Biogenesis of Transverse Tubules: Immunocytochemical Localization of A Transverse Tubular Protein (TS28) and a Sarcolemmal Protein (SL50) In Rabbit Skeletal Muscle Developing In Situ

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Abstract. To study the biogenesis of transverse tubules, the temporal appearance and distribution of TS28 (a specific marker of transverse tubules absent from the sarcolemma in adult skeletal muscle; 28,000 M_r) and SL50 (specifically associated with the sarcolemma and absent from the region of the transverse tubules in adult rabbit skeletal muscle) (Jorgensen, A. O., W. Arnold, A. C.-Y. Shen, S. Yuan, M. Gaver, and K. P. Campbell. 1990. *J. Cell Biol.* 110:1173–1185) were determined in rabbit skeletal muscle developing in situ (day 17 of gestation to day 15 newborn) by indirect immunofluorescence labeling.

The results presented show that the temporal appearance and subcellular distribution of TS28 is distinct from that of SL50 at the developmental stages examined. TS28 was first detected in some, but not all, multinucleated myotubes on day 17 of gestation. At this stage of development, SL50 and the Ca^{2+}-ATPase of the sarcoplasmic reticulum were already present in all myotubes.

TS28 first appeared in discrete foci mostly confined to the cell periphery of the myotubes. At subsequent stages of development (days 19–24 of gestation), TS28 was also found in short finger-like structures extending obliquely and transversely from the cell periphery towards the center of the myotubes. 1–2 d after birth, TS28 was observed in an anastomosing network composed of transversely oriented chickenwire-like networks extending throughout the cytoplasm and interconnected by longitudinally oriented fiber-like structures. As development proceeded, the transversely oriented network became increasingly dominant. By day 10 of postnatal development, the longitudinally oriented component of the tubular network was not regularly observed. At none of the developmental stages examined was TS28 observed to be uniformly distributed at the cell periphery.

SL50, like TS28, first appeared in discrete foci at the cell periphery. However, shortly after its first appearance it appeared to be distributed along the entire cell periphery. Although the intensity of SL50 labeling increased with development, it remained confined to the sarcolemma and was absent from the interior regions of the myofibers, where transverse tubules were present at all subsequent developmental stages examined.

Immunoblotting of cell extracts from skeletal muscle tissue at various stages of development showed that SL50 was first detected on day 24 of gestation, while TS28 was not detected until days 1–2 after birth.

Comparison of these results with previous ultrastructural studies of the formation of transverse tubules supports the idea that the temporal appearance and subcellular distribution of TS28 correspond very closely to that of the distribution of forming transverse tubules in rabbit skeletal muscle developing in situ. The possibility that the de novo biogenesis of transverse tubules occurs via sequential fusion of newly formed TS28 containing vesicles to preformed caveolae (the "add-on" model) is discussed.

TRANSVERSE tubules are essential for excitation-contraction coupling in skeletal muscle (22). Ultrastructural studies of adult mammalian skeletal muscle have shown that the transverse tubular membranes are continuous with the sarcolemma from which they extend transversely to the center of the myofiber surrounding the myofibrils at the level of the interface between the A- and I-band region (1, 2). The transverse tubules are closely apposed and physically connected to the terminal cisternae of the sarcoplasmic reticulum which in turn releases Ca^{2+} to the cytosol, thus causing a contraction.

At present, our knowledge of the formation of transverse tubules in situ is very sparse. Ultrastructural studies of developing rabbit skeletal muscle in situ have shown that the formation of transverse tubules in rabbit skeletal muscle does

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not begin until days 16–17 of gestation (14). They first appear as short sarcolemmal invaginations. At later stages of development, the forming transverse tubules extend further toward the center of the muscle fiber. 3 d after birth, junctional complexes between transverse tubules and sarcoplasmic reticulum, also called triads, are regularly arranged in the interfibrillar space at the A-band–I-band interface (14).

A detailed ultrastructural study of the formation of transverse tubules and sarcoplasmic reticulum in developing rat skeletal muscle in situ reported that caveolae became more numerous at the onset of the formation of transverse tubules and were sometimes observed to be arranged either in clusters or as short beaded transverse tubules. Most of these transverse tubules were oriented parallel to the long axis of the myofiber and were mainly present in the subsarcolemmal region of the myotubes. As development proceeded, the transverse orientation of the transverse tubules became more prominent, and the number of junctions between the sarcoplasmic reticulum and transverse tubules increased considerably (12).

Due to the fact that comparatively little is known about the structure and function of transverse tubular proteins, it has been difficult to obtain antibodies to transverse tubular marker proteins suitable for probing transverse tubular assembly in situ.

Taking advantage of the hybridoma technology, we have recently used monoclonal antibodies to identify, partially characterize, and determine the subcellular distribution of novel markers of transverse tubules (TS28) and of the sarcolemma (SL50) in rabbit skeletal muscle (10). TS28, a 28,000-D protein, was shown by immunoelectron microscopy to be confined to transverse tubules and to some subsarcolemmal vesicles, possibly corresponding to the subgroup of caveolae connecting transverse tubules with the sarcolemma. TS28 was not detected on the lateral[1] portion of the sarcolemma. In contrast, SL50 was shown by confocal immunofluorescence microscopy to be confined to the cell periphery. Specific immunofluorescence labeling of the A-band–I-band interface or other subcellular regions was not detected, implying that SL50 is absent from transverse tubules and other subcellular compartments.

To begin to understand how transverse tubular proteins assemble into functional transverse tubules in situ, we have determined the temporal appearance and distribution of TS28 (a specific marker of transverse tubules in adult skeletal muscle) and compared it with that of SL50 (a specific marker of the sarcolemma in adult skeletal muscle) during the de novo formation of transverse tubules in developing rabbit skeletal muscle in situ (day 17 of gestation to day 15 after birth) by immunofluorescence labeling.

The results support the idea that the temporal appearance and subcellular distribution of TS28 (*a*) correspond very closely to that of transverse tubules as determined by previous ultrastructural studies of rabbit (14) and rat skeletal muscle developing in situ (12) and (*b*) are distinct from that of SL50, which appears before TS28 and is confined to the cell periphery, where it first appears in discrete foci but shortly after is uniformly distributed at all subsequent stages of development.

[1]In the present study, we have used the term lateral portion of the sacrolemma to denote sacrolemmal membrane less caveolae membrane.

Materials and Methods

Antibodies to Transverse Tubular Membrane Vesicles and Sarcolemmal Membrane

mAb IXE11$_2$ to TS28 and mAb IVD3$_1$ to SL50 of rabbit skeletal muscle were prepared, purified, and characterized as previously described (10).

Antibodies to the Ca^{2+}-ATPase of the Sarcoplasmic Reticulum

mAb IIH11 to the Ca^{2+}-ATPase of rabbit skeletal muscle sarcoplasmic reticulum (8) and affinity-purified polyclonal antibodies to the Ca^{2+}-ATPase of rat skeletal muscle sarcoplasmic reticulum used in this study were prepared, purified, and characterized as previously described (5).

Dissection and Cryosectioning

Hind limb muscle tissues were dissected from rabbits at different stages of development ranging from day 17 of gestation to day 15 after birth. Some bundles of myofibers from 10- and 15-d-old gracilis muscle were tied to tooth picks at 100–120% of rest length, fixed in 2% paraformaldehyde, and infused with 0.6 M sucrose as previously described (6). Small blocks of fixed and unfixed muscle tissue were cryofixed in isopentane precooled in liquid N_2. Cryostat sections (6–8 μm) were cut, fixed in 70% ethanol for 5 min, air dried, and stored in a desiccator at −20°C until used.

Indirect Immunofluorescence Labeling

Immunofluorescence labeling of cryostat sections of fixed and unfixed developing rabbit skeletal muscle tissue was carried out as described previously (6). Briefly, cryosections were first labeled with either mAb IXE11$_2$ to rabbit skeletal TS28 (1.74 mg/ml) (10), mAb IVD3$_1$ to rabbit skeletal protein SL50 (50 μg/ml) (10), mAb IIH11 to the fast form of the Ca^{2+}-ATPase of rabbit sarcoplasmic reticulum (10 μg/ml) (8), or affinity-purified polyclonal antibodies to the Ca^{2+}-ATPase of rat skeletal sarcoplasmic reticulum (5) in PBS (50 mM NaH_2PO_4, 0.9% NaCl, pH 7.4) containing 0.1% BSA. The affinity-purified F(ab')$_2$ fragments of rabbit anti-mouse gamma-globulin conjugated to either fluorescein or rhodamine (1:40 and 1:80 dilution; Jackson Immunoresearch Laboratories, Inc., West Grove, PA) were used as the secondary reagents. Some sections from 17-d fetal rabbit skeletal muscle were double labeled with either mAb IXE11$_2$ to TS28 or with mAb IVD3$_1$ to SL50 and polyclonal antibodies to the Ca^{2+}-ATPase of the sarcoplasmic reticulum (45 μg/ml). The immunofluorescence cryostat sections were examined in a photomicroscope (Carl Zeiss, Inc., Thornwood, NY) provided with epifluorescence attachment. Where indicated, immunolabeled sections were also examined with a confocal fluorescence imaging system (Lasersharp MRC-500; Bio-Rad Laboratories Ltd., Toronto, Canada) (24).

Preparation of Muscle Extracts and Transverse Tubules

Bundles of myofibers were dissected from hind limb of adult and developing rabbit skeletal muscle. The tissues were quickly frozen in liquid N_2 and powdered with a precooled mortar and pestle. The extraction buffer (10% SDS, 10 mM EDTA, 0.1 M Tris, pH 8.0) was added to the powdered muscle tissue at the ratio of 200 mg/ml (wet wt/vol). The extracts were centrifuged for 15 min at 12,800 *g* (Eppendorf centrifuge 5412; Brinkmann Instruments Co., Westbury, NY). The supernatant of the muscle extracts was used immediately for SDS-PAGE. Transverse tubular membranes were prepared by the method of Rosemblatt et al. (20). Protein concentrations were determined by the method of Lowry et al. (17) as modified by Peterson (19) using BSA as standard. SDS-PAGE on 7.5–15% gradient gels was performed by the method of Laemmli (15).

Immunoblotting

Immunoblotting was performed according to the general method of Towbin et al. (23) using 5% nonfat dry milk in TBS (blotto) for blocking the immunoblots as described by Johnson et al. (4). Briefly, the blots were first incubated with either mAb IVD3$_1$ to SL50 or with mAb IXE11$_2$ to TS28 in 2.5% blotto in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) containing 0.05% Tween 20. The blots were then washed to remove unbound antibody and incubated with affinity-purified goat anti-mouse IgG conjugated to alkaline phosphatase (1:3,000

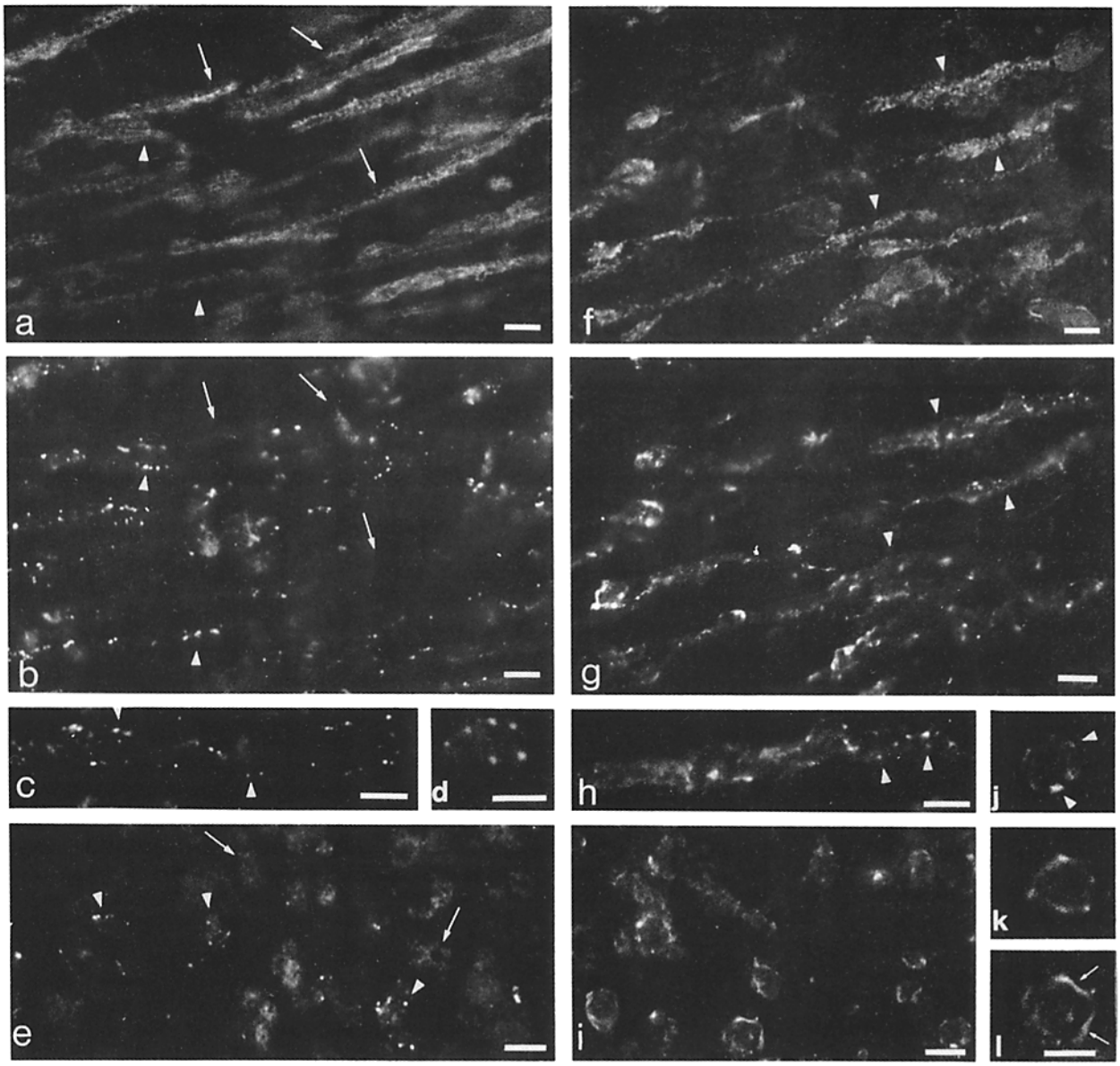

Figure 1. Immunofluorescence localization of TS28 and SL50 in cryosections of unfixed 17-d-old fetal rabbit skeletal muscle. A longitudinal section was double immunolabeled with affinity-purified antibodies to the Ca^{2+}-ATPase of the sarcoplasmic reticulum (*a*) and with mAb $IXE11_2$ to TS28 (*b* and *c*) by the indirect immunofluorescence labeling technique (see Materials and Methods). Similarly, a longitudinal section was double immunolabeled with affinity-purified antibodies to the Ca^{2+}-ATPase of the sarcoplasmic reticulum (*f*) and with mAb $IVD3_1$ to SL50 (*g* and *h*). Transverse sections were immunolabeled with either mAb $IXE11_2$ to TS28 (*d* and *e*) or with mAb $IV3D_1$ to SL50 (*i–l*). All myotubes positively labeled for the Ca^{2+}-ATPase of the sarcoplasmic reticulum (*f, arrowheads*) are also positively labeled for SL50 (*g, arrowheads*). In contrast, only some of the myotubes positively labeled for the Ca^{2+}-ATPase of the sarcoplasmic reticulum (*a, arrowheads*) are also positively labeled for TS28 (*b, arrowheads*), while others positively labeled for the Ca^{2+}-ATPase of the sarcoplasmic reticulum (*a, arrows*) are not labeled for TS28 (*b, arrows*). Labeling for TS28 is confined to the cell periphery, where it is present in a few discrete foci (*b–e, arrowheads*). Labeling for SL50 is also confined to the cell periphery. However, although labeling for SL50 appears in discrete foci in some cells (*h* and *j, arrowheads*), the fluorescence foci in other cells appear to be more diffuse (*k*), and in others fluorescence labeling is fairly homogeneously distributed along a considerable portion of the cell periphery (*l, arrows*). Bars, 5 μm.

dilution; Bio-Rad Laboratories Ltd.) in 2.5% blotto in TBS containing 0.05% Tween 20. The blots were incubated for another reaction cycle (primary antibody, secondary antibody) to enhance the signal as described by Linsenmyer et al. (16). The blots were rinsed twice with TBS containing 0.05% Tween 20 for 5 min each and TBS for 10 min. Color development was completed using BCIP/NBT substrate reagents. All procedures were performed at room temperature with gentle agitation. The reaction was terminated by thoroughly rinsing the blots with water and allowing them to air dry.

Results

Subcellular Distribution of TS28, SL50, and the Ca^{2+}-ATPase in Developing Muscle

Ultrastructural studies have reported that de novo formation of transverse tubules was first observed in myofibers in the

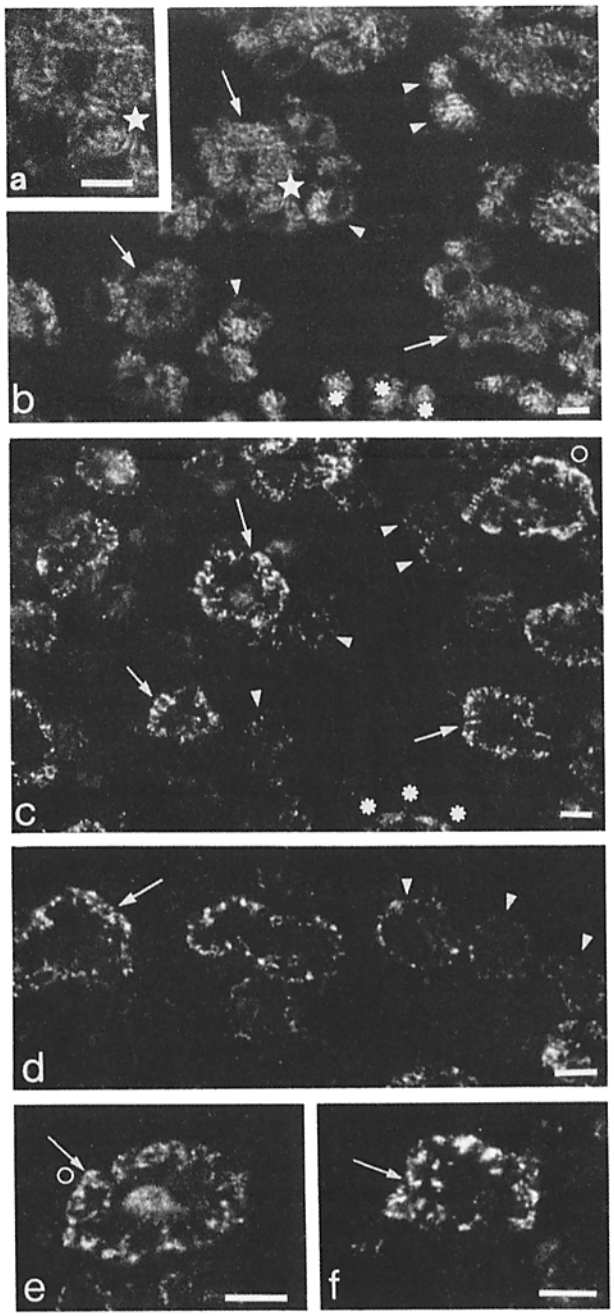

Figure 2. Immunofluorescence localization of TS28 in transverse cryosections of unfixed 19–24-d-old fetal rabbit skeletal muscle. Serial transverse sections were labeled with affinity-purified antibodies to the Ca^{2+}-ATPase of the sarcoplasmic reticulum (*b*) and with mAb IXE11$_2$ to TS28 (*c*) by the indirect immunofluorescence labeling technique. The group of cells in *b* marked by a star is shown at a higher magnification in *a*. Transverse sections (*d–f*) containing both primary (*arrows*) and secondary myotubes (*arrowheads*) were labeled with mAb IXE11$_2$ to TS28. Specific labeling for the Ca^{2+}-ATPase appears as a network throughout the cytoplasm of both primary (*a* and *b*, *arrows*) and secondary myotubes (*a* and *b*, *arrowheads*). Labeling for TS28 is present in all large primary myotubes (*c*, *arrows*) and most of the small secondary myotubes (*c*, *arrowheads*). However, some of the small secondary myotubes positively labeled for the Ca^{2+}-ATPase (*b*, *asterisks*) are not positively labeled for TS28 (*c*, *asterisks*). Labeling for TS28 in large primary myotubes (*c–f*, *arrows*) is most pronounced at the cell periphery, where it sometimes appears to be distributed in discrete foci (*f*). At other times, labeling for TS28 appears to be fairly homogeneously distributed along a considerable portion of the cell periphery (*c* and *e*, *open circle*). In addition, rod-like structures extending from the cell periphery toward the center of the cell and discrete foci were also observed in the cytosol (*e* and *f*). In the smaller, secondary myotubes, labeling for TS28 is confined to the cell periphery, where it is present as discrete foci (*c–d*, *arrowheads*). Bars, 5 μm.

hind limb of rabbit skeletal muscle aged between fetal day 17 and day 2 after birth (14). To determine the temporal appearance and subcellular distribution of TS28 (a specific marker of the adult transverse tubules) and SL50 (specifically associated with the sarcolemma) (10) in relation to the de novo formation for transverse tubules, cryosections from developing rabbit skeletal muscle (day 17 of gestation to day 15 after birth) were examined after indirect immunofluorescence labeling for either TS28 or SL50.

To identify multinucleated myofibers in cryosections from skeletal muscle tissue at various stages of development, the distribution of the Ca^{2+}-ATPase of the sarcoplasmic reticulum (a specific marker of the sarcoplasmic reticulum in adult skeletal muscle) (7) was also examined in the cryosections. The rationale behind this approach is that immunofluorescence studies of developing rat skeletal muscle cells in vitro (5) showed that the Ca^{2+}-ATPase of the sarcoplasmic reticulum first appears in mononucleated myotubes just before fusion and is present in all multinucleated myotubes at subsequent stages of development. Similarly, an immunofluorescence study of the temporal appearance of the fast and slow isoforms of the Ca^{2+}-ATPase in developing chicken pectoral muscle showed that both slow and fast Ca^{2+}-ATPase of the sarcoplasmic reticulum were present in both primary and secondary myotubes on embryonic day 6 of development in situ (11). On the basis of these results, we assumed that sectioned muscle cells positively labeled for the Ca^{2+}-ATPase of the sarcoplasmic reticulum represent multinucleated myotubes.

Fetal Day 17

Examination of longitudinal cryosections from fetal day-17 rabbit skeletal muscle after double labeling with antibodies to the Ca^{2+}-ATPase (Fig. 1 *a*) and to TS28 (Fig. 1 *b*) showed that labeling for TS28 was present in most (Fig. 1 *b*, *arrowheads*) but not all of the myotubes (Fig. 1 *b*, *arrows*) positively labeled for the Ca^{2+}-ATPase of the sarcoplasmic reticulum (Fig. 1 *a*, *arrows* and *arrowheads*). In longitudinally sectioned myotubes, specific immunofluorescence labeling for TS28 was confined to the cell periphery of the positively labeled myotubes (Fig. 1, *b* and *c*), where it was distributed in a few discrete foci. In contrast, specific labeling of the Ca^{2+}-ATPase was present throughout the cytoplasm of all myotubes (Fig. 1 *a*), where it was observed to be distributed in a network-like pattern in some cells. In transverse sections, most of the specific labeling for TS28 also appeared in discrete foci at the cell periphery of some (Fig. 1, *d* and *e*, *arrowheads*) but not all myotubes (Fig. 1 *e*, *arrows*).

Examination of longitudinal cryosections double labeled with antibodies to the Ca^{2+}-ATPase (Fig. 1 *f*) and to SL50 (Fig. 1 *g*) showed that all the myotubes positively labeled for

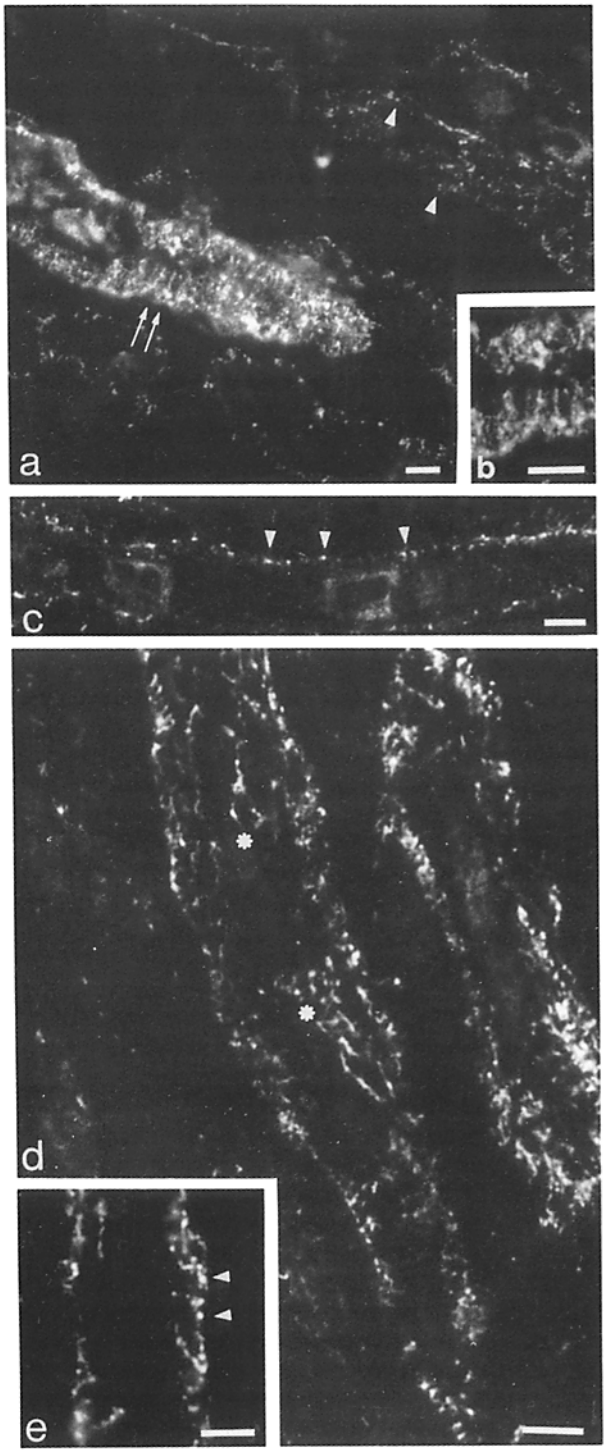

Figure 3. Immunofluorescence localization of TS28 in longitudinal cryosections of 19–24-d-old fetal rabbit skeletal muscle. Longitudinal cryosections were immunolabeled with mAb IXE11$_2$ to TS28 (*a–e*) by the indirect immunofluorescence labeling technique. Labeling for TS28 in large primary myotubes is most pronounced at the cell periphery (*a* and *b*, *arrows*). In addition, transversely oriented rod-like structures extend from the cell periphery toward the center of the cell (*a* and *b*). In the smaller secondary myotubes, TS28 labeling is mostly confined to discrete foci at the cell periphery (*a*, *c*, and *e*, *arrowheads*). A few short fluorescent strands oriented obliquely or longitudinally to the long axis of the cell were occasionally observed (*e*). In other cells, labeling for TS28 distributed in an anastomosing network is occasionally observed over the central region of the secondary fibers (*d*, *asterisks*). Bars, 5 μm.

the Ca^{2+}-ATPase (Fig. 1 *f*, *arrowheads*) were also positively labeled for SL50 (Fig. 1 *g*, *arrowheads*). Specific labeling for SL50 was confined to the sarcolemmal region of the myotubes (Fig. 1, *g–l*), while the Ca^{2+}-ATPase labeling was present throughout the cytoplasm (Fig. 1 *f*). Although specific labeling for SL50, like the labeling for TS28 (Fig. 1, *b–e*), was confined to the cell periphery, the distribution of the labeling for SL50 varied from cell to cell (Fig. 1, *g–l*). In some transversely sectioned myotubes, the labeling for SL50 was present in discrete foci at the cell periphery (Fig. 1 *j*, *arrowheads*); in others, the labeling in discrete foci appeared more diffuse (Fig. 1 *k*). In other myotubes, again faint but specific labeling for SL50 was present along extensive portions of the cell periphery (Fig. 1 *l*, *arrows*).

Fetal Days 19–24

A mixture of myofibers with large and small diameters was observed in transverse sections at this stage of development. Generally, the large myofibers were surrounded by several small myofibers. We presume that the large and small myofibers, respectively, correspond to the primary generation ("primary") and the secondary generation ("secondary") of myotubes previously described in developing rat (13) and chick skeletal muscle (18). Since the developmental stage of primary myotubes is ahead of that of the surrounding secondary myotubes, a mixture of myofibers at various stages of development can be observed in sections from rabbit hind limbs between fetal day 19 and day 24 of gestation.

Examination of two serial transverse sections labeled with either mAb IIH11 to the Ca^{2+}-ATPase (Fig. 2 *b*) or with mAb IXE11$_2$ to TS28 (Fig. 2 *c*) showed that the labeling for the Ca^{2+}-ATPase was present in all primary (Fig. 2 *b*, *arrows*) and secondary myofibers (Fig. 2 *b*, *arrowheads*). Except for a few small secondary myofibers (Fig. 2 *c*, *asterisks*), all myofibers positively labeled for the Ca^{2+}-ATPase (Fig. 2 *b*) were also labeled for TS28 (Fig. 2 *c*). Primary fibers were strongly labeled for TS28 (Fig. 2, *c–f*, *arrows*). Most of the labeling was confined to the peripheral region of the myofibers, where it was mostly present in either discrete foci or in rod-like structures (Fig. 2 *c*). Some fluorescently labeled structures also appeared to extend from the cell periphery approximately halfway toward the center of the myofibers (Fig. 2, *c–f*, *arrows*). This was clearly observed in longitudinally sectioned primary myofibers (Fig. 3, *a* and *b*, *arrows*). Secondary myofibers (Fig. 2, *c* and *d* and Fig. 3, *a*, *c*, and *e*, *arrowheads*) were less strongly labeled for TS28 than the primary myofibers (Fig. 2, *c–f*, and Fig. 3, *a* and *b*, *arrows*). Most of the labeling was confined to the cell periphery, where it was mainly present in discrete foci in both transverse (Fig. 2, *c* and *d*, *arrowheads*) and longitudinal sections (Fig. 3, *a*, *c*, and *e*, *arrowheads*). It is noteworthy that the distribution of TS28 in secondary fibers in fetal day-24 hind limb is very similar to that observed in fibers from fetal day-17 muscle. This observation supports the view that the developmental process in secondary fibers, with respect to the temporal appearance and distribution of TS28, represents a delayed repeat of this process in primary fibers. In some longitudinally sectioned cells, labeling in the interior region was distributed in an anastomosing network (Fig. 3 *d*, *asterisks*). In contrast, specific labeling for the Ca^{2+}-ATPase was present as a network throughout the cytoplasm in both primary and secondary myofibers (Fig. 2, *a* and *b*).

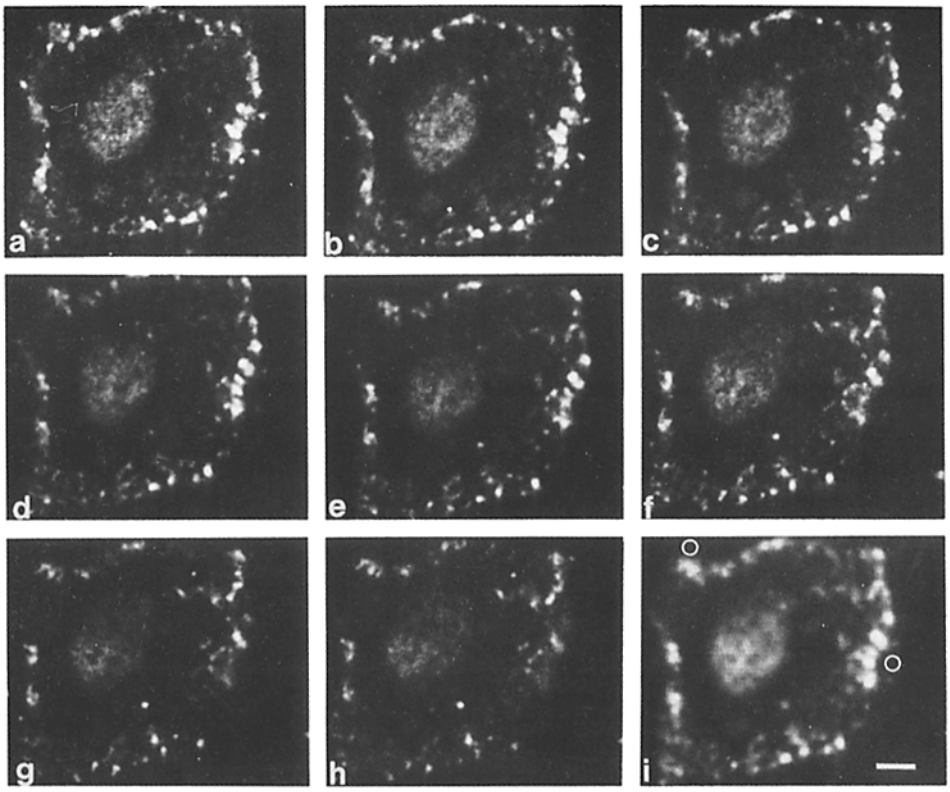

Figure 4. Immunofluorescence localization of TS28 in a series of 0.2-μm optical sections from 24-d-old fetal skeletal muscle. A 6–8-μm transverse cryosection was immunofluorescently labeled with mAb IXE11$_2$ to TS28. Images of ten serial optical sections (0.2 μm) were obtained from the central region of the 6–8-μm cryosection by confocal microscopy. The summary image of these ten sections (total of 2 μm) is shown in *i*. Eight of the serial sections are shown in *a–h*. The immunofluorescence labeling is mostly confined to the cell periphery, although a few discrete foci are seen in the central region of the cells. The regions of the cell periphery of the summary image (*i*), where the distribution of the immunofluorescence labeling approaches continuity (*i*, *open circles*), can in the series of 0.2-μm optical sections be seen to be resolved into distinct foci or short rod-like structures at the cell periphery. Some of the foci at the cell periphery are seen in a few optical sections and some are seen in all eight optical sections, suggesting that most of the foci at the cell periphery represent a tubular structure in the subsarcolemmal region of the myofiber, where it is generally oriented parallel to the long axis of the myofiber. Bar, 2 μm.

The intensity of the Ca^{2+}-ATPase labeling was quite similar in both types of myofibers (Fig. 2, *a* and *b*, *arrows* and *arrowheads*).

Since the distribution of TS28 in some of the primary myofibers appeared to approach continuity in some regions of the cell periphery (Fig. 2, *c* and *e*, *open circle*), it became important to determine whether this was due to the lack of resolution along the *Z*-axis or whether TS28 was indeed becoming uniformly distributed along the sarcolemma as development progressed. Thus, transverse cryosections (6–8 μm) of fetal 24-d skeletal muscle tissue, labeled for TS28, were examined using a confocal imaging system. A series of eight confocal images (Fig. 4, *a–h*; recorded with a stage movement of 0.2 μm between successive sections) taken through the central region of a single 6–8-μm-thick cryosection is shown in Fig. 4. Examination of a photograph of a summary projection (Fig. 4 *i*) of the individual images (Fig. 4, *a–h*) showed that specific labeling for TS28 is mostly distributed in discrete foci but also approaches continuity in some regions of the cell periphery (Fig. 4 *i*, *open circle*). In contrast, examination of the distribution of TS28 labeling in each of the confocal images of the series of optical sections (Fig. 4, *a–h*) showed that the regions of the cell periphery that appeared continuously labeled in the summary projection had been resolved into discrete foci or short rod-like structures in the confocal images.

Comparison between two serial transverse sections (6 μm) from 24-d fetal skeletal muscle labeled with antibodies to either TS28 (Fig. 5 *a*) or SL50 (Fig. 5 *b*) showed that the distribution of labeling for SL50 is distinct from that for TS28. As described above, specific labeling for TS28 was most densely distributed at the cell periphery, where labeling for the most part was present as discrete foci or rod-like structures (Fig. 5 *a*, *arrows*). In contrast, the labeling for SL50 appeared to be more or less continuously distributed along a considerable portion of the cell periphery of some cells (Fig. 5 *b*, *solid circle*). However, the degree of staining intensity varied considerably.

To better assess whether SL50 was uniformly distributed along the cell periphery, the distribution of labeling for SL50 in 6–8-μm cryosections imaged by conventional immunofluorescence microscopy (Fig. 5 *b*) was compared with that of 2-μm (Fig. 5 *c*) and of 0.2-μm (Fig. 5 *d*) optical sections imaged by confocal fluorescence microscopy. The results showed that the proportion of the cell periphery homogeneously labeled for SL50 increases considerably as the thickness of the imaged section decreases from 6–8 μm (Fig. 5 *b*) to 2 μm (Fig. 5 *c*) and then to 0.2 μm (Fig. 5 *d*). This finding supports the idea that SL50 is indeed uniformly distributed along the entire cell periphery. Thus, the nonhomogeneous distribution of labeling for SL50 at the cell periphery is most likely due to obliquely oriented sarcolemma in the weakly labeled regions of the cell periphery (Fig. 5 *b*, *open circle*).

Newborn: 1–2 d

Examination of serial transverse cryosections from 1–2-d-old rabbit skeletal muscle immunolabeled for the Ca^{2+}-ATPase (Fig. 6 *a*) and TS28 (Fig. 6 *b*) showed that all myofibers were

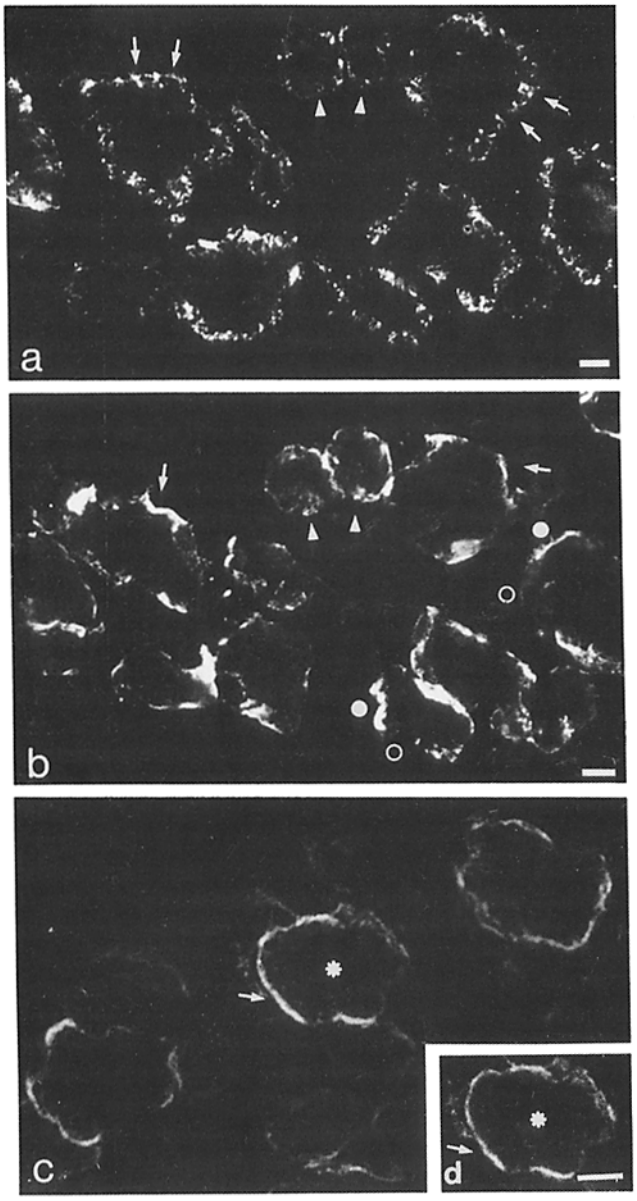

Figure 5. Comparison between the immunofluorescence localization of TS28 and SL50 in cryosections of 24-d-old fetal rabbit skeletal muscle. Serial transverse sections were labeled with mAb IXE11$_2$ to TS28 (*a*) and with mAb IVD3$_1$ to SL50 (*b*) and imaged by conventional fluorescence microscopy (6–8-μm sections). A transverse section (6–8 μm) was labeled with mAb IVD3$_1$ to SL50 and imaged by confocal fluorescence microscopy (*c* and *d*). The image in *c* represents a summary image of ten 0.2-μm optical sections obtained from the central region of a 6–8-μm cryosection. The cell shown in *d* shows a portion of one of the ten 0.2-μm optical sections used to compose the central cell (*c*, *asterisk*). As previously shown in Fig. 2, *c–f*, labeling for TS28 is most prominent at the cell periphery, where it is distributed in discrete foci in both primary (*a*, *arrows*) and secondary myotubes (*a*, *arrowheads*). In addition, labeling for TS28 is present in short rod-like structures extending from the cell surface toward the interior region of the cell and is also present in primary myotubes (*a*, *arrows*). Labeling for SL50 is also confined to the cell periphery. However, the intensity of labeling along the cell periphery appears to be very irregularly distributed. Thus, while the intensity of labeling may be relatively strong along some portions of the sarcolemma (*b*, *solid circles*), it may be relatively weak along other regions of the same myofiber (*b*, *open circles*). As the thickness of the section imaged decreases from 6–8 μm (*b*) to 2 μm (*c*) and then to 0.2 μm (*d*), the labeling for SL50 becomes increasingly homogeneous along extensive regions of the cell periphery, suggesting that the lack of homogeneity in the distribution of labeling in the 6–8-μm-thick sections (*b*) might be due to obliquely oriented sarcolemma in the regions of the cell periphery, where intensity of SL50 labeling is relatively low. Bars, 5 μm.

strongly labeled for both of these proteins. The Ca^{2+}-ATPase immunolabeling appeared as a chickenwire-like network throughout the cytoplasm of all myofibers (Fig. 6 *a*). The immunolabeling for TS28 (Fig. 6 *b*), like that for the Ca^{2+}-ATPase (Fig. 6 *a*), was also distributed in a chickenwire-like pattern throughout the cytoplasm; however, the distribution of the immunolabeling for TS28 appeared as a less complete network than that for the Ca^{2+}-ATPase. Specific immunolabeling for SL50 was confined to the cell periphery, where it was fairly uniformly distributed along the entire circumference of the cell (Fig. 6 *c*). However, the intensity of labeling was somewhat nonuniform. Specific immunolabeling for SL50 was not detectable in the interior regions of the myofibers (Fig. 6 *c*).

Examination of longitudinal cryosections after immunolabeling for TS28 (Fig. 6, *d* and *e*) showed that the labeling was distributed in an anastomosing network of which the transversely (Fig. 6 *e*, *arrows*) and longitudinally oriented components (Fig. 6, *d* and *e*) were equally prominent, suggesting that the transverse tubular membrane system at this stage of development is composed of a series of transversely oriented chickenwire-like networks that are interconnected by numerous longitudinally oriented transverse tubules.

Newborn: 10–15 d

Examination of transverse cryosections from 10-d (Fig. 7, *a* and *b*) and 15-d-old rabbit muscle (Fig. 7, *f* and *g*) immunolabeled for TS28 showed that all fibers were labeled at both stages of development. In 10-d-old rabbits, the intensity of labeling was similar in all fibers (Fig. 7 *a*). Although most fibers were relatively strongly labeled in 15-d-old rabbits (Fig. 7 *f*, *arrowheads*), some fibers were relatively weakly labeled (Fig. 7 *f*, *arrows*). Since slow (type I) fibers in adult skeletal muscle are relatively weakly labeled for TS28 compared with those of fast (type II) fibers (10), we conclude that the myofibers weakly labeled for TS28 in 15-d-old muscle correspond to slow (type I) myofibers.

Examination of longitudinal cryosections from 10-d (Fig. 7, *d* and *e*) and 15-d-old rabbits (Fig. 7, *i* and *j*) immunolabeled for TS28 showed that the labeling was confined to transversely oriented strands (two per sarcomere) (Fig. 7, *e* and *j*, *arrows*) positioned at the A-band–I-band interface, as demonstrated by imaging the same field using phase-contrast microscopy (results not shown).

Comparison of serial transverse cryosections from 10-d (Fig. 7, *b* and *c*) and 15-d-old (Fig. 7, *g* and *h*) rabbit skeletal muscle immunolabeled for either TS28 (Fig. 7, *b* and *g*) or SL50 (Fig. 7, *c* and *h*) showed that immunolabeling for TS28 in both 10- (Fig. 7 *b*) and 15-d-old skeletal muscle fibers (Fig. 7 *g*) was confined to the chickenwire-like network in the cytosol and absent from the cell periphery. In contrast, immunolabeling for SL50 was fairly uniformly distributed

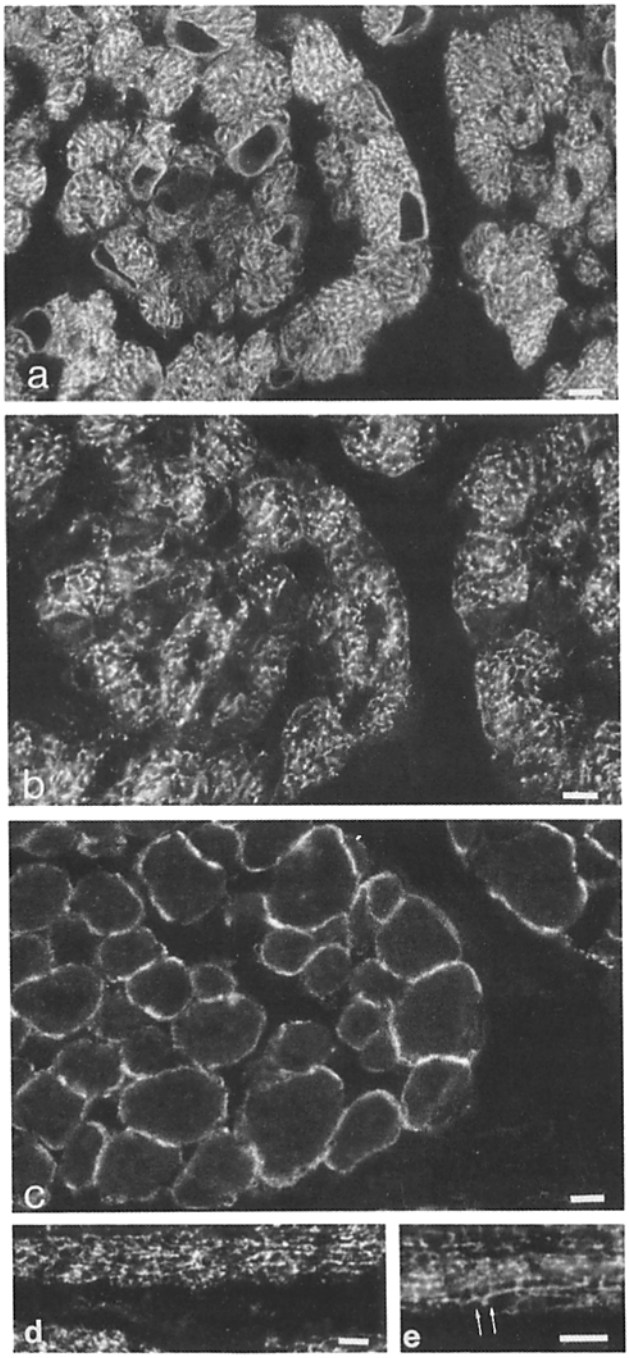

Figure 6. Immunofluorescence localization of TS28 and SL50 in cryosections from 1-2-d newborn rabbit skeletal muscle. Transverse (*a–c*) and longitudinal sections (*d* and *e*) were labeled with mAb IIH11 to the fast form of the Ca^{2+}-ATPase of the sarcoplasmic reticulum (*a*), with mAb $IXE11_2$ to TS28 (*b*, *d*, and *e*), and with mAb $IVD3_1$ to SL50 (*c*). The sections shown in *a* and *b* are serial. In transverse sections, immunofluorescence labeling for the Ca^{2+}-ATPase (*a*) and for TS28 (*b*) is distributed in a chickenwire-like network throughout the cytosol. In contrast, the immunofluorescence labeling for SL50 is confined to the cell periphery, where it appears to be fairly homogeneously distributed along the entire circumference (*c*). In longitudinal sections, labeling for TS28 (*d* and *e*) is distributed in an anastomosing network composed of both longitudinally and transversely (*e*, *arrows*) oriented fiber-like structures. Bars, 5 μm.

along the cell periphery but apparently absent from the interior regions of the cytosol, where transverse tubules are located in both 10- (Fig. 7 *c*) and 15-d-old (Fig. 7 *h*) rabbit skeletal muscle fibers.

Temporal Expression of TS28 and SL50

The immunofluorescence studies of fetal day-17–24 muscle tissue suggested that SL50 appears before TS28 during the in situ differentiation of hind limb myotubes (Fig. 1). To further assess this possibility, the relative amounts of TS28 and SL50 were evaluated by immunoblotting of SDS-PAGE separated proteins in extracts from rabbit skeletal muscle at different stages of development.

The results presented in Fig. 8 show that mAb $IVD3_1$, specific for SL50 in adult skeletal muscle (10), detected a 50,000-D component in muscle extracts from day-24 fetal rabbits (Fig. 8 *b*, lane *2*) and from 1–2-d-old rabbits (Fig. 8 *b*, lane *3*) but not from day-17 fetal rabbits (Fig. 8 *b*, lane *1*). mAb $IVD3_1$ to SL50 also recognized a minor band of 48,000 D in these extracts from 24-d fetal (Fig. 8 *b*, lane *2*) and from 1–2-d-old rabbits (Fig. 8 *b*, lane *3*). The 48,000-D protein was not detected by mAb $IVD3_1$ to SL50 in either extracts (10) or isolated transverse tubular membranes (Fig. 8 *b*, lane *4*) of adult skeletal muscle. Since previous studies suggested that SL50 is a wheat germ agglutinin binding protein (10), it is likely that the 48,000-D band recognized by mAb $IVD3_1$ in extracts from developing muscle represents incompletely glycosylated SL50. In contrast, mAb $IXE11_2$, specific for TS28 in adult skeletal muscle (10), first detected a 28,000-D component in extracts from 1–2-d-old muscle (Fig. 8 *a*, lane *3*) but not in extracts from day-17 (Fig. 8 *a*, lane *1*) and day-24 fetal skeletal muscle tissue (Fig. 8 *a*, lane *2*).

The apparent discrepancy between the results of the immunoblotting experiments showing that SL50 and TS28 first appear in fetal day 24 and days 1–2 after birth, respectively, and the immunofluorescence studies suggesting that SL50 is present in all myofibers while TS28 is only present in some myofibers on day 17 of gestation is most likely due to the fact that the immunofluorescence labeling technique is more sensitive than the immunoblotting technique. Since the development of the hind limb muscle fibers is not synchronized, the relative rather than the absolute time of appearance of these two proteins is important for our ability to determine the sequence of events required for transverse tubular membrane assembly. Thus, both sets of experiments support the conclusion that the first appearance of SL50 in a particular cell precedes that of TS28.

Discussion

We have recently reported that TS28 is confined to transverse tubules and that SL50 is confined to the region of the sarcolemmal membrane in adult skeletal muscle (10). In the present study, we have determined the temporal appearance and distribution of TS28 and SL50 during the de novo biogenesis of transverse tubules in rabbit skeletal muscle by indirect immunofluorescence labeling.

The immunofluorescence studies showed that on day 17 of gestation TS28 was detected in some but not all myotubes, whereas SL50 and the Ca^{2+}-ATPase of the sarcoplasmic re-

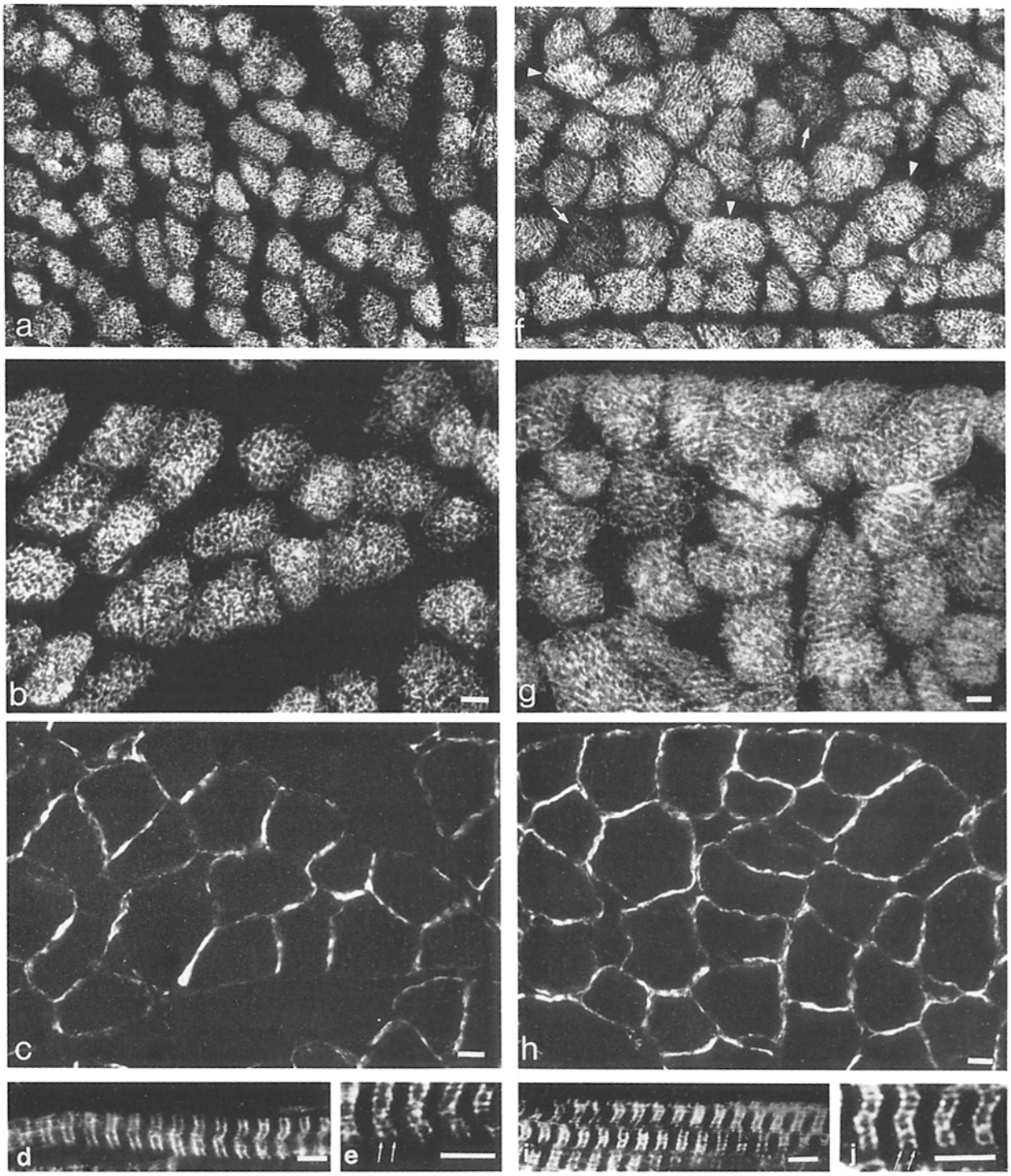

Figure 7. Immunofluorescence localization of TS28 and SL50 in cryosections from 10- and 15-d-old rabbit skeletal muscle. Transverse sections from 10- (*a–c*) and 15-d-old rabbit muscle (*f–h*) were labeled with mAb IXE11$_2$ to TS28 (*a*, *b*, *f*, and *g*) or with mAb IVD3$_1$ to SL50 (*c* and *h*). The sections in *b* and *c*, or *g* and *h* are serial. In transverse sections from 10- (*c*) and 15-d-old (*h*) rabbit muscle, immunofluorescence labeling for SL50 is observed to be fairly uniformly distributed along the cell periphery and absent from the interior regions of the cytosol. In contrast, immunofluorescence labeling for TS28 in both 10- and 15-d-old muscle are distributed in a complete chickenwire-like network present throughout the cytosol (*a*, *b*, *f*, and *g*). The intensity of immunofluorescence labeling for TS28 is similar in all fibers in 10-d-old muscle (*a*). However, in 15-d-old muscle, two classes of fibers can be distinguished. Of these, one class is relatively intensely labeled (*f*, *arrowheads*), while the second class is very weakly labeled for TS28 (*f*, *arrows*). In longitudinal sections from 10- (*d* and *e*) and 15-d-old muscle (*i* and *j*), immunofluorescence labeling for TS28 is confined to transversely oriented strands, at the A-band–I-band interface (*e* and *j*, *arrows*). Bars, 5 μm.

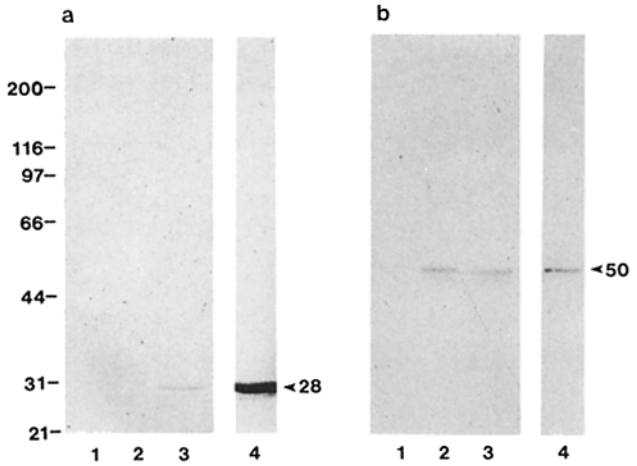

Figure 8. Detection of SL50 and TS28 in muscle extracts from rabbit skeletal muscle at different stages of development by immunoblotting. Isolated transverse tubular membrane vesicles (*a* and *b*, lane *4*; 10 μg) and rabbit skeletal muscle extracts from day-17 fetal (*a* and *b*, lane *1*; 30 μl), day-24 fetal (*a* and *b*, lane *2*; 30 μl), and 2-d-old animals (*a* and *b*, lane *3*; 30 μl) were prepared, separated by SDS-PAGE, and immunoblotted with mAb IXE11$_2$ to TS28 (*a*) and with mAb IVD3$_1$ to SL50 (*b*) as described in Materials and Methods. SL50 was first detected in skeletal muscle extracts from 24-d fetal rabbits (*b*, lane 2) and subsequently from 1–2-d newborn animals (*b*, lane *3*). In contrast, TS28 was first detected in skeletal muscle extracts from 1–2-d-old animals (*a*, lane *3*).

ticulum were present in all myotubes. These results imply that the temporal appearance of SL50 and the Ca^{2+}-ATPase in a particular cell precede that of TS28. This conclusion is supported by the immunoblotting experiment (Fig. 8) showing that SL50 was first detected in muscle extracts from fetal day-24 muscle, while TS28 was not detected in the very same extracts until 1–2 d after birth.

The first appearance of TS28 on days 16–17 of gestation is concurrent with the onset of the de novo formation of transverse tubules as previously determined by ultrastructural studies (14). The presence of TS28 in discrete foci sparsely distributed at the cell periphery in both longitudinal and transverse sections suggests that TS28 first appears in caveolae previously shown to be densely distributed at the cell surface at the onset of the formation of transverse tubules (12). The presence of TS28 in rod-like structures later on supports the idea that TS28 is present in short tubular invaginations of the sarcolemma. This conclusion is also supported by the results of the serial confocal images of transverse sections from 24-d-old fetal muscle, suggesting that the discrete foci at the cell periphery represent fiber-like structures. The presence of an anastomosing network seen in longitudinal sections of 19–24-d fetal myofibers labeled for TS28 supports the conclusion that TS28 is associated with fiber-like structures that grow longer and branch as development proceeds.

Combining the results of the distribution of TS28 observed in longitudinal and transverse sections of 19–24-d-old fetal muscle indicates that TS28 is associated with subsarcolemmal fiber-like structures oriented parallel to the long axis of the myofiber. Only later (1–2 d after birth) did TS28 appear to be associated with a denser anastomosing network composed of both longitudinally and transversely oriented fiber-like components, and at 10–15 d after birth TS28 was almost exclusively associated with transversely oriented chickenwire-like networks indistinguishable from those of adult fibers (10). These results are in agreement with previous ultrastructural studies of the formation of sarcoplasmic reticulum and transverse tubules in developing rat skeletal muscle

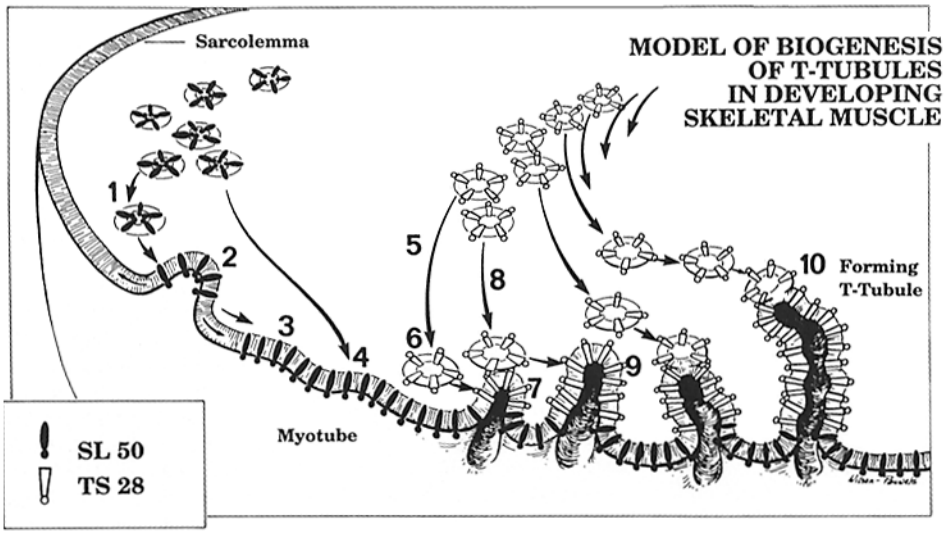

Figure 9. Model for the assembly of SL50 and TS28 into sarcolemma and transverse tubules. First, SL50 begins to be synthesized on membrane-bound polysomes and then is transported to the subsarcolemmal region of the myotubes (step *1*). Next, SL50-containing vesicles fuse with the sarcolemma, resulting in, first, a nonuniform distribution of SL50 (step *2*). Then the SL50 quickly diffuses laterally into the lipid bilayer of the sarcolemma, where it remains uniformly distributed during all subsequent stages of myofiber development (step *3*). As this continues to occur (step *4*), TS28 synthesized on membrane-bound polysomes is transported to the subsarcolemmal region in transfer vesicles (step *5*). To initiate the formation of a particular transverse tubule, a TS28-containing vesicle (step *6*) fuses with the sarcolemma, forming a TS28-containing caveola (step *7*). However, TS28 in the lipid bilayer of the caveola is somehow prevented from lateral diffusion into the lateral portion of the sarcolemma. Subsequently, additional TS28-containing transfer vesicles (step *8*) fuse first with the TS28-containing caveola (step *7*), forming short tubular invaginations in the subsarcolemmal region (step *9*). Repeated fusions of TS28-containing transfer vesicles with the short TS28-containing tubular invaginations result in the formation of further extended transverse tubules (step *10*). These are, at first, oriented parallel to the long axis in the subsarcolemmal region of the myotubes (not shown). Eventually they become organized into a transversely oriented chickenwire-like network surrounding the myofibrils present throughout the cytosol.

in situ (12, 21). These studies reported that the transverse tubular membrane system is mostly oriented parallel to the long axis of the myofiber when they first appear (15–18 d of gestation) in rat and are mainly present in the subsarcolemmal region of the myotubes (12). As development proceeds, the transverse orientation of the transverse tubules becomes more prominent.

On the basis of the results presented and previous ultrastructural studies of developing skeletal muscle (12, 14, 21), we conclude that the temporal appearance and subcellular distribution of TS28 as determined by immunofluorescence labeling correspond very closely to that of transverse tubules forming de novo in developing rabbit and rat skeletal muscle. This conclusion implies that TS28 is fairly uniformly distributed in forming transverse tubules and absent from the sarcolemma at the onset of formation of transverse tubules and at all subsequent stages of the development of transverse tubules.

Although SL50 was observed in all myotubes on day 17 of gestation, where it was confined to the cell periphery, the distribution of SL50 at the cell periphery varied from cell to cell. In some myotubes SL50 appeared as discrete foci, in others it appeared as diffuse foci, and in others it appeared to be homogeneously distributed along either one portion or along the entire circumference of the myofiber. On day 24 of gestation and in 1–2-, 10-, and 15-d-old rabbit skeletal fibers, SL50 was observed to be rather uniformly distributed along the entire length of the cell periphery. The absence of labeling for SL50 in the interior regions of the myofibers from fetal day-17 to newborn day-15 muscle supports the idea that SL50 is not a component of the forming transverse tubules at any developmental stage examined.

Regarding the mechanism of the formation of transverse tubules, two models have been proposed on the basis of ultrastructural studies of muscle developing in vitro (3) and in vivo (12). One of the models proposes that transverse tubules are formed by sequential fusions between a preformed caveola and newly formed membrane-bound vesicles (the "add-on" model) (3). The other model proposes that the formation of transverse tubules occurs by continuous extension of short sarcolemmal membrane invaginations toward the center of the cell. This model proposes that the increase in the surface area of the sarcolemma is provided by random fusion of newly synthesized membrane-bound vesicles with the sarcolemma (the "swept-in" model) (12).

If the add-on model is correct, one would expect a marker of the transverse tubules to be confined to transverse tubules and their precursors but absent from the lateral portions of the sarcolemma. This model would also predict a marker of the sarcolemma to be confined to the sarcolemma and thus absent from transverse tubules at all stages of the de novo biogenesis of transverse tubules. These predictions furthermore assume that proteins in caveolae and transverse tubules are prevented from diffusing into the lateral region of the sarcolemma. If the swept-in model is correct, one would predict a marker of the sarcolemma present in the sarcolemma before the formation of transverse tubules to also be a component of the forming transverse tubules, just as a marker of transverse tubules would first arrive at the sarcolemma by vesicular transport, then become incorporated into sarcolemma, and subsequently be swept into the forming transverse tubules by an as yet unknown mechanism.

The results presented strongly support the add-on model of the de novo transverse tubule formation (Fig. 9). On the basis of the results presented and current ideas about intracellular vesicular transport of membrane proteins, we propose the following model for the assembly of SL50 and TS28 into the sarcolemma and the transverse tubules, respectively. Assuming that the different patterns of distribution of SL50 along the cell periphery represent different stages of myotube development, the results presented support the idea that SL50, after its biosynthesis on membrane-bound polysomes, is transported in "transfer vesicles" to the cell periphery where the transfer vesicles fuse with the sarcolemma. Thereby, SL50 is first briefly distributed in discrete foci and later, as it diffuses into the lateral portions of the sarcolemma, becomes homogeneously distributed in the sarcolemma. SL50 is prevented from diffusing into transverse tubules at all stages of development by an as yet unknown mechanism. The finding that TS28 is distributed in discrete foci and not observed to be uniformly distributed at the cell periphery is consistent with the idea that TS28, after its biosynthesis on membrane-bound polysomes, is transported in transfer vesicles directly to the subsarcolemmal region. After fusion of the transfer vesicle with the sarcolemma, the transfer vesicle becomes a caveola. TS28 in the caveolae is prevented by an unknown mechanism from diffusing laterally and, thus, from becoming a component of the lateral portion of the sarcolemma. Subsequently, additional TS28-containing transfer vesicles fuse first with the TS28-containing caveola, forming short tubular invaginations in the sarcolemmal region. Repeated fusions of TS28-containing transfer vesicles with the short TS28-containing tubular invaginations results in the formation of branching and further extending transverse tubules. To test this hypothesis, identification of the subcellular organelles labeled with antibodies to TS28 and SL50 at different stages of development by immunoelectron microscopical labeling would be an important step toward understanding the role of these organelles in transporting TS28 and SL50 from their site of synthesis to their site of assembly—into the transverse tubules and the sarcolemma, respectively. To further elucidate the sequence of events leading to the de novo biogenesis of functional transverse tubules, it will be important to compare the temporal appearance and subcellular distribution of TS28 with those of the α_1-subunit of the 1,4-dihydropyridine receptor, previously shown to be a specific marker of transverse tubules (9).

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