

Identification of Novel Proteins Unique to Either Transverse Tubules (TS28) or the Sarcolemma (SL50) in Rabbit Skeletal Muscle

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Abstract. Novel proteins unique to either transverse tubules (TS28) or the sarcolemma (SL50) have been identified and characterized, and their in situ distribution in rabbit skeletal muscle has been determined using monoclonal antibodies.

TS28, defined by mAb IXE11₂, was shown to have an apparent relative molecular mass of 28,000 D. Biochemical studies showed that TS28 is a minor membrane protein in isolated transverse tubular vesicles. Immunofluorescence and immunoelectron microscopic studies showed that TS28 is localized to the transverse tubules and in some subsarcolemmal vesicles possibly corresponding to the subgroup of caveolae connecting the transverse tubules with the sarcolemma. In contrast, TS28 is absent from the lateral portion of the sarcolemma. Immunofluorescence studies also showed that TS28 is more densely distributed in type II (fast) than in type I (slow) myofibers. Although TS28 and the 1,4-dihydropyridine receptor are both localized to transverse tubules and subsarcolemmal vesicles, TS28 is not a wheat germ agglutinin (WGA)-binding glycoprotein and does not appear to copurify with the 1,4-dihydropyridine recep-

tor after detergent solubilization of transverse tubular membranes.

SL50, defined by mAb IVD3₁, was shown to have an apparent relative molecular mass of 50,000 D. Biochemical studies showed that SL50 is not related to the 52,000-D (β subunit) of the dihydropyridine receptor but does bind to WGA-Sepharose. Immunofluorescence labeling imaged by standard and confocal microscopy showed that SL50 is associated with the sarcolemma but apparently absent from the transverse tubules. Immunofluorescence labeling also showed that the density of SL50 in type II (fast) myofibers is indistinguishable from that of type I (slow) myofibers.

The functions of TS28 and SL50 are presently unknown. However, the distinct distribution of TS28 to the transverse tubules and subsarcolemmal vesicles as determined by immunocytochemical labeling suggests that TS28 may be directly involved in excitation-contraction coupling. Our results demonstrate that, although transverse tubules are continuous with the sarcolemma, each of these membranes contain one or more unique proteins, thus supporting the idea that they each have a distinct protein composition.

EXCITATION-CONTRACTION coupling in skeletal muscle requires a message elicited during depolarization of the sarcolemma to cross the gap between the transverse tubules and the terminal cisternae of the sarcoplasmic reticulum. This message induces a release of Ca²⁺ from the sarcoplasmic reticulum, thus resulting in contraction. To understand the specific function of transverse tubules in excitation-contraction coupling, it is important to identify and characterize the structure and function of transverse tubular membrane proteins.

Presently, two distinct approaches are used to purify transverse tubules. According to one procedure, transverse tubular membrane vesicles are obtained by French press dissociation of isolated triads (6, 28). Alternatively, transverse tubules are purified from calcium phosphate-loaded sarcoplasmic reticulum by sucrose density centrifugation (40).

Both transverse tubular membrane preparations are characterized by a high density of high affinity 1,4-dihydropyridine receptors. However, they differ considerably with respect to their content of enzyme activities (e.g., [Na⁺,K⁺]-ATPase and Mg²⁺-ATPase), considered to be markers of the plasma membrane. To date, the reason for these differences has not been elucidated.

The finding that the high affinity 1,4-dihydropyridine receptor is 30-fold more densely distributed in transverse tubular membrane vesicles than in sarcolemmal membrane vesicles from skeletal muscle suggested that this protein is confined to transverse tubular membrane (13). Recent electrophysiological evidence supports the view that the 1,4-dihydropyridine receptor acts as a voltage sensor essential for excitation-contraction coupling (38, 44). Determination of the subcellular distribution of the 1,4-dihydropyridine

receptor in adult rabbit skeletal muscle *in situ* by immunocolloidal gold labeling confirmed that the α_1 subunit of the 1,4-dihydropyridine receptor is indeed densely distributed in the transverse tubules and apparently absent from the lateral portion of the sarcolemma. Unexpectedly, the α_1 subunit of the 1,4-dihydropyridine receptor was also found to localize over some subsarcolemmal vesicles, possibly corresponding to a subpopulation of sarcolemmal invaginations called caveolae (25). Thus, it appears that immunocytochemical labeling *in situ* provides an alternative and powerful approach to determining whether proteins present in purified transverse tubules are indeed localized to transverse tubules *in situ* and if so whether they are unique to transverse tubules or also present in other skeletal muscle membranes (i.e., caveolae). To identify and characterize the structure, function, and subcellular distribution of other proteins present in isolated transverse tubular membranes, we have begun to prepare monoclonal antibodies to isolated transverse tubular membrane vesicles.

In this paper, we report on the identification, characterization, and subcellular localization of two novel rabbit skeletal muscle proteins each defined by a monoclonal antibody prepared against isolated transverse tubular membrane vesicles by hybridoma technology. Immunocytochemical studies showed that mAb IXE11₂ defines a novel skeletal muscle protein with an apparent relative molecular mass of 28,000 D. Immunocytochemical studies showed that this protein localizes to the transverse tubules but is apparently absent from the sarcolemmal membranes of the skeletal muscle. It is also present in some subsarcolemmal vesicles, possibly representing a subgroup of caveolae connecting the transverse tubules with the sarcolemma. The mAb IVD3₁ defines another novel rabbit skeletal muscle protein with an apparent relative molecular mass of 50,000 D. Immunocytochemical studies showed that this protein is associated with the sarcolemma but apparently absent from transverse tubules of adult rabbit skeletal muscle.

Materials and Methods

Preparation of Skeletal Muscle Extract, Triads, Transverse Tubular Membranes, and the 1,4-Dihydropyridine Receptor

Skeletal muscle extracts represent the postnuclear supernatant obtained from tissue homogenates during the preparation of light sarcoplasmic reticulum (3). Triads were prepared as described by Mitchell et al. (34) with slight modification (42). Transverse tubular membranes were prepared by the method of Roseblatt et al. (40). Light sarcoplasmic reticulum was prepared as previously described (3). The 1,4-dihydropyridine receptor was purified as described by Campbell and Kahl (2). Protein concentrations were determined by the method of Lowry et al. (31) as modified by Peterson (35) using BSA as a standard.

Preparation of Monoclonal Antibodies

Monoclonal antibodies were prepared by hybridoma technology as previously described (29) using spleens from mice immunized with isolated transverse tubular membranes. Hybridoma supernatants were screened by an immunodot assay (4) against the following membrane and protein fractions purified from rabbit skeletal muscle: light sarcoplasmic reticulum, triads, transverse tubules, and 1,4-dihydropyridine receptor. Supernatants positive for transverse tubules and triads but negative for light sarcoplasmic reticulum and 1,4-dihydropyridine receptor were further screened by immunoblotting and immunofluorescence labeling. mAb VD2₁, to the β subunit

of the 1,4-dihydropyridine receptor, was prepared as previously described (30).

Immunoblotting

SDS-PAGE was performed by the method of Laemmli (27) in either 3–12% or in 7.5–12% acrylamide gels. Transblotting of proteins from SDS-PAGE gels and immunoblotting was performed according to the methods of Towbin et al. (45) with minor modifications as outlined in immunoblotting procedures A and B (see below).

Procedure A. Nitrocellulose blots were blocked in 5% nonfat dry milk in TBS (20 mM Tris, 500 mM NaCl [pH 7.4]) (19). The antibody incubation medium included 2.5% nonfat dry milk in TBS. An affinity-purified goat anti-mouse IgG-alkaline phosphatase conjugate (1:3,000; Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) was used as secondary antibody for immunoblotting with mAb IXE11₂ according to the procedure of Blake et al. (1). Immunoblotting with mAb IVD3₁ to SL50 was carried out as referenced above except that a triple layered procedure was used. Thus, the incubation with mAb IVD3₁ (1:1,000) was followed first by affinity-purified F(ab)₂ fragments of goat anti-mouse IgG (Fc specific) conjugated to biotin (1:3,000 dilution; Jackson Immunoresearch Laboratories Inc., West Grove, PA) and then by avidin-conjugated alkaline phosphatase (1:1,000; Bio-Rad Laboratories Ltd.).

Procedure B. Nitrocellulose blots were blocked for 1 h in blotto (50 mM sodium phosphate, pH 7.4 150 mM sodium chloride, 5% nonfat dry milk). The blots were incubated for 1 h with mAb IXE11₂ or overnight with mAb IVD3₁ in blotto at a dilution of 1:1,000. Nitrocellulose blots were washed three times and then incubated with peroxidase-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) at a dilution of 1:1,000. They were then washed three times with blotto and developed using 4-chloro-1-naphthol as a substrate.

Preparation of Cross-linked Monoclonal Antibody Beads

Protein A-Sepharose beads (Sigma Chemical Co.) were hydrated overnight in TBS (150 mM NaCl, 50 mM Tris [pH 7.4]). 2 ml of beads were then incubated with 10 ml of hybridoma supernatant from the cell line IXE11₂ for 24 h with three changes of media. The antibody was then covalently linked to the protein A beads by the method of Harlow and Lane (17) using dimethyl suberimidate (14) as a cross-linking agent.

Affinity Chromatography on Protein A-Sepharose: mAb IXE11₂ Beads

1 mg of isolated transverse tubular membrane vesicles was solubilized in 2 ml of 1% digitonin, 0.5 M sucrose, 0.5 M NaCl, 50 mM Tris (pH 7.4), 0.83 mM benzamidine. The supernatant (750 μ l) was then incubated with 1 ml of protein A-IXE11₂ cross-linked beads as prepared above or with 1 ml of wheat germ agglutinin (WGA)¹-Sepharose beads. The affinity column void or the WGA-Sepharose void were then run on SDS-PAGE and either stained with Coomassie brilliant blue or transferred and immunoblotted with IXE11₂ using procedure B as described above.

Dissection, Fixation, and Sectioning of Adult Skeletal Muscle

Fixed and unfixed bundles of skeletal muscle fibers from rabbit gracilis, diaphragm and psoas muscle were prepared as previously described (22). Briefly, bundles of myofibers were dissected and quickly frozen in liquid nitrogen-cooled isopentane. Bundles of myofibers to be fixed were dissected and immediately tied to applicator sticks (for chemical fixation) or stainless steel loops (for cryofixation) at 100–120% of their rest length and allowed to recover for 30 min in a modified Krebs-Henseleit buffer (145 mM NaCl, 2.6 mM KCl, 5.9 mM CaCl₂, 1.2 mM MgSO₄, 25 mM NaHCO₃, and 10 mM glucose saturated with a mixture of 95% O₂ and 5% CO₂). The bundles of myofibers to be used for immunofluorescence studies were fixed for 3 h in ice-cold 2% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.4). Sucrose infusion, storage, and cryosectioning (6–8 μ m) were carried out as previously described (22). The bundles of myofibers used to demonstrate the ultrastructural features of the cryofixed (36) and freeze-dried (9, 33) myofibers were prepared as described by Jorgensen and Mc-

1. Abbreviation used in this paper: WGA, wheat germ agglutinin.

Guffee (procedure III of reference 20), with minor modification (24). The bundles of myofibers to be used for immunocolloidal gold labeling were cryofixed (36), freeze-dried (9, 33), and low temperature embedded in Lowicryl K4M according to the procedure of Chiovetti et al. (8, 9) as modified by Jorgensen and McGuffee (procedure II of reference 20) and Jorgensen et al. (24). Thin sections (60–80 nm) were collected on nickel grids coated with formvar.

Immunofluorescence Labeling

Immunofluorescence staining of 6–8- μ m-thick cryosections from unfixed adult rabbit diaphragm muscle was carried out as previously described (22). The sections were first labeled with one of the following mAbs: IXE11₂ to TS28 or IVD3₁ to SL50. The secondary antibody was F(ab')₂ fragments of affinity-purified goat anti-mouse IgG conjugated to FITC (Jackson Immunoresearch Laboratories Inc.). It was used at a dilution of 1:40. Conventional fluorescence microscopy was carried out with a photomicroscope (Carl Zeiss, Inc., Thornwood, NY) provided with an epifluorescence attachment and a phase-contrast condenser. Confocal microscopy was carried out with a photomicroscope (Nikon Inc., Garden City, NY) provided with a confocal fluorescence imaging system (Lasersharp MRC-500; courtesy of Bio-Rad Laboratories Ltd.) using an argon laser for illumination (46).

Histochemical Staining

Transverse sections of unfixed rabbit diaphragm muscles were stained for myosin ATPase after alkaline preincubation (pH 10.4) as described by Guth and Samaha (15). This method specifically labels the myosin ATPase of type II (fast) skeletal muscle fibers.

Immunocolloidal Gold Labeling

Immunocolloidal gold labeling of thin sections of cryofixed, freeze-dried, and Lowicryl K4M-embedded rabbit psoas muscle was carried out as previously described (20, 25), except that a triple layered immunolabeling technique was used. Briefly, the sections were first labeled with ammonium sulphate-fractionated mAb IXE11₂ (0.5 mg/ml in PBS [pH 7.4]). Then an affinity-purified rabbit anti-mouse (Fc fragment) gamma globulin (Jackson Immunoresearch Laboratories Inc.) was used at 25 μ g/ml in TBS containing 3% BSA. Finally, the sections were incubated with affinity-purified goat anti-rabbit gamma globulin-colloidal gold conjugate (3–7 nm) at 0.5 mg/ml TBS containing 3% BSA (Janssen Pharmaceutica, Beerse, Belgium).

To assess the immunolabeling specificity of the thin sections of Lowicryl K4M-embedded tissue, mouse gamma globulin purified from preimmune serum (10 μ g/ml PBS) was substituted for mAb IXE11₂ in the immunocolloidal gold labeling procedure. After immunolabeling, the sections were first stained for 15 s in saturated aqueous uranyl acetate and then for 15 s in lead citrate (20). The sections were examined in a transmission electron microscope (7000; Hitachi Ltd., Tokyo, Japan).

Results

Biochemical Characterization of TS28 and SL50

mAb IXE11₂ and mAb IVD3₁ were obtained from a panel of hybridoma clones whose supernatants recognized isolated transverse tubular membrane vesicles but neither light sarcoplasmic reticulum vesicles nor purified 1,4-dihydropyridine receptor as determined by immunodot blotting (data not shown). Immunoblotting of SDS-PAGE separated purified membrane fractions from adult rabbit psoas muscle showed that mAb IXE11₂ specifically binds a single band with an apparent relative molecular mass of 28,000 D present in both isolated triads (Fig. 1 a, lane 2) and isolated transverse tubules (Fig. 1 a, lane 3) but absent from light sarcoplasmic reticulum (Fig. 1 a, lane 1). The results presented in Fig. 1 b showed that mAb IVD3₁ specifically binds a single band with an apparent relative molecular mass of 50,000 D present in isolated triads (Fig. 1 b, lane 2) and isolated transverse tubular membrane fractions (Fig. 1 b, lane 3) but absent

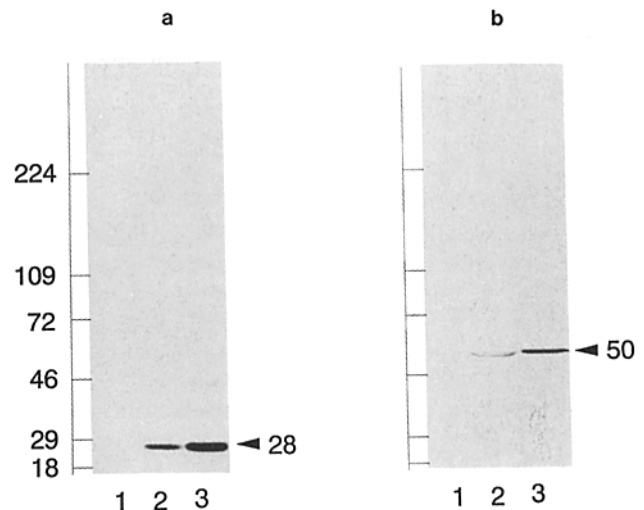


Figure 1. Immunoblot staining of rabbit skeletal muscle membrane fractions. Rabbit skeletal muscle light sarcoplasmic reticulum membranes (lane 1), triads (lane 2), and transverse tubular membranes (lane 3) were prepared as described in Materials and Methods and separated by SDS-PAGE (30 μ g/lane) followed by immunoblotting with monoclonal antibodies. (a) Stained with mAb IXE11₂ to TS28; (b) stained with mAb IVD3₁ to SL50.

from light sarcoplasmic reticulum vesicles (Fig. 1 b, lane 1). The antigens defined by mAb IXE11₂ and mAb IVD3₁ will be referred to as TS28 and SL50, respectively.

To determine whether TS28 represents a major or minor component of the purified transverse tubular membrane vesicles, an mAb IXE11₂ affinity column was prepared and used to remove TS28 from detergent-solubilized transverse tubular membrane vesicles. TS28 was solubilized from isolated transverse tubular membranes using 1% digitonin and 0.5 M NaCl as previously described for the 1,4-dihydropyridine receptor (29). Comparison of the protein composition of the same fractions separated by SDS-PAGE and stained with Coomassie blue showed that the protein composition of the solubilized transverse tubular vesicles (Fig. 2 a, lane 1) was indistinguishable from that of the unadsorbed fraction (Fig. 2 a, lane 2). However, comparison of digitonin-solubilized transverse tubules immunoblotted with mAb IXE11₂ before (Fig. 2 b, lane 1) and after adsorption to the mAb IXE11₂ affinity column (Fig. 2 b, lane 2) showed that TS28 was completely removed by the affinity column. These results show that TS28 retained on the column represents a minor protein component of the transverse tubular membrane vesicle fraction.

Since TS28 has a molecular mass similar to that of the δ subunits 24–33 kD (43) of the 1,4-dihydropyridine receptor, we examined whether mAb IXE11₂ would stain the purified 1,4-dihydropyridine receptor and whether TS28 from digitonin-solubilized transverse tubules (Fig. 3 b, lane 1), like the 1,4-dihydropyridine receptor, would be retained on a WGA-Sepharose column. Immunoblotting showed that mAb IXE11₂ did not recognize any of the subunits of the 1,4-dihydropyridine receptor under either nonreducing (Fig. 3 a, lane 2) or under reducing conditions (not shown) and that TS28 is not retained on the WGA-Sepharose column (Fig. 3 a, lane 3). These results show that TS28 is not related to any of the subunits of the 1,4-dihydropyridine receptor, is not a

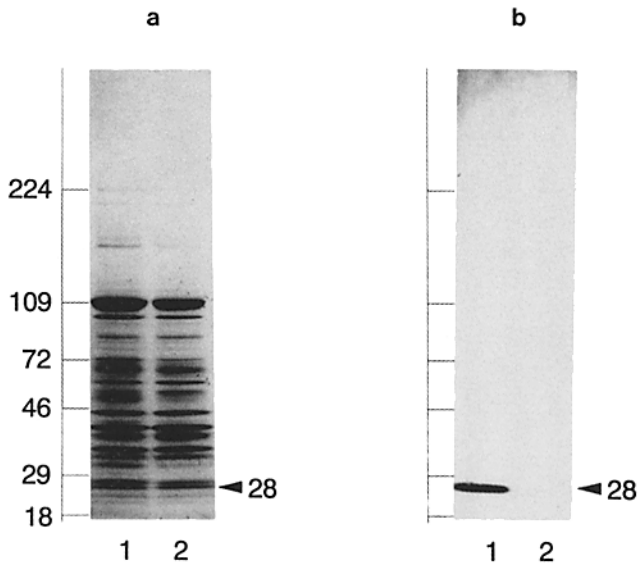


Figure 2. Coomassie blue staining and immunoblotting of rabbit skeletal muscle transverse tubular membranes adsorbed with a protein A-mAb IXE11₂ complex. Digitonin-solubilized transverse tubular membranes from rabbit skeletal muscle before (lane 1) and after adsorption to mAb IXE11₂ conjugated to protein A (lane 2) were prepared as described in Materials and Methods and separated by SDS-PAGE (40 μg/lane) followed by either Coomassie blue staining (a) or immunoblotting with mAb IXE11₂ to TS28 (b).

WGA-binding glycoprotein, nor is tightly associated with a WGA-binding glycoprotein after detergent solubilization.

Similarly, it was examined whether SL50 was related to the β subunit (52 kD) of the 1,4-dihydropyridine receptor (Fig. 3 b, lane 2) or retained on WGA-Sepharose (Fig. 3 b, lane 3). The results show that SL50 is indeed retained on the

WGA-Sepharose column (Fig. 3 b, lane 3) and therefore imply that either SL50 itself or a protein to which it is tightly associated is a WGA-binding glycoprotein like the 1,4-dihydropyridine receptor. However, immunoblotting of SDS-PAGE separated subunits of the 1,4-dihydropyridine receptor with either mAb VD2₁ to the β subunit of the 1,4-dihydropyridine receptor (Fig. 3 c, lane 2) or with mAb IVD3₁ to SL50 (Fig. 3 b, lane 2) showed that mAb IVD3₁ to SL50 does not bind to either the β subunit or to any other subunits of the WGA-binding 1,4-dihydropyridine receptor.

The specificity of mAb IXE11₂ for the skeletal muscle component TS28 with a relative molecular mass of 28,000 D (Fig. 4 a, lane 1) was demonstrated by its ability to bind only one polypeptide band present in a SDS-PAGE immunoblot of a postnuclear supernatant from rabbit psoas muscle (Fig. 4 a, lane 2). Similarly the specificity of mAb IVD3₁ for a component with a relative molecular mass of 50,000 D (Fig. 4 b, lane 1) was demonstrated by its ability to bind only one polypeptide band present in SDS-PAGE immunoblots of a postnuclear supernatant from adult rabbit skeletal muscle (Fig. 4 b, lane 2).

Distribution of TS28 and SL50 in Adult Rabbit Skeletal Muscle In Situ

Serial transverse cryosections from adult rabbit diaphragm muscle containing both type I (slow) and type II (fast) myofibers were labeled with mAb IXE11₂ to TS28 (Fig. 5 a) and mAb IVD3₁ to SL50 (Fig. 5 b). It is noteworthy that examination of arterial vessels present in the cryosections after immunolabeling showed mAb IXE11₂ to TS28 did label arterial smooth muscle but neither fibroblasts nor endothelial cells, while mAb IVD3₁ to SL50 did not label either of these three cell types (results not shown). These results indicate that both antibodies are specific for muscle tissue. Examination of the sections labeled with mAb

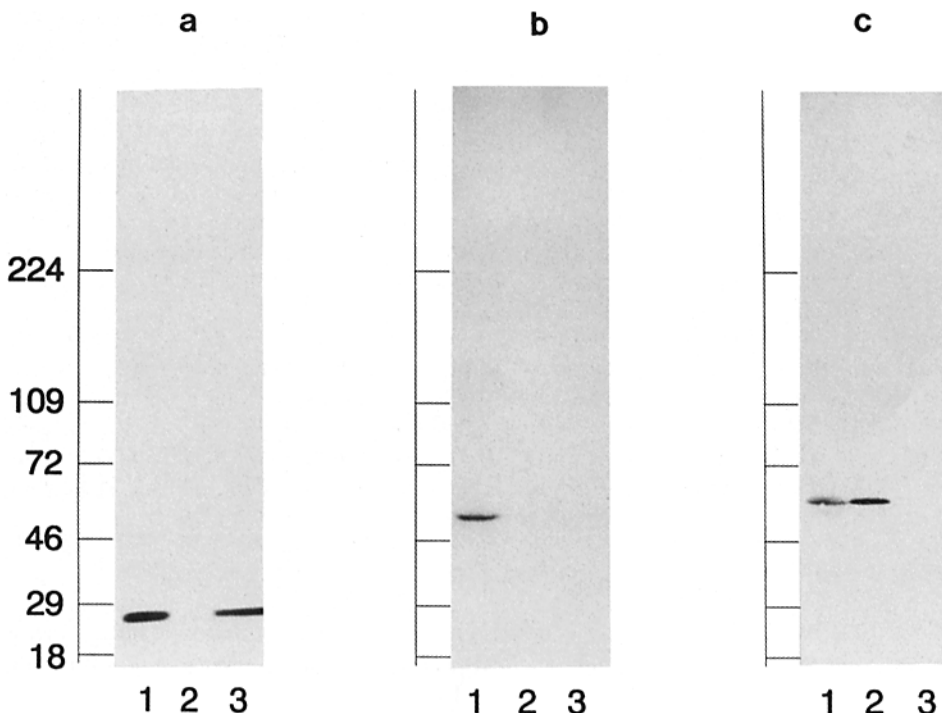


Figure 3. Immunoblot staining of rabbit skeletal muscle transverse tubular membranes before and after adsorption with WGA-Sepharose and of the 1,4-dihydropyridine receptor. Transverse tubular membranes (lane 1), the purified 1,4-dihydropyridine receptor (lane 2), and the void of the WGA-Sepharose column (lane 3) were prepared as described in Materials and Methods and separated by SDS-PAGE followed by immunoblotting with monoclonal antibodies (80 μg/lane). (a) Stained with mAb IXE11₂ to TS28; (b) stained with mAb IVD3₁ to SL50; (c) stained with mAb VD2₁ to the β subunit of the 1,4-dihydropyridine receptor.

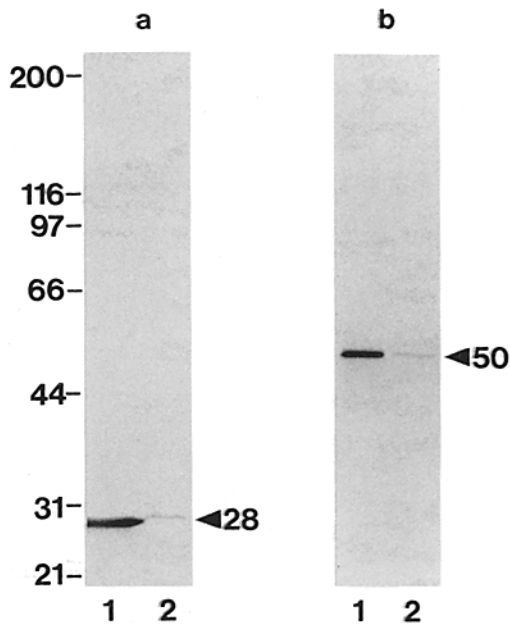


Figure 4. Immunoblot staining of rabbit skeletal muscle postnuclear supernatant and membrane fractions. Rabbit skeletal muscle transverse tubular membranes (15 μ g; *a* and *b*, lane 1) and postnuclear supernatant (600 μ g; *a* and *b*, lane 2) were prepared as described in Materials and Methods and separated by SDS-PAGE followed by immunoblotting with monoclonal antibodies. *a* and *b* are immunoblots of the gel stained with mAbs IXE11₂ to TS28 and IVD3₁ to SL50, respectively.

IXE11₂ showed that all skeletal myofibers were specifically labeled (Fig. 5 *a*). However, some myofibers were two- to threefold more intensely labeled than the rest of the myofibers (Fig. 5 *a*). Examination of the sections labeled with mAb IVD3₁ showed that all myofibers appeared to be labeled with the same intensity (Fig. 5 *b*). To determine the fiber type distribution, additional serial sections were histochemically stained for alkaline stable myosin ATPase, a specific marker of type II (fast) fibers (Fig. 5 *c*) (15). The results suggest that mAb IXE11₂ labels type II (fast) approximately two- to threefold more intensely than it labels type I (slow) fibers. In contrast the intensity of mAb IVD3₁, labeling of type II (fast) is indistinguishable from that of type I (slow) fibers.

Subcellular Distribution of TS28 and SL50 in Adult Skeletal Muscle In Situ

TS28. Examination at higher magnification of transverse cryosection of rabbit gracilis labeled with mAb IXE11₂ (Fig. 6, *b* and *d*) showed a polygonal staining pattern throughout the cytoplasm of skeletal muscle fibers. Centers of neighboring polygons were separated by a distance ranging from 1.1 to 1.5 μ m (Fig. 6 *d*). The immunofluorescence staining pattern observed in longitudinal cryosections of fixed gracilis muscle after labeling with mAb IXE11₂ appeared as transversely oriented rows of discrete bright foci (Fig. 7, *a* and *c*). The distribution of the transversely oriented row of foci (Fig. 7 *a*) corresponded to the interphase between the

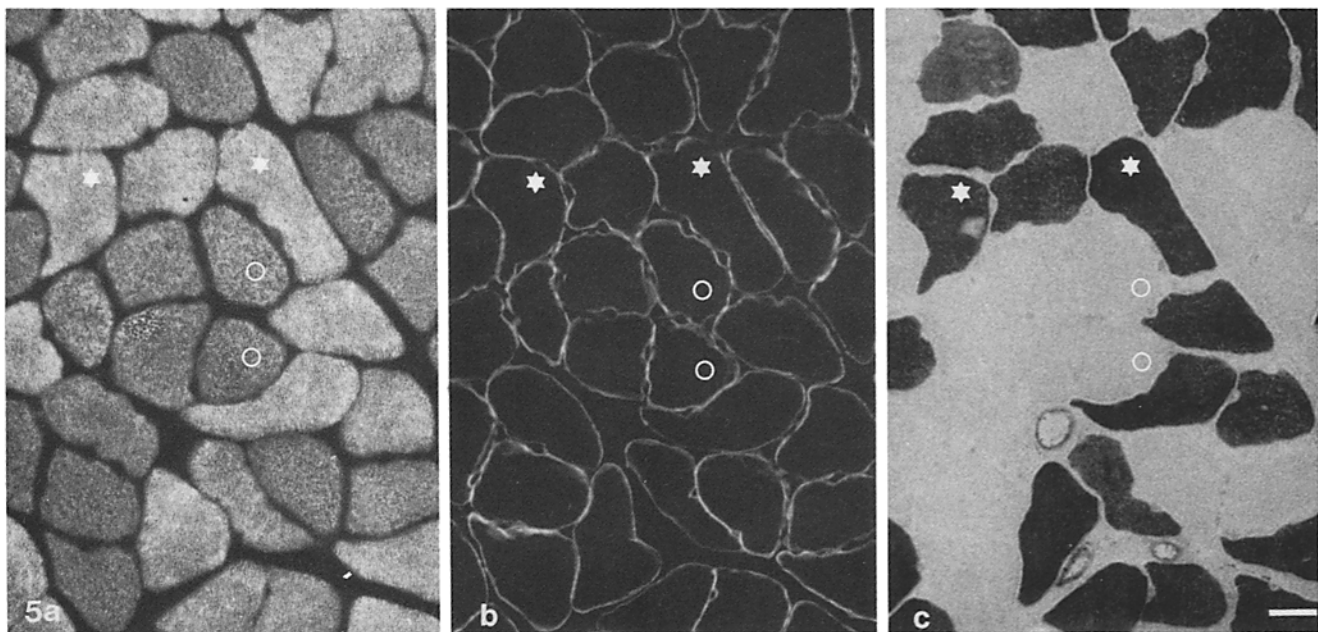


Figure 5. Distribution of TS28 and SL50 in type I (slow) and type II (fast) myofiber of rabbit diaphragm muscle. Serial transverse cryosections of unfixed rabbit diaphragm muscle were immunofluorescently labeled with mAb IXE11₂ to TS28 (*a*) and mAb IVD3₁ to SL50 (*b*) and histochemically stained for alkali-stable myosin ATPase, a marker for type II (fast) myofibers (*c*). Two classes of fibers can be distinguished after labeling with mAb IXE11₂. The class of fibers labeled relatively intensely with mAb IXE11₂ (*a*, *star*) correspond to type II (fast) fibers (*c*, *star*), while the less intensely labeled class of fibers (*a*, *circle*) correspond to type I fibers (*c*, *circle*). By contrast, the intensity of labeling of the cell periphery with mAb IVD3₁ of type I (slow) (*b*, *circle*) is indistinguishable from that of type II (fast) (*b*, *star*) fibers. Bar, 20 μ m.

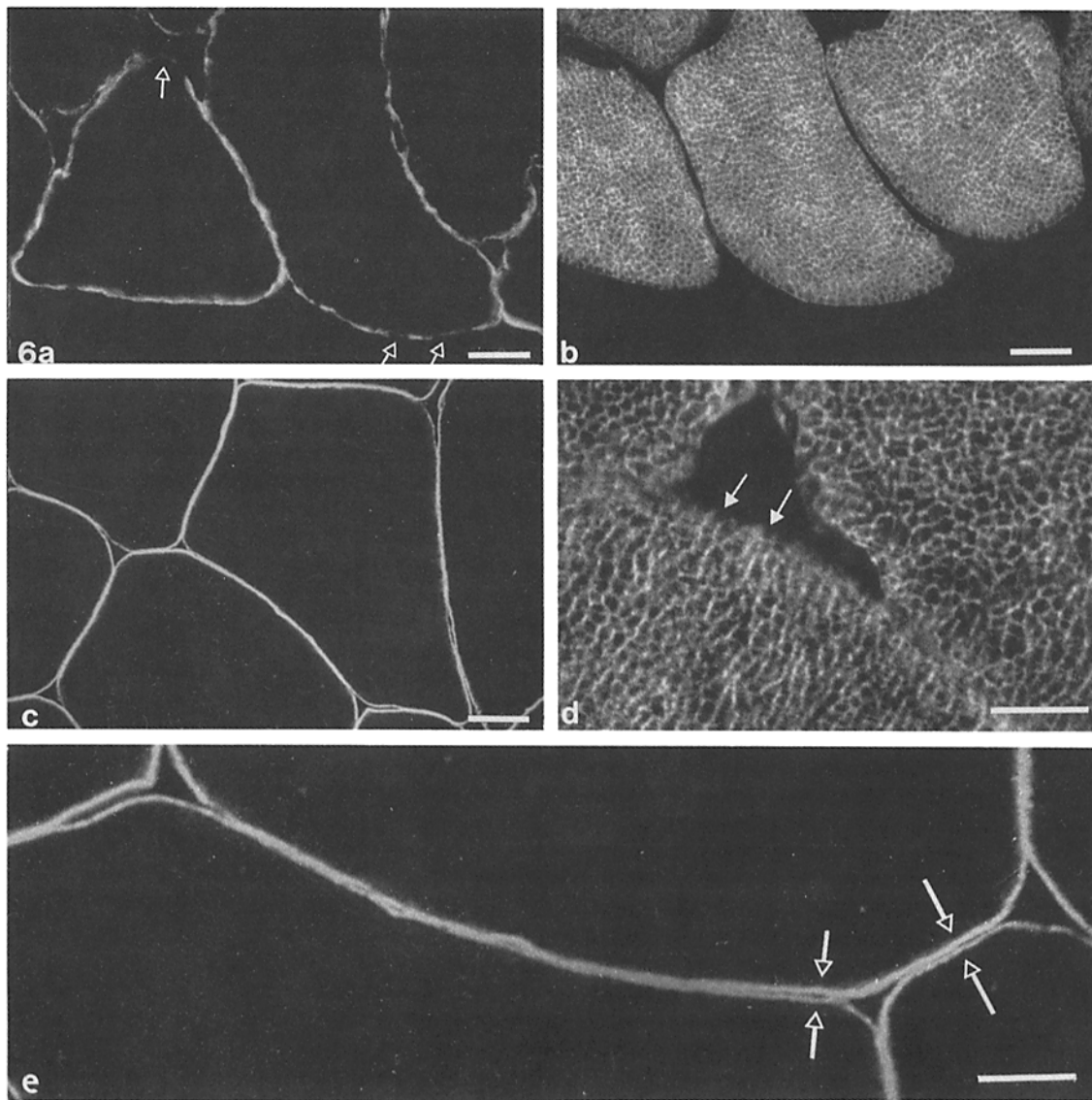


Figure 6. Subcellular distribution of TS28 and SL50 in type II (fast) myofibers of rabbit gracilis muscle. Transverse sections (5–8 μm) of unfixed rabbit skeletal muscle were labeled with mAb IVD3₁ to SL50 (*a*, *c*, and *e*) and with mAb IXE11₂ to TS28 (*b* and *d*) and examined with a conventional (*a*, *b*, and *d*) and a confocal (thickness of optical section 0.5 μm) UV photomicroscope (*c* and *e*). The mAb IVD3₁ specifically labeled the cell periphery, while specific labeling was not observed in either the internal regions of the fibers (*a*, *c*, and *e*) or in the connective tissue between the fibers (*e*, arrows). The apparent discontinuity of labeling at some regions of the cell periphery observed with conventional microscopy was not apparent when the same section was viewed by confocal microscopy. In contrast, a polygonal staining pattern was present throughout the cytoplasm of the myofiber after labeling with mAb IXE11₂ to TS28 (*b* and *d*). However, specific labeling of the cell periphery was not apparent (*d*, arrows). Bars, 5 μm .

A-band and the I-band as observed by viewing the same field by phase-contrast microscopy (Fig. 7 *b*). Generally, the intensity of immunofluorescence labeling of the sarcolemma was indistinguishable from that of the extracellular space after labeling with mAb IXE11₂ (Fig. 7 *c*). This is particularly evident in the A-band regions of the sarcolemma, where immunofluorescence demarcation of the sarcolemma is absent. Specific labeling of the sarcolemma was not observed in either transverse or longitudinal sections (Fig. 6, *b* and *d*, and Fig. 7 *c*).

SL50. Examination of a transverse cryosection of rabbit skeletal muscle (Fig. 6 *a*) immunolabeled with mAb IVD3₁,

showed that specific labeling was confined to the cell periphery and apparently absent from the interior regions of the skeletal muscle fibers. To test the possibility that specific labeling of transverse tubular membranes was lacking because positive labeling of transverse tubules (30 nm diameter) might not provide a fluorescent signal of sufficient intensity in comparison with that obtained from the sarcolemma in a 6–8- μm cryosection, the distribution of immunofluorescence labeling in an 0.5- μm optical section was examined by confocal microscopy (46). The results shown in Fig. 6, *c* and *e*, demonstrated that an 0.5- μm optical section from the same 5–8- μm immunolabeled section did not

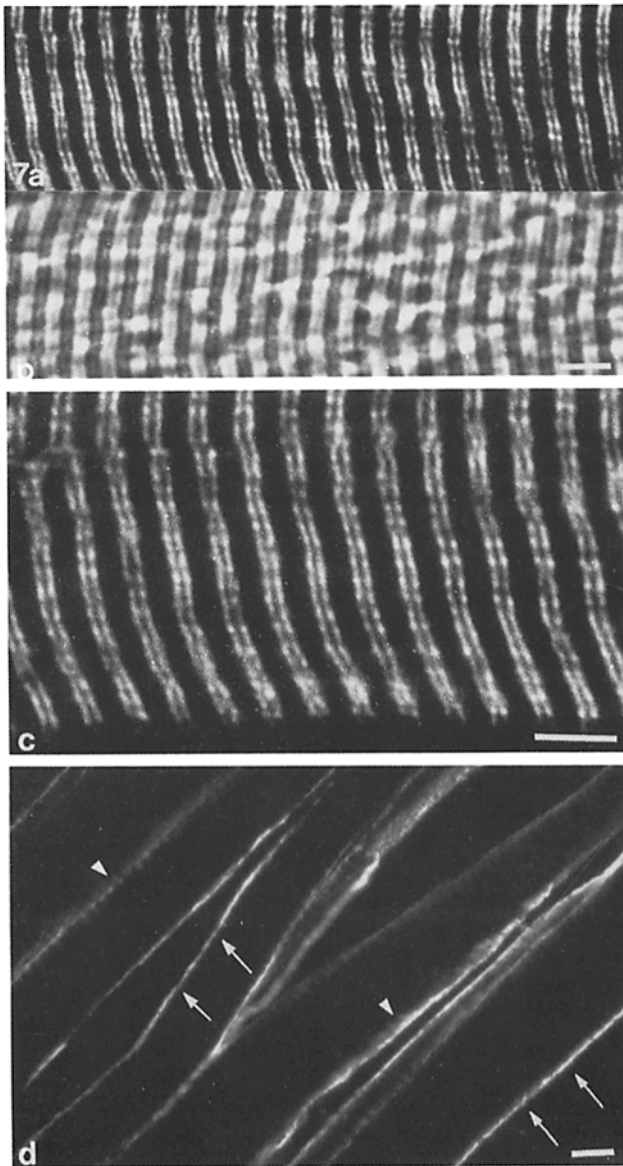


Figure 7. Subcellular distribution of TS28 and SL50 in longitudinal sections of type II myofibers of rabbit gracilis muscle. Longitudinal cryosections of paraformaldehyde fixed (2%) adult rabbit skeletal muscle tissue were labeled with mAb IXE11₂ to TS28 (*a* and *c*) and mAb IVD3₁ to SL50 (*d*). The immunofluorescence staining pattern in *a* was compared with the position of the A- and I-bands in the same respective field (mirror image) (*b*) as viewed by phase-contrast microscopy. Regular fluorescent staining appeared as small bright foci in the interphase between the A- and I-bands after labeling with mAb IXE11₂ to TS28 (*a* and *c*). The intensity of labeling with mAb IXE11₂ of the sarcolemma was generally indistinguishable from that of the extracellular space (*c*). In contrast, mAb IVD3₁ labeled only the cell periphery (*d*). Bars, 5 μ m.

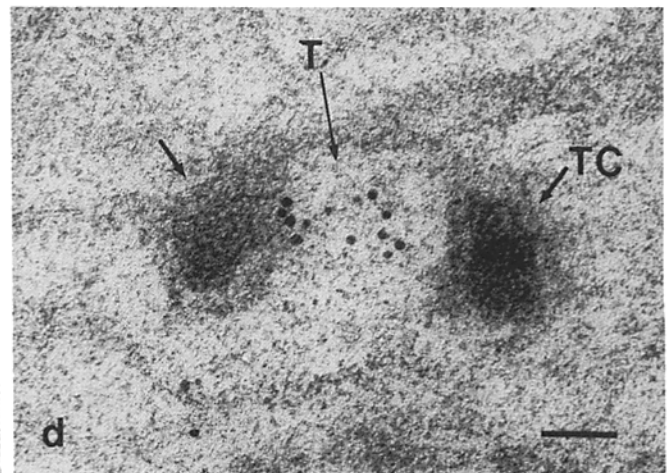
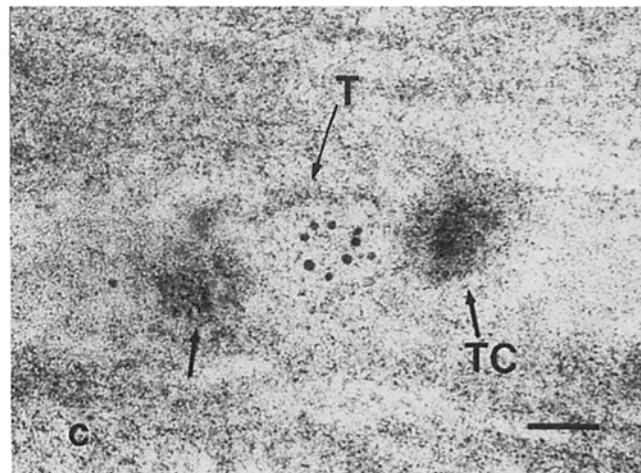
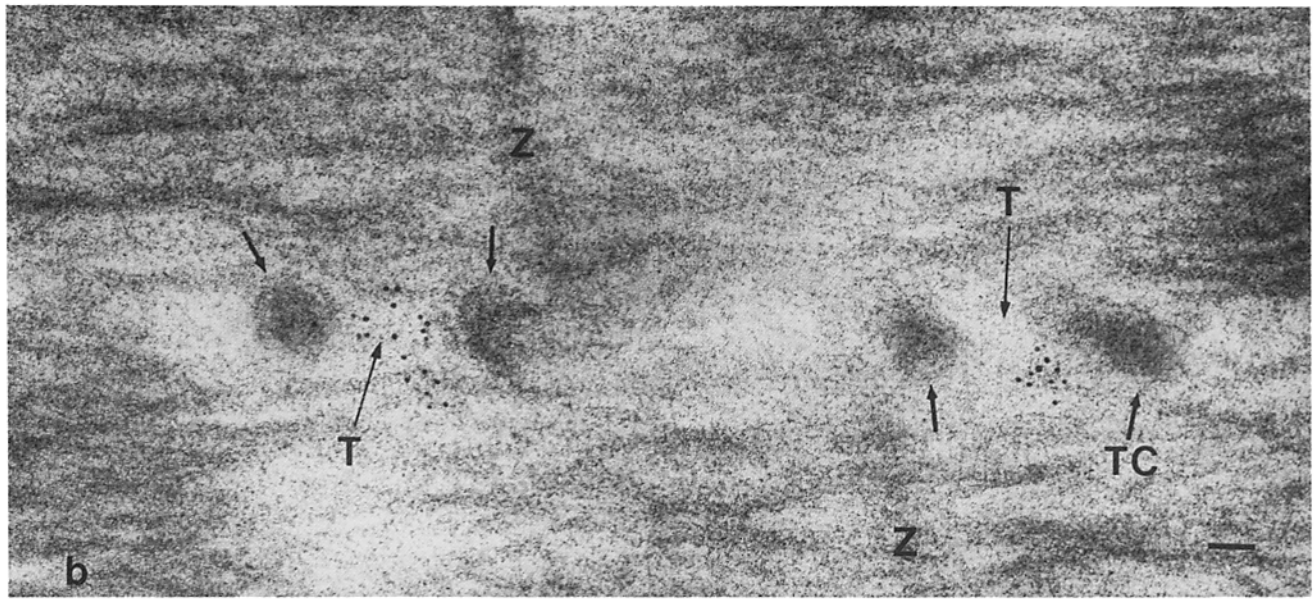
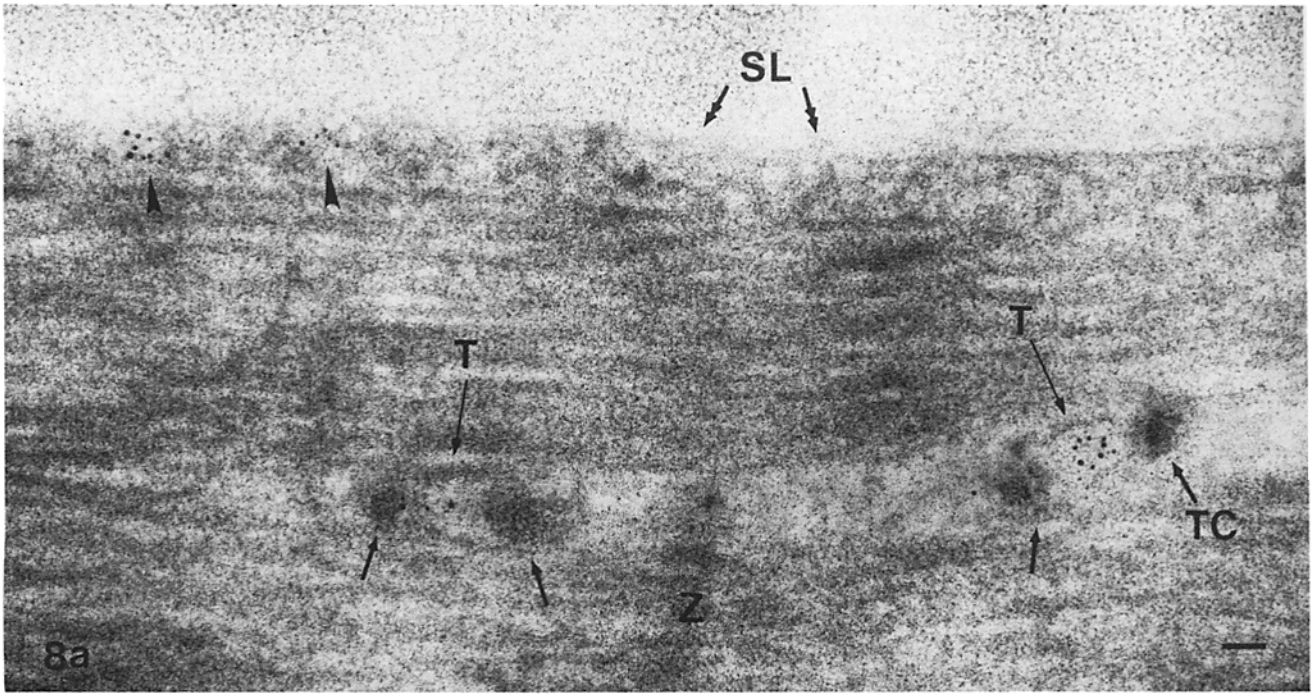
reveal any specific labeling of the interior regions of the muscle cells as would be expected if transverse tubules were labeled. It is noteworthy that the immunofluorescence labeling observed in the 0.5- μ m optical section by confocal microscopy is continuous and of uniform intensity along the entire cell periphery. The immunofluorescence staining pattern observed in longitudinal cryosections of fixed gracilis muscle

after labeling with mAb IVD3₁, showed that specific labeling was confined to the cell periphery where the labeling was generally fairly uniformly distributed (Fig. 7 *d*, *arrows*). Some sarcolemmal regions were less uniformly labeled (Fig. 7 *d*, *arrowheads*). We believe that these regions of the sarcolemma represent imaging of obliquely sectioned sarcolemma. It is also possible that SL50 is more densely distributed in the region of the sarcolemma corresponding to the A-band than in that corresponding to the I-band.

Immunoelectron Microscopical Labeling

The distribution of the TS28 and SL50 in rabbit skeletal muscle as determined by immunofluorescence labeling is consistent with the idea that TS28 is densely distributed in the transverse tubular membrane but absent from the sarcolemma and that SL50 is associated with the sarcolemmal membrane but absent from the transverse tubular membrane. To determine whether TS28 is confined to the transverse tubules the studies were extended to include the immunoelectron microscopical localization of TS28 in rabbit psoas muscle. Preliminary studies showed that inclusion of low concentrations of glutaraldehyde (0.3%) in the 2% paraformaldehyde fixative decreased the intensity of immunofluorescence labeling to a level indistinguishable from that of the background. To optimize the preservation of the antigenicity of TS28, the muscle tissue was cryofixed, freeze-dried, and low temperature embedded in Lowicryl K4M (24). As previously shown for skeletal (25) and cardiac (19, 24) muscle tissue, cellular membranes are well preserved and readily visualized in cryofixed and freeze-dried muscle tissue, provided the tissue is vapor osmicated before embedding in Spurr. Although the osmication step is compatible with the immunoelectron microscopical localization of proteins present in the lumen of subcellular organelles (e.g., 10, 24), it unfortunately blocks immunoelectron microscopical localization of TS28 in Spurr-embedded tissue. Since osmication of cryofixed tissue before low temperature embedding in Lowicryl K4M is not feasible, visualization of membranes in Lowicryl K4M-embedded tissue is variable and less than optimal. However, the sarcolemma (Fig. 8, *a*, *e*, and *f*, *SL*), terminal cisternae (Fig. 8, *a-d*, *TC*), and transverse tubules (Fig. 8, *a-d*, *T*) can be identified. Occasionally, subsarcolemmal vesicles (Fig. 8, *a*, *e*, and *f*, *arrowheads*) can be discerned while longitudinal sarcoplasmic reticulum is rarely visualized. The dilation of the transverse tubules is very likely due to the differential effect of the cryoprotectant PVP (24) on the osmolarity of the cytosol and the lumen of the transverse tubules. The dilated transverse tubular membranes are well delineated in the osmicated specimen (see Fig. 4 of reference 25).

Examination of electron micrographs of thin sections of muscle fibers labeled with mAb IXE11₂ showed that >95% of the colloidal gold particles in the interior regions of the myofiber were distributed over the transverse tubules (Fig. 8, *a-d*). Of these >80% were within 35 nm of the transverse tubular membranes. The remaining 20% of the colloidal gold particles were found in the lumen of the transverse tubules but >35 nm from the transverse tubular membrane. It is, however, very likely that these colloidal gold particles were within 35 nm of tangentially cut transverse tubular membrane not visualized in the images.



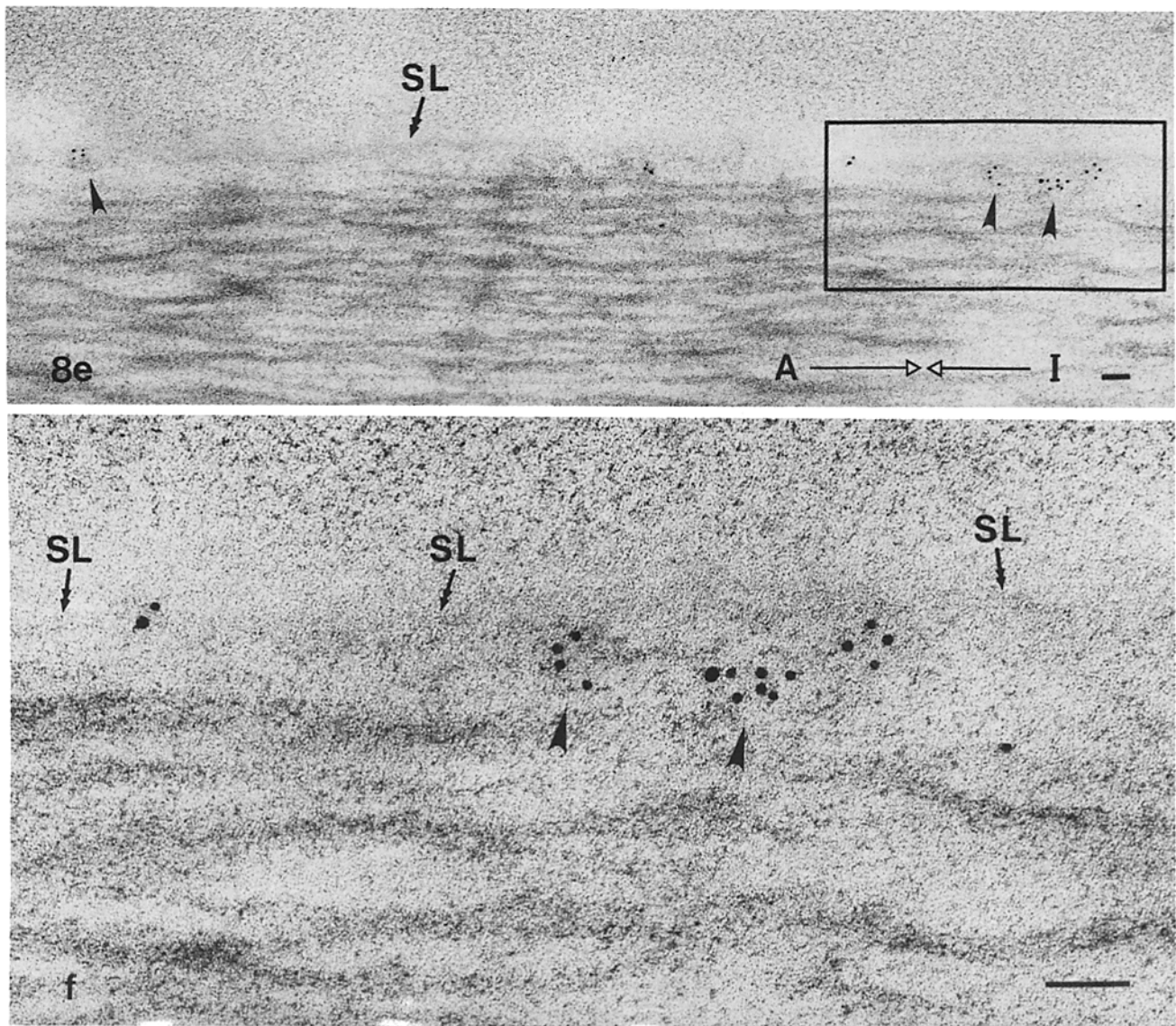


Figure 8. Electron micrograph of longitudinal sections of cryofixed, freeze-dried, and Lowicryl K4M-embedded rabbit psoas muscle labeled with mAb IXE11₂ to TS28 by the triple layered immunocolloidal gold staining technique described in Materials and Methods. A majority of the colloidal gold particles were distributed over the lumen of the transverse tubules (*a-d*, *T*, long thin arrow). Occasionally, clusters of colloidal gold particles were distributed in the subsarcolemmal region of the muscle fiber (*a*, *e*, and *f*, arrowhead). *f* shows a higher magnification of the boxed in area in *e*. Some of the clusters of colloidal gold particles in the subsarcolemmal region appeared to be membrane bound (*f*, arrowhead). The lateral portion of the sarcolemma (*a*, *e*, and *f*, *SL*, double-headed arrow) and the terminal cisternae (*d*, *TC*, arrow) were labeled at the level of the background. *Z*, Z-line. Bars, 0.05 μm .

At the peripheral region of the myofibers, discrete clusters of colloidal gold particles were present in the subsarcolemmal region of the muscle fibers (Fig. 8, *a*, *e*, and *f*). On occasion, these clusters were observed to be localized over caveolae-like structures (Fig. 8 *a*, arrowheads), and membrane-bound structures with a diameter of 80–150 nm (Fig. 8, *e* and *f*, arrowheads). Examination of several long stretches of sarcolemma suggested that these clusters were distributed in a non-uniform manner. Determination of the relative distribution of the clusters of colloidal gold particles in relation to the A- and I-bands showed that ~50% of these clusters were localized within 0.3 μm of the A-band–I-band interface, while the remaining 50% were localized in the A-band regions. In con-

trast, colloidal gold particles were very sparsely distributed over the lateral regions of the sarcolemma (Fig. 8, *a*, *e*, and *f*, *SL*) (<1 colloidal gold particle/10 μm). Similarly, the myofibrils and the interfibrillar spaces, where the longitudinal sarcoplasmic reticulum is densely distributed, were labeled at a level similar to that of the background (<1 colloidal gold particle/10 μm).

Attempts to immunolocalize the SL50 protein at the ultrastructural level of resolution were unsuccessful in that immunolabeling was indistinguishable from that of the background. Since reduction of the transverse tubular membranes before SDS-PAGE and nitrocellulose blotting prevent mAb IVD3₁ from binding to SL50, we anticipate that the conformation

of the epitope on nonreduced SL50 recognized by mAb IVD3₁ is not recognized by this mAb after the light-induced polymerization of Lowicryl K4M.

Discussion

Two novel skeletal muscle membrane proteins, TS28 and SL50, have been identified and partially characterized, and their subcellular distribution in adult skeletal muscle in situ has been determined using two monoclonal antibodies prepared against isolated transverse tubular membrane vesicles.

One of these proteins, TS28, has an apparent relative molecular mass of 28,000 D and is a minor component of the isolated transverse tubular membranes. The association of TS28 with isolated transverse tubular membranes is very strong since TS28 is not removed during washing with high ionic strength buffers carried out during the isolation of these membranes. In addition, TS28 does not appear to be a soluble protein present in the lumen of isolated transverse membrane vesicles since saponin did not remove TS28 (results not shown) like it removes rabbit serum albumin from these membrane vesicles (26). Immunoblotting showed that TS28 is neither a WGA-binding glycoprotein nor is tightly associated with a WGA-binding glycoprotein, implying that TS28 is distinct from the δ subunits (24–32 kD) of the WGA-binding 1,4-dihydropyridine receptor (43). This conclusion was further supported by the finding that sucrose gradient centrifugation of digitonin-solubilized transverse tubular membranes results in the separation of the 1,4-dihydropyridine receptor complex from the TS28 (results not shown). This result also showed that TS28 is not tightly associated with the 1,4-dihydropyridine receptor complex after detergent solubilization. Thus, biochemical studies indicate that TS28 is either an integral membrane protein of the transverse tubular system or attached to an integral membrane protein of this membrane system.

The immunofluorescence and immunoelectron microscopical studies in situ showed that TS28 is localized to the transverse tubules and in some subsarcolemmal vesicles possibly corresponding to a subgroup of caveolae. In contrast, TS28 is absent from the lateral regions of the sarcolemma as well as from the sarcoplasmic reticulum. The ultrastructural study of Ezerman and Ishikawa (12) of developing chick skeletal muscle in culture was the first to show the connection between caveolae and the transverse tubules. Similar studies of guinea pig (37) and frog skeletal muscle (48) have shown that transverse tubules frequently terminate in caveolae, which in turn are continuous with other caveolae and the sarcolemma. Thus, it is possible that the TS28-labeled subsarcolemmal vesicles represent caveolae connecting transverse tubules with the sarcolemma.

Immunofluorescence labeling of skeletal muscle fibers in situ suggested that TS28 was two- to threefold more densely distributed in type II (fast) than in type I (slow) fibers. This finding might in part be explained by the results of ultrastructural studies showing that the density of transverse tubules is approximately twofold higher in type II (fast) fibers than in type I fibers (11). However, further studies will be required to determine whether or not this is the case. Comparison of these results with our previous immunofluorescence and immunoelectron microscopical studies of the subcellular distribution of the α_1 subunit of the 1,4-dihydropyridine receptor

in rabbit skeletal muscle (25) suggest that both TS28 and the α_1 subunit of the 1,4-dihydropyridine receptor are present in the transverse tubules and in some caveolae but absent from the lateral portion of the sarcolemma. Although the function of TS28 is presently unknown, these results support the possibility that TS28 may regulate or function in the excitation-contraction coupling.

The other protein, SL50, has an apparent relative molecular mass of 50,000 D. Immunoblotting showed that SL50 is either a WGA-binding glycoprotein or that it like the β subunit (52,000 D) of the 1,4-dihydropyridine receptor is tightly associated with a WGA-binding glycoprotein (5, 7). However, immunoblotting of the SDS-PAGE separated subunits of the 1,4-dihydropyridine receptor showed that SL50 is distinct from the β subunit of the 1,4-dihydropyridine receptor.

The conventional and confocal immunofluorescence studies of skeletal muscle in situ showed that SL50 is associated with the sarcolemma but apparently absent from the transverse tubules. Regarding the apparent absence of SL50 in transverse tubules it is important to consider whether or not this finding is due to inaccessibility of mAb IVD3₁ to SL50 in transverse tubules especially since recent immunocytochemical studies of cultured myotubes (Jorgensen, A. O., and W. Arnold, unpublished results) showed that the epitope of SL50 labeled by mAb IVD3₁ is exposed to the extracellular side of the sarcolemma. Since calsequestrin localized in the lumen of junctional sarcoplasmic reticulum (23, 32) can be immunolocalized in cultured developing myotubes fixed and air dried by a procedure (21) equivalent to that used to prepare the cryostat sections for immunolabeling in the present study, it is highly unlikely that the observed absence of SL50 in transverse tubules of adult skeletal muscle is due to the fact that SL50 is not accessible to mAb IVD3₁. Although the resolution of immunofluorescence microscopy does not permit one to conclude whether SL50 is an integral or an associated component of the sarcolemma, the inability to extract SL50 from isolated membrane vesicles, unless detergent was added to the extraction buffer, supports the contention that SL50 is an integral component of the sarcolemma. Immunofluorescence studies in situ suggested that the density of SL50 in type II (fast) fibers was indistinguishable from that of type I (slow) fibers.

On the basis of biochemical studies, it has been reported that a protein with an apparent relative molecular mass of 28,000–30,000 D is a major component of the transverse tubular membrane vesicles purified either according to the procedure of Roseblatt et al. (40) or by ion-free sucrose density centrifugation as described by Horgan and Kuypers (18). Recently, Roseblatt and Scales (39) identified four transverse tubular proteins using immunochemical and immunocytochemical studies (Table I). They concluded that two of these, a 38,000-D and a 53,000-D protein, were present in transverse tubules but absent from the sarcolemma. In contrast the two other proteins with apparent relative molecular mass values of 27,500 and 105,000 D were present in both transverse tubules and the sarcolemma.

Since TS28 (28,000 D) described in the present paper is a minor component of transverse tubules and absent from the sarcolemma, we conclude that TS28 is a novel component distinct from both the 28,000–30,000-D proteins previously reported to be a major component of purified transverse tu-

Table I. Comparison of Characteristics of Transverse Tubular and Sarcolemmal Proteins in Skeletal Muscle*

Name	Structural			Subcellular distribution						
	Relative molecular mass	WGA glycoprotein binding	Tight association with 1,4-dihydropyridine receptor	Immunoblotting (isolated membrane)		Immunocytochemical (in situ)				
				TT‡	SL	Light microscopy		Electron microscopy		
						TT	SL	TT	SL	
	<i>kD</i>									
T28	28	No	No	+	ND	+	-	+	-	
SL50	50	Yes	No	+	ND	-	+	ND	ND	
1,4-dihydropyridine receptor subunits (5, 7)		Yes		Yes	No					
α_1	170	No	Yes	Yes	?	+	-	+	-	
α_2	175	Yes	Yes	Yes	?	ND	ND	ND	ND	
β	52	No	Yes	Yes	?	+	-	ND	ND	
γ	32	Yes	Yes	Yes	?	ND	ND	ND	ND	
δ	22-33	Yes	Yes	Yes	?	ND	ND	ND	ND	
MSA55 (41)§	55	ND	ND	ND	ND	-	+	ND	ND	
TT proteins (39)	28	ND	ND	+	+	+	+	ND	ND	
	37	ND	ND	+	-	+	-	ND	ND	
	53	ND	ND	+	-	+	-	ND	ND	
	100	ND	ND	+	+	+	+	ND	ND	
Na ⁺ channel (16)	260	ND	ND	ND	+	+	+	+	+	
	260	ND	ND	ND	+	-**	+	ND	ND	

* Demonstrated by immunoblotting and/or immunocytochemical studies.

‡ TT, transverse tubular membrane; SL, sarcolemmal membrane.

§ Does not bind Con A-Sepharose.

|| Slow skeletal muscle.

** Fast skeletal muscle.

bules (18, 40) and from the 27,500-D protein reported to be equally distributed in the transverse tubules and the sarcolemma (39). In contrast, SL50 (50,000-D), although identified in isolated transverse tubules and isolated triads, was shown by immunocytochemical labeling to be associated with the sarcolemma but apparently absent from the internal regions of skeletal muscle fibers and thus absent from transverse tubules. We conclude that SL50 is confined to the sarcolemma and thus distinct from the 53,000-D protein identified by Roseblatt and Scales (39) and reported to be present in the region of the transverse tubules but absent from the sarcolemma.

Recently Schafer and Stockdale (41) identified a sarcolemma-associated antigen in chicken skeletal muscle with an apparent relative molecular mass of 55,000 D named MSA55 (Table I). Like SL50 described in the present paper, the density of MSA55 in type I (slow) fibers was indistinguishable from that of type II (fast) fibers. Since mAb IVD3₁ does not label chicken skeletal muscle fibers, it is not feasible at the present time to determine whether MSA55 and SL50 are analogous proteins.

The distinct distribution of TS28 and the α_1 subunit of the 1,4-dihydropyridine receptor (25) to the transverse tubular membrane and of SL50 to the sarcolemmal region of the skeletal muscle in situ shows directly that each of these separate but continuous regions of the plasma membrane contain unique proteins.

Thus, our present and previous studies (25) of the subcellular distribution of transverse tubular and sarcolemmal proteins by immunocytochemical labeling of skeletal muscle tissue in situ show directly that each of these distinct but continuous membranes contain some unique proteins. These results support the concept that transverse tubular membranes and sarcolemmal membranes are specialized to carry out distinct functions.

In agreement with this conclusion, Roseblatt and Scales (39) used immunofluorescence labeling to demonstrate that two other proteins present in isolated transverse tubular membranes were localized to the A-band-I-band interphase where transverse tubules are present but were absent from the sarcolemma (Table I). Similarly, Haimovich et al. (16) demonstrated that an epitope on the 260,000-D subunit of the Na⁺ channel is localized to the sarcolemma but absent from interior regions of fast rat skeletal fibers (Table I).

The finding that TS28 is confined to the transverse tubular membrane and that SL50 is confined to the sarcolemmal region as determined by immunocytochemical labeling suggests that these proteins may serve as markers for the two distinct but continuous regions of the skeletal muscle plasma membrane and thus provide a tool for assaying the purity of isolated sarcolemmal and transverse tubular membrane preparations. The presence of SL50 in isolated transverse tubular membranes is most likely due to contamination of these membranes with sarcolemmal membrane vesicles.

This finding suggests that it would be prudent to consider the membrane preparations used in the present studies to be enriched in triadic and transverse tubular membranes rather than purified triads and transverse tubular membrane vesicles.

Finally, we have used mAbs to TS28 (47) and SL50 to study the biogenesis of the transverse tubular membranes and the differentiation of the sarcolemma in developing skeletal muscle cells in situ. The results are presented in the accompanying paper (47).

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