structure, function, and subcellular distribution of the dystrophin-glycoprotein complex in cardiac muscle tissues is relatively limited. Biochemical[18] and immunocytochemical studies[7,19–21] have suggested that dystrophin in cardiac muscle as in skeletal muscle is closely associated with the surface SL but absent from T tubules and intercalated discs. Northern blotting and immunoblotting studies have so far only detected the expression of rabbit ventricular muscle proteins analogous to 156-DAG and 43-DAG in skeletal muscle.[15] To determine whether cardiac muscle contains other DAPs analogous to those in skeletal muscle and, if so, whether one or more of these DAPs codistribute with dystrophin in this tissue, the subcellular distribution of 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG in rabbit and sheep papillary myofibers as well as in sheep Purkinje fibers was determined and compared with that of dystrophin by single and double immunofluorescence labeling. Since 156-DAG binds to laminin, the distribution of laminin and components of the dystrophin-glycoprotein complex were also compared.

Our results demonstrate that DAPs, dystrophin, and laminin codistribute not only at the free surface sarcolemma but also in regions where T tubules are present. These results are consistent with the idea that free surface sarcolemma and T tubules in cardiac myofibers have several membrane proteins in common that are localized to the sarcolemma but are absent from T tubules in skeletal muscle, implying that the protein composition and possibly the function of cardiac T tubules is distinct from that of skeletal T tubules.

Materials and Methods

Dissection, Cryosection, and Sectioning of Sheep and Canine Myocardium

Tissues from adult rabbit, sheep, and canine papillary muscles and from rabbit and sheep diaphragm muscles were dissected, quickly frozen in liquid nitrogen–cooled isopentane, and stored in liquid nitrogen. Cryosections (6–8 μm) were either cut and stored as previously described[22] or cut and collected on Superfrost Plus microscope slides (Fisher Scientific, Toronto), air-dried, and used within 6 hours.

Preparation of Microsomes From Cardiac and Skeletal Muscle

Microsomes from rabbit, sheep, and canine ventricular muscle as well as from rabbit psoas muscle were prepared as described by Ohlendieck et al,[6] except that cardiac tissues were homogenized three times for 30 seconds each in 4 vol buffer A (20 mM sodium pyrophosphate, 20 mM sodium phosphate monohydrate, 1 mM $MgCl_2$, 0.303 M sucrose, and 0.5 mM EDTA, pH 7.0) in the presence of the following protease inhibitors: aprotinin (76.8 μM), leupeptin (1.1 μM), pepstatin A (0.7 μM), benzamidine (76.8 μM), iodoacetamide (1 mM), and phenylmethylsulfonyl fluoride (0.23 mM). An Ultra-Turrox homogenizer was used for the preparations.

Antibodies: Preparation and Characterization

The specificity and characterization of the primary antibodies used in the present studies are summarized in Table 1.

Monoclonal Antibodies

50-DAG. Monoclonal antibody (MAb) $IVD3_1$ to the 50-kd glycoprotein (50-DAG) of the dystrophin-glycoprotein complex of rabbit skeletal muscle was prepared in the mouse, and its specificity for rabbit skeletal sarcolemma was characterized as previously described.[2,12]

Polyclonal Antibodies

156-DAG. Preparation and characterization of sheep affinity-purified antibodies to fusion protein D (156-DAG) was carried out as described by Ibraghimov-Beskrovnaya et al.[15]

59-DAP, 50-DAG, 43-DAG, and 35-DAG. Preparation and characterization of sheep affinity-purified antibodies specific for the 59-, 50-, 43-, and 35-kd DAPs were carried out according to the procedures described by Ohlendieck and Campbell.[23]

Dystrophin. Polyclonal antibodies to the C-terminal decapeptide of the predicted human skeletal muscle dystrophin (Pro-Gly-Lys-Pro-Met-Arg-Gln-Asp-Thr-Met)[14] were produced in rabbits and characterized as previously described.[2] Affinity-purified antibodies to this decapeptide of dystrophin were prepared as previously described[22,24] using the purified decapeptide photochemically coupled to bovine serum albumin[25] and blotted onto Immobilon-P. The affinity-purified antibodies to dystrophin do not cross-react with a synthetic peptide corresponding to the C-terminal 12 amino acids of the chromosome 6 dystrophin-related protein.[26]

Laminin. Polyclonal rabbit antiserum to laminin purified from mouse Engelbreth-Holm-Swarm (EHS) sarcoma was purchased from GIBCO Laboratories, Mississauga, Canada. This antibody is specific for EHS laminin previously shown to contain epitopes shared with mouse heart laminin.[27]

Gel Electrophoresis and Immunoblotting

Membrane proteins were separated on 3–12% sodium dodecyl sulfate (SDS)–polyacrylamide gels[28] and transelectrophoresed to nitrocellulose paper according to the general method of Towbin et al[29] using 5% nonfat dry milk in Tris-buffered saline (TBS, Blotto, 100 mM Tris, 2.5 mM sodium chloride, pH 7.5) for blocking the immunoblots as described by Johnson et al.[30]

Immunoblotting procedure A. Immunoblot staining with MAb $IVD3_1$ to 50-DAG or rabbit antiserum to the C-terminal decapeptide of human skeletal dystrophin was carried out as previously described,[12] except that a double-layered procedure was used.

Immunoblotting procedure B. Immunoblot staining with affinity-purified antibodies to each of the following DAPs—156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG—was carried out as previously described.[2]

Indirect Cytochemical Fluorescence Labeling

Single[31] and double[32] fluorescence labeling of 6–8-μm cryosections of unfixed muscle tissues was carried out as previously described and is briefly outlined below.

Single labeling. Sections were first labeled with one of the following primary reagents: 1) MAb $IVD3_1$ to rabbit skeletal protein 50-DAG; 2) affinity-purified rabbit antibodies to the C-terminal decapeptide of human dystrophin (50 μg/ml); 3–7) affinity-purified sheep antibodies to 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG; 8) wheat germ agglutinin (WGA) conjugated to biotin (5 μg/ml, Vector Laboratories, Inc., Burlingame, Calif.); and 9) polyclonal rabbit antiserum to laminin purified from mouse EHS sarcoma (1/100, GIBCO). Sections were subsequently labeled with the corresponding secondary reagents: 1) the gamma globulin fraction of goat anti-mouse heavy chain immunoglobulin (Ig) G_1, conjugated to Texas Red (12.5 μg/ml, Sera Lab, Copthorne, England); 2) affinity-purified $F(ab)_2$ fragments of donkey anti-rabbit gamma-globulin conjugated to Texas Red or fluorescein (20 μg/ml, Jackson ImmunoResearch Laboratories, Inc., Westgrove, Pa.); 3–7) affinity-purified $F(ab')_2$ fragments of rabbit anti-sheep Fc fragment, conjugated to fluorescein (12.5 μg/ml, Jackson ImmunoResearch Laboratories); 8) streptavidin conjugated to Texas Red (10 μg/ml, Vector Laboratories); and 9) affinity-purified $F(ab)_2$ fragments of donkey anti-rabbit gamma globulin conjugated to fluorescein (20 μg/ml, Jackson ImmunoResearch Laboratories).

For the control assay, affinity-purified antibodies (50 μg/ml) to the C-terminal decapeptide of dystrophin were incubated for adsorption as previously described with 0 and 1.25 μg/ml of the purified C-terminal decapeptide of dystrophin conjugated to bovine serum albumin (17:1).[25] Thus, the molar ratio of peptide/antibody was ≈4 for the adsorption. The supernatants obtained by centrifugation were used as the primary reagent in the single indirect immunolabeling assay.

As a negative control for mouse MAb $IVD3_1$ (IgG_1) to 50-DAG, mouse MAb IIH11 (IgG_1) specific for the Ca^{2+}-ATPase of the sarcoplasmic reticulum in fast skeletal muscle was used.[33] Furthermore, as a control for the specificity of WGA (5 μg/ml), WGA was preincubated with *N*-acetyl-D-glucosamine (200 mM, No. A3286, Sigma Chemical Co., St. Louis, Mo.) for 30 minutes before fluorescence labeling.

Double labeling. Double labeling of 6–8-μm cryosections was carried out in sequential steps as follows.

For 50-DAG and dystrophin, double-labeling procedure A was composed of single-labeling procedure 1 for 50-DAG followed by single-labeling procedure 2 for dystrophin, except that fluorescein-conjugated secondary antibodies (10 μg/ml, affinity-purified IgG of goat anti-mouse IgG_1, Sera Lab) were used to label 50-DAG.

For 50-DAG and WGA, double-labeling procedure B was composed of single-labeling procedure 1 for 50-DAG and procedure 8 for labeling with WGA, except that fluorescein-conjugated secondary antibodies (10 μg/ml, affinity-purified gamma globulin of goat anti-mouse IgG_1, Sera Lab) were used to label 50-DAG.

For dystrophin and WGA, double-labeling procedure C was composed of single-labeling procedure 2 for dystrophin and procedure 8 for labeling with WGA, where fluorescein-conjugated secondary antibodies (20 μg/ml, affinity-purified donkey antibodies to rabbit IgG, Jackson ImmunoResearch Laboratories) were used to label dystrophin.

For 50-DAG and laminin, double-labeling procedure D was composed of single-labeling procedure 1 for 50-DAG followed by single-labeling procedure 9 for laminin.

For laminin and WGA, double-labeling procedure E was composed of single-labeling procedure 9 for laminin and procedure 8 for labeling with WGA.

Imaging

Conventional fluorescence microscopy was carried out with a Zeiss photomicroscope provided with an epifluorescence attachment and a phase-contrast condenser. Confocal fluorescence microscopy was carried out with a photomicroscope (Nikon Canada Inc., Mississauga, Canada) provided with a confocal fluorescence imaging system[34] (Laser Sharp MRC, Bio-Rad Laboratories Ltd.) using a krypton-argon laser for illumination.

Results

Identification of Cardiac DAPs

It has previously been reported that the skeletal muscle dystrophin-glycoprotein complex is composed of 156-DAG, 59-DAP, 50-DAG, 43-DAG, 35-DAG, 25-DAG, and dystrophin and that the components of this complex codistribute at the surface sarcolemma but are absent from T tubules in skeletal muscle.[2,6,12] To determine whether DAPs analogous to skeletal DAPs are also present in cardiac muscle, rabbit cardiac and skeletal microsomes separated and transblotted by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 1A) were stained with affinity-purified sheep antibodies to rabbit skeletal 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG. Bands with apparent M_rs of 59,000, 50,000, and 35,000, respectively, were identified in rabbit cardiac microsomes (Figure 1A). Except for the 152,000 cardiac protein detected by antibodies to the skeletal 156-DAG, the apparent M_r values of the cardiac proteins identified by antibodies to each of the skeletal DAPs are indistinguishable. These results show that rabbit cardiac microsomes contain proteins with similar apparent M_rs that are antigenically related to each of the components of the dystrophin-glycoprotein complex in skeletal muscle. Furthermore, the relative abundance of each of these DAPs in skeletal microsomes (Figure 1A) appears to be very similar to that of the corresponding proteins in cardiac muscle (Figure 1A). Similarly, SDS-PAGE–separated and –transblotted sheep, canine, and rabbit cardiac microsomes were stained with MAb IVD3$_1$ to rabbit skeletal 50-DAG. The results presented (Figure 1B) show that MAb IVD3$_1$ to 50-DAG detected a protein doublet with apparent M_rs of 50,000 and 52,000 in sheep microsomes (Figure 1B, lane 1) and a protein with an M_r of 50,000 in rabbit cardiac microsomes (Figure 1B, lane 3). No band was detected in canine cardiac microsomes by MAb IVD3$_1$ (Figure 1B, lane 2). These results strongly support the conclusion that MAb IVD3$_1$ principally identifies a 50-kd protein in rabbit and a 50–52-kd protein doublet in sheep cardiac microsomes antigenically related to the 50-kd glycoprotein subunit of the dystrophin-glycoprotein complex in skeletal muscle called 50-DAG.

The antigens in rabbit cardiac muscle labeled by the affinity-purified sheep antibodies to skeletal 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG will be referred to as 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG. Similarly, the antigens in sheep and rabbit cardiac muscle defined by MAb IVD3$_1$ will be referred to as 50-DAG.

Specificity of Antibodies to Skeletal Dystrophin Toward Dystrophin in Cardiac Muscle Microsomes

Immunoblotting of SDS-PAGE–separated and –transblotted microsomal membranes from sheep and canine ventricular muscle showed that dystrophin antiserum also specifically labels a single band in cardiac microsomes from sheep (Figure 1C, lane 1) ventricular muscle. This band has an apparent M_r of 400,000 corresponding to that of dystrophin in canine cardiac microsomal membranes (Figure 1C, lane 2). This finding strongly supports the conclusion that the antiserum to the C-terminal decapeptide of human dystrophin specifically binds to dystrophin in microsomes isolated from rabbit and sheep ventricular muscle.

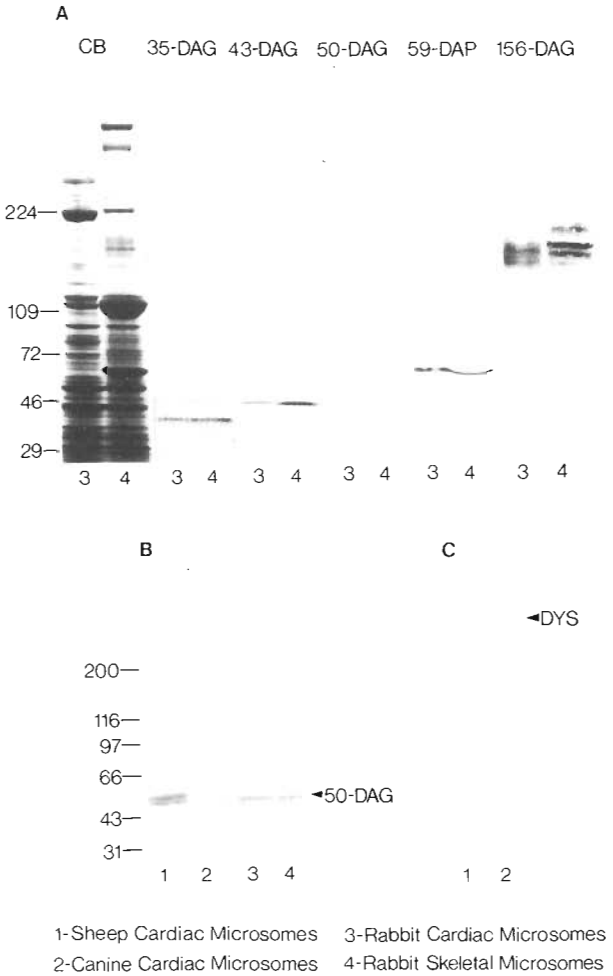

FIGURE 1. *Western blotting of cardiac and skeletal microsomal membranes. CB, Coomassie blue; DAG, dystrophin-associated glycoprotein; DAP, dystrophin-associated protein; DYS, dystrophin. Microsomes from various muscle tissues were isolated as described in "Materials and Methods" and separated by 3–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by either Coomassie blue staining (panel A) or immunolabeling with affinity-purified sheep antibodies to 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG from rabbit skeletal muscle (panel A, procedure A), monoclonal antibody IVD3$_1$ to 50-DAG (panel B, procedure B) and affinity-purified antibodies to the C-terminal decapeptide of dystrophin (panel C, procedure B). The lanes in panel A contain 250 μg rabbit cardiac (lanes 3) and rabbit skeletal (lanes 4) microsomes. The lanes in panels B and C contain 150 μg microsomes except lane 1 in panel B, which contains 25 μg.*

Subcellular Distribution of Components of the Dystrophin-Glycoprotein Complex in Transverse Cryosections of Papillary Myofibers

DAPs. Confocal imaging of transverse cryosections from rabbit papillary muscle showed that immunofluorescence labeling for 156-DAG (Figures 2a and 2c), 59-DAP (Figures 2b and 2d), 50-DAG (Figures 2e and

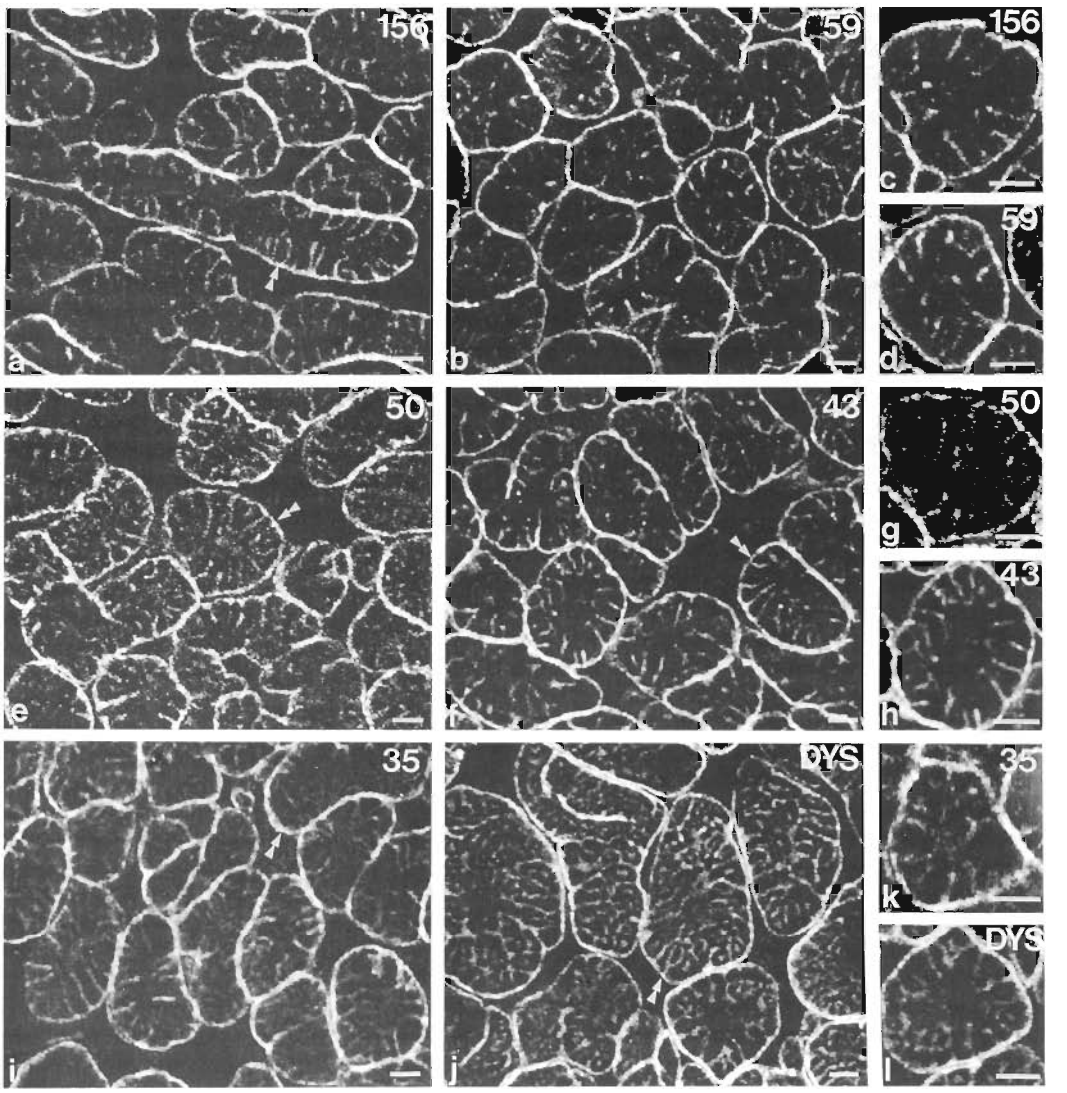

FIGURE 2. *Subcellular distribution of components of the dystrophin-glycoprotein complex in papillary myofibers by immunofluorescence labeling and confocal imaging. Transverse cryosections from adult rabbit (panels a–i and k) and sheep papillary muscle (panels j and l) were immunolabeled with affinity-purified antibodies to dystrophin-associated glycoproteins (DAGs) 156-DAG (panels a and c), 59-DAP (panels b and d), 50-DAG (panels e and g), 43-DAG (panels f and h), and 35-DAG (panels i and k) and to the C-terminal decapeptide of human dystrophin (DYS, panels j and l). Confocal imaging shows that each of these components of the dystrophin-glycoprotein complex is distributed along the cell periphery of papillary myofibers (double arrowheads) as well as in an anastomizing network extending from the cell periphery toward the central regions of the myofibers. Bar, 5 μm.*

2g), 43-DAG (Figures 2f and 2h), and 35-DAG (Figures 2i and 2k) were fairly homogeneously distributed along the entire cell periphery of papillary myofibers. In addition, immunofluorescence labeling for each of these components of the dystrophin-glycoprotein complex was distributed in a networklike pattern present throughout the cytoplasm of the papillary myofibers (Figures 2a–i and 2k). These results are consistent with the conclusion that 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG are distributed not only at the surface sarcolemma but also in interior regions of the cytoplasm where T tubules are present in papillary myofibers.

Dystrophin. Previous immunofluorescence[7,19–21] and immunocolloidal gold[21] studies have shown that dystrophin is localized in cardiac sarcolemma in mouse, human, and rat ventricular myocardium. However, the presence of dystrophin in cardiac T tubules has not been reported. Since dystrophin and the DAPs codistribute in rabbit skeletal muscle, where they are confined to the sarcolemma and apparently absent from T tubules, it was important to determine whether dystrophin and the DAPs also codistribute in cardiac myofibers.

Examination of confocal images of transverse cryosections of sheep papillary muscle immunolabeled for dystrophin shows that dystrophin like the DAPs (Figures 2a–2i and 2k) is distributed not only at the cell periphery but also in a networklike pattern present throughout the cytoplasm of these myofibers (Figures 2j and 2l). Furthermore, comparison of the distribution of dystrophin (Figure 3b) and 50-DAG (MAb IVD3$_1$) (Figure 3a) by double immunofluorescence labeling shows that these two proteins codistribute at both the

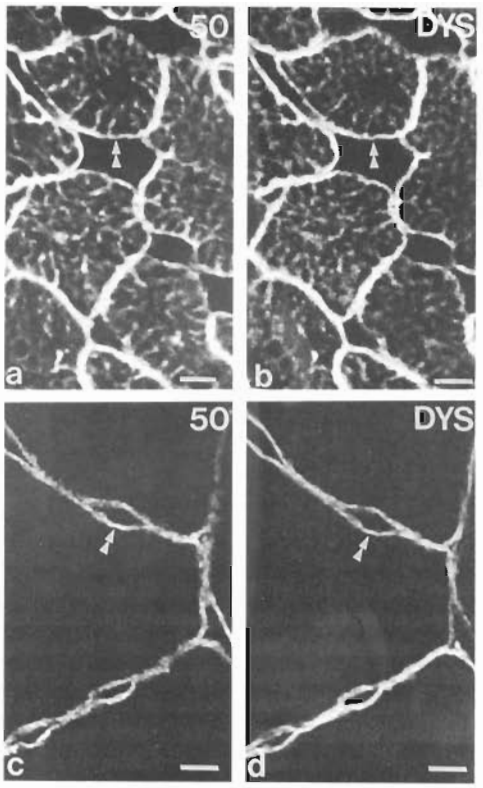

FIGURE 3. *Comparison between the subcellular distribution of dystrophin-associated glycoprotein (DAG) 50-DAG and dystrophin (DYS) in sheep cardiac and skeletal muscle by double immunofluorescence labeling and confocal imaging. Transverse cryosections from sheep papillary (panels a and b) and skeletal (diaphragm) muscle (panels c and d) were double-immunolabeled for 50-DAG (monoclonal antibody IVD3$_1$) (panels a and c) and DYS (panels b and d) as described in "Materials and Methods." Comparison of the staining pattern for 50-DAG (panel a) with that for DYS (panel b) in cardiac muscle shows that they codistribute not only at the cell periphery (panels a and b, double arrowhead) but also in the anastomizing networklike structure extending from the cell periphery toward the center of the myofibers (panels a and b). By contrast, specific staining for 50-DAG (panel c) and DYS (panel d) was confined to the cell periphery (panels c and d, double arrowhead) but absent from interior regions of skeletal myofibers where T tubules are present. Bar, 5 μm.*

cell periphery and in the networklike structures in the cytoplasm. By contrast, confocal imaging of transverse sections of sheep skeletal muscle confirms that specific labeling for dystrophin (Figure 3d) and 50-DAG (Figure 3c) is confined to the surface SL but absent from interior regions of the cytoplasm in skeletal muscle where T tubules are present.

Similarly, immunofluorescence labeling shows that dystrophin and 50-DAG (MAb IVD3$_1$) codistribute at both the free surface SL and the interior regions of the cytoplasm where T tubules are present in canine cardiac muscle but only at the free surface SL in canine skeletal muscle (Table 2). Since an affinity-purified site-specific antibody to 50-DAG also identifies a 50-kd protein in rabbit cardiac microsomes and localizes it to regions of

TABLE 2. Summary of the Distribution of Dystrophin, Dystrophin-Associated Proteins, and Laminin in Adult Cardiac and Skeletal Muscle In Situ, Demonstrated by Immunocytochemical Labeling

	T tubules	"Free" surface SL	Surface SL closely apposed to the SL of neighboring myofibers*
Present study			
Adult cardiac muscle			
Papillary myofibers			
Dystrophin (S,C)	+	+	−
50-DAG (R,S,C)	+	+	−
DAPs (R)	+	+	−
Laminin (S)	+	+	−
Purkinje fibers			
Dystrophin (S)	NA	+	−
50-DAG (S)	NA	+	−
Laminin (S)	NA	+	−
Adult skeletal muscle			
Dystrophin (S,C)	−	+	NA
50-DAG (S,C)	−	+	NA
Laminin (S)	−	+	NA
Previous studies			
Adult cardiac muscle			
Dystrophin (H,Rt,M)	−	+[7,19–21]	−[21]
Adult skeletal muscle			
Dystrophin[2,5–11]	−	+	NA
DAPs[2,5,6,12]	−	+	NA
Laminin[39]	−	+	NA

SL, sarcolemma; S, sheep; C, canine; +, present; −, absent; DAG, dystrophin-associated glycoprotein; R, rabbit; DAP, dystrophin-associated protein; NA, not applicable; H, human, Rt, rat; M, mouse.

*Represents intercalated discs in papillary myofibers.

rabbit cardiac myofibers corresponding to the position of the SL and T tubules, it appears to be highly unlikely that the labeling of T tubules in canine cardiac muscle with MAb to 50-DAG is due to cross-reactivity of this MAb with an unrelated protein in canine cardiac T tubules. The lack of labeling of a Western blot of canine microsomes with MAb 50-DAG is more likely due to a slight difference between the particular epitope of 50-DAG in canine and rabbit cardiac muscle, which after SDS-PAGE renders 50-DAG in canine cardiac microsomes undetectable by Western blotting.

The intensity of immunofluorescence labeling following staining with an irrelevant mouse Mab (IIH11) specific for fast skeletal sarcoplasmic reticulum Ca^{2+}-ATPase[33] corresponded to that of the secondary reagent alone (results not shown).

Subcellular Distribution of Dystrophin and DAPs in Longitudinal Sections of Papillary Myofibers

If the network-like distribution of components of the dystrophin-glycoprotein complex indeed represent labeling of T tubules in papillary myofibers, specific immunofluorescence labeling for these components should be distributed not only at the surface SL but also in the center of the I band where T tubules are present in these myofibers. To determine if this is indeed the

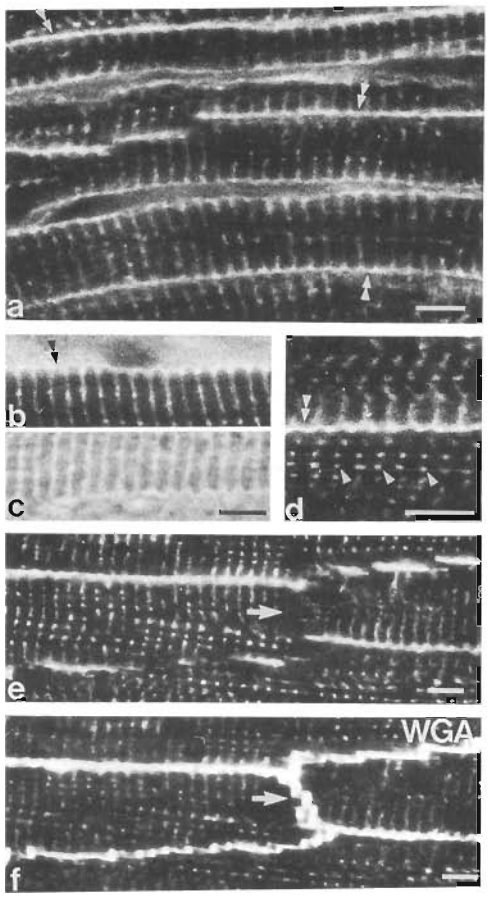

FIGURE 4. *Subcellular distribution of dystrophin-associated glycoprotein (DAG) 50-DAG and wheat germ agglutinin (WGA) in sheep papillary myofibers by fluorescence labeling. Longitudinal cryosections of adult sheep papillary muscle were single-labeled with monoclonal antibody IVD3$_1$ to 50-DAG (panels a, b, and d) and double-labeled with monoclonal antibody IVD3$_1$ to 50-DAG (panel e) and WGA (panel f). The immunofluorescence staining pattern in panel b was compared with the position of the A and I bands in the mirror image of the same field (panel c) imaged by phase-contrast microscopy. Specific immunofluorescence staining for 50-DAG was distributed densely at the cell periphery (panels a, b, and d; double arrowheads) and less densely in transversely oriented fluorescent strands present in the center of the I bands where T tubules are present in cardiac muscle (panels a, b, and d). Arrowheads in panel d point to regions in the myofiber where transversely oriented fluorescent strands are resolved into rows of discrete fluorescent foci. Comparison between the staining patterns in panels e and f show that labeling for 50-DAG (panel e) and WGA (panel f) codistributes along the surface sarcolemma and in the transversely oriented rows of fluorescent foci and strands in the center of the I band. However, specific labeling for 50-DAG was not detected over intercalated discs (panel e, large arrow) very prominently labeled with WGA (panel f, large arrow). Bar, 5 μm.*

case, the distribution of DAPS and dystrophin were compared in longitudinal cryosections of sheep and rabbit papillary muscle.

50-DAG. Examination of longitudinal cryosections from sheep papillary muscle showed that specific labeling for 50-DAG with MAb IVD3$_1$ was fairly homogeneously distributed at the cell periphery of papillary myofibers (Figures 4a, 4b, and 4d; double arrowheads). In addition, less intense transversely oriented fluorescent narrow bands (Figures 4a and 4b) that occasionally resolved into rows of discrete bright foci (Figure 4d, arrowheads) were also observed. The position of the transversely oriented narrow fluorescent bands (Figure 4b) corresponded to the center of I bands as observed by viewing the mirror image of the same field by phase-contrast microscopy (Figure 4c). Since T tubules in mammalian ventricular myofiber are mostly confined to the interfibrillar spaces corresponding to the level of the I band, these results are consistent with the conclusion that 50-DAG is localized to both the free surface SL and T tubules in sheep papillary myofibers.

50-DAG and WGA. The distribution of 50-DAG in relation to the SL and its extensions, the T tubules, was also determined by double fluorescence labeling of longitudinal cryosections from sheep papillary muscle using WGA and MAb IVD3$_1$ to 50-DAG. Since WGA binds specifically to *N*-acetyl-D-glucosamine and sialic acid moieties and thus to the external side of cell surfaces including the SL and T tubules of isolated rat myocardial fibers in situ,[35] it is assumed that labeling of cardiac cryosections with WGA delineates the cell periphery of cardiac myofibers, which is composed of surface SL, T tubules, and intercalated discs.[36] The results showed that labeling for 50-DAG (Figure 4e) and WGA (Figure 4f) codistributed along the cell periphery parallel to the longitudinal axis of the myofibers as well as along transversely oriented narrow bands at the level of the Z line. Furthermore, it was observed that the intercalated discs strongly labeled with WGA (Figure 4f, arrow) were not specifically labeled for 50-DAG (Figure 4e, arrow). In addition, specific labeling with WGA was greatly reduced when WGA was incubated with *N*-acetyl-D-glucosamine before fluorescence labeling (not shown).

DAPs. Examination of longitudinal cryosections from rabbit papillary muscle showed that immunolabeling for 156-DAG, 59-DAP, 43-DAG, and 35-DAG was localized at both the free surface SL and the center of the I band but not detected at the intercalated discs (Table 2).

Dystrophin. Examination of longitudinal cryosections of sheep papillary myofibers (Figure 5a) labeled with polyclonal antibodies to the C-terminus of human dystrophin showed that specific labeling for dystrophin was present at both the free surface SL (Figures 5a and 5b, double arrowheads) and in transversely oriented narrow bands of the myofibers (Figures 5a and 5b) corresponding to the center of I band (Figure 5c) where T tubules are present in papillary myofibers.

Dystrophin and WGA. Comparison between the distribution of dystrophin and WGA by double fluorescence labeling confirmed that dystrophin (Figure 5e), like 50-DAG, codistributes with WGA (Figure 5f) in the center of the I band where T tubules are localized. Furthermore, in agreement with previous studies,[21] comparison between the distribution of dystrophin and WGA in longitudinal cryosections showed that dystrophin (Figure 5e, arrows), like 50-DAG, was not detected in the region of the SL where intercalated discs are present (Figure 5f, arrows). The intensity of labeling for dystrophin as seen in Figure 5b was greatly diminished when affinity-purified antibod-

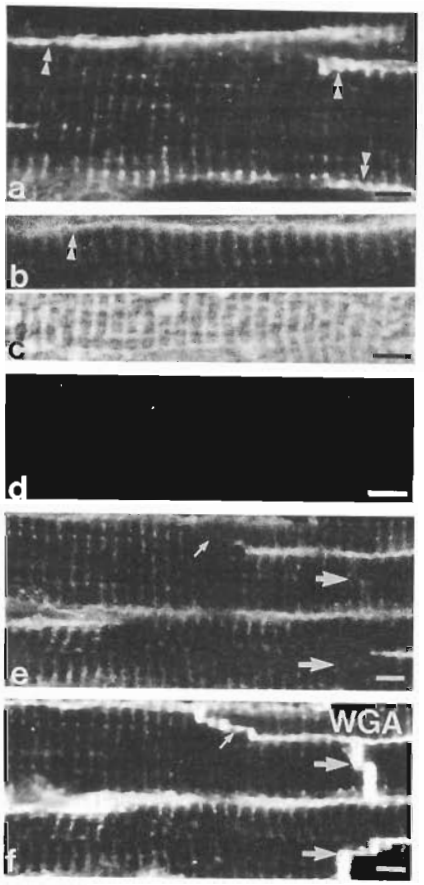

FIGURE 5. *Subcellular distribution of dystrophin and wheat germ agglutinin (WGA) in sheep papillary myofibers by fluorescence labeling. Longitudinal cryosections of adult sheep papillary myofibers were single-labeled with affinity-purified antibodies to the C-terminal decapeptide of human dystrophin before (panels a and b) and after preadsorption with the decapeptide to human dystrophin conjugated to bovine serum albumin (panel d) and double-labeled for dystrophin (panel e) and with WGA (panel f) as described in "Materials and Methods." The immunofluorescence staining pattern in panel b was compared with the position of the A and I bands in the same field as imaged by phase-contrast microscopy (panel c). In longitudinal sections, fluorescent staining was distributed densely at the cell periphery (panels a and b, double arrowheads) and less densely in transversely oriented fluorescent strands present in the center of the I bands where T tubules are present in cardiac muscle (panels a and b). The intensity of labeling for dystrophin as observed in panel a was greatly diminished when the dystrophin antibodies were preadsorbed with the C-terminal peptide to human dystrophin conjugated to bovine serum albumin (panel d). Comparison between the staining patterns observed after double labeling for dystrophin (panel e) and WGA (panel f) showed that they codistribute along the surface sarcolemma and in the center of the I bands where T tubules are present. However, specific labeling for dystrophin was either very faint (panel e, large arrows) or not observed (panel e, small arrows) over intercalated discs, which are strongly labeled for WGA (panel f, large and small arrows, respectively). Bar, 5 μm.*

ies to dystrophin were adsorbed with the C-terminal decapeptide of human dystrophin before immunofluorescence labeling (Figure 5d).

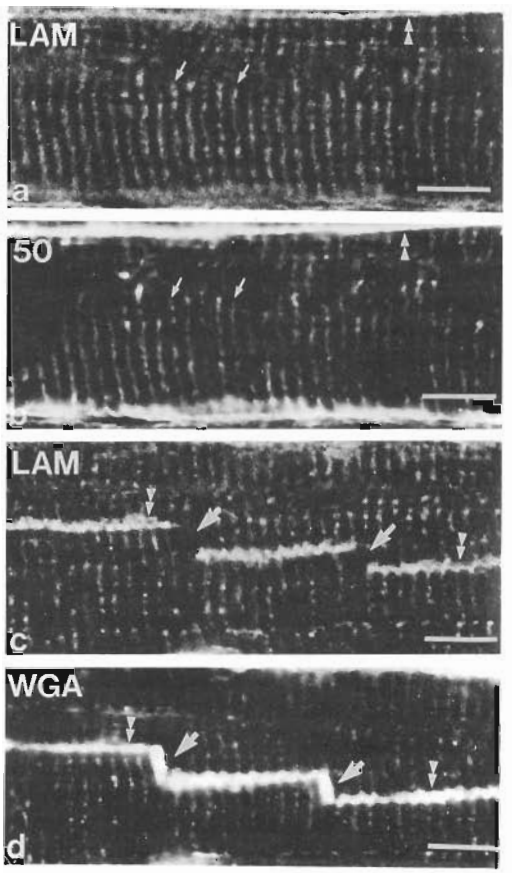

FIGURE 6. *Comparison between the subcellular distribution of dystrophin-associated glycoprotein (DAG) 50-DAG, laminin, and wheat germ agglutinin (WGA) in sheep papillary myofibers. Longitudinal sections of sheep papillary myofibers were double-labeled for 1) laminin (LAM, panel a) and 50-DAG (panel b) and for 2) LAM (panel c) and WGA (panel d). Comparison between the staining patterns for panels a and b show that labeling for laminin and 50-DAG codistributes along the surface sarcolemma (double arrowheads) and in transversely oriented rows of fluorescent strands (panels a and b, small arrows). Similarly, comparison between the staining pattern shown in panels c and d shows that labeling for LAM (panel c) codistributes with WGA (panel d) at the surface sarcolemma (panels c and d, double arrowheads) and in transversely oriented fluorescent strands. However, although WGA labeled intercalated discs very strongly (panel b, arrows), specific labeling for LAM was not observed over intercalated discs (panel c, arrows). Bar, 10 μm.*

These results strongly support the conclusion that dystrophin and the DAPs codistribute in both the free surface SL and T tubules but are absent from the intercalated discs of rabbit, sheep, and canine papillary myofibers (Table 2).

Comparison of the Distribution of 50-DAG and Laminin in Papillary Myofibers

It has recently been reported[15] that the extracellular 156-DAG of the dystrophin-glycoprotein complex binds to laminin, the major glycoprotein of the basal lamina.[37,38] Thus, it was of interest to determine whether the dystrophin-glycoprotein complex codistributes with

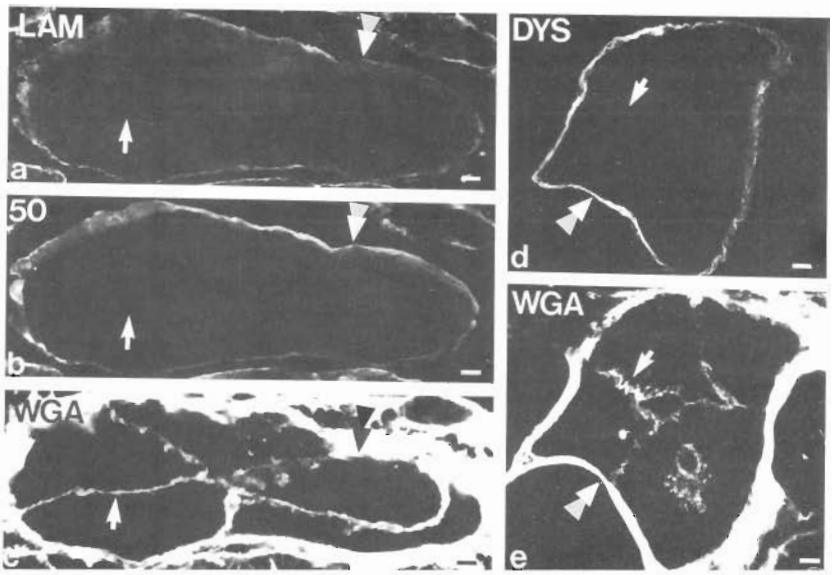

FIGURE 7. *Subcellular distribution of dystrophin-associated glycoprotein (DAG) 50-DAG, dystrophin (DYS), laminin (LAM), and wheat germ agglutinin (WGA) in sheep Purkinje fibers. Transverse cryosections of sheep papillary muscle containing bundles of Purkinje fibers were double-labeled with either 1) antibodies to LAM (panel a) and monoclonal antibody IVD3$_1$ to 50-DAG (panel b) or 2) antibodies to DYS (panel d) and WGA (panel e) by fluorescence labeling. Panel c is a serial section to that shown in panels a and b labeled with WGA. Labeling for 50-DAG (panel b) and laminin (panel a) is uniformly distributed along the region of the Purkinje fiber sarcolemma not closely apposed to other Purkinje fibers (panels a and b, double arrowheads) but absent from the region of the sarcolemma closely apposed to other Purkinje fibers (panels a and b, arrow) and visualized by labeling with WGA, which stains the entire circumference of each Purkinje fiber within the same bundle (panel c, arrow). Comparison between the distribution of labeling for DYS and WGA shows that DYS (panel d), like 50-DAG (panel b), is confined to the region of Purkinje fibers not closely apposed to other Purkinje fibers (panel d, double arrowhead) while apparently absent from the region of the sarcolemma where neighboring Purkinje fibers are closely apposed (panel d, arrow), as visualized by double labeling with WGA (panel e, arrow). Bar; 20 μm.*

laminin in the SL and the T tubules in papillary myofibers as it does in the SL but not the T tubules of skeletal myofibers.[39]

Papillary myofibers. Examination of double-labeled longitudinal cryosections from sheep papillary myofibers shows that the staining for laminin (Figure 6a) and for 50-DAG (Figure 6b) codistributes at the free surface SL (Figures 6a and 6b, double arrowheads) and in transverse strands corresponding to the position of the I band where most T tubules are present (Figures 6a and 6b, small arrows). Examination of cryosections double-labeled with antibodies to laminin and with WGA shows that labeling for laminin (Figure 6c) and WGA (Figure 6d) codistributes along the cell periphery parallel to the longitudinal axis of the myofibers (Figures 6c and 6d, double arrowheads) as well as along transversely oriented narrow strands at the level of the Z line. However, as demonstrated above for 50-DAG (Figure 4e, arrows) and dystrophin (Figure 5e, arrows), labeling for laminin (Figure 6c, arrows) was not detected in the region of intercalated discs strongly labeled with WGA (Figure 6d, arrows). These results show that 50-DAG and dystrophin codistribute with laminin and that they are confined to regions of the SL in papillary myofibers associated with a basement membrane. These results show that 50-DAG, dystrophin, and laminin codistribute in sheep papillary myofibers and imply that the components of the dystrophin-glycoprotein complex are confined to regions of the SL in papillary myofibers associated with a basement membrane.

Subcellular Distribution of 50-DAG, Dystrophin, and Laminin in Sheep Purkinje Fibers In Situ

If the networklike distribution of components of the dystrophin-glycoprotein complex represent labeling of T tubules in papillary myofibers, specific immunofluorescence labeling for these components should be absent in interior regions of myofibers that lack T tubules, such as sheep Purkinje fibers.[36] To determine if this is indeed the case, the subcellular distribution of 50-DAG, laminin, and dystrophin was determined in these myocardial fibers.

Examination of transverse cryosections containing bundles of sheep Purkinje fibers after double immunofluorescence labeling shows that specific labeling for laminin (Figure 7a) and 50-DAG (Figure 7b) is fairly homogeneously distributed along the free surface SL of Purkinje fibers (Figures 7a and 7b, double arrowhead) yet apparently absent from the region of the SL closely apposed to the SL of other Purkinje fibers (Figures 7a and 7b, arrows), as visualized by labeling a serial cryosection with WGA (Figure 7c, arrow). Similarly, examination of cryosections containing transversely oriented bundles of sheep Purkinje fibers double-labeled for dystrophin and with WGA showed that dystrophin (Figure 7d, double arrowhead), like 50-DAG and laminin, is confined to the free surface of SL of Purkinje fibers but apparently absent from the region of SL closely apposed to the SL of other Purkinje fibers (Figure 7e, double arrowheads). These results show that 50-DAG, dystrophin, and laminin codistribute in sheep

tubules than in free surface SL of isolated guinea pig ventricular myofibers.[49]

The functional significance of the presence of DAPs and dystrophin not only in the free surface SL but also in T tubules is currently unknown; however, it is the first evidence to directly show that free surface SL and T tubules in rabbit, sheep, and canine papillary myofibers have several membrane proteins in common, which in skeletal muscle of these species are confined to the SL but apparently absent from T tubules. These results point to the possibility that the function of T tubules in cardiac muscle may not be specialized to carry out functions distinct from those of the SL as they are in skeletal muscle. This possibility is consistent with results of ultrastructural studies suggesting that the structural characteristics of the surface SL and T tubules in cardiac muscle studied so far are very similar.[36] Thus, both regions show a similar distribution of integral membrane proteins, have a basement membrane, and form junctional complexes with junctional sarcoplasmic reticulum. The observation that some myocardial cells (e.g., mammalian Purkinje fibers and avian ventricular myofibers) lack T tubules further supports the possibility that unique functions may not reside in cardiac T tubules.

Although morphological, biochemical, and immunocytochemical studies so far have not revealed distinct differences between the free surface SL and T tubules, the studies presented here nonetheless support the idea that cardiac SL is composed of at least two structurally distinct but continuous domains. One of these domains (domain I) corresponds to the regions of cardiac SL not closely apposed to the SL of other myocardial fibers and includes free surface SL and T tubules (when present). The DAPs and dystrophin colocalize in this region. Furthermore, domain I is associated with a basal lamina, as suggested by the presence of laminin as shown here and in previous ultrastructural studies.[36] The other structurally distinct region of the SL (domain II) corresponds to surface SL closely apposed to the SL of neighboring myocardial cells. Domain II includes the intercalated discs of papillary myofibers and the region of SL of Purkinje fibers closely apposed to the SL of neighboring Purkinje fibers. Domain II appears to lack components of the dystrophin-glycoprotein complex but contains up to three distinct cell–cell junctional complexes[50] and appears to lack a basal lamina, as suggested by the absence of laminin from this region of the SL.

Presently, the exact function of the dystrophin-glycoprotein complex is unknown. However, a model of the organization of the dystrophin-glycoprotein complex in the skeletal SL has recently been presented.[5] In this model, dystrophin was proposed to link the actin filament containing cytoskeleton to the extracellular 156-DAG of the dystrophin-glycoprotein complex via a transmembrane complex containing four other identified DAPs. In view of the similarity between the organization of the dystrophin-glycoprotein complex[5] and that of the cadhedrins[51] and integrins,[52] it was proposed that the dystrophin-glycoprotein complex might bind to components of the extracellular matrix or to a molecule on a closely apposed cell.[5] In strong support of this hypothesis, it was recently demonstrated that 156-DAG skeletal muscle binds laminin[15] and that dystrophin contains regions that are both structurally and functionally homologous to the actin-binding regions of α-actinin.[16] In this regard, it is noteworthy that the presence of dystrophin and DAPs in distinct regions of the SL and/or T-tubular membranes in cardiac and skeletal myofibers are correlated with the presence of laminin and thus presumably a basal lamina on the extracellular side of those regions of the SL (i.e., skeletal SL,[39] cardiac free surface SL, and cardiac T tubules, Table 2). Similarly, the apparent absence of dystrophin and DAPs in other regions of SL in these myofibers correlate with the absence of laminin (i.e., cardiac SL closely apposed to a neighboring myofiber and skeletal T tubules,[39] Table 2). This correlation may indicate that the subcellular distribution of laminin in distinct domains of cardiac and skeletal muscle SL dictates the subcellular distribution of the dystrophin-glycoprotein complex in these muscle cells. Studies are in progress to determine if this is indeed the case.

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Purkinje fibers, where they are densely distributed along the free surface SL but apparently absent from the regions of the SL closely apposed to the SL of other Purkinje fibers.

The lack of specific labeling for both 50-DAG and dystrophin in interior regions of the Purkinje fibers is consistent with previous ultrastructural studies showing that Purkinje fibers lack T tubules.[36]

Discussion

We have identified cardiac muscle proteins antigenically related to 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG of the dystrophin-glycoprotein complex in rabbit skeletal muscle.[2,6,12] The subcellular distribution of the 59-DAP, 50-DAG, and 35-DAG from rabbit ventricular microsomes electrophorese as single bands on SDS–polyacrylamide gels with M_rs of 59,000, 50,000, and 35,000, respectively. The 50-DAG from sheep ventricular muscle migrates as a doublet band with apparent M_rs of 50,000 and 52,000. Thus, the apparent M_rs of the cardiac proteins detected by antibodies to 59-DAP, 50-DAG, and 35-DAG from skeletal muscle are very similar to those of the corresponding DAPs in skeletal muscle.[2,12] These results support the idea that 59-DAP, 50-DAG, and 35-DAG in cardiac muscle is structurally related to the corresponding DAPs detected in skeletal muscle. Similarly, it has previously been demonstrated by Northern blotting and immunoblotting that cardiac muscle contains proteins antigenically related to the 156-DAG and the 43-DAG from skeletal muscle.[15] The apparent M_rs of the corresponding proteins in cardiac muscle are 152,000 and 43,000, respectively. Taken together with the results presented here, we conclude that cardiac muscle contains at least five DAPs that are structurally and thus perhaps functionally very similar to the 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG in skeletal muscle.

We demonstrate by immunofluorescence studies that 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG of the dystrophin-glycoprotein complex are localized not only at the free surface SL but also in interior regions of the myofibers where T tubules are present in rabbit papillary myofibers. None of these DAPs was detected in regions of the SL corresponding to the intercalated discs. Furthermore, we demonstrate that dystrophin and laminin colocalize with 50-DAG at both the free surface SL and in interior regions of sheep papillary myofibers where T tubules are present. Like the DAPs, dystrophin and laminin were not detected at the intercalated discs. The suggestion that 50-DAG and dystrophin are indeed associated with both the surface SL and with T tubules in cardiac myofibers is further supported by the finding that 50-DAG and dystrophin also codistribute with WGA at the surface SL and in the I band region of the myofiber, where the distribution of WGA is assumed to image T tubules. Finally, the observation that 50-DAG, dystrophin, and laminin colocalize at the free surface SL but are not detected in interior regions of sheep Purkinje fibers that lack T tubules is consistent with the conclusion that labeling for 50-DAG and dystrophin in interior regions of papillary myofibers is associated with T tubules as opposed to other cellular components present in these regions of the myofibers. In summary, the single– and double–fluorescence labeling studies strongly support the conclusion that the dystrophin-glycoprotein complex codistributes with laminin both at the free surface SL and T tubules but not in intercalated discs in rabbit, sheep, and canine papillary myofibers (Table 2).

Previous immunocytochemical studies observed that dystrophin was localized to the surface SL in mouse, human, and rat ventricular myocytes[7,19–21] but was not detected in T tubules and intercalated discs.[21] The laminin also codistributes at the surface SL and in T tubules in rat papillary muscle. Thus, one explanation for the differences between results of the present and previous studies of rat cardiac muscle may be that the intensity of labeling of the surface SL for geometric reasons is considerably higher than that of T tubules, making it difficult to image T tubules.

The finding that the DAPs and dystrophin colocalize at the free surface SL and in regions where T tubules are present in cardiac papillary myofibers was also surprising in view of previous immunocytochemical studies in skeletal muscle. These studies demonstrated that although dystrophin[2,6,8–11] and DAPs[2,5,6,12] colocalize in skeletal myofibers, they are confined to the SL and are absent from the T tubules. We demonstrate here that 50-DAG and dystrophin are also localized to the surface SL but are absent from interior regions where T tubules are present in sheep and canine skeletal muscle.

The distribution of the components of the dystrophin-glycoprotein complex in skeletal muscle is consistent with previous studies demonstrating that skeletal surface SL and T tubules each have distinct ultrastructural features,[40,41] contain several unique proteins,[2,12,42,43] and carry out distinct functions related to excitation–contraction coupling.[44,45] Since mammalian ventricular myofibers contain both surface SL and T tubules,[36] some investigators have assumed that the function and thus the protein composition of these two regions of the cardiac SL would be distinct and perhaps analogous to those of skeletal SL and T tubules, respectively.[46–48] Subfractionation of cardiac SL vesicles by sucrose density centrifugation suggested that the 1,4-dihydropyridine receptor (1,4-DHPR) is fairly uniformly distributed in isolated T tubules and surface SL vesicles,[47] whereas subfractionation of cardiac SL by WGA suggested that the 1,4-DHPR is densely distributed in the surface SL but very sparse or absent from T tubules.[48] The interpretations of these results contradicted each other and differed from the anticipated results, namely that the 1,4-DHPR would be densely distributed in T tubules in cardiac muscle but absent from the SL as it is in skeletal muscle.[42,43] Thus, identification of membrane proteins unique to either surface SL or T tubules in cardiac muscle has not yet been determined, although a recent immunocytochemical study concluded that the Na^+-Ca^+ exchanger is more densely distributed in T

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