Sequential Docking, Molecular Differentiation, and Positioning of T-Tubule/SR Junctions in Developing Mouse Skeletal Muscle

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Skeletal muscle Ca$^{2+}$ release units (CRUs) are junctions of the surface membrane/T-tubule system and the sarcoplasmic reticulum (SR) that function in excitation-contraction coupling. They contain high concentrations of dihydropyridine receptors (DHPRs) in the T-tubules and of ryanodine receptors (RyR) in the SR and they are positioned at specific locations in the sarcomere. In order to characterize the sequence of developmental steps leading to the specific molecular and structural organization of CRUs, we applied a range of imaging techniques that allowed us to follow the differentiation of the membrane compartments and the expression of junctional proteins in developing mouse diaphragm muscle. We find that docking of the two membrane systems precedes the incorporation of the RyRs into the junctions, and that T-tubule/SR junctions are formed and positioned at the I-A interface at a stage when the orientation of T-tubule is predominantly longitudinal. Thus, the sequence of developmental events is first the docking of T-tubules and SR, secondly the incorporation of RyR in the junctions, thirdly the positioning of the junctions in the sarcomere, and only much later the transverse orientation of the T-tubules. These sequential stages suggest an order of inductive processes for the molecular differentiation and structural organization of the CRUs in skeletal muscle development. © 2001 Academic Press

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INTRODUCTION

The rapid rise in intracellular Ca$^{2+}$ during muscle activation is controlled by the close interactions of two separate membrane systems, the transverse (T) tubules, which are invaginations of the plasmalemma, and the sarcoplasmic reticulum (SR; Porter and Palade, 1957), the intracellular Ca store. SR and T tubules are structurally and functionally joined at Ca$^{2+}$ release units (CRUs). The CRUs are the sites at which Ca$^{2+}$ is rapidly released from the SR via the Ca$^{2+}$ release channel, or ryanodine receptors (RyRs), under command of the T tubule membrane potential, which is sensed by dihydropyridine receptors (DHPRs; reviewed in Franzini-Armstrong and Protasi, 1997). The close spatial relationship between these membrane systems and the myofibrils and the regular arrangement of the CRUs allows for the rapid availability of Ca$^{2+}$ to the contractile machinery upon muscle activation and its effective removal during relaxation.

SR-T tubule junctions and their association with myofibrils develop in a series of consecutive steps over a period of several weeks (reviewed in Flucher, 1992; Flucher et al., 1993; Takekura et al., 1993, 1994). ER differentiates into SR by the gradual displacement of generic ER proteins by SR-specific proteins (SERCA 1 and 2, calsequestrin, and RyRs), and by a massive increase in the concentration of SERCA in the free SR membrane (Martonosi et al., 1980; Flucher et al., 1993). Next, SR proteins need to be segregated into two functional domains, the free SR containing the Ca$^{2+}$ pump and junctional SR containing RyRs and calsequestrin (Jorgensen et al., 1985). In parallel to this...
differentiation, formation of the T tubules and the sequestration of DHPRs into junctional domains participating in CRUs takes place (reviewed in Flucher and Franzini-Armstrong, 1996). The formation of junctions between the two membrane systems and the molecular differentiation of the junctional membrane domains occur concurrently, suggestive of a highly coordinated and possibly interdependent process (Yuan et al., 1991; Flucher et al., 1994). Neither the aggregation within restricted membrane domains of these Ca$^{2+}$ channels nor interactions between them are required for CRU formation. Despite numerous observations, it is not known whether all components of the junction are simultaneously assembled during normal development. Answering this question will provide indication of the inductive processes, and show whether junction formation is necessary for RyR and DHPR assembly or whether these processes are independent.

In addition to the questions concerning the association of the two membrane systems, the membrane-cytoskeleton interactions leading to the specific arrangement of CRUs relative to the myofibril cross striation are still elusive. The transverse orientation of T tubule networks and the final precise location of triads and longitudinal SR elements at specific bands of the sarcomere are late developmental events (Veratti, 1902; Schiaffino and Magreth, 1969; Edge, 1970; Kelly, 1971; Flucher et al., 1991, 1993; Franzini-Armstrong, 1991; Takekura et al., 1993; Franzini-Armstrong and Jorgensen, 1994 for a review). Hitherto, it has been unclear whether the reorientation of T tubules drives the arrangement of triads at the A-I interface or whether independent processes exist for this specific membrane-cytoskeleton association.

Our aim is to correlate the development of the individual membrane structures and to determine the sequence of events leading to their specific interactions with one another and with the myofibrils. For this, we have used labeling of surface membrane with a fluorescent lipid soluble dye to image T tubules, immunolabeling to detect SERCA distribution and clustering of RyRs, and electron microscopy to detect formation, location, and orientation of CRUs. The results indicate that SR-T tubule docking clearly precedes trapping of RyRs in CRUs and location of T tubules in a transverse network follows the specific positioning of CRUs relative to the myofibrils and may be dependent on it. These findings suggest that docking of T tubules and SR initiates molecular changes in both membrane systems which lead to the molecular differentiation of the junctional membrane domains as well as to their specific interactions with the myofibrillar apparatus.

**MATERIALS AND METHODS**

**Experimental Animals**

This study was conducted with mouse (C57BL/6J) embryos at 14, 15, 16, 17, and 18 days of gestation (E14–E18, plug day; 0 day); young mice at 1, 2, 7, and 14 days after birth (D1–D14); and adult mice.

**T-Tubule Labeling**

The fluorescent lipid analog DiIC$_{16}$[3] was used to trace the T tubule development. DiIC$_{16}$[3] (Molecular Probes, Eugene, OR) was diluted from a stock solution of 2.5 mg/ml DiIC$_{16}$[3] in absolute ethanol into 85 mM sodium cacodylate buffer (pH 7.2) containing 85 mM sucrose, to a final concentration of 12.5 mg/ml immediately before use (Terasaki et al., 1986; Sanger et al., 1989; Flucher et al., 1991, 1993).

The resulting micellar suspension of dye was forcibly pipetted against both surfaces of the unfixed diaphragm, after partially removing the connective tissue, for 5–10 min. The muscles were washed and mounted for immediate observation in the cacodylate-sucrose buffer. Samples were observed in a scanning laser confocal microscope equipped with Ar-Kr laser (MRC-600; BioRad, Hercules, CA). The technique was used only on embryonal and early postnatal mice, because it does not work in the adult due to the thickness of the basal lamina.

**Immunohistochemical Labeling**

For immunofluorescent labeling, whole diaphragms were fixed in 1% paraformaldehyde in phosphate buffered saline (PBS) containing 0.5% Triton X-100 for 1 h at room temperature. The muscles were blocked for 1 h in PBS containing 1% bovine serum albumin (BSA) and 10% goat serum, and then incubated overnight at 4°C in the primary antibody. After washing three times for 10 min in PBS/BSA, the muscle bundles were incubated in Texas Red-conjugated goat anti-mouse IgG (Cappel Products, Durham, NC) for 1 h at room temperature, and then washed again. The muscles were mounted in glycerol containing 0.0025% para-phenylenediamine, 0.25% 1,4-diazobicyclo-2,2,2-fluorene, and 5% N-propyl-gallate to retard photo-bleaching. Specimens were observed in a scanning laser confocal microscope equipped with Ar-Kr laser (LSM510; Carl Zeiss, Germany). The two monoclonal antibodies were 34C (Airey et al., 1990; purchased from Developmental Studies Hybridoma Bank, University of Iowa), which recognizes all RyR isoforms, and anti fast Ca$^{2+}$-ATPase (Kaprielian et al., 1986; Sanger et al., 1989, 1991, 1993).

**Electron Microscopy**

Diaphragms were fixed in situ by injection of 2.5% glutaraldehyde (in 100 mM sodium cacodylate buffer, pH 7.2) into thoracic and abdominal cavities of dead mice in proximity of the muscle. The diaphragm was then dissected, further immersed in cold 2.5% glutaraldehyde in sodium cacodylate buffer (pH 7.2) for 1–2 h at 4°C. Thin sections (~50 nm) stained with uranyl acetate and lead solutions were examined by using an electron microscope at 80 kV (JEOL-2000EX; JEOL, Japan).

**Quantitative Measurements**

Using randomly collected photographs from transverse and longitudinal sections, we determined the relative abundance of peripheral versus internal couplings, and the disposition of CRUs relative to the bands of the sarcomere. The same photographs were used to measure the total length of peripheral and internal coupling profiles (including both dyads and triads), the length of profiles occupied by arrays of feet

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and the width of the junctional gap. For the latter parameter, five randomly selected positions were measured and averaged for each junctional gap. The quantitative observations were statistically treated with Student’s t-test or multivariate analysis of variance (ANOVA). The significant level of $P < 0.05$ (95% confidence) was used.

RESULTS

The Time Course of Key Events during Early Differentiation of the Mouse Diaphragm

Myofibrillar development begins at day 14 of embryonic development (E14) in myotubes with the formation of a peripheral ring of myofibrils and centrally located nuclei. Myotube to myofiber transition occurs at E16–E17, and myofibrils fill the entire cross section of the fibers and are fairly well aligned by E17 (Fig. 1A).

The SR is present from the early stages and its nonjunctional (free) domains show a preferential association with the Z-lines as soon as the first myofibrils appear. By E17, this specific association results in a distinct banding pattern of discrete SR networks at the level of the I-bands of myofibrils (Fig. 1B, see also Flucher et al., 1993).

T Tubules Have a Predominantly Longitudinal Orientation throughout Embryonic Development

We have used labeling of the surface membrane in whole diaphragm with the fluorescent lipid dye analogue DiIC$_{16}$[3]...
to detect T tubules. Small tubular invaginations of the surface membrane appear at E15 (arrows, Fig. 1C). At this stage, the tubules are few and are preferentially found within the peripheral ring where the myofibrillar bundles begin to form. Myoplasm and nuclei occupy the centers of the myotubes. At E16, the myotubes are considerably larger and T tubules are longer, more frequent and penetrate more deeply into the fiber. At this age, the great majority of T tubules run in an almost perfect longitudinal orientation and are connected to the surface by short transverse segments (arrows, Fig. 1D). Between E16 and E17, the myotubes turn into myofibers, that is the entire cells cross section is occupied by myofibrils (Okazaki and Holtzer, 1966) and concomitantly nuclei migrate to the periphery. The cross striations are fairly well aligned across the young fibers (Figs. 1A and 1B), but the T tubules, which now penetrate to the center, still have a predominantly longitudinal orientation, with only occasional cross connections (Fig. 1E). SERCA labeling obtained independently on diaphragm at the same age shows that the free SR at this stage is already aligned with the myofibrils, marking the striations (compare with Figs. 1A and 1B).

Birth occurs after 19 days of gestation. One day after birth (D1), the T tubules are evenly distributed throughout the myotube and the cross connections are more regularly spaced. However the majority of T tubules are still longitudinally oriented (Fig. 1F). The transition to a full transverse orientation in the postnatal period of mouse muscle has previously been described to occur between birth and 3 weeks (Edge, 1970; Franzini-Armstrong, 1991).

In summary, T tubule development in mouse diaphragm begins at E15, progressively filling the myotube with a system of interconnected tubules that maintain a longitudinal orientation for a considerable period of time after myofibrils and SR have become cross aligned.

Cross Alignment of CRUs Is an Early Event, in Parallel to Myofibrillogenesis

Confocal images from fibers immunolabeled against RyRs show small, intense RyR-positive foci representing CRUs. At E15, punctate RyR foci are relatively sparse and it is not possible to tell whether they have a specific distribution relative to the myofibrils either near the surface of the fibers (arrows, Fig. 2A), or deep into the myotube (double arrows, Fig. 2A). One day later, at E16, RyR clusters are abundant throughout most myotubes (Fig. 2B). At this stage, the labeling intensity of the RyR clusters is uneven and weak. In some portions of the cell, few adjacent clusters are transversely aligned, forming doublets that mark a sarcomere-related periodicity, consistent with EM data below. This indicates that some CRUs are already located in a sarcomere-related pattern. Figure 2B shows an extreme case of this early cross alignment at E16.

As the fibers mature (E17 and E18; Figs. 2C and 2D), the RyR clusters become brighter and the transverse alignment becomes the dominating feature of the distribution pattern. The cross bands of RyR cluster doublets correspond to pairs of triads located at the A-I border at either side of the Z-line (see below). This overall pattern remains basically unaltered, but becomes more pronounced as muscle differentiation proceeds (Figs. 2D–2F). Small areas in which RyRs do not show this clear banding pattern are more frequent at or before E17 (Fig. 2C), and become virtually absent after birth (Fig. 2F). Sudden longitudinal displacements in the transversely aligned bands can be seen at all ages. These correspond to similar displacements between the cross striations of groups of myofibrillar bundles (cf. Fig. 1B). Thus, the striking increase in the expression and clustering of RyRs at E16/17 are temporally coordinated with the arrangement of RyR-containing membrane domains relative to the cross striation.

Transition between Peripheral and Internal Location of CRUs Parallels Early Formation of T Tubules: Burst of CRU Formation at E17–E18

Electron microscopy shows that CRUs are present as soon as myofibrils develop, but they initially are limited to the immediate proximity of the surface membrane. At E14, 42% (n = 79 junctions) of the CRUs are peripheral couplings and the rest are junctions between the SR and very shallow, wide invaginations of the surface membrane. At E15, the frequency of peripheral couplings is reduced to 17% (n = 94 junctions), and internal junctions are present both close to the fiber periphery and more deeply. This coincides with the appearance of the first definitive T tubules at E15 (Fig. 1C). Overall density of junctions is low at both E14 and E15. Between E16 and E18, a dramatic surge in the T tubule development (Figs. 1D and 1E) and the formation of numerous junctions at all depths of the fiber occur in parallel, while peripheral couplings completely disappear. Peripheral couplings are 2% of CRUs at E17 and are not detectable at E18 and later. In addition to the increased frequency, junction types also shift from peripheral couplings and dyads, which contain a single SR sac and dominate the early stages (constituting 88% of the CRUs at E14), to triads which contain two SR sacs and constitute 87% of all CRUs at E17.

Association of CRUs with the A-I Junction Precedes Their Transverse Orientation

In agreement with the confocal observations, electron microscopy shows that the distribution of newly formed T tubule/SR junctions is initially random and that CRUs acquire a sarcomere-related location by becoming associated with the edges of the A bands (A-I junction) of the adjacent myofibrils (see Fig. 1A and Table 1). At E16, the location of CRUs is poorly related to the banding of the myofibrils, and only 22% are associated with the A-I junctions. Twenty-four hours later, at E17, the A-I junction association has dramatically increased to 68% and at E18 it reaches 91%. Note that CRU-myofibril association pre-
FIG. 2. Sarcomeric organization of RyR clusters in developing mouse diaphragm. Immunofluorescence labeling of RyRs shows the first RyR clusters at E15 at or close to the periphery of individual myotubes (arrows, A). Some clusters are also present internally (double arrows) but show no regular organization. At E16, strings of RyRs clusters are detected throughout the myotubes (B). In some areas, the clusters are in doublets (double arrows) that have a two-per-sarcomere spacing and are transversely aligned across several adjacent myofibrillar bundles. However, a less ordered arrangement is also often seen at this age. At E17, the majority of RyR clusters are arranged in doublets and in obvious transverse bands (C). While this general arrangement persists throughout development, the doublets of RyR clusters become more discrete, denser, and better aligned with age (D–F). Small areas of disorder are common at E17, but become scarce at E18 (D) and are virtually absent at later stages (E, F). Occasional jumps in the cross alignment of RyR spots indicate that they follow the myofibrils' banding pattern.
cedes the rearrangement of the T tubule network from longitudinal to transverse, which is still quite poor at D1 (see Fig. 1F). This rapid (re)organization of CRUs relative to the sarcomeres indicates the existence of a potent organizing principle that captures T tubule/SR junctions as soon as they have formed.

The disposition of triads during early differentiation is predominantly longitudinal (Fig. 3A and Table 1) and it becomes transverse with maturation (Figs. 3C). The transition from longitudinal to transverse orientation of CRUs is gradual, going through oblique stages (Fig. 3B). Reorientation of the CRU axis lags behind CRU-myofibril association, being only 44% complete at E18 (Table 1). The reorientation of CRUs seems to follow that of the entire T tubule network. Longitudinal and oblique junctions and T tubules are still present at birth (Fig. 3) and postnatally (Edge, 1970; Franzini-Armstrong, 1991) in mouse and after hatching in bird (Takekura et al., 1993), but both essentially disappear few weeks after birth.

SR-T Tubule Docking and Insertion of Feet Are Two Sequential Events in the Formation of CRUs

Adult CRUs have four easily recognizable ultrastructural features: a close association of an SR vesicle with the exterior membrane; a junctional gap of uniform width; the presence of RyRs in the junctional gap; and the presence of a density within the SR vesicle (calsequestrin, Jorgensen et al., 1985). The cytoplasmic domains of the RyR homotetramers can be seen in electron microscopic preparations as regularly arranged electron-dense structures (also called feet) spanning the gap between SR exterior membranes (Fig. 4B). Calsequestrin’s association with the junctional SR membrane, presumably via junctin and/or triadin, results in the presence of periodic luminal densities in close proximity to the junctional SR membrane (Franzini-Armstrong et al., 1987; Zhang et al., 2000). These features are quite distinctive, so differentiating CRUs can be identified as such, even when feet are missing (e.g., see Fig. 4A), due to the presence of calsequestrin in the SR.

The content of feet within CRUs in differentiating muscles is variable. Some CRUs, particularly at the early developmental stages, do not have any feet (Figs. 4A and 4D). Some triads have feet only on one side but not on the other (Fig. 4B), and those junctions that contain feet are frequently not fully occupied by them (Fig. 4C). The width of the junctional gap in the junctions and/or portions of junctions without feet (5.7 ± 1.6 nm, mean ± 1 S.D., n, number of junctions = 277) is only about half that of the feet-occupied gap (9.6 ± 1.8 nm, n = 467), indicating that

### TABLE 1
The Position and Orientation of CRUs Relative to the Bands of the Nearest Myofibril in Developing Mouse Diaphragm

<table>
<thead>
<tr>
<th>Age</th>
<th>Position of CRUs relative to myofibrils (%)</th>
<th>Orientation of CRUs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-I junction</td>
<td>Z-line</td>
</tr>
<tr>
<td>E16 (n = 165)</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>E17 (n = 197)</td>
<td>68</td>
<td>8</td>
</tr>
<tr>
<td>E18 (n = 219)</td>
<td>91</td>
<td>3</td>
</tr>
</tbody>
</table>

Note. n, Number of junctions counted. Abbreviations: CRUs, Ca\(^{2+}\) release units.

FIG. 3. Changes in the orientation of the triad in developing mouse diaphragm. Examples of various triad orientations relative to the myofibrils in diaphragm just before birth. All images are shown with the myofibril axis vertical. Longitudinal (A), oblique (B), and transverse (C) triads are shown. Triad orientation shifts from predominantly longitudinal at E16 to completely transverse in the adult. A good portion of this shift occurs in the critical E16–E18 period (see Table 1). All triads shown are near the A-I junction of the myofibrils. However, at earlier stages of development, the triad may have a random position relative to the cross striation.
FIG. 4. Examples of CRUs showing variable occupancy of the junctional gap by feet (RyRs). Two examples of junctions completely lacking feet are derived from myotubes at E14 (A) and E16 (D). The triad in (B), from a myotube at AE15, has feet on one side (arrows) and none on the other (between arrowheads). The elongated dyad in (C), at E16, has alternate junctional SR segments with and without feet. Triads completely occupied by feet are shown in (E–G). Occupancy of the gap by feet increases with developmental age (see Fig. 5). The width of the junctional gap in the absence of feet (between arrowheads, A, B, and D) is smaller than where feet are present (see text). Accumulation of electron dense material in the SR sacs (presumably calsequestrin) precedes the presence of feet in the junctional gap.
the feet are truly absent (compare with Takekura et al., 1995).

Occupancy of CRUs by feet increases during the course of development (Figs. 4A–4G). At E14, many junctions have either none or few feet; between E15 and D14, most junctions have at least some feet, and at D14 and later all junctions are completely filled by feet. In order to quantitate this parameter, we measured the total length of junctional SR membrane profiles in each sectioned junction (e.g., the length of membrane between double arrowheads in Fig. 4C) and the total length of membrane associated with feet (e.g., the length of membrane associated with arrows in Fig. 4C). The data are shown in Fig. 5. The total length of junction (open circles, Fig. 5) increases very slowly between E14 and E18 (close to birth) and more rapidly after birth, probably due to differentiation of fast fibers. The feet-occupied length (filled circles, Fig. 5) increases more rapidly than the total length between E15 and E18 and it increases more rapidly than the total length, so that at E18 the difference is small, even though still significant (P < 0.01). At D7 and later, the two lengths are not significantly different. The ratio between feet occupied and total length (triangles, right ordinate) increases rapidly between E16 and E18, indicating rapid filling of the junctions during this period. Values are means ± 1S. D. *, Significant differences (P < 0.01) between the total length and feet occupied length.

**DISCUSSION**

The highly regular arrangement of organelles and membrane systems in the sarcomere is a striking feature of striated muscle that has important consequences for the efficiency of excitation–contraction coupling. The order is of importance both at the level of the organelle and at the molecular level. The CRUs are precisely positioned at the borders of the A- and I-bands allowing for the rapid supply of Ca\(^{2+}\) for the activation of the adjacent myofibrils. The free, Ca\(^{2+}\) pumping SR is linked to the Z lines and thus maintained in proximity of the myofibrils (Walker et al., 1968, 1969; Edge, 1970; Walker and Edge 1971; Flucher et al., 1993). At the molecular level, surface membrane DH-PRs are associated with RyRs within CRUs, thus allowing their rapid interaction, while calsequestrin in the adjacent SR lumen maintains a high total luminal Ca\(^{2+}\) content.

Our studies reveal a highly coordinated differentiation plan, in which key events have a specific temporal sequence. By unraveling this sequence, we gain insight into causal relationships that are responsible for the final assembly of a highly ordered skeletal muscle fiber.

Figure 6 gives a comparison of the parameters analyzed here with a range of fluorescent and electron microscopic techniques. It becomes clear that, whereas the myofibrils and the SR differentiate continuously over an extended period, key events in the structural and molecular differentiation of the CRUs occur rapidly during a critical period.
The mechanism of triad formation is still elusive. Studies in muscle of null-mutant mice have demonstrated that interactions between the RyR in the junctional SR and the DHP-receptor in the junctional T tubules are not required for the structural formation of a specific junction between SR and exterior membranes. Dysgenic (lacking DHPRs, Powell and Fambrough, 1973; Franzini-Armstrong et al., 1991; dyspedic (lacking RyRs, Takeshima et al., 1995; Takekura et al., 1995; Takekura and Franzini-Armstrong, 1994) initiated by the onset of T tubule formation at E15. This period of coordinated T tubule and junction formation in mouse diaphragm muscle is reminiscent of the development of the excitation-contraction coupling apparatus in avian muscle (Flucher et al., 1993; Takekura et al., 1994). Paramount to this differentiation period is the establishment of the differentiated architecture of CRUs and of their association with the myofibrils. Further growth after this period of time, while resulting in major increase in fiber size, entails only a modest amount of structural rearrangement (e.g., the final completion of the T tubule network’s transverse orientation, and the differentiation between CRUs of fast and slow fibers). However, even during this rapid differentiation, detailed comparison of the time course of the various CRU differentiation events show that they are not exactly in parallel. Close examination of subtle temporal differences contributes new insights in two important aspects of triad formation: the sarcomeric arrangement of the CRU and the sequence of events in the course of junction formation.

Since mature T tubules penetrate the muscle fiber at the plane of the A-I border, one might assume that the junctions form at this place because this is where the SR and T tubules meet. Instead, we show that the SR initially meets T tubules at random locations, resulting in CRUs that are irregularly distributed in the fiber. In addition, RyR-containing CRUs are specifically positioned at the A band edges at a developmental stage when T tubules are mostly running along a longitudinal path. This is particularly obvious in the E17 diaphragm, in which longitudinal T tubules show only few transverse connections, but the cross-striated alignment of RyR clusters and of the corresponding association of CRU with the A-I border of the myofibrils is quite striking (asterisks in Fig. 6). Indeed, when triads and dyads assume the specific position at the A-I border of the myofibrils, the orientation of their junctional surfaces is still predominantly longitudinal, indicating that the junctions are not restricted to the short transverse connecting tubules. Thus, it is not the position of the T tubules that determines the position of the CRUs relative to the bands of the sarcomeres but vice versa. By contrast, the final positioning of the overall T tubule network into transverse planes is a slow and relatively late process (Franzini-Armstrong, 1991; Flucher, 1992 for a review; Flucher et al., 1993). From these observations, we conclude that a linkage between the CRUs and myofibrils presumably through the SR component is the primary event, which may be the cause for the subsequent slow movement of T tubules into a transverse orientation. To this date, no candidates for the protein–protein interactions between the CRUs and myofibrils shown here or for the SR-myofibril interaction at the Z lines have been identified. However, based on our present observations, we now know that at least one of these proteins must be located in the junctional SR.
1999; Barone et al., 1998) and even double knock out junctions (Felder et al., 2001) are formed. In agreement with these published data, we show that during normal in vivo development of skeletal muscle RyR are recruited into preformed junctions. Similar observations have been made previously in cardiac muscle (Protasi et al., 1996) and in developing mouse leg muscles (Walker et al., 1975). Thus, docking of the SR to T tubule must precede the incorporation of feet within the junction and perhaps even provides the inductive signal for it (Flucher and Franzini-Armstrong, 1996). Candidates for docking proteins have been proposed and these may very well be the molecules that initiate the formation of junctions and the recruitment of other components (Takeshima et al., 1998, 2000; Komazaki et al., 1999). RyRs have been shown to possess an inherent ability for self-aggregation in isolated preparations (Yin and Lai, 2000). Thus, we expect that the newly formed junction may provide RyRs with the positional clue that allows them to be retained, but not necessarily with the signal for forming a semicrystalline arrangement.

During the early part of differentiation (E15–E16), T tubule/ SR docking outpaces the rate of RyR incorporation leading to numerous partially occupied junctions. In the subsequent period (E17–E18), filling of the junctional gap with feet catches up with the formation of new junctions, indicating that RyR expression occurs at a higher rate than junction formation. After E18, both junction formation and filling by RyRs occur in parallel at similar rates, leading to a phase of continuing triad growth, which keeps pace with the overall fiber diameter increase.

The present data suggest the following sequence of events for the differentiation of mature CRU in skeletal muscle. T tubule formation triggers the formation of T tubule/ SR junctions and perhaps a sudden increase in the expression of junctional proteins like the RyR and those proteins that are responsible for specific membrane–cytoskeletal interactions. As these proteins are incorporated into the junctions, the CRU assume their mature triadic composition and their position in the sarcosomes. All these processes occur during a brief time window that is followed by a period of secondary growth and trimming, including the transverse orientation of the T system. The specific developmental sequences suggest distinct mechanisms for various events: the T tubule/ SR docking; the docking of SR to T tubules; the acquisition of RyR in the junctions; and the positioning of these junctions at the A-I border independently of the orientation of the T tubules. The molecular bases for these specific processes remain to be identified.

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