Caveolin-3 Associates with Developing T-tubules during Muscle Differentiation

Robert G. Parton,* Michael Way,‡ Natasha Zorzi,* and Espen Stang*

*Centre for Microscopy and Microanalysis, Department of Physiology and Pharmacology, and Centre for Molecular and Cellular Biology, University of Queensland, Queensland 4072, Brisbane, Australia; and ‡European Molecular Biology Laboratory, D69012 Heidelberg, Germany

Abstract. Caveolae, flask-shaped invaginations of the plasma membrane, are particularly abundant in muscle cells. We have recently cloned a muscle-specific caveolin, termed caveolin-3, which is expressed in differentiated muscle cells. Specific antibodies to caveolin-3 were generated and used to characterize the distribution of caveolin-3 in adult and differentiating muscle. In fully differentiated skeletal muscle, caveolin-3 was shown to be associated exclusively with sarcolemmal caveolae. Localization of caveolin-3 during differentiation of primary cultured muscle cells and development of mouse skeletal muscle in vivo suggested that caveolin-3 is transiently associated with an internal membrane system. These elements were identified as developing transverse-(T)-tubules by double-labeling with antibodies to the α_1 subunit of the dihydropyridine receptor in C2C12 cells. Ultrastructural analysis of the caveolin-3–labeled elements showed an association of caveolin-3 with elaborate networks of interconnected caveolae, which penetrated the depths of the muscle fibers. These elements, which formed regular reticular structures, were shown to be surface-connected by labeling with cholera toxin conjugates. The results suggest that caveolin-3 transiently associates with T-tubules during development and may be involved in the early development of the T-tubule system in muscle.

THE plasma membrane of mammalian cells is divided into a number of different structural and functional microdomains. Much recent interest has been focused on one such domain, the caveola, a surface invagination with unique morphology which is readily identifiable by electron microscopy (Parton, 1996). Caveolae are extremely abundant in endothelial cells, adipocytes, and smooth muscle cells. In endothelia, caveolae appear to play a major role in transport across the endothelial monolayer (Ghitescu et al., 1986; Schnitzer et al., 1994). Other work has suggested a role for caveolae in signal transduction (Lisanti et al., 1994), in specialized endocytic uptake pathways (Anderson, 1993), and in calcium homeostasis (Fujimoto, 1993; Fujimoto et al., 1992).

The diversity of the proposed functions of caveolae raises the question of whether they have a single function, as does a clathrin-coated pit, or whether they are structural units used for many different purposes. Caveolae are enriched in cholesterol (Montesano et al., 1982; Rothberg et al., 1990) and in glycosphingolipids (Parton, 1994), and increasing evidence suggests that caveolae are built up around sphingolipid–cholesterol rafts (Simons and Ikonen, 1996). The plasma membrane of all mammalian cells appears to contain such rafts which, upon detergent treatment, can be isolated as insoluble glycosphingolipid-enriched complexes (DIGs;[1] Parton and Simons, 1995). DIGs and caveolae share many features, but caveolae appear to be more restricted in distribution, being undetectable in some cell types (Fra et al., 1994; Gorodinsky and Harris, 1995). Caveolin-1, the major protein of caveolae in mammalian cells (Kurzchalia et al., 1994; Parton, 1996), is a 21-kD integral membrane protein which has been shown to bind cholesterol and to interact with glycosphingolipids (Fra et al., 1995*a*; Murata et al., 1995). Heterologous expression of caveolin-1 in cells lacking caveolae causes formation of surface invaginations with many of the features of caveolae (Fra et al., 1995*b*). The de novo produced caveolae were the same size and shape as endogenous caveolae, and cross-linked glycosylphosphatidylinositol-anchored proteins were shown to be concentrated within these invaginations. These results suggest that caveolin-1 has the capacity to interact with DIGs and create the characteristic caveolar invagination and to generate a microdomain with

Address all correspondence to R.G. Parton, Centre for Microscopy and Microanalysis, University of Queensland, Queensland 4072, Australia. Tel.: 61-7-3365-6468. Fax: 61-7-3365-4422. e-mail: R.Parton@mailbox.uq.oz.au

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1. *Abbreviations used in this paper*: CT-B, cholera toxin binding subunit; DIG, detergent-insoluble glycosphingolipid-enriched complex; DHPR, dihydropyridine receptor; T-tubule, transverse-tubule.

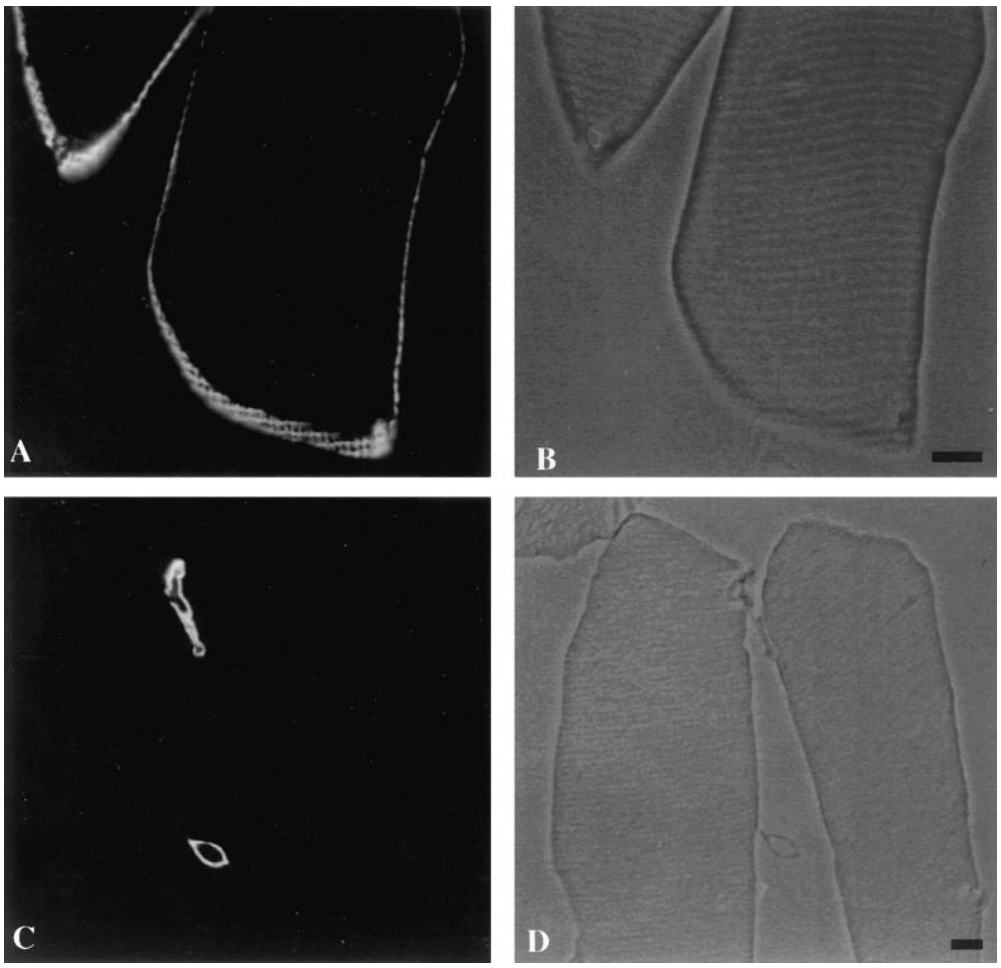
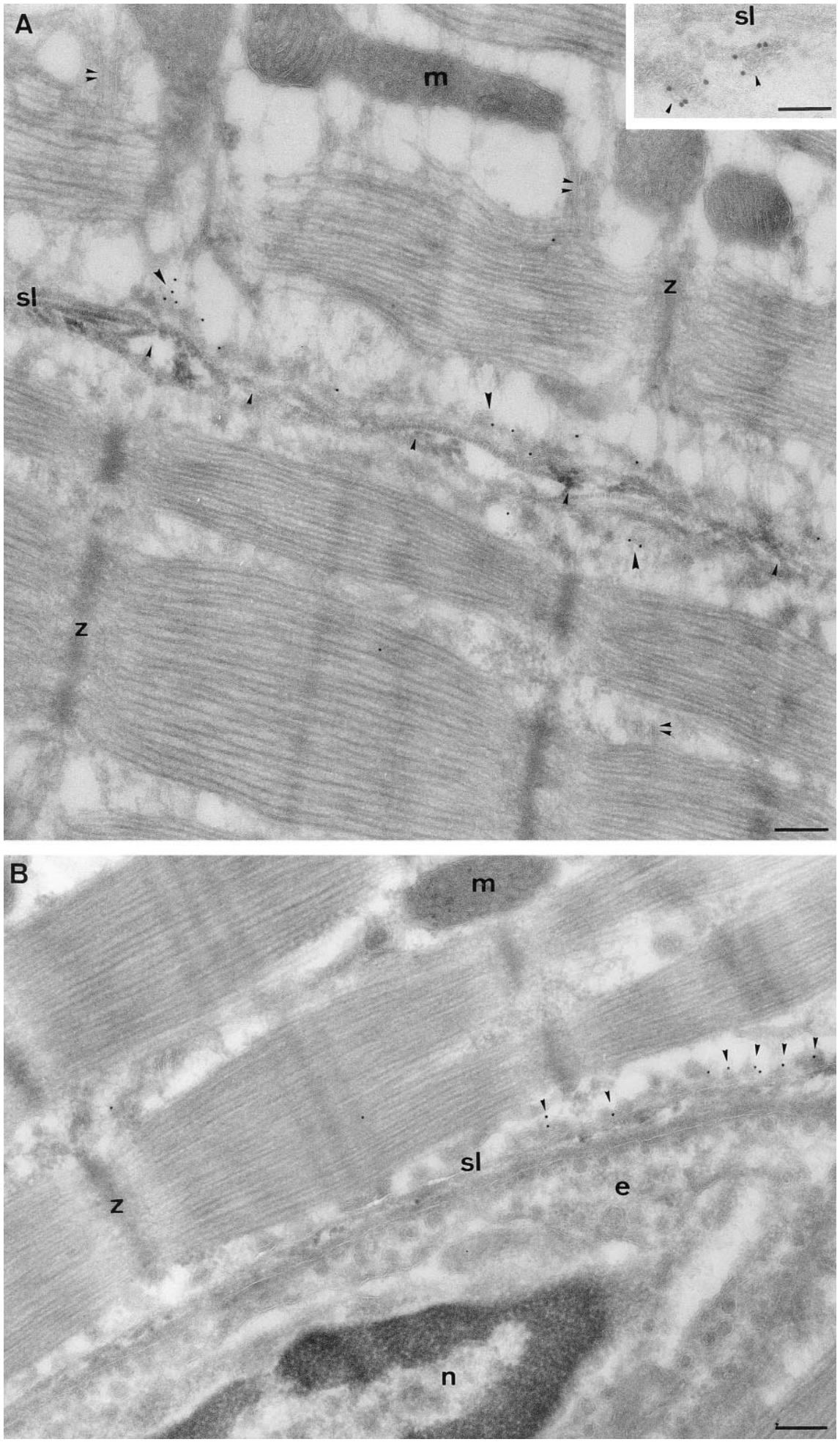


Figure 1. Immunolocalization of caveolin-1 and caveolin-3 in adult mouse skeletal muscle. 0.5 μm frozen sections of mouse skeletal muscle (*A–D*) were labeled with antibodies to caveolin-3 (*A*) or caveolin-1 (*C*). *B* and *D* show the corresponding phase images for *A* and *C*, respectively. (*A*) Caveolin-3 labels the periphery of the fiber with negligible internal staining. Note the regular meshwork of labeled elements at the ends of the muscle fiber. (*B*) In contrast to caveolin-3, antibodies to caveolin-1 specifically label the endothelial cells of muscle capillaries rather than the muscle fibers. Bars, 5 μm.

a distinct lipid composition (Parton and Simons, 1995). Recent work has identified two additional caveolin family members which share many structural features with caveolin-1 (Way and Parton, 1995; Scherer et al., 1996; Tang et al., 1996). The role of caveolin-1 and other caveolins is not restricted to generating and maintaining caveolar structure. In vitro studies have shown specific functional interactions with trimeric G protein α subunits (Li et al., 1995). These interactions may hold the G protein in an inactive state on the cytoplasmic face of the membrane.

We have recently cloned and characterized a novel muscle-specific homologue of caveolin-1, termed M-caveolin or caveolin-3 (Way and Parton, 1995). Expression of caveolin-3 is induced upon muscle differentiation, and mRNA is undetectable in undifferentiated C2C12 cells (Way and Parton, 1995; Tang et al., 1996). Caveolin-3 is ~60% homologous to caveolin-1 with the major differences occurring in the NH_2-terminal portion of the protein. The identification of a muscle-specific caveolin protein raises the question of the specific role of this protein, and of caveolae generally, in muscle. Caveolae of muscle cells (also termed subsarcolemmal vesicles or microvesicles) have been studied using a host of morphological techniques (Gabella, 1978). Several different hypotheses were proposed for the role of caveolae in muscle function and development. The three main theories proposed roles for caveolae in (*a*) provision of extra membrane during muscle extension (Prescott and Brightman, 1976), (*b*) calcium entry (Popescu, 1974), and (*c*) in the formation of the transverse (T)-tubule system during muscle development (Ishikawa, 1968). The first "stretch" theory has largely been discounted by subsequent studies suggesting that caveolae do not contribute membrane during muscle extension in vivo (Dulhunty and Franzini-Armstrong, 1975; Gabella and Blundell, 1978). However, the second theory, proposing a role for muscle caveolae in calcium intake or homeostasis, remains relevant, as recent studies have shown a striking concentration of two putative calcium-regulating molecules within caveolae of several different cell types, including muscle cells (Fujimoto, 1993; Fujimoto et al., 1992). The third theory, invoking a role for caveolae in formation of the T-tubule system, was suggested from

Figure 2. Immunoelectron microscopic localization of caveolin-3 in adult mouse skeletal muscle. Ultrathin frozen sections of mouse skeletal muscle were labeled with antibodies to caveolin-3. Specific labeling is associated with sarcolemmal caveolae (*small arrowheads* indicate the sarcolemmal region; *large arrowheads* indicate labeled caveolae), as shown at higher magnification in the inset. An endothelial cell (*e*) in *B* is unlabeled. Double arrowheads indicate regions of the T-tubule system which generally show negligible labeling. *m*, mitochondria; *sl*, sarcolemma; *n*, nucleus; *z*, Z-line. Bars: (*A* and *B*) 200 nm; (*inset*) 100 nm.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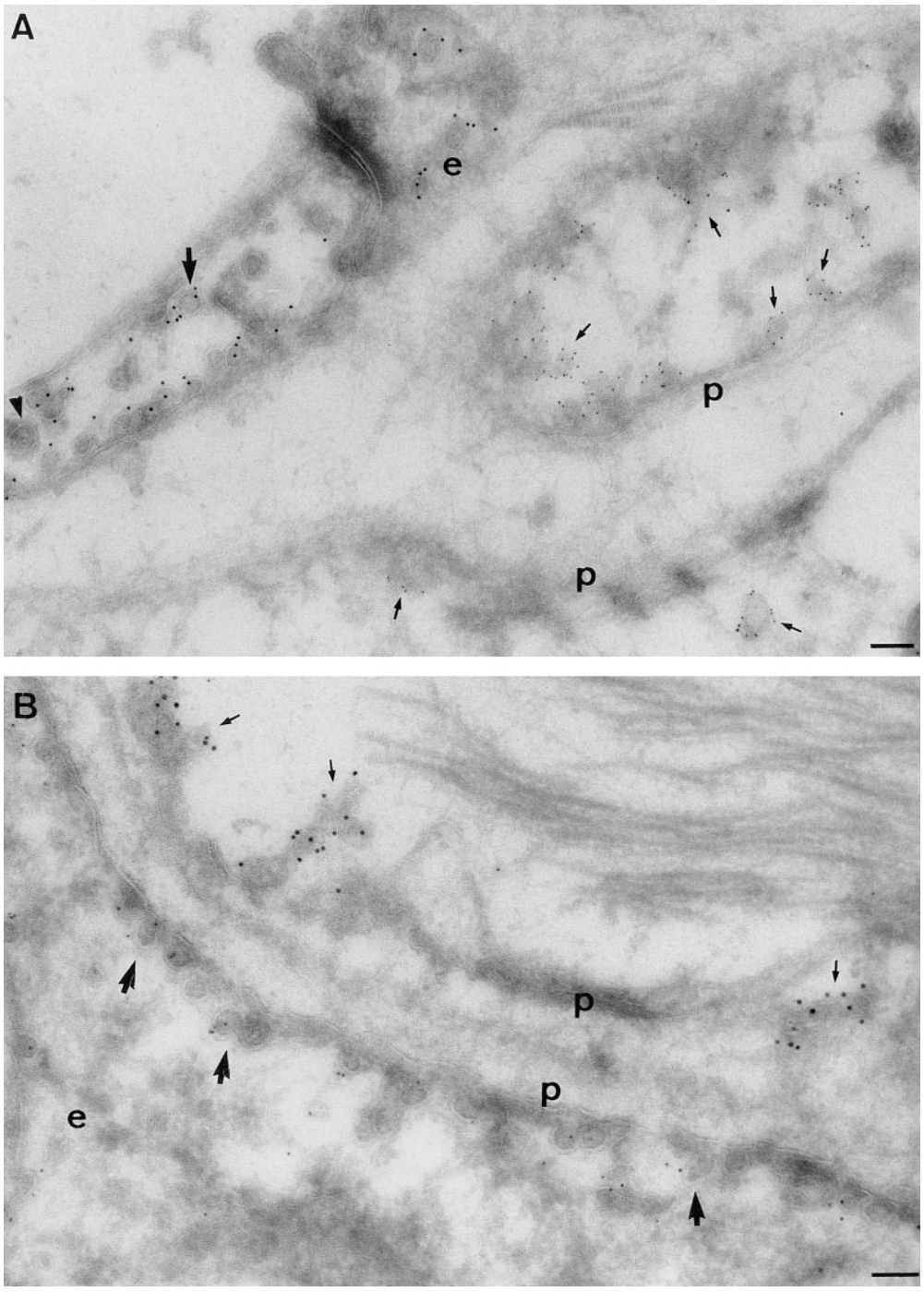


Figure 3. Immunoelectron microscopic localization of caveolin-3 and caveolin-1 in adult cardiac tissue. Ultrathin frozen sections of mouse atrium were labeled with antibodies to caveolin-3 and caveolin-1. Small gold in *A* represents labeling for caveolin-3 and in *B*, indicates labeling for caveolin-1. Caveolin-1 labeling (*large arrows*) is only detectable on caveolae of endothelial cells (*e*), whereas labeling for caveolin-3 is only evident within the cardiac muscle cells, showing the specificity of the two antibodies. In each case, labeling is associated with uncoated plasma membrane invaginations with the characteristics of caveolae. The arrowhead in *A* indicates an unlabeled clathrin-coated pit. *p*, plasma membrane. Bars, 100 nm.

early studies of the development of the T-tubule system in cultured myotubes (Ezerman and Ishikawa, 1967; Ishikawa, 1968). The T-tubules are an extensive surface-connected system of membranes which develop and maintain a protein and lipid composition distinct from the sarcolemma (for review see Flucher, 1992). Elegant morphological studies suggested that T-tubules form from the repeated budding of caveolae. Initially the caveolae formed short, beaded, tubular structures, and then these developed into extensive, three-dimensional networks which appeared to be formed from interconnected arrays of caveolae (Ezerman and Ishikawa, 1967; Ishikawa, 1968). Later studies of mouse muscle development in vivo showed a similar association of caveolae-like structures with forming T-tubules, at an early developmental stage (Franzini-Armstrong, 1991). These results were consistent with studies showing that inhibition of T-tubule formation resulted in an accumulation of caveolae-like structures (Schiaffino et al., 1977). This model is still favored by many investigators as some caveolar components are shared with T-tubules (Yuan et al., 1991). However, other studies using membrane-impermeant lipid probes also provided evidence for an internal T-tubule compartment which subsequently fuses with the sarcolemma (Flucher et al., 1991). In the absence of specific markers for muscle caveolae, all of these models have been difficult to test.

We have examined the localization of caveolin-3 in C2C12 cells, primary mouse muscle cultures, and mouse skeletal and cardiac muscle during development in vivo. We show that caveolin-3 is associated with the T-tubule system of developing muscle. We postulate that caveolae and specifically, caveolin-3, may play a role in the formation of the T-tubule system of muscle. In addition, we speculate that the underlying principles of organization and formation of T-tubules and caveolae may be similar.

Materials and Methods

Cell Culture, Transfection, and Immunofluorescence

Media and reagents for cell culture were purchased from GIBCO BRL (Eggenheim, Germany). C2C12 cells were cultured as described previously (Way and Parton, 1995). Transfection was carried out using Lipofectin (GIBCO BRL) according to the manufacturer's instructions, using caveolin-3-HA in the CB6 vector described by Way and Parton (1995). Cell lines were selected using G418 (GIBCO BRL). Colonies were subcloned and analyzed for caveolin-3 expression by immunofluorescence, as described below.

Primary cultures of mouse muscle were prepared from 18-d embryos exactly as described by Chu et al. (1995). Briefly, muscle fibers from the limbs of 18-d post coitus embryos were incubated in trypsin and the cells dissociated by trituration. Debris was removed by filtering the cells through gauze, and the dissociated cells were plated on matrigel (GIBCO BRL) or on calf skin collagen (Sigma Chemical Co., New South Wales, Australia) according to manufacturer's recommendations.

Antibodies

A peptide corresponding to the 15 NH_2-terminal amino acids of mouse caveolin-3 but, with the addition of a COOH-terminal cysteine residue and a bridging glycine residue, was synthesized (CGMTEEHTDLEARIIKD). The cysteine residue was used for coupling to activated keyhole limpet hemocyanin before injection into rabbits using the Imject Activated Immunogen Conjugation Kit (Pierce, Rockford, IL). The conjugated peptides were separated from free peptide using a Presto Desalting column (Pierce) and the concentration of the pooled conjugates determined using the Bio-Rad Laboratories (Richmond, CA) assay with BSA as a standard. Antisera were affinity purified on a column prepared by coupling the peptide through the cysteine residue (Harlow and Lane, 1988). Affinity-purified antibodies were characterized by immunofluorescence and Western blotting; in each case a specific signal was only detected in differentiated C2C12 cells, and this signal was competed by preincubation of the antibody with the specific caveolin-3 peptide. Antibodies to the NH_2 terminus of caveolin-1 (VIP21-caveolin) have been characterized previously (Dupree et al., 1993). Mab427 to the α_1 subunit of the dihydropyridine receptor (DHPR) was purchased from Chemicon Intl., Inc. (Temecula, CA). Antibodies to cholera toxin were raised in rabbits using fixed cholera toxin binding subunit (Sigma Chemical Co.) as immunogen.

Immunofluorescence

C2C12 cells were grown on glass coverslips coated with laminin (Sigma Chemical Co.) according to manufacturer's instructions. Cells were either fixed in cold methanol or in paraformaldehyde and then permeabilized with 0.1% saponin, as described previously (Parton et al., 1994). After immunolabeling cells were mounted in Mowiol (Hoechst, Frankfurt, Germany) and examined using fluorescence microscopes (Axiovert; Zeiss, Inc.). Confocal microscopy was performed using the EMBL confocal microscope or the confocal microscope (model MRC600 head and laser; Bio-Rad Laboratories; Axioscope, Zeiss, Inc.; used at the Vision, Touch and Hearing Research Centre University of Queensland, Brisbane, Australia). In each case, optical sections were 0.5 μm in the z-plane.

Preparation and Immunolabeling of Frozen Sections for Light and Electron Microscopy

Mice were killed by cervical dislocation, and small pieces of muscle from the leg or atrium were rapidly excised and fixed by immersion in either

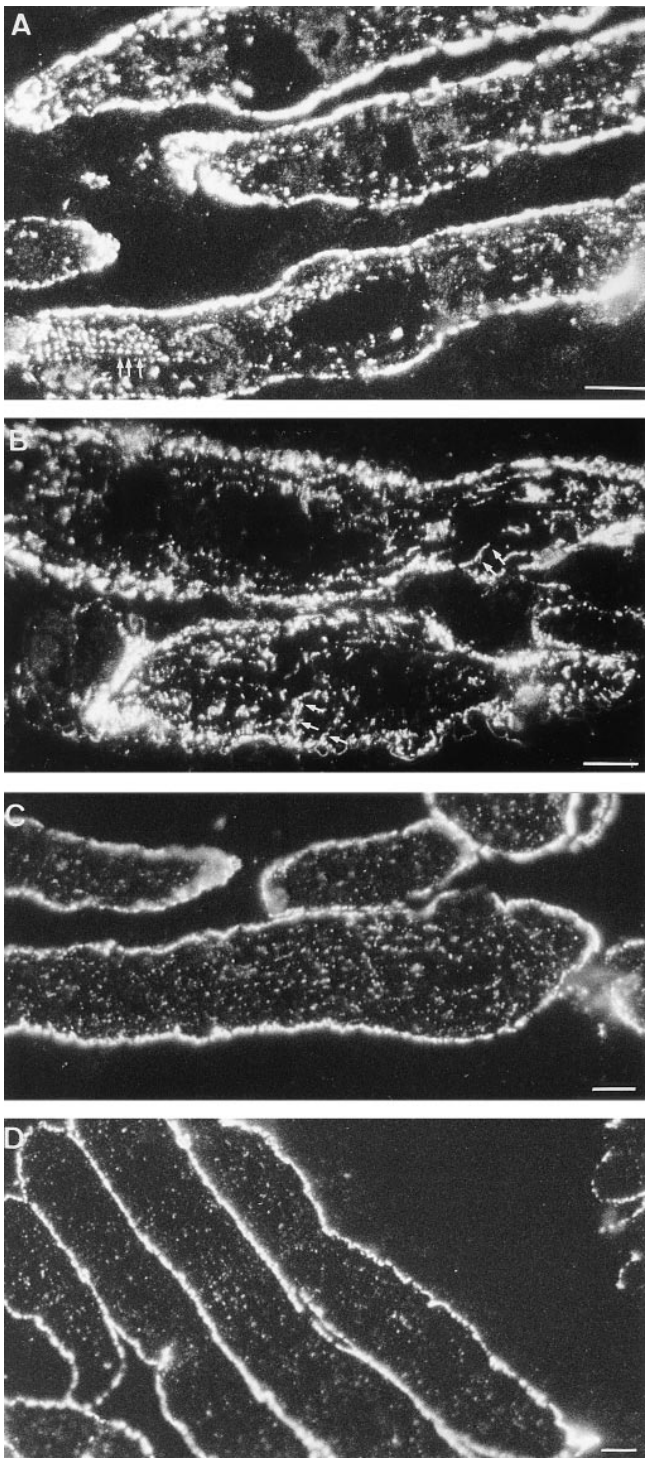


Figure 4. Immunolocalization of caveolin-3 during muscle development. 0.5 μm frozen sections of mouse skeletal muscle were fixed by immersion at various stages of embryonic development (embryonic day 16, *A*; embryonic day 18, *B*), immediately after birth (*C*) or 3 d after birth (*D*). All sections were labeled at the same time, and images were prepared with the same exposure and development times. Specific labeling is associated with the periphery of the muscle cells at all stages. In addition, internal labeling is evident from embryonic day 16 up to birth. Labeling is often evident as punctate dots of labeling aligned along the longitudinal axis of the muscle cells (*A*, *arrows*). In the embryonic day 18, muscle tubular structures, which apparently lead from the sarcolemma, are labeled (*B*, *arrows*). In the newborn muscle and especially 3 d after birth, the internal labeling is decreased. Bars, 5 μm.

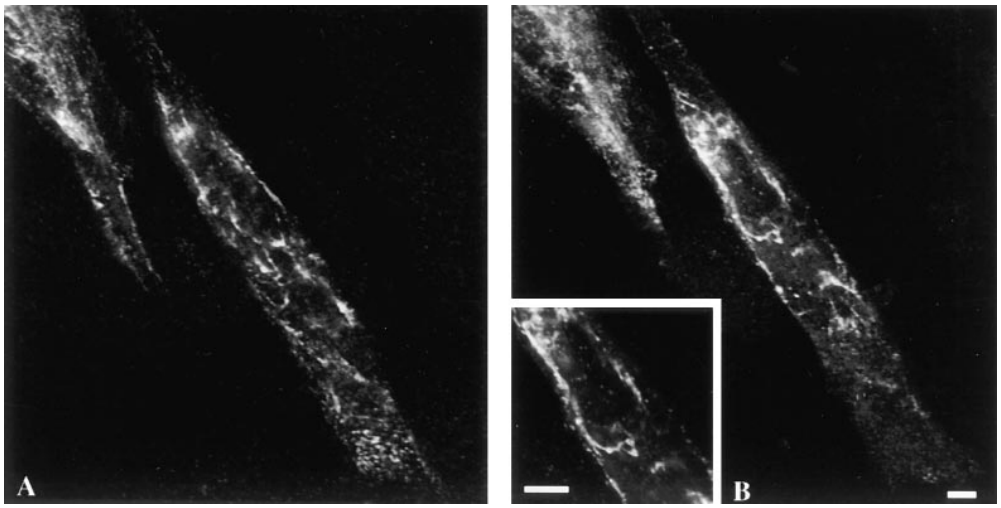


Figure 5. Immunolocalization of caveolin-3 in C2C12 cells. C2C12 cells were maintained in culture in differentiating medium for 6–8 d after reaching confluency. Cells were fixed with paraformaldehyde and processed for immunofluorescent localization of caveolin-3. Cells were viewed by confocal microscopy. *A* and *B* show two sections of two C2C12 myotubes at different planes through the cell: (*A* close to the base of the cell; *B* midway through the cell). Tubules and reticular structures (*inset*) run throughout the entire depth of the cell and are mainly orientated in the longitudinal direction. Specific labeling is associated with the differentiated C2C12 cells, whereas the underlying layer of undifferentiated cells (in the plane of the section shown in *A*) show low labeling. Bars, 5 μm.

8% paraformaldehyde in 100 mM phosphate buffer, pH 7.35, or the same fixative containing 0.1% glutaraldehyde. Muscle pieces were embedded in gelatin and were then infiltrated with polyvinylpyrrolidone/sucrose overnight and processed for ultrathin, frozen sectioning (Griffiths, 1993). Semi-thick (0.5–1 μm) and ultrathin sections (50–60 nm) were cut on a Leica Ultracut with FCS attachment. Thick sections were transferred to poly-lysine-coated coverslips and were then labeled immediately or stored until needed, at −20°C with identical results. Labeling of ultrathin sections for electron microscopy or thick sections for light microscopy was performed as described previously (Lütcke et al., 1994; Parton et al., 1989).

C2C12 cells were labeled with cholera toxin binding subunit (10 μg/ml; Sigma Chemical Co.) or with cholera toxin binding subunit/horseradish peroxidase (10 μg/ml; Sigma Chemical Co.) at 4°C as described previously (Parton, 1994), except that all incubations were increased to 2 h to allow time for diffusion into all surface-connected compartments. Labeling of frozen sections or embedding in Epon were performed as described previously (Parton, 1994). Grids were viewed using an electron microscope (model 1010; Jeol, Japan) in the Centre for Microscopy and Microanalysis, University of Queensland (Brisbane, Australia).

Results

Localization of Caveolin-3 in Adult Skeletal and Cardiac Muscle

As a first step to understanding the function of caveolin-3, we examined its localization in muscle tissues. A peptide corresponding to the NH_2-terminal portion of caveolin-3, a region of the protein which is not shared with other members of the caveolin family (Way and Parton, 1995), was used to immunize rabbits, and the resulting antiserum was affinity purified on a peptide column. The affinity-purified antibody (anti-cav3-N) recognized a band of ~20 kD, which was present in differentiated but not undifferentiated C2C12 cells, and was competed by the specific peptide to which it was raised (results not shown). We investigated the distribution of caveolin-3 and caveolin-1 in fully differentiated skeletal and cardiac muscle cells. Skeletal muscle tissue from adult mice was fixed and processed for semi-thick or ultrathin sections. Sections were labeled with anti-cav3-N, followed by fluorescent second antibodies or protein A–gold. Transverse or longitudinal muscle sections of adult skeletal muscle tissue showed strong peripheral staining of the muscle fibers for caveolin-3 (Fig. 1, *A* and *B*). At the ends of muscle fibers, a clearly organized network of labeling was apparent (Fig. 1 *A*). Immunogold labeling of ultrathin sections showed that caveolin-3 was localized to sarcolemmal caveolae with low labeling of the intervening membrane or the interior of the muscle fiber (Fig. 2). Caveolin-1 was undetectable in the myofibers by immunofluorescence (Fig. 1, *C* and *D*) but showed a strong labeling of endothelial cells (Fig. 1 *C*). Similar results were obtained with cardiac tissue. Double labeling immunoelectron microscopy of thin, frozen sections from mouse atrium with antibodies to caveolin-1 and caveolin-3 confirmed the lack of colocalization of the two proteins; caveolin-3 was restricted to caveolae of cardiomyocytes, whereas caveolin-1 was only detected in capillary endothelia (Fig. 3). In contrast to skeletal muscle where caveolin-3 was only observed in association with sarcolemmal caveolae, caveolin-3 positive caveolae were observed to be associated with both the plasma membrane and the T-tubules of cardiac muscle (not shown).

Caveolin-3 Distribution during Muscle Development In Vivo

Earlier work has suggested a role for caveolae in the formation of the T-tubule system. We therefore examined the localization of caveolin-3 during muscle development in vivo and in vitro.

Electron microscopy studies have shown that the T-tubule system of mouse skeletal muscle starts to develop in the period before birth (Franzini-Armstrong, 1991). At this time T-tubules are orientated in the longitudinal direction along the length of the fiber. After birth the T-tubule system is reorganized to form the transverse arrangement characteristic of adult tissue (for review see Flucher, 1992). We therefore examined the distribution of caveolin-3 in mouse skeletal muscle between embryonic day 16 and 3 d after birth by immunofluorescence (Fig. 4). In embryonic mouse leg muscle, strong labeling for caveolin-3 was detectable around the periphery of muscle fibers. In addi-

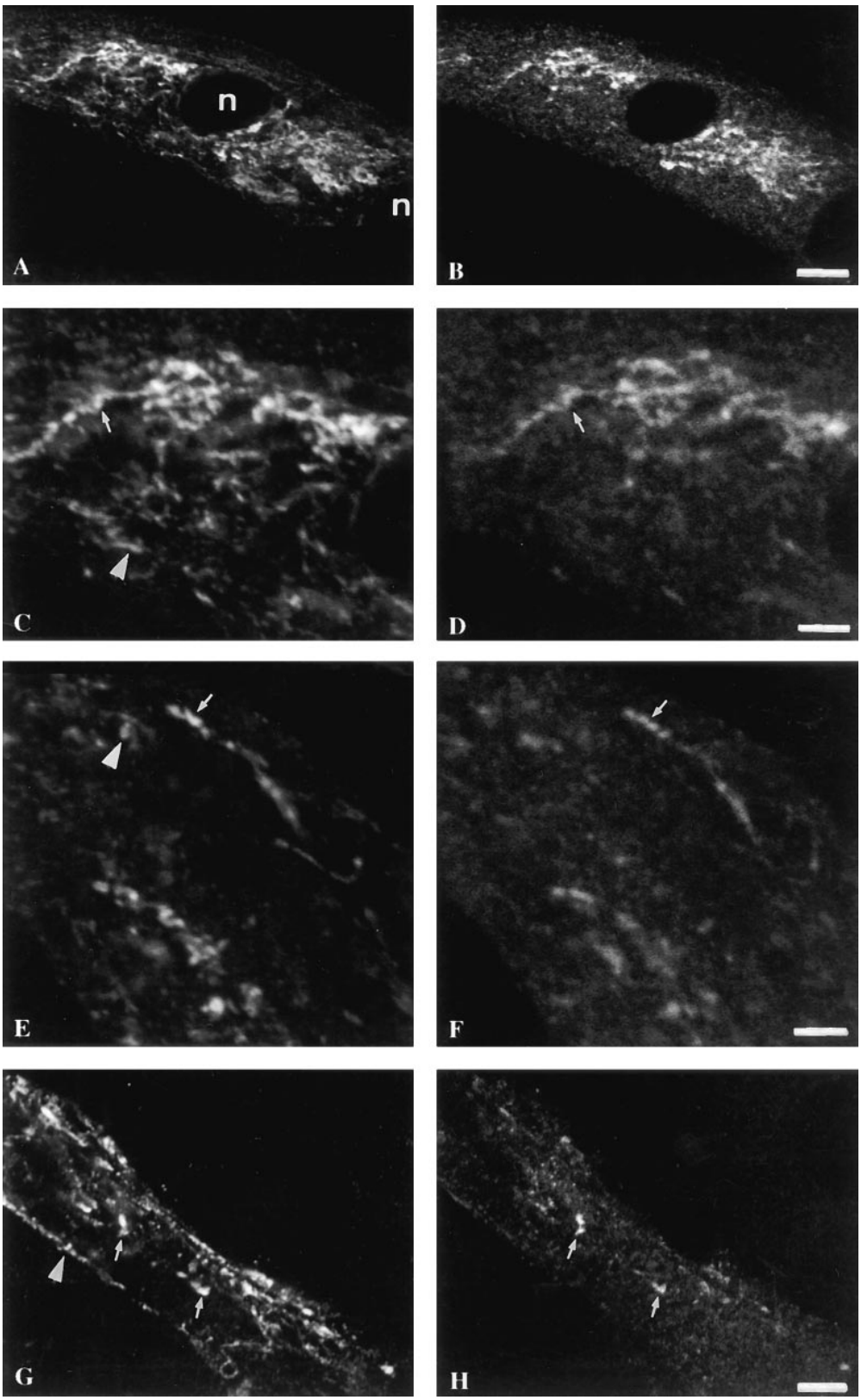


Figure 6. Caveolin-3 colocalizes with a T-tubule marker in C2C12 cells. C2C12 cells were cultured as described in the legend to Fig. 5. Cells were fixed with paraformaldehyde and double labeled for caveolin-3 (rhodamine, *A*, *C*, *E*, and *G*) and for the T-tubule specific marker α1-DHPR (FITC, *B*, *D*, *F*, and *H*). Caveolin-3 colocalizes with α1-DHPR in many tubular/reticular structures throughout the cell (*arrows*). Note that not all caveolin-3 positive structures are α1-DHPR positive (*arrowheads*). Note the variation in the labeling for α1-DHPR (for example see the relatively low level of labeling of the cell in *F*) but the clear colocalization with caveolin-3. Bars: (*A–D*) 5 μm; (*E* and *F*) 2.5 μm.

tion, at early stages of development, labeling was apparent within punctate structures throughout the cell (Fig. 4 *A*). A characteristic feature of the labeling at this developmental stage was lines of regularly spaced puncta close to the surface membrane (Fig. 4 *A*). Internal staining was particularly striking in 18-d embryonic muscle. At this stage, clearly defined tubules, which apparently extended from the sarcolemmal region into the muscle fiber, were labeled by caveolin-3 antibodies (Fig. 4 *B*). Such structures are reminiscent of forming T-tubules. However, available an-

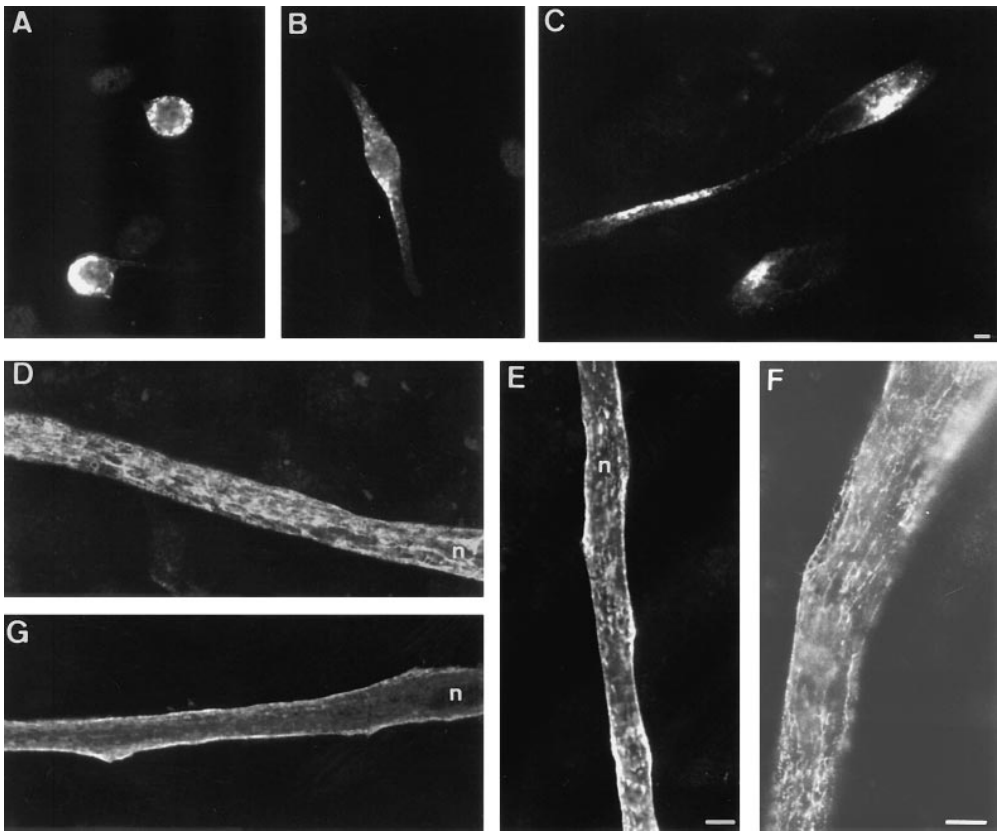


Figure 7. Immunolocalization of caveolin-3 during differentiation of primary muscle cells in culture. Mouse muscle cells from embryonic day 18 were cultured for various periods at 37°C before fixation and labeling for caveolin-3, as described in Materials and Methods. Cells were fixed 1 day after plating (*A*) or at various times after adding differentiation medium; 1 d (*B* and *C*), 5 d (*D*), 11 d (*E* and *F*), and 24 d (*G*). Cells were viewed by confocal (*A–E* and *G*) or conventional microscopy (*F*). Specific labeling is associated with the perinuclear and peripheral regions of day 0 myoblasts (*A–C*). From day 5 onwards, labeling is apparent within the putative T-tubule reticulum of fused myotubes (e.g., compare *F* with Fig. 12 *F*). At later stages, an increasing number of cells show surface labeling but low intracellular labeling (*G*). *n*, nuclei. Bars: (*A–C*) 5 μm; (*D–G*) 10 μm.

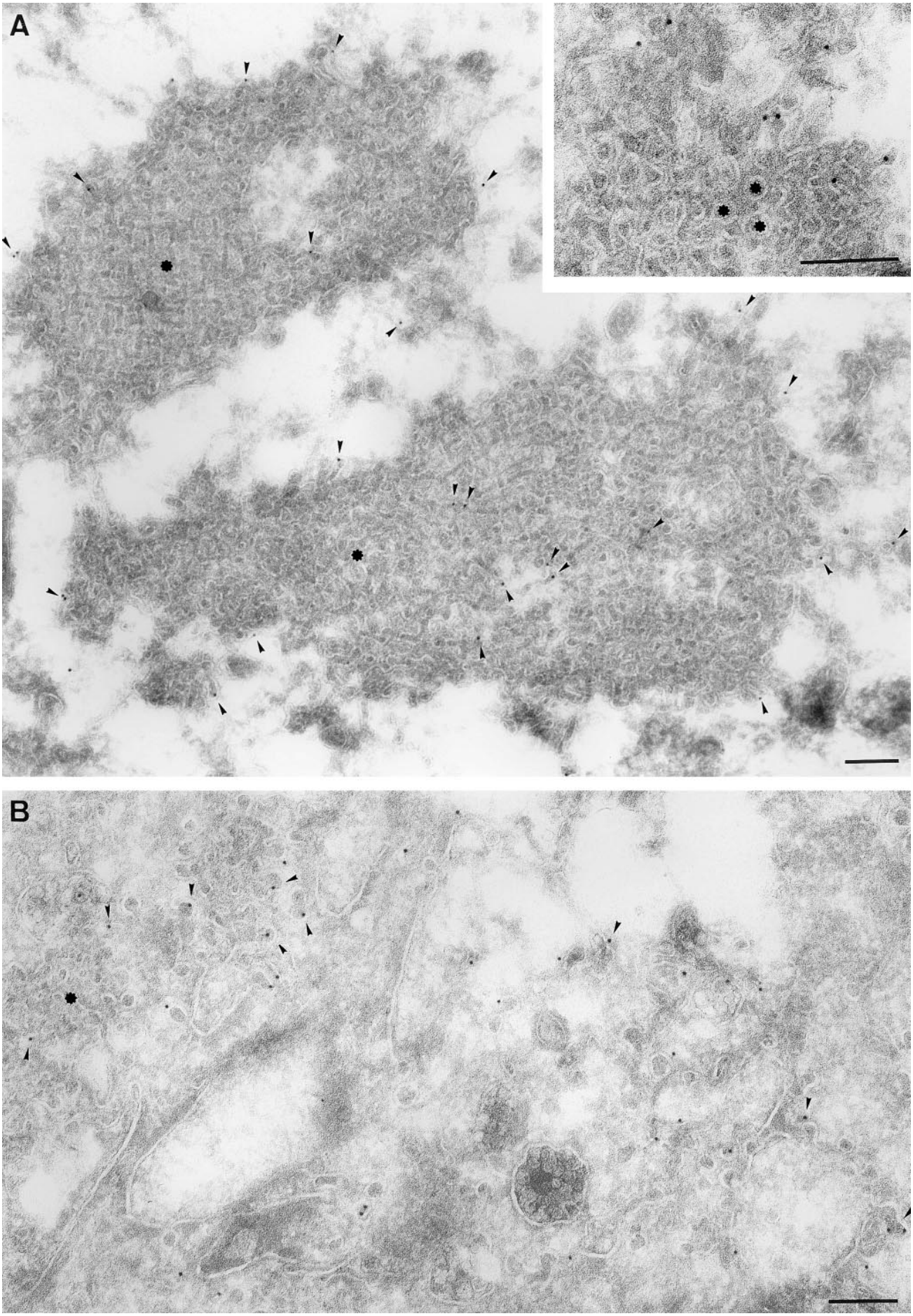
tibodies to the DHPR gave low labeling of embryonic muscle tissue, consistent with a low expression level before birth (Morton and Froehner, 1989). Internal labeling for caveolin-3, although decreased, was also evident in newborn mouse muscle (Fig. 4 *C*). In 3-d-old mice, internal labeling was barely detectable (Fig. 4 *D*), consistent with the lack of internal labeling in mature muscle.

Immunolocalization of Caveolin-3 in C2C12 Cells and Primary Cultured Skeletal Muscle Cells

The above results suggest that caveolin-3 associates with an internal compartment during muscle development. To investigate this process further, we varied the use of cell culture systems to study caveolin-3 during muscle development. We examined caveolin-3 distribution in two well-characterized culture systems: the C2C12 mouse myoblast/myotube cell line and primary cultured mouse muscle cells. C2C12 cells are a well-characterized model system for studies of muscle differentiation which, in the differentiated state, express caveolin-3 (Way and Parton, 1995). The distribution of caveolin-3 in C2C12 cells was examined using the affinity-purified anti-cav3-N antibodies and immunofluorescence microscopy. After fusion of C2C12 cells to form myotubes, caveolin-3 antibodies labeled an extensive tubular network within the cytoplasm (Fig. 5). As shown by confocal microscopy, the tubules are present within the depth of the muscle fiber, extend over many micrometers, and form a complex branching network throughout the cytoplasm. Labeling was specific for caveolin-3, as it was inhibited by the specific peptide to which the antibody was raised (results not shown). In addition, identical labeling was observed in a C2C12 cell line expressing epitope-tagged caveolin-3 (see Fig. 12).

We next examined the nature of the labeled organelles. In view of the morphology of the labeled elements we used antibodies to a well-characterized T-tubule marker, the α_1 subunit of the DHPR (α_1-DHPR). As shown in Fig. 6, the two markers showed a high degree of colocalization, as analyzed by confocal microscopy. Some peripheral structures were labeled by antibodies to caveolin-3 but not anti-DHPR, but, most of the tubular internal structures were labeled with both markers. The labeled elements were predominantly orientated in the longitudinal direction,

Figure 8. Immunoelectron microscopic localization of caveolin-3 in C2C12 cells. C2C12 cells were cultured as described in the legend to Fig. 5 and then fixed with a glutaraldehyde-containing fixative. The cells were then processed and immunolabeled for caveolin-3 followed by protein A–gold. Note the specific labeling (gold particles indicated by *arrowheads*) of extensive regular interconnected arrays of labeled membranes (*asterisks*). These structures are made up of unit structures with similar dimensions to caveolae (*asterisks* mark units of a reticulum in the *inset*). In the less compact arrays, (shown at higher magnification in *B*), the labeling is stronger, apparently due to greater access to caveolin-3 epitopes, and the individual caveolae-like elements are clearly evident (*arrows*). Note the similarity of these structures to caveolae clusters of nonmuscle cells. Bars, 200 nm.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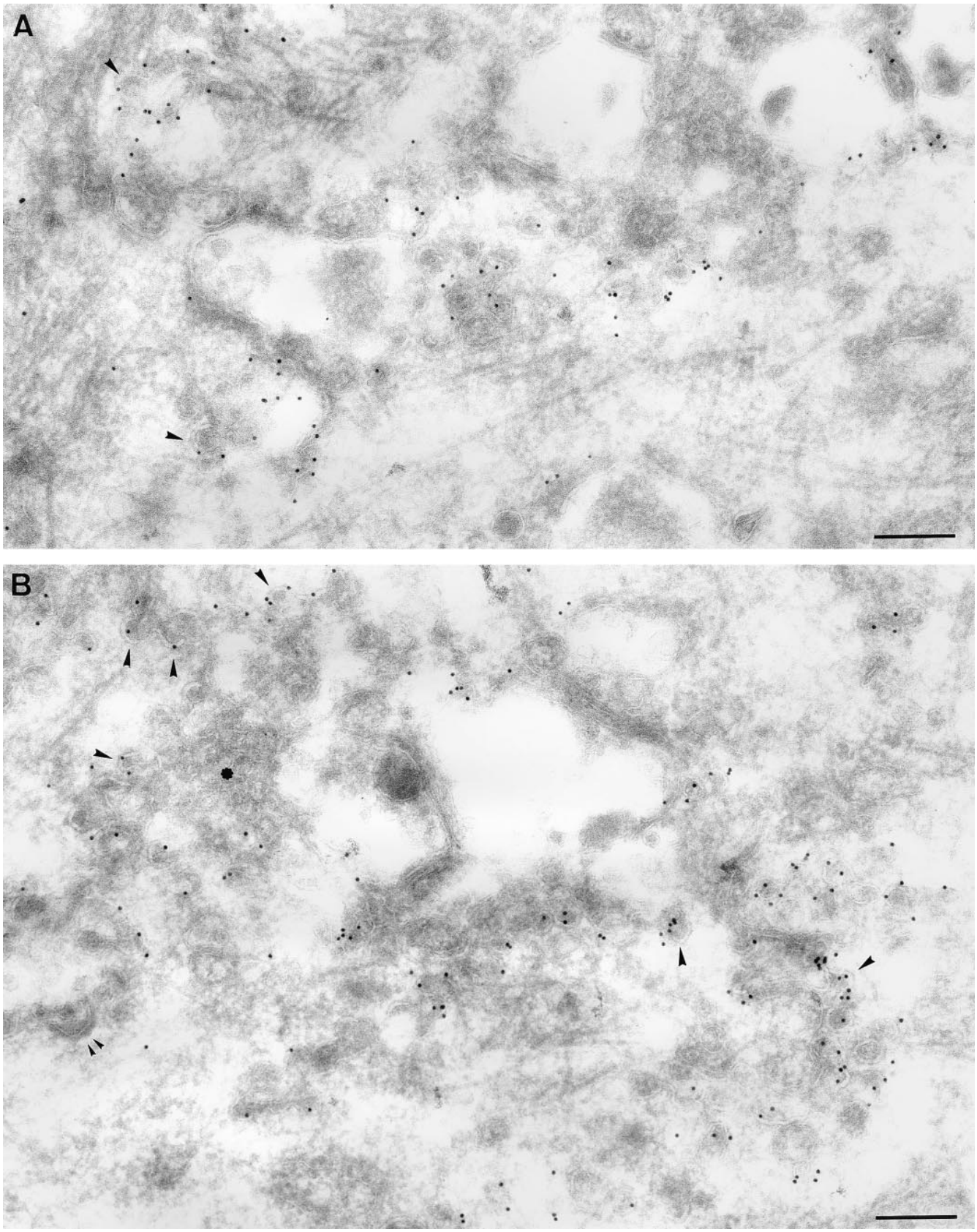


Figure 9. Immunoelectron microscopic localization of caveolin-3 in C2C12 cells. C2C12 cells were cultured and processed for frozen sectioning as described in the legend to Fig. 8, except that the cells were fixed in paraformaldehyde. Sections were immunolabeled with affinity-purified antibodies to caveolin-3. Specific immunolabeling for caveolin-3 is associated with 50–60 nm budding profiles with characteristic caveolar morphology (*arrowheads*). The caveolin-3 labeled elements form complex, extended arrays which penetrate the cen-

characteristic of T-tubules in incompletely differentiated muscle cells in vivo (Franzini-Armstrong, 1991). C2C12 cells maintained for longer periods in culture (>10 d), when some of the myotubes showed spontaneous contractile activity showed lower internal labeling (not shown).

To investigate the association of caveolin-3 with putative T-tubule elements in a more physiological culture situation, we examined the distribution of caveolin-3 in primary cultured mouse muscle cells. Primary cultured myotubes have been used extensively for studies of muscle differentiation and T-tubule formation and show a defined sequence of development in culture (Flucher et al., 1992). Muscle cells from the limbs of 18-d-old mouse embryos were dissociated by trypsin treatment and cultured for up to 24 d in vitro. Three days after plating, the culture medium was changed to differentiation medium, and two days later the cells started to show spontaneous contractile activity. Cells at different developmental stages were labeled for immunofluorescent detection of caveolin-3 and viewed by confocal microscopy. From the first day after plating, caveolin-3 was detectable in a small number of putative myoblasts (Fig. 7 *A*). Labeling first appeared in the perinuclear region of these cells. As the cells differentiated more, caveolin-3 labeling was observed in the cell periphery (Fig. 7, *B* and *C*). After fusion of myoblasts to form myotubes, caveolin-3 labeling greatly increased and labeling appeared within an extensive system of tubular/reticular elements which penetrated the entire cytoplasm (Fig. 7, *D–F*) and appeared identical to the T-tubule labeling of C2C12 cells. Consistent with the studies of developing skeletal muscle tissue with longer times in culture an increasing number of cells was observed to show predominantly peripheral staining with a reduction in the level of internal T-tubule labeling (Fig. 7 *G*). The results show that in both primary cultured muscle cells and C2C12 cells, caveolin-3 associates with developing T-tubules. In differentiated cells and in mature muscle, caveolin-3 is no longer detectable within the T-tubule system but is associated with sarcolemmal caveolae.

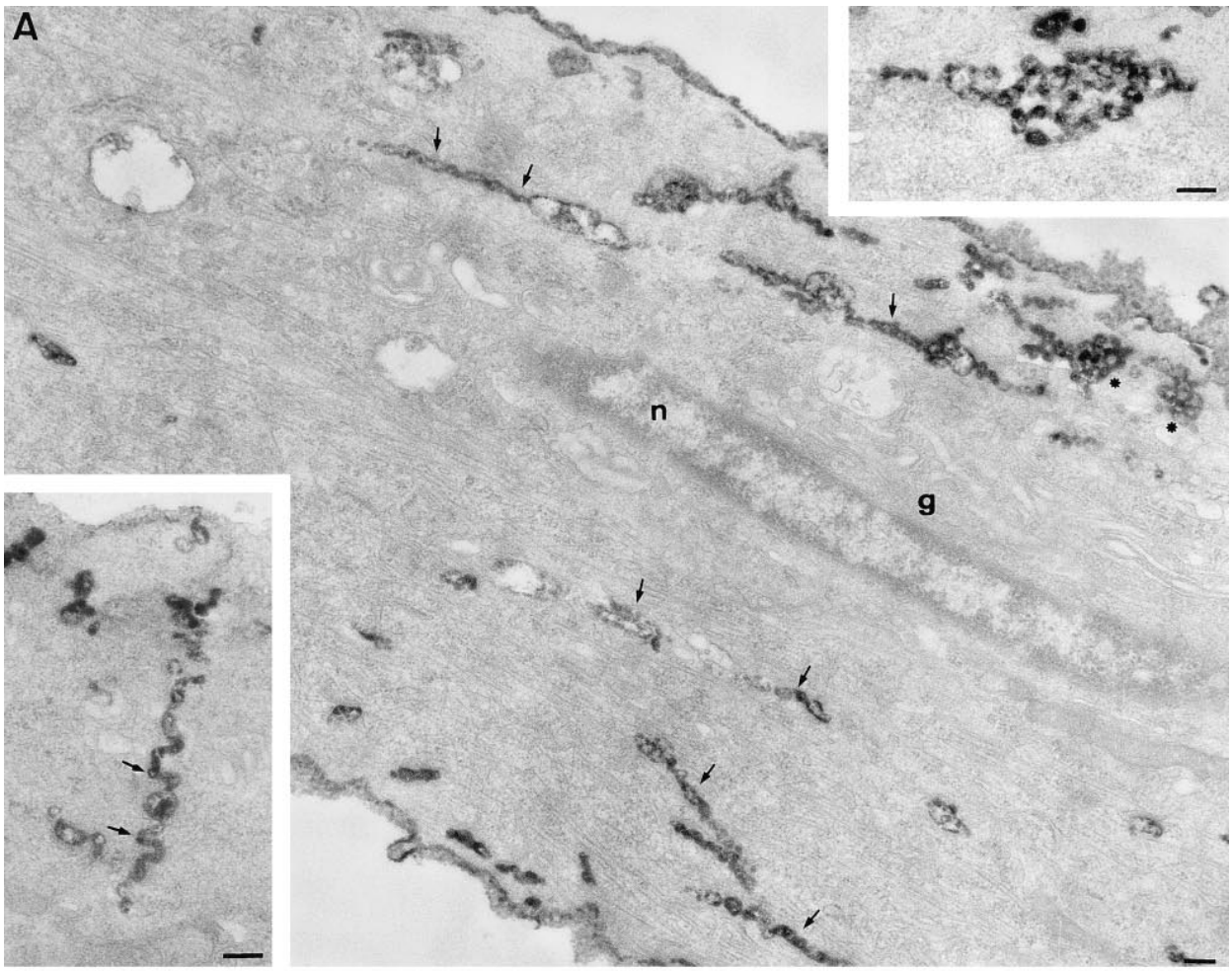
Ultrastructural Localization of Caveolin-3 in C2C12 Cells

The distribution of caveolin-3 in differentiating C2C12 cells was further examined by immunoelectron microscopy. C2C12 cells were fixed with glutaraldehyde and then processed for frozen sectioning. Sections were labeled with affinity-purified anti-cav3-N antibodies. Negligible labeling was found in undifferentiated cells, but in fused cells, caveolin-3 labeling was associated with elaborate networks of interconnected membranes apparently deep within the cytoplasm of the myotubes (Fig. 8). The networks were composed of regular repeating units which, at low magnification, almost had a crystalline appearance. Labeling for caveolin-3, although low, was apparent over these entire networks and was not detectable on other intracellular membranes or the plasma membrane. Labeling appeared to be higher over the periphery of these networks and in the less tightly clustered regions. In these areas the individual units of the reticulum were apparent and, as shown in Fig. 8 *B*, strongly resembled caveolae. The dimensions of the individual units of the reticulum (Fig. 9 *A*, *inset*) suggest that these networks may be composed of fused caveolae or caveolae which have formed repeatedly but have not been released through a fission event.

We examined this further in paraformaldehyde-fixed cells. As shown in Fig. 9, the reticular structures appeared to be less well preserved under these fixation conditions, as they were less extensive and more irregular. However, labeling for caveolin-3 was clearly increased compared to glutaraldehyde-fixed cells and was associated with large clusters of caveolae-like elements. Specific labeling was invariably associated with caveolae-like profiles and not with any intervening flat membrane. The labeled structures extended over several micrometers into the center of the cells (Fig. 9). The labeled elements also had associated clathrin-coated pits, consistent with their identification as T-tubule elements.

The similarity of the caveolin-3–labeled elements to the previously described precursor T-tubule elements (Ishikawa, 1968) and the colocalization of caveolin-3 with a T-tubule marker by confocal microscopy, strongly support the idea that caveolin-3 associates with developing T-tubules. However, the available antibodies to the DHPR gave weak labeling by electron microscopy. To confirm that these elements were surface connected, we incubated differentiating C2C12 cells with peroxidase-labeled cholera toxin binding subunit (CT-B) at 4°C, a temperature at which endocytosis is blocked. The plasma membrane receptor for cholera toxin, GM1, has previously been shown to be enriched in caveolae (Montesano et al., 1982; Parton, 1994). Surface-labeled cells were fixed and embedded in Epon. Semi-thick sections were cut parallel to the culture substratum. As shown in Fig. 10, CT-B–peroxidase labeled the cell surface as well as long tubules and reticular structures throughout the cell. The labeled tubular elements were preferentially orientated in the longitudinal direction, characteristic of developing T-tubules. Both the tubular and reticular elements (Fig. 10, *insets*) showed the characteristic morphology of "fused" caveolar elements and appeared similar to those labeled with caveolin-3. We then repeated the above experiment using unlabeled CT-B and processed the cells for frozen sectioning. Thawed sections were labeled with antibodies to caveolin-3 and CT-B. As shown in Fig. 11, labeling for CT-B was associated with the cell surface and also with the caveolin-3 positive putative T-tubule elements. These results confirm that a large number of the labeled elements are indeed surface connected. Taken together, the results suggest that during muscle differentiation, caveolin-3 associates with developing T-tubules.

ter of the muscle cell. The complex, clustered arrangement of these structures, as viewed in thin sections, suggests that in three dimensions, the caveolae form large clusters resembling "bunches of grapes." In some regions, reticular elements are evident (Fig. 4 *B*, *asterisk*) which resemble forming T-tubules, as described in early morphological studies (Ishikawa, 1968), but are less compact and regular than those seen in glutaraldehyde-fixed cells. Note that the caveolin-3 labeling is typically associated with the bud-like profiles rather than the tubular interconnecting regions. The double arrowhead indicates a clathrin-coated pit. Bars, 200 nm.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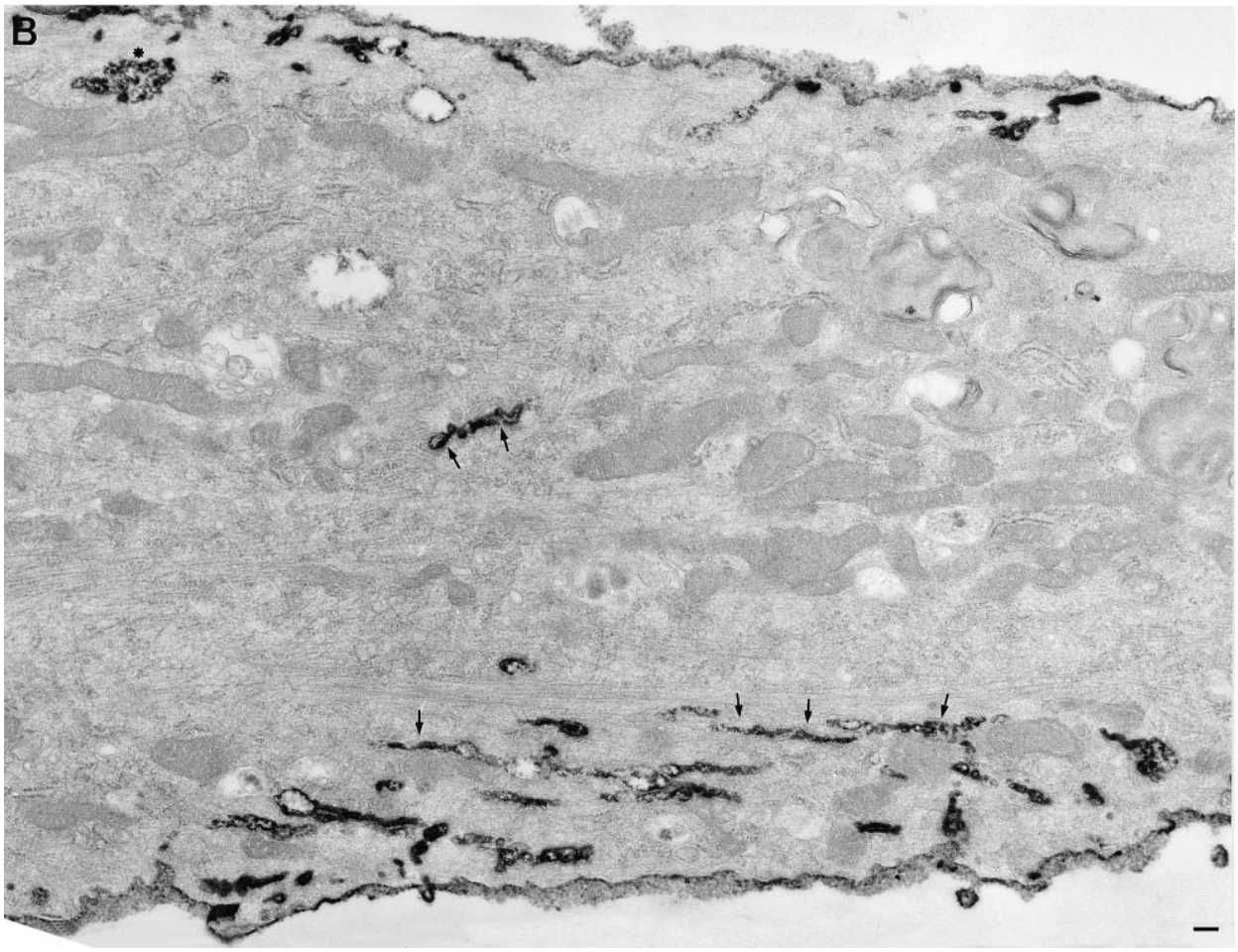

B

The developing T-tubule system takes the form of a reticulum made up of individual units with morphological features and components characteristic of caveolae. These observations are consistent with the involvement of repeated caveolae formation in T-tubule development.

Muscle-specific Sorting of Caveolin-3

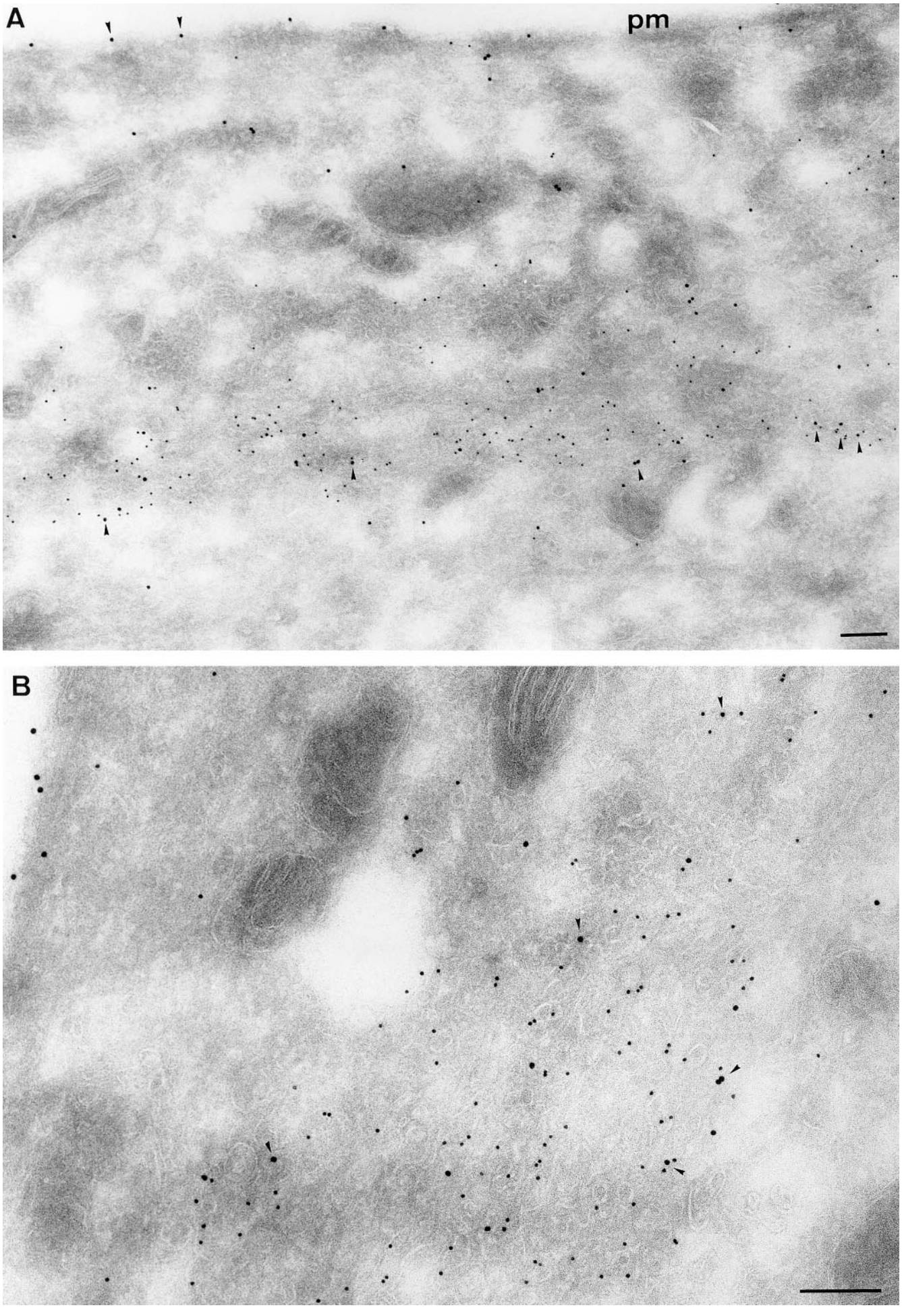
Finally we sought to examine the localization of caveolin-3 with respect to caveolin-1. We have previously shown that caveolin-3 and caveolin-1 colocalize when expressed in fibroblasts. To examine the distribution of the two proteins in myoblasts, which express caveolin-1 but not caveolin-3 (Way and Parton, 1995), a stable C2C12 cell line containing caveolin-3 with a COOH-terminal HA tag was generated. We examined the distribution of epitope-tagged caveolin-3 and of caveolin-1 during differentiation by double labeling with antibodies to the NH_2 terminus of endogenous caveolin-1 (Dupree et al., 1993) and the 12CA5 antibody against the HA tag. In undifferentiated C2C12-CAV3HA cells 24 h after plating there was clear colocalization of caveolin-1 and epitope-tagged caveolin-3 at the cell periphery (Fig. 12, *A* and *B*). Therefore, in these undifferentiated muscle cells, as in fibroblasts (Way and Parton, 1995), caveolin-3 is apparently directed to caveolae. In contrast, 48 h after plating, but before fusion into myotubes, a number of cells started to show a different labeling pattern for caveolin-1 and caveolin-3 (Fig. 12, *C* and *D*), with caveolin-1 generally showing a more diffuse labeling pattern. In fused C2C12-CAV3HA cells epitope-tagged caveolin-3 was localized to the T-tubule system running throughout the cytoplasm (Fig. 12, *E*, *F*, and *H*). In contrast, caveolin-1 showed negligible labeling in the differentiated cells (Fig. 12 *G*), suggesting that the expression of caveolin-1 is reduced upon muscle differentiation. It therefore appears that caveolin-3 expressed in C2C12 cells colocalizes with caveolin-1 in the nondifferentiated state, but, as the cells differentiate, the two markers are separated. Caveolin-1 is sorted away from caveolin-3, which eventually associates with the T-tubule system.

Discussion

The T-tubule system of mammalian cells is an extensive membranous system which penetrates the entire muscle fiber but is continuous with the muscle plasma membrane. The protein and lipid composition of the T-tubule system is distinct from that of the sarcolemma. How this system develops and maintains its unique composition is a fundamental problem in cell biology. In the present study we have shown that caveolin-3, a member of a family of integral membrane proteins proposed to be involved in organizing membrane form and composition, is associated with precursor T-tubule elements in skeletal muscle. Our studies of caveolin-3 and caveolin-1 in C2C12 cells, primary cultured myotubes, and developing muscle in vivo suggest the following series of events. As myoblasts fuse to form myotubes, expression of caveolin-1 starts to decrease, and expression of the muscle-specific protein, caveolin-3, is dramatically increased. Caveolin-3 then appears in association with tubular elements which penetrate the entire cytoplasm of the myotubes but are predominantly orientated longitudinally along the length of the muscle fiber. These tubules are labeled by antibodies to the α_1 subunit of the dihydropyridine receptor. By electron microscopy, the caveolin-3–labeled structures resemble elaborate, regular arrays of interconnected clusters of caveolae and are connected to the cell surface. These elements therefore have the characteristics of precursor T-tubules (Flucher et al., 1992). Around the time of birth, the level of caveolin-3 associated with the T-tubules starts to decrease, and in adult muscle, caveolin-3 is no longer detectable within the T-tubule system but is highly concentrated in sarcolemmal caveolae. The association of caveolin-3 with T-tubules is therefore restricted to a precursor T-tubule stage in which the forming T-tubules appear to consist of interconnected caveolae-like elements. These results raise the intriguing possibility that caveolae, and in particular caveolin-3, are involved in the biogenesis of the T-tubule system during muscle differentiation.

Early electron microscopic studies were the first to suggest a role for caveolae, identified purely on morphological grounds, in early T-tubule formation in cultured myotubes (Ezerman and Ishikawa, 1967; Ishikawa, 1968). These studies suggested that caveolae form repeatedly but keep their connectivity with the surface, leading to the generation of extensive, regular arrays of interconnected caveolae-like structures. The resulting reticulum was proposed to represent the precursor T-tubule system based on its connectivity with the cell surface and its junctions with the sarcoplasmic reticulum. Later studies showed that these structures contained markers characteristic of the T-tubule system but excluded sarcolemmal components (Flucher et al., 1993). In vivo studies also confirmed the association of caveolae-like structures with developing T-tubules during embryonic muscle development (Franzini-Armstrong, 1991). Moreover, in regenerating muscle fibers, the reforming T-tubule system was shown to be composed of caveolar elements often having a "honeycomb" appearance (Miike et al., 1984). Taken together, these studies strongly argue for a role for caveolae-like structures at an early stage in T-tubule formation, but their identification as caveolae was based on morphology alone. The findings of the present study clearly show that these elements are indeed caveolae, as demonstrated by labeling with the caveolin-3 antibody. The reticular precursor T-tubule elements described in the above studies are apparently identical to the structures labeled with antibodies to caveolin-3 in C2C12 cells in the present study (e.g., compare Figs. 8 and 9 with Figs. 2 or 10 of Ishikawa, 1968) and show for the first

Figure 10. Cholera toxin peroxidase labeling of differentiating C2C12 cells. C2C12 cells cultured as described in the legend to Fig. 5 were incubated with CT-B-peroxidase for 2 h at 4°C and then fixed and processed for embedding in epon. Semi-thick sections (~200 nm) were cut parallel to the substratum. Peroxidase-labeled elements are seen within the depths of the cell (*arrows*). The labeled structures are composed of individual bud-like elements of ~60 nm diam, which form chains or reticula of interconnected structures (*insets*). Bars, 200 nm.


A
pm
B

time that a caveolin protein is associated with the developing T-tubule system. We have also shown that at least some of these structures are accessible to and labeled by a cholera toxin conjugate administered in the extracellular medium. Our results are therefore consistent with the proposed endocytic model for T-tubule formation (Flucher, 1992). A second model for T-tubule formation invokes a role for exocytic, rather than endocytic transport. In this model, the precursor T-tubule system is formed as a result of exocytic transport and is not initially connected to the plasma membrane. Recent studies provided evidence for a combination of these models, as some T-tubule precursor elements were shown to be discontinuous with the cell surface at early stages of development (Flucher et al., 1991). Further work will be required to ascertain how this compartment relates to the caveolin-labeled elements described here. At present we cannot rule out that newly formed caveolae can fuse with each other to form clusters which subsequently fuse with the cell surface. In many respects, the caveolae clusters shown here resemble the caveolin-1 positive caveolae in many nonmuscle cells. Caveolae in many cell types form extensive arrays of interconnected structures which penetrate the cytoplasm (Parton, 1996). This is particularly evident after okadaic acid treatment, when caveolae form large clusters which appear to be pulled into the center of the cell in an actin- and microtubule-dependent manner (Parton et al., 1994). These structures show a remarkable resemblance to the caveolin-3 positive caveolae clusters seen in the present study (Figs. 8 and 10). Taken together, these studies suggest that caveolae have a propensity to form such structures, and in muscle cells, these structures form the basis for the development of the T-tubule system.

In view of the known characteristics of caveolins, the postulated role of caveolae in T-tubule formation, and the transient detection of caveolin-3 within the developing T-tubule system, we speculate that caveolin-3 might be required to generate the unique protein and lipid composition of the T-tubule system. We have previously shown that caveolin-1 expression in caveolae-deficient cells causes de novo formation of caveolae (Fra et al., 1995*b*). The high density of caveolin-1 in the caveolar membrane as well as the need for a threshold level of caveolin-1 in the plasma membrane to produce caveolae (Parton, 1996), both argue for a structural role of caveolin in caveolae formation. Two recently described properties of caveolin-1 might be important in caveolar-domain formation. First, caveolin-1 self-associates to form oligomers (Monier et al., 1995; Sargiacomo et al., 1995). Second, caveolin is a cholesterol-binding protein (Murata et al., 1995). Cholesterol is essential for caveolae form and function (Rothberg et al., 1990, 1992), and it has been proposed that the interaction of caveolin with cholesterol in glycosphingolipid-enriched domains may be necessary for caveolae formation (Parton and Simons, 1995). Caveolin-3 appears to show a similar propensity to form oligomers (Tang et al., 1996) and shows high sequence homology in that region of the caveolin molecule postulated to be involved in oligomer formation (Sargiacomo et al., 1995). The region of the molecule involved in cholesterol binding is still unknown, but the three known caveolins have particularly high homology in the intramembrane and membrane proximal regions (Way and Parton, 1995; Tang et al., 1996). Thus, we speculate that caveolins may be general modulators of the plasma membrane, being able to generate the unique protein and lipid composition of caveolae, or, in the case of caveolin-3, the precursor T-tubule domain. Intriguingly, T-tubules, like caveolae, are known to be enriched in cholesterol (Hidalgo et al., 1983; Horgan and Kuypers, 1987), and this has even been used as a marker in fractionation studies (Knudson and Campbell, 1989). In addition, in the present study we have shown that the precursor T-tubule system is labeled by cholera toxin conjugates, which are concentrated in caveolae of other cells (Parton, 1994). While further work will be required to ascertain whether the receptor for cholera toxin, the ganglioside GM1, is actually concentrated within T-tubules, a logical extension of this model for T-tubule formation is that the protein and lipid composition of the T-tubule system may be maintained by principles similar to those of caveolae. Both caveolae and T-tubules represent membrane systems which are continuous with the plasma membrane but have a distinct composition. Caveolin-3 might be involved in the initial process of generating the T-tubule domain, and then cytoskeletal elements would assume the role of maintaining this structure in its precise alignment with each side of the Z-lines. Perhaps caveolae at the neck of the T-tubules in mature muscle (Franzini-Armstrong et al., 1975; Zampighi et al., 1975) act as barriers to prevent lipids and proteins of the sarcolemma and T-tubules from intermixing, as previously hypothesized (Flucher, 1992). In view of the importance of lipid-based sorting mechanisms in a number of different aspects of cellular organization (Simons and Ikonen, 1996), the hypothesis that the distinct sarcolemmal and T-tubule compositions may be generated using principles similar to those of caveolae, clearly warrants further attention.

In mature skeletal muscle, caveolin-3 is restricted to sarcolemmal caveolae and is no longer detectable in the T-tubule system. A decrease in internal labeling was already apparent around birth. From birth onwards, the T-tubules are gradually reorganized from longitudinally orientated tubules to the regularly spaced radially orientated T-tubules characteristic of mature muscle (Franzini-Armstrong, 1991). Our observations of developing mouse muscle, primary mouse muscle cultures, and C2C12 cells suggest that the association of caveolin-3 with the T-tubule system is restricted to the predominantly longitudinal precursor T-tubules. This longitudinal arrangement has been shown to persist for many days in cultured C2C12 cells and in primary cultures but appears to be short-lived in vivo (Flucher, 1992),

Figure 11. Immunoelectron microscopic localization of caveolin-3 in cholera toxin surface-labeled C2C12 cells. C2C12 cells cultured as described in the legend to Fig. 5 were incubated with CT-B for 2 h at 4°C before fixation with paraformaldehyde. Ultrathin frozen sections were immunolabeled with antibodies to cholera toxin, followed by 15 nm protein A–gold (*arrowheads*) and to caveolin-3 detected with 10 nm protein A–gold. Cholera toxin labeling is evident on the cell surface and in the extensive caveolin-3–positive tubulovesicular elements. *pm*, plasma membrane. Bars, 200 nm.

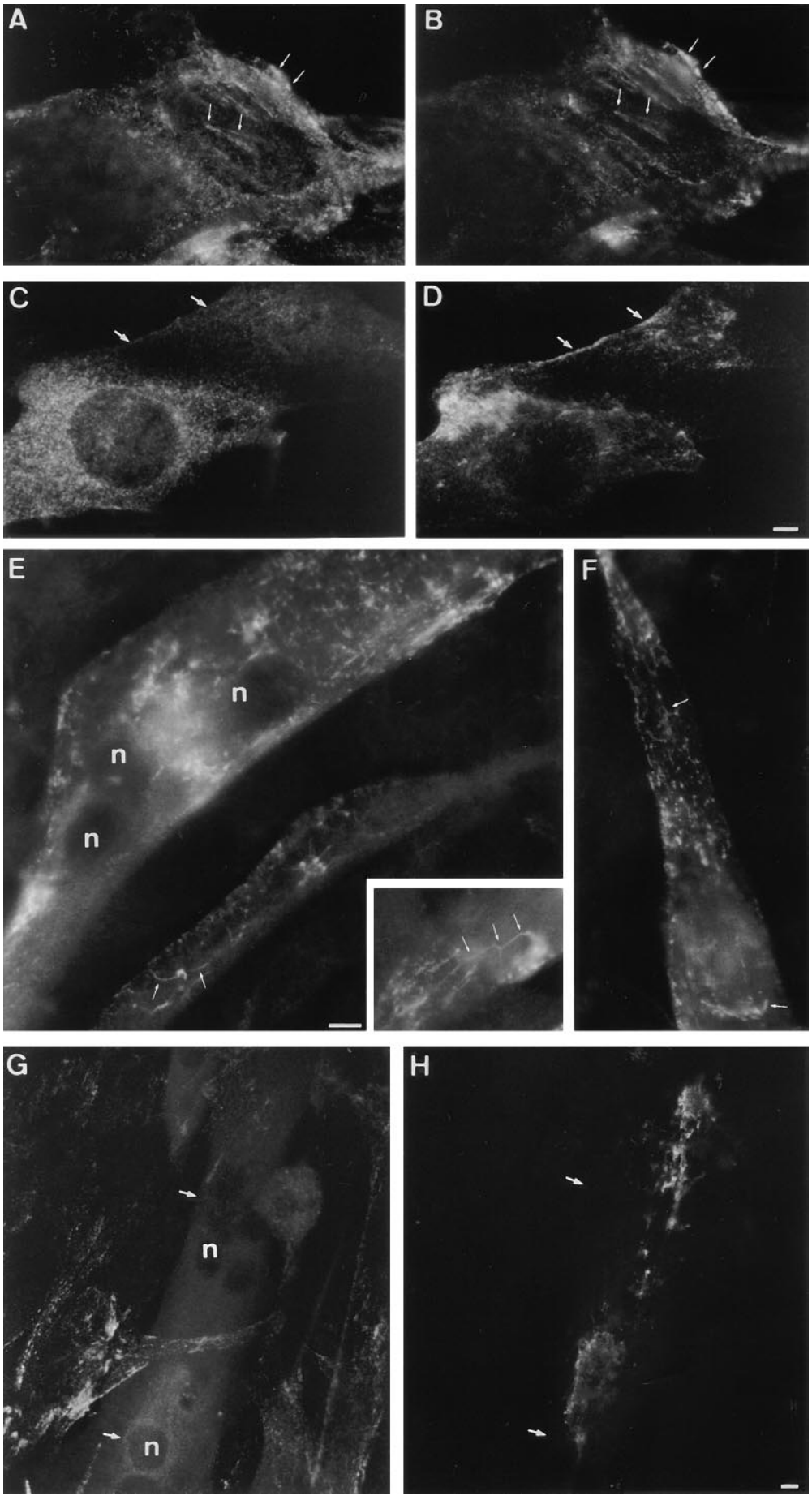


Figure 12. Immunolocalization of epitope-tagged caveolin-3 and endogenous caveolin-1 in C2C12 cells. C2C12 cells expressing caveolin-3 with a COOH-terminal HA tag were fixed after 1 d (*A* and *B*), 2 d (*C* and *D*), or after 6–8 d, 4–6 d after the cells reached confluency and differentiation medium was added), (*E–H*). The cells were then labeled with antibodies to caveolin-1 (*A*, *C*, and *G*) or to the HA tag (*B*, *D*, *E*, *F*, and *H*). *A–D* and *G* and *H* show cells double labeled for caveolin-1 and the epitope-tagged caveolin-3. In day 1 myoblasts (*A* and *B*), the endogenous caveolin-1 and expressed caveolin-3 colocalize (*arrows*). At later times (*C* and *D*), some cells clearly show a different labeling pattern for the two caveolin proteins (*arrows* indicate comparable regions of the two cells which are labeled for caveolin-3 but not caveolin-1). After fusion of myoblasts to form myotubes, the epitope-tagged caveolin-3 is present within the T-tubule system which runs thoughout the cell (*E*, *F*, and *inset*, *arrows*). *G* and *H* show cells double labeled for caveolin-1 and epitope-tagged caveolin-3. While caveolin-1 labeling is present in neighboring, undifferentiated myoblasts, labeling is very low in the multinucleate myotube (*arrows*). Weak staining represented background labeling as shown by peptide inhibition (not shown). *n*, nucleus. Bars: (*A–D*) 2 μm (same magnification); (*E–H*) 5 μm (*E* and *F*, *G* and *H*, same magnifications).

fitting well with the results of the present study. As the T-tubules reorganize, caveolin-3 may be removed from the T-tubules, by some unknown recycling mechanism, or newly synthesized protein may be directed away from the T-tubule system to the sarcolemma. Alternatively, the epitope recognized by the antibody, at the NH_2 terminus, may be masked. A precedent exists for the latter, as intracellular labeling for caveolin-1 is not detectable within the *trans*-Golgi network of fibroblasts with antibodies to the NH_2 terminus of the protein, but only with antibodies to the COOH terminus (Dupree et al., 1993). However, as the morphological features characteristic of caveolae are not detectable within the T-tubules of skeletal muscle, we favor the view that caveolin-3 is not present in the mature T-tubule system but only during its development. The fact that caveolin-3 is present in T-tubules during development but not in the final differentiated state, when it is restricted to sarcolemmal caveolae, suggests that caveolin-3 might have two distinct functions: as a morphogenetic element involved in forming the T-tubule domain, and as a component of sarcolemmal caveolae. The role in sarcolemmal caveolae is presumably similar to that of caveolin-1 in other cells, that is, maintenance of caveolar form and signal transduction. The functional interaction of caveolin-3 with trimeric G protein α subunits is consistent with a role in signaling in muscle cells similar to that of caveolin-1 in nonmuscle cells (Tang et al., 1996). It should be noted in addition that caveolin-3 is also present in smooth muscle cells which do not have a T-tubule system (Song et al., 1996). This again indicates that the function of caveolin-3 is not restricted to T-tubule formation.

Caveolin-1 was not detectable in differentiated C2C12 cells nor in adult muscle tissue by immunofluorescence, although endothelial capillaries were heavily labeled (Fig. 12). However, undifferentiated myoblasts express caveolin-1, and we show here that expression of caveolin-3 in these cells results in initial colocalization and then segregation. Future studies should establish the signals involved in the sorting of the two proteins to different cellular compartments. While caveolin-3 associates with the T-tubules, the level of caveolin-1 decreases, suggesting that caveolin-3 replaces caveolin-1 as the major caveolin of differentiated muscle cells. The absence of caveolin-1 in differentiated cultured muscle cells is consistent with two previous studies (Munoz et al., 1996; Tang et al., 1996). However, despite the absence of caveolin in differentiated cultured cells, Munoz et al. (1996) detected caveolin-1 within muscle tissue by Western blotting. This apparent discrepancy may be explained by the abundance of caveolin-1 in endothelial cells in the muscle tissue.

In conclusion, we speculate that caveolin-3 plays a role in T-tubule formation analogous to that of caveolin-1 in caveolae formation, and that caveolae and T-tubules may represent different manifestations of lipid-based sorting phenomenon. In the final mature muscle, caveolin-3 has an additional role in sarcolemmal caveolae where, as in other cells, it is presumably involved in interactions with signaling molecules. These studies raise the intriguing possibility that T-tubules and caveolae may use similar principles to generate and maintain their form and composition.

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