Developmental Reorganization of the Skeletal Framework and Its Surface Lamina in Fusing Muscle Cells

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ABSTRACT The skeletal framework of cells, composed of internal structural fibers, microtrabeculae, and the surface lamina, is revealed with great clarity after extraction with detergent. When muscle cells fuse to form a multinucleated myotube, their skeletal framework reorganizes extensively.

When myoblasts prepare to fuse, the previously continuous surface lamina develops numerous lacunae unique to this stage. The retention of iodinated surface proteins suggests that the lacunae are not formed by the extraction of lamina proteins. The lacunae appear to correspond to extensive patches that do not bind concanavalin A and are probably regions of lipid bilayer devoid of glycoproteins. The lacunae appear to be related to fusion and disappear rapidly after the multinucleated myotube is formed.

When muscle cells fuse, their internal structural networks must interconnect to form the framework of the myotube. Transmission electron microscopy of skeletal framework whole mounts shows that proliferating myoblasts have well developed and highly interconnected internal networks. Immediately before fusion, these networks are extensively reorganized and destabilized. After fusion, a stable, extensively cross-linked internal structure is reformed, but with a morphology characteristic of the myotube. Muscle cells therefore undergo extensive reorganization both on the surface and internally at the time of fusion.

In the interior of vertebrate cells, there are dense, heterogeneous and highly interconnected networks; these include the well known structural filaments (micro- and intermediate filaments, microtubules) and the extensive system of highly cross-linked microtrabeculae described by Porter and co-workers in intact cells (2, 3, 6, 8, 9, 20, 21). These networks appear largely unaffected by extraction with Triton or glycerol under gentle conditions (4, 5, 13, 17-19). Such extraction permits much clearer visualization of many aspects of internal cell structure because the detergent removes most phospholipid and obscuring soluble cytoplasmic proteins. An important aspect of cell structure seen after extraction with Triton is the surface lamina, an external protein sheet derived from the plasma membrane, that retains the overall morphology and many details of the intact cell. We have suggested that this extracted structure, bounded by the protein sheet or lamina formed by the plasma membrane proteins (1), be designated the "skeletal framework" to distinguish it from a more narrowly defined cytoskeleton.

The formation of muscle fibers by myoblasts fusing is a striking example of the skeletal framework reorganizing during development. Myoblasts fuse to form multinucleated myotubes which then synthesize and organize the banded actomyosin contractile apparatus. Cells develop under optimum in vitro conditions to mature, twitching muscle fibers. To form the multinucleated myotube, plasma membranes of fusion-competent myoblasts must meld: at this time, large openings, or lacunae, in the surface lamina appear. The junction formed at fusion then disappears and the internal structural networks reorganize to form a continuous structure oriented along the muscle fiber axis. The specialization of the skeletal framework and its surface lamina that accompanies these highly specific events is described here.

MATERIALS AND METHODS

Muscle Cultures

Primary chick embryonic muscle cultures were made from 11- to 13-d-old (usually 12-d-old) chick embryo pectoral muscles following the method of Paterson and Prives (14). The cultures were maintained at 37°C on 5% CO2 in a medium containing 88% Dulbecco's Modified Eagle's Medium (DMEM), 2% chick embryo extract, 10% horse serum, and penicillin and streptomycin. Under
these conditions, cultures kept from 6 to 10 d generally displayed spontaneous twitching. This medium, which allows one to a few rounds of mitosis before cells prepare to fuse, is called fusion medium, to distinguish it from a richer medium also used. Growth medium, which contained 30% fetal calf serum and 10% chick embryo extract in Dulbecco's MEM, maintained myoblasts proliferating for several days; <10% fusion was seen at 48 h. Thus, at 24 h on growth medium, >95% of the myoblasts were proliferating; at the same time on fusion medium, most of the cells were at some stage of preparing for fusion and will be called "postproliferative myoblasts."

**Staining Intact Cells with Fluorescent Concanavalin A**

Myoblasts and fibroblasts that were to be stained intact with fluoresceinilated concanavalin A (Con A) were rinsed three times with cold phosphate-buffered saline (PBS), covered with a cold solution of 100 μg/ml of fluoresceinilated Con A in PBS, and maintained in a humid atmosphere at 4°C for 30 min. After being exposed to Con A, cells were rinsed three times in cold PBS. The cells were fixed, intact, in cold 3% formaldehyde in PBS for 1 h, rinsed with PBS and distilled water, dehydrated through a graded ethanol series, and then air-dried. No differences in staining were seen when cells were fixed and then stained; in both cases, debris being cleared from the cell surface was associated with bright patches and strands.

**Scanning Electron Microscopy**

Cells for scanning electron microscopy were grown on circular glass cover slips that had been ethanol sterilized and soaked in PBS for 30 min before being used for cell growth. Cells were prepared for scanning electron microscopy by being rinsed several times with PBS, left intact or extracted with lysis buffer as described below, and then fixed for 30 min in 2% glutaraldehyde in lysu buffer. They were rinsed twice with lysis buffer and then fixed with 0.5% osmium tetroxide for 5 min on ice. Cells were rinsed with distilled water, dehydrated through a graded ethanol series, critical point dried, and sputter-coated with gold and paladium.

**Radioiodination of External Proteins**

Cells to be radioiodinated were rinsed three times with PBS and were then incubated at room temperature for 10 min with a reaction mixture that contained 5 mM glucose, 0.1 U/ml glucose oxidase, 20 μg/ml horseradish peroxidase, and 400 μCi/ml Na[125I] in PBS. After iodination, the cells were rinsed three times with phosphate-buffered iodide solution. The soluble proteins were removed by a 1-min room temperature extraction with lysis buffer. The remaining proteins were delved off the plate in SDS buffer. All samples were acetone precipitated with 2 vol of cold acetone and washed three times with cold acetone to remove free iodine. Samples were suspended in running buffer as described below for polyacrylamide gel electrophoresis. Control dishes treated with collagen and epithelial cells (1) is nearly continuous. Myoblasts that are closer to preproliferative myoblasts may participate in fusion.

**RESULTS**

**Cell Surface Lamina of Fibroblasts and Myoblasts**

Myoblasts for these studies are dissociated from breast muscle of 12-d chicken embryos. During the first 24 h in culture, the myoblasts proliferate and then leave the proliferative cycle and prepare for cell fusion. Under proper conditions, cells fuse, and the resulting myotubes continue to mature. The morphology and surface structures of postproliferative cells close to fusion are unique and probably related to the act of fusion and later orderly development.

The distribution of cell surface proteins in proliferating and postproliferative myoblasts is clearly visible in the skeletal framework prepared by detergent extraction. After extraction, plasma membrane proteins constitute a lamina that retains much of the configuration of the plasma membrane of the intact cell. This surface lamina retains most plasma membrane proteins and surface-specific structures such as binding sites for virus and lectins (1). However, the surface lamina of postproliferative myoblasts is quite unlike the surface structure of either fibroblasts or proliferating myoblasts, in that it contains lacunae, apparently corresponding to lipid-rich regions of the plasma membrane and concentrated in regions that apparently may participate in fusion.

The surface lamina seen in cultured mammalian fibroblasts and epithelial cells (1) is nearly continuous. Myoblasts that are still proliferating also form an almost continuous surface lamina (Fig. 1). Freshly explanted myoblasts can be maintained in...
the proliferative state and prevented from fusing by using highly enriched culture medium. These proliferating myoblasts are typically short, bipolar cells with ruffled or knobbed tips; their surface lamina is almost as continuous (Fig. 1) as that of the fibroblasts described earlier (1). A few small perforations, <0.2 μm, can be seen near the nucleus; occasionally, the lamina over the tip of the cell may have one or two perforations that are as large as 1 μm. Similar small perforations were seen in fibroblasts (1).

In sharp contrast, both the morphology and surface lamina of postproliferative myoblasts show marked specialization. The myoblasts take on a new morphology as they prepare to fuse and form multinucleated myotubes, becoming elongated and more spindlelike. One or both cell tips generally have a complex ruffled and knobbed configuration; the other end may be pointed. The remainder of the plasma membrane appears relatively smooth. The surface lamina of these postproliferative myoblasts is revealed by detergent extraction (Fig. 2). The extracted structure retains the overall shape of the intact myoblast. However, unlike the almost continuous surface lamina of the proliferative myoblast, many large openings or lacunae are seen in the surface lamina of the postproliferative myoblast.

The extremities of the postproliferative myoblast framework have largely collapsed and the surface lamina in these regions has a very discontinuous, reticulate appearance. At higher magnification, an extracted tip (Fig. 2C) shows the typical

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**Figure 2** (A) Scanning electron micrograph of skeletal framework of postproliferative myoblast: maintained for 1 d in culture on fusion medium containing 10% horse serum and 2% chick embryo extract. Cell is more elongated; surface lamina shows many lacunae, particularly at the tip, revealing the underlying filaments. (B) The lacunae near the nucleus and the underlying cables. (C) Tip of myoblast showing the filamentous network.
fibrillar substructure underlying the lacunae. In many regions where the surface lamina is absent, prominent fibrous structures are seen in the exposed layer (Fig. 2B). The lamina appears more continuous in regions between the periphery and the nucleus, but often some lacunae are clustered in the nuclear region itself. The lacunae in the surface lamina of the postproliferative myoblasts are very likely related to the formation of cell-to-cell junctions during fusion and are found principally in regions where junction formation is expected. The lacunae seen in postproliferative myoblasts disappear quickly after fusion, and the lamina becomes almost entirely continuous. The skeletal framework of a day-2 myotube is shown in Fig. 3; the lamina is a smooth, continuous sheet, with few breaks. No lacunae are visible at this time.

**Lacunae in the Surface Lamina Arise from Protein-deficient Regions of the Plasma Membrane**

Lacunae in the surface lamina appear in the postproliferative myoblasts and disappear shortly after fusion. Because the surface lamina is formed by plasma membrane proteins, these lacunae indicate that myoblasts organize plasma membrane proteins in an exceptional and characteristic way. Such lacunae could arise either from unstable membrane regions that release proteins extensively during extraction with detergent or from protein-deficient, lipid-rich domains in the plasma membrane.

The partitioning of surface proteins between the skeletal framework and the soluble, detergent-extracted phase is measured by iodinating the surface proteins of the intact cell with 

\[^{125}\text{I}\] and then analyzing the iodinated polypeptides released from and retained by the skeletal framework during extraction with detergent. The electropherogram in Fig. 4 shows that the skeleton fraction of postproliferative and fusing myoblasts almost completely retains surface iodinated proteins (days 1 and 2), even though lacunae in the surface lamina of the postproliferative myoblast often cover nearly a fifth of the cell. Clearly, surface proteins that can be iodinated are not extensively released. Myotubes after fusion (day 3), that have developed a continuous surface lamina, retain a similar amount of surface proteins (Fig. 4, lane 3B). Indeed, the results in Fig. 4 for all days are similar to those obtained using fibroblasts, which retain ~95% of their surface proteins after detergent extraction (1). The myoblast lacunae do not, therefore, appear to result from extensive surface protein extraction.

If the lacunae in the myoblast surface lamina result from extraction of lipid-rich, protein-poor areas in the plasma membrane, then such areas would also have few surface glycoproteins and should bind lectins poorly. The distribution of Con A bound to the surfaces of intact cells is visualized by fluorescence microscopy using fluorescein isothiocyanate (FITC)-Con A. Surfaces of intact fibroblasts display a relatively uniform distribution of Con A, with occasional bright patches and strands where debris (visible by phase-contrast optics) is being cleared from the cell surface (Fig. 5A). The surface of intact proliferating myoblasts stains diffusely; high magnification reveals, in addition, a finely punctate pattern of bright staining (Fig. 5B). Although a similar pattern of staining is seen over most of the intact postproliferative myoblast, these postproliferative cells have peripheral regions that are free of Con A sites (arrows, Fig. 5C), such Con A-free regions have only been seen in these postproliferative myoblasts. Thus, there are unstained regions in the intact myoblast plasma membrane that correspond in size and location to the lacunae seen in the surface lamina after extraction; these regions very likely represent the postulated protein-poor membrane regions.

The fusion-related lacunae persist for only a brief time after cell fusion and are essentially gone by day 2-3 in culture. Scanning electron microscopy of the extracted myotube skeletal framework at this time shows an essentially continuous surface lamina (Fig. 3). The disappearance of the large lipid-rich, protein-deficient regions should accompany a uniform distribution of surface proteins and, probably, of lectin binding sites. No lectin-free regions are seen in the intact cell at day 2, just shortly after fusion (Fig. 5D). The absence of dark regions on the surfaces of intact cells at day 2 indicates that these lacunae represent the postulated protein-poor membrane regions.

**FIGURE 4** Polyacrylamide gel electrophoresis of externally 

\[^{125}\text{I}\] -iodinated proteins of myoblasts, day 1-3 of culture. Lanes 1, 2, and 3 represent days 1, 2, and 3, respectively, in culture. Lane A for each day contains the soluble fraction, proteins extracted by a gentle Triton extraction. Lane B contains the skeletal fraction, those proteins that remain with the skeletal framework after the gentle Triton extraction.

**FIGURE 3** Scanning electron micrograph of skeletal framework of day-2 myotube. The cell shown contained at least four nuclei. Surface lamina has become intact, with no lacunae visible.
the surface of intact day-2 myotubes is consistent with the postulated direct relation between lacunae seen by scanning microscopy before and during fusion and the distribution of surface proteins in the plasma membrane.

FIGURE 5. Fluorescence micrographs of intact cells stained with fluoresceinated Con A and fixed without extraction. All bars represent 5 μm. (A) Intact chicken embryonic fibroblast; bright patches are associated with debris (visible by phase-contrast optics). (B) Intact proliferating myoblasts at day 1 in culture on growth medium. High magnification reveals diffuse staining with punctate brightness. (C) Intact postproliferative myoblasts at day 1 in culture on fusion medium. Arrows indicate Con A-free regions seen only at this stage at locations where lacunae are seen after extraction. (D) Intact myotubes at day 2 in culture. No Con A-free regions are visible.
Transitional State of the Internal Skeletal Networks during Myoblast Fusion

At the time that postproliferative myoblasts show profound surface modifications, the elaborate structural networks that fill the cytoplasmic space also undergo extensive changes. These networks must rearrange after fusion and become continuous in the maturing myotube.

FIGURE 6. Transmission electron micrographs of whole mounted muscle cell skeletal frameworks. (A) The skeletal framework of a proliferating myoblast grown on growth medium. Note the well-integrated and extensively cross-linked microtrabecular framework. (B) Inset at higher magnification. (C) Post-proliferative myoblast, grown on fusion medium. Note the sparsely cross-linked skeletal framework and the cables running the length of the cell. (D) Inset at higher magnification. (E) Peripheral tip of multinucleated myotube taken on day 2 in culture. Note the increased integrity of the skeletal framework and the extensive cross-linking between the cables that run the length of the cell.
The internal networks can be best seen in muscle cells grown on grids and Triton extracted and then examined in peripheral regions of the cytoplasm; here, the thinness of the cell minimizes confusion from overlapping filaments. The electron micrographs of extracted whole mounts (Fig. 6) show the major stages during which internal networks are reorganized in the muscle cell. The proliferating myoblast (Fig. 6A and B) has a dense and highly interconnected trabecular network that resembles (but is not identical to) that seen in fibroblasts.

Preparation for fusion occurs rapidly when the myoblasts are switched from the medium supporting proliferation to the medium inducing fusion. The cytoplasmic network at the periphery of a postproliferative myoblast is shown in Fig. 6C and D. The myoblasts in this culture are the same age as that
shown in Fig. 6A but were shifted to fusion medium 24 h before the sample was prepared. The organization of the filamentous networks has changed drastically. Large, nearly empty regions are now seen, with only a few trabecular structures, and there are now major cables aligned along the long axis of the cell, which often splay at the periphery. The cytoplasmic networks are either depolymerized in the intact cell at this stage or are more labile during extraction.

Notable structures in the postproliferative myoblast are the major filament bundles or cables extending from deep within the cytoplasmic structure to the outer cell periphery. These cables, traversing large, nearly empty regions, are characteristic of and always found in postproliferative myoblasts. The cables, much less prominent in the proliferative myoblast (Fig. 6A), apparently form or increase in size as cells prepare for fusion.

After myoblasts fuse and form multinucleated cells, further changes occur rapidly in the networks of the skeletal framework. These changes can be seen in whole mounts at those tips distal to the junctions; the junction regions themselves are too thick for transmission electron microscopy without sectioning. In such a cell tip (Fig. 6E), the fiber networks are either reformed or stabilized against extraction and now form a dense, interconnected complex, distinct from the pattern seen in postproliferative myoblasts (Fig. 6C). The major filament cables are still present, but now the interlinked trabecular network appears connected with and organized around these cables.

The cytoplasmic filaments and microtrabeculae clearly undergo a profound change in organization and stability when the myoblast prepares for fusion. Not surprisingly, the protein content of the skeletal framework relative to total cytoplasmic protein is lowest at this time and rises sharply after fusion. The electropherograms in Fig. 7A show the partition of proteins between the skeletal framework and the soluble phase, for muscle cells in fusion-inducing medium at days 1 (postproliferative) through 4 (fully fused). The fraction of cytoplasmic protein in the skeletal framework is plotted as a function of days in culture in Fig. 7B. The framework preparation here includes both the cytoplasmic networks and surface lamina; nuclei and their protein content, also a component of the framework, have been removed by a second fractionation. The
The extent of disruption of cytoplasmic structural networks during preparation for fusion is indicated by the partial release of a normally unextractable structural component, the presumptive 58,000-mol wt intermediate-filament protein. Intermediate filaments are completely retained in the skeletal frameworks of all cells examined, with the sole exception of the day-1 postproliferative myoblast. The transient extractability of intermediate-filament protein in Triton buffer is seen in the electropherograms in Fig. 7A. The band corresponding to the 58,000-mol wt protein, indicated by the arrow on the electropherogram, is partially extracted and partially retained on day 1; it is completely retained in the skeletal framework at all subsequent times.

**DISCUSSION**

The skeletal frameworks of postproliferative myoblasts revealed by gentle detergent extraction undergo profound alterations in preparation for and after fusion. Many of these changes in cellular organization are not easily visualized in the intact cell but are seen with great clarity in the Triton-extracted structures.

The surface lamina, formed by plasma membrane proteins remaining after extraction, has been described previously in cultured fibroblasts and epithelial cells (1) and appears to be part of the skeletal framework. Both the internal networks and surface lamina in the myoblast undergo profound, yet distinct, changes in preparation for and after fusion.

In contrast to both proliferating myoblasts and mature muscle fibers, the surface lamina of the postproliferative myoblast has many clustered lacunae. The nearly complete retention in the skeletal framework of surface proteins labeled by iodination suggests that these lacunae are not formed by extensive loss of surface protein. Furthermore, only the postproliferative myoblasts show extensive dark patches when stained with FITC-Con A; these two findings suggest that the lacunae in the surface lamina correspond to regions deficient in lectin binding protein that are presumably lipid-rich domains in the plasma membrane.

It seems likely that the lacunae in the surface lamina relate to the requirements for cell fusion. Myotube formation requires juxtaposing fusion-competent cell surfaces. The lacunae seen in the postproliferative myoblast may reflect an early stage of this process. The act of melding together two cell surfaces must require unusual membrane properties, perhaps met by the lipid-rich patches characteristic of this stage of muscle development.

Transient lipid-rich patches, relatively free of protein, may explain the observation that myoblast lipid fluidity changes with fusion (16). Fluorescence depolarization measurements have indicated a large increase in lipid fluidity just before fusion followed by a decline to normal values after fusion. This fluidity change probably does not result from alterations in cell lipids, because the phospholipid composition and cholesterol content of cultured muscle cells remain relatively constant (10). Recent reports of greater fluidity at the cell tips and nuclei (7)
and of similarly located, particle-free regions in the freeze-fractured myoblast plasma membrane (11) suggest that the lacunae seen in the surface lamina result from these regions of protein-free, highly fluid lipid; thus, the change in plasma lamina organization may account for the transient increase in lipid fluidity.

The structural networks underlying the surface lamina undergo profound changes in organization during fusion, changes best studied in whole mounts at the flattened cell tips. Three distinct stages of internal cytoskeletal structure organization are discerned. The dense, highly interconnected filamentous network of the proliferative myoblast resembles the microtrabecular networks of fibroblasts and other cell types. When cells prepare to fuse, they have a sparse skeletal structure, lightly cross-linked and nearly empty, with a few major filamentous cables that terminate at the cell periphery. These cables, observed by Pudney and Singer (17), are characteristic of this specific stage in muscle development. In the cells observed by Pudney and Singer, these cables were predominantly peripheral. This difference in location and the differences in cell flattening are likely due to the different substrates and media used. Finally, after fusion, a dense interconnected framework re-forms but with a pattern distinctly different from the proliferating myoblast; this stage is characterized by a significant increase in the fraction of cytoplasmic protein associated with the skeletal framework.

The relatively empty framework of the postproliferative myoblast appears to result, in part, from the extraction of skeletal elements, as at least one major skeletal component can be extracted only during this brief transition stage. Intermediate-filament protein is partially removed from the day-1 myoblast (Fig. 7A). Shortly after fusion (day 2), the 58,000-molecular-weight protein is no longer extractable but appears well anchored in the skeletal framework. Thus, in myoblasts preparing to fuse, both the surface lamina and the internal networks show highly specific spatial rearrangement; in addition, the internal networks become more extractable. After fusion, both the internal networks and the surface lamina rapidly reorganize in a stable arrangement as the muscle cell begins to construct the extensive contractile apparatus. Thus, a critical stage in muscle development is accompanied by rapid, extensive, and transient reorganization of the skeletal framework and its surface lamina.

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