

## Immunolocalization of Caveolin-1 and Caveolin-3 in Monkey Skeletal, Cardiac and Uterine Smooth Muscles

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**ABSTRACT.** Caveolin, a 20–24 kDa integral membrane protein, is a principal component of caveolar domains. Caveolin-1 is expressed predominantly in endothelial cells, fibroblasts, and adipocytes, while the expression of caveolin-3 is confined to muscle cells. However, their localization in various muscles has not been well documented. Using double-immunofluorescence labeling and confocal laser microscopy, we examined the localization of caveolins-1 and 3 in adult monkey skeletal, cardiac and uterine smooth muscles and the co-immunolocalization of these caveolins with dystrophin, which is a product of the Duchenne muscular dystrophy gene. In the skeletal muscle tissue, caveolin-3 was localized along the sarcolemma except for the transverse tubules, and co-immunolocalized with dystrophin, whereas caveolin-1 was absent except in the blood vessels of the muscle tissue. In cardiac muscle cells, caveolins-1 and -3 and dystrophin were co-immunolocalized on the sarcolemma and transverse tubules. In uterine smooth muscle cells, caveolin-1, but not caveolin-3, was co-immunolocalized with dystrophin on the sarcolemma.

**Key words:** caveolin/skeletal muscle/cardiac muscle/smooth muscle/dystrophin

Caveolin is a major component of the caveolae which are small invaginations of the plasma membrane. Three distinct mammalian caveolins have been identified to date: caveolin-1, -2, and -3 (Way and Parton, 1995; Scherer *et al.*, 1996; Tang *et al.*, 1996). Caveolins-1 and 3 are closely related based on primary sequence homology, showing 65% identity and 85% similarity (Tang *et al.*, 1996). Caveolins-1 and 2 are co-expressed in many cell types, such as endothelial cells, fibroblasts and adipocytes, where they form hetero-oligomers (Scherer *et al.*, 1996). In contrast, the expression of caveolin-3 is muscle-specific (Tang *et al.*, 1996). In smooth muscle cells of the taenia coli, caveolin has been reported to be localized on the sarcolemma (North *et al.*, 1993). However, it has not been well documented whether the caveolin expressed in smooth muscle cells is

caveolin-1 or -3. Recently, caveolin-3 was detected in tissue homogenate of the small intestine but not of the uterus in mice (Li *et al.*, 2001).

Caveolae are abundant in endothelial cells, adipocytes and muscle cells and have been implicated in a variety of cellular functions, including endothelial transcytosis, calcium uptake and signal transduction (Fujimoto, 1993; Lisanti *et al.*, 1994; Parton, 1996; Couet *et al.*, 1997; Anderson, 1998; Kurzchlija and Parton, 1999). In skeletal muscle cells, caveolae are often associated with the opening of transverse (T-) tubules and have been implicated in their formation. Based on studies of cultured chicken skeletal muscle cells (Ezerman and Ishikawa, 1967; Ishikawa, 1968) and developing mouse cardiac muscle cells (Ishikawa and Yamada, 1975), caveolae are proposed to play a role in the formation of the T-tubule system during muscle development. The T-tubules appear first as caveolae that form tubules through caveolar proliferation and into interconnected arrays of three-dimensional networks. These previous observations are supported by a recent study that caveolin-3 is associated with T-tubules at early stages of development, while caveolin-3 is no longer observed on the T-tubules in adult skeletal muscles (Parton *et al.*, 1997). However, in caveolin-3

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Abbreviations: T-tubules, transverse tubules; DAP, dystrophin-associated proteins; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; TRITC, tetra-methyl-rhodamine isothiocyanate.

knockout mice, T-tubules can be formed, although some abnormalities in the T-tubule have been reported (Galbiati *et al.*, 2001).

After caveolin-3 was first characterized, Song *et al.* (1996) biochemically observed the presence of dystrophin in a caveolin-3-rich fraction. Dystrophin is a protein associated with actin filaments, which form the membrane cytoskeletal network, and with  $\beta$ -dystroglycan (Hoffman *et al.*, 1987; Suzuki *et al.*, 1992). Subsequently, McNally *et al.* (1998) reported that caveolin-3 is present in a preparation of the complex of dystrophin and dystrophin-associated proteins (DAP). These authors considered caveolin-3 to be a new member of the DAP family. Furthermore, transgenic overexpression of caveolin-3 in skeletal muscle cells caused downregulation of dystrophin (Garbiati *et al.*, 2000). However, Crosbie *et al.* (1998) observed that the amount of caveolin-3 recovered decreases with the progress of purification of the dystrophin-glycoprotein complex and that they did not consider caveolin-3 as a DAP.

Mutations of the caveolin-3 gene have been reported to be involved in a type of limb-girdle muscular dystrophy inherited in an autosomal dominant manner (Minetti *et al.*, 1998). However, because some apparent mutations of caveolin-3 gene have turned out to be harmless, the problem is raised whether deficiency of caveolin-3 causes dystrophic phenotypes or not. Recently, we developed caveolin-3-null mice that have a mild muscular dystrophic phenotype, although the disease is inherited in an autosomal recessive manner (Hagiwara *et al.*, 2000). This was followed by a similar report (Galbiati *et al.*, 2001). These findings clearly show that loss of caveolin-3 causes dystrophic changes.

To better understand the roles of caveolins in muscle cells, we compared the localization of caveolins-1 and 3 in skeletal, cardiac and smooth muscle and the cellular colocalization of the caveolins and dystrophin by immunohistochemistry.

## Materials and Methods

### Tissues

Limb skeletal, heart ventricular and uterine muscles were obtained from adult monkeys (*Macaca fascicularis*) and immediately frozen in isopentane cooled by liquid nitrogen and stored in deep-freezer at  $-80^{\circ}\text{C}$  until use. These tissues were provided from the National Institute for Infectious Diseases, Japan.

### Antibodies

Rabbit polyclonal and mouse monoclonal antibodies against a sequence of rat caveolin-3, TEEHTDLEARIKDIHCKEIDL (aa. 3–24), and against an 11.1-kDa protein fragment corresponding to the cytoplasmic domain (aa. 1–79) of human caveolin-1 were purchased from Transduction Laboratories (Lexington, KY, USA). A mouse monoclonal anti-dystrophin antibody (NCL-DYS2) specific

for C-terminus was purchased from Novocastra Laboratories (Newcastle, UK).

### Immunoblot analysis

Immunoblot analysis was performed as described previously (Hagiwara *et al.*, 1995). Briefly, cryostat sections of the muscle tissue were suspended with 10 volumes of 70 mM Tris-HCl (pH 6.7), 10% SDS, 10 mM ethylene-diamine tetraacetic acid and 5%  $\beta$ -mercaptoethanol. The suspensions were boiled for 5 min, and then centrifuged at 15,000 rpm for 15 min at  $4^{\circ}\text{C}$ . The supernatants were subjected to 14% polyacrylamide gel electrophoresis in the presence of 1% SDS according to the method of Laemmli (1970). After electrophoresis, the proteins were electroblotted onto an immobilon-P membrane (Millipore, Bedford, MA, USA) according to the method of Kyhse-Anderson (1984). The membrane was incubated in a blocking solution and then allowed to react with the primary antibody at  $37^{\circ}\text{C}$  for 1 h. Horse radish peroxidase-labeled IgG, a secondary antibody, was applied to the membrane at room temperature for 30 min. Signals were detected using an ECL detection kit (Amersham Pharmacia Biotech, Little Chalfont, UK).

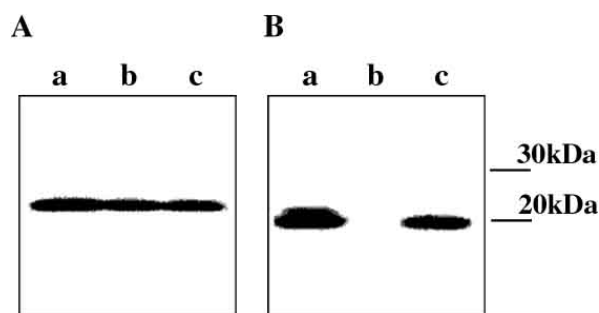
### Immunohistochemistry

Cryosections of 6  $\mu\text{m}$  thickness were analyzed by immunofluorescence staining using specific antibodies as described previously (Hagiwara *et al.*, 1998). Briefly, the sections were dried and pretreated with chilled acetone for 10 min. After incubation in a blocking solution containing 0.1% casein/0.1% gelatin in phosphate-buffered saline (PBS, pH 7.4) for 1 h at  $4^{\circ}\text{C}$ , the sections were incubated overnight with the primary antibodies at  $4^{\circ}\text{C}$ . The bound antibodies were detected with fluorescein isothiocyanate (FITC)-conjugated goat F(ab') anti-rabbit IgG (G+L) (TAGO Inc., Burlingame, CA, USA) or tetra-methyl-rhodamine isothiocyanate (TRITC)-conjugated mouse IgG (H+L) (KPL, Inc., Gaithersburg, MD, USA) as the secondary antibodies. Confocal laser scanning microscopy was performed using an Axioplan 2 microscope on an LSM 510 system (Carl Zeiss, Oberkochen, Germany) and M system (Leica, Heidelberg, Germany).

## Results

Homogenates of limb skeletal, heart ventricular and uterine muscle from a monkey were examined by immunoblot analysis using antibodies against caveolins-1 and 3. As shown in Figure 1, caveolin-1 was detected in all of the tissues examined (Fig. 1A), whereas caveolin-3 was detected in skeletal and heart muscle but not in uterine muscle (Fig. 1B).

The skeletal muscle was examined immunohistochemically using anti-caveolin-1 and -3 antibodies. In the longitudinal and transverse sections of the limb skeletal muscle, the sarcolemma but not the T-tubules was stained by the caveolin-3 antibody (Figs. 2a and 2c). In some longitudinal sections cut tangentially at the sarcolemma, caveolin-3 was labeled as lattice regularly arranged in a checkerboard pattern (Fig. 2b). A close observation of the sarcolemma revealed



**Fig. 1.** Immunoblot analysis of caveolins-1 and -3 in various muscle tissues. Tissue homogenates were prepared from heart (a), uterus (b) and skeletal muscles (c) and subjected to immunoblot analysis using rabbit polyclonal anti-caveolin-1 (A) and mouse monoclonal anti-caveolin-3 (B) antibodies. Note that caveolin-1 is present in all tissue homogenates, whereas caveolin-3 is found in heart and skeletal muscle.

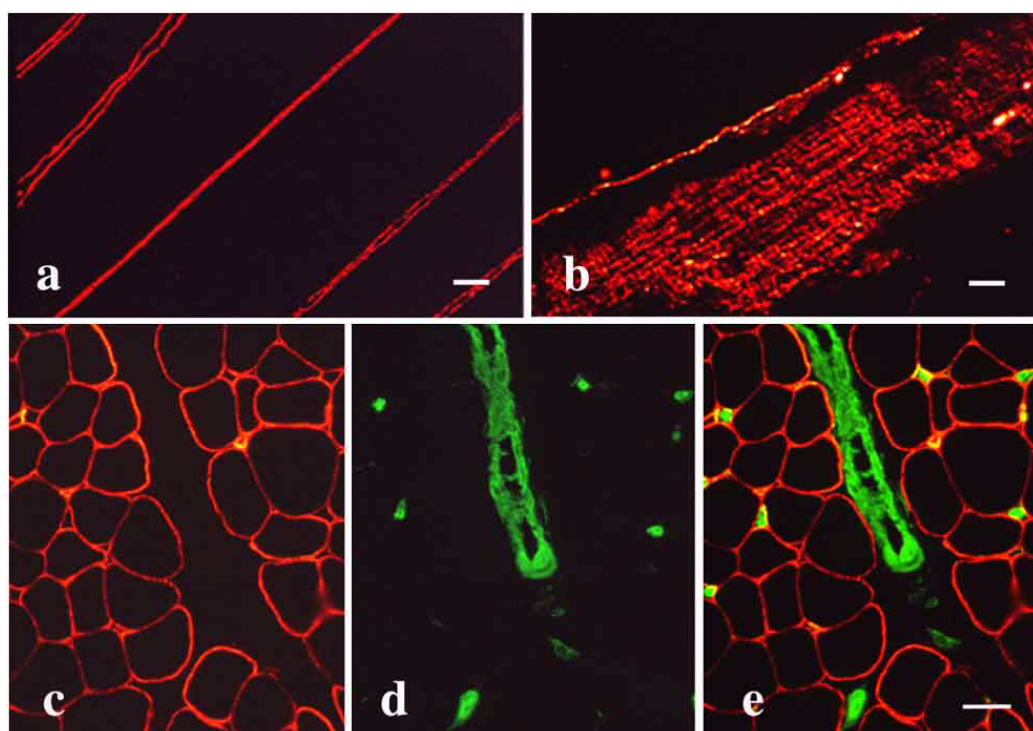
that caveolin-3 was also labeled as a series of numerous dots fused to each other to form a continuous line. On the other hand, caveolin-1 was not labeled in muscle cells but present in blood vessels (Fig. 2d). The merged image shows that the muscle cells and blood vessels are specifically reacted with caveolin-3 and -1 antibodies, respectively (Fig.

2e). Therefore, caveolin-1 detected in the skeletal muscle tissue homogenate (Fig. 1A) is derived from the blood vessels.

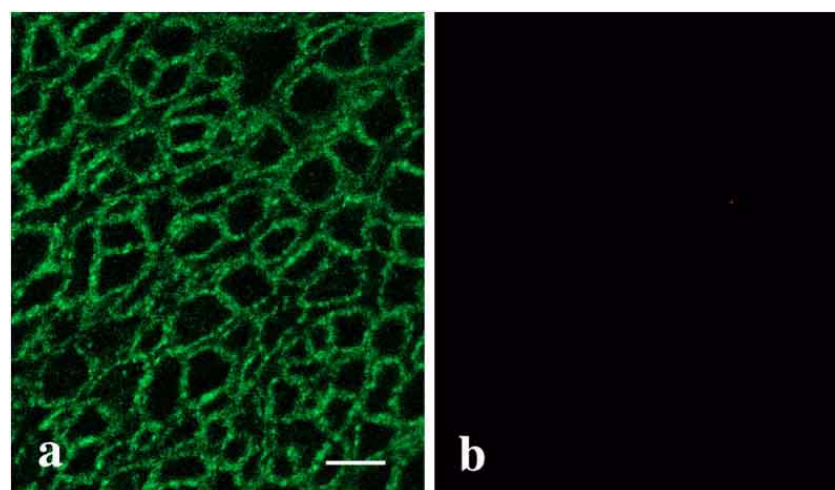
In the uterine muscle tissue stained with the antibodies against caveolins-1 and -3, the sarcolemma of smooth muscle cells was stained by the caveolin-1 antibody as dots (Fig. 3a) but not by the caveolin-3 antibody (Fig. 3b).

In the heart muscle tissue reacted with the antibodies against caveolins-1 and -3, the sarcolemma and some intracellular structures of the cardiac muscle cells were positive with both antibodies (Fig. 4). In merged images (Figs. 4Ac and 4Bc), it can be seen that caveolins-1 (Fig. 4; green) and -3 (Fig. 4; red) were labeled in cardiac muscle cells at the same loci. T-tubules of skeletal muscle cells were not stained (Fig. 2) whereas those in the cardiac muscle cells did react (Fig. 4A; arrows and Fig. 6). Longitudinal sections of the heart examined in the same manner revealed that the intercalated discs of cardiac muscle cells were stained with both antibodies (Fig. 4B; arrows). These results are summarized in Table. I.

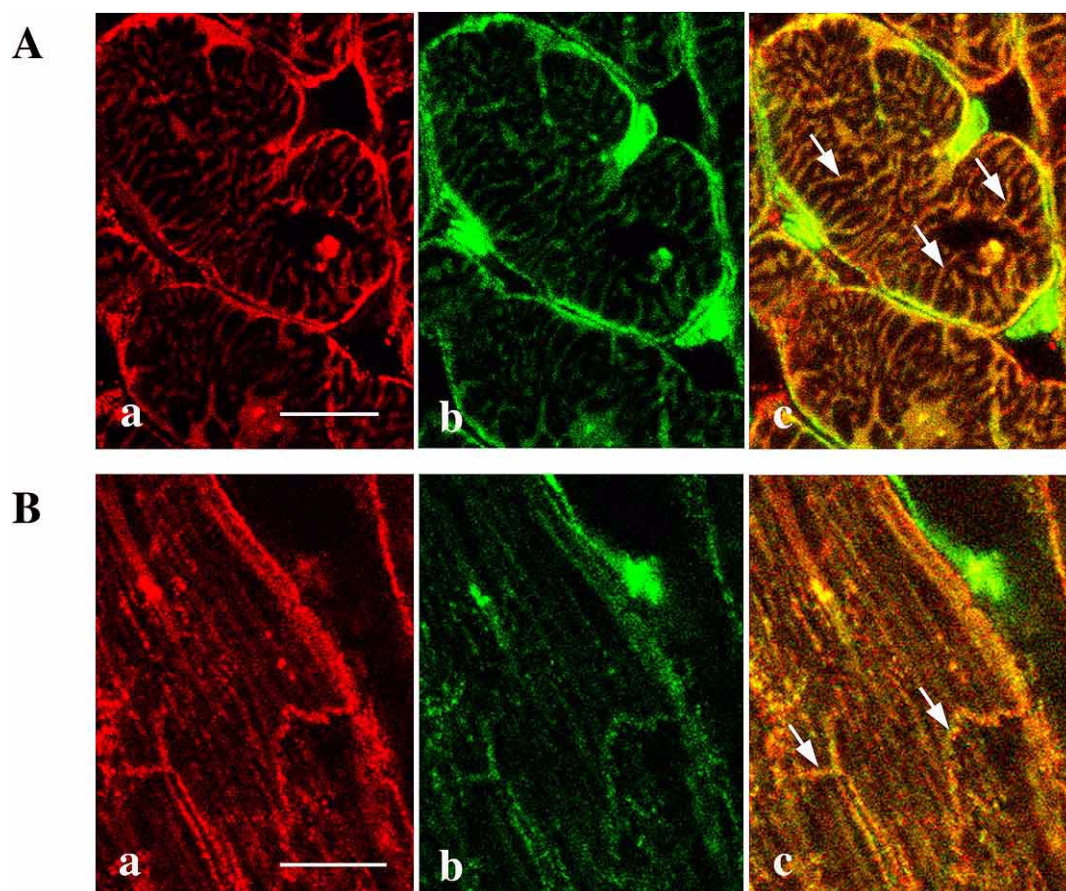
To analyze the colocalization of caveolin-3 and dystrophin, double immunofluorescence staining was performed. In the longitudinal section of the limb skeletal muscle, caveolin-3 and dystrophin were labeled on the sarcolemma



**Fig. 2.** Confocal laser microscopy of skeletal muscle cells. Longitudinal (a and b) and transverse (c, d and e) sections of frozen monkey skeletal muscle were examined by double-immunofluorescence staining of caveolin-1 (d) and caveolin-3 (a, b and c) using the same antibodies as those of Figure 1. Note that caveolin-3 labeling is observed on the sarcolemma of the muscle cell, with negligible internal staining (a and c). In a tangential section of a muscle cell, caveolin-3 is labeled as dots (b). Caveolin-1 is not detectable in muscle cells whereas it is strongly labeled in capillaries and arteries (d). (e) is a merged image of (c) and (d). Bars (a) 10  $\mu$ m; (b) 10  $\mu$ m; (e) 50  $\mu$ m.



**Fig. 3.** Confocal laser microscopy of smooth muscle cells. A transverse section of frozen monkey uterine muscle was examined by double-immunofluorescence staining of caveolin-1 (a) and caveolin-3 (b) using the same antibodies as those of Figure 1. Note that caveolin-1 labeling is observed on the smooth muscle plasma membrane, whereas caveolin-3 is not stained. Bar 5  $\mu$ m.



**Fig. 4.** Confocal laser microscopy of cardiac muscle cells. Transverse (A) and longitudinal (B) sections of frozen monkey cardiac muscle were examined by double-immunofluorescence staining of caveolin-3 (a) and caveolin-1 (b) using the same antibodies as those of Figure 1. Note that the merged image (Ac and Bc) shows colocalization (yellow) of caveolins-1 and 3 on the sarcolemma, T-tubules (Ac: arrows) and intercalated discs (Bc: arrows). Bars 10  $\mu$ m.



**Table I.** EXPRESSION OF CAVEOLIN-1 AND CAVEOLIN-3

	uterine smooth muscle	cardiac muscle		skeletal muscle	
	sarcolemma	sarcolemma	T-tubules	sarcolemma	T-tubules
caveolin-1	+	+	+	—	—
caveolin-3	—	+	+	+	—

(Figs. 5a and 5b). The merged image shows that both proteins are colocalized (Fig. 5c).

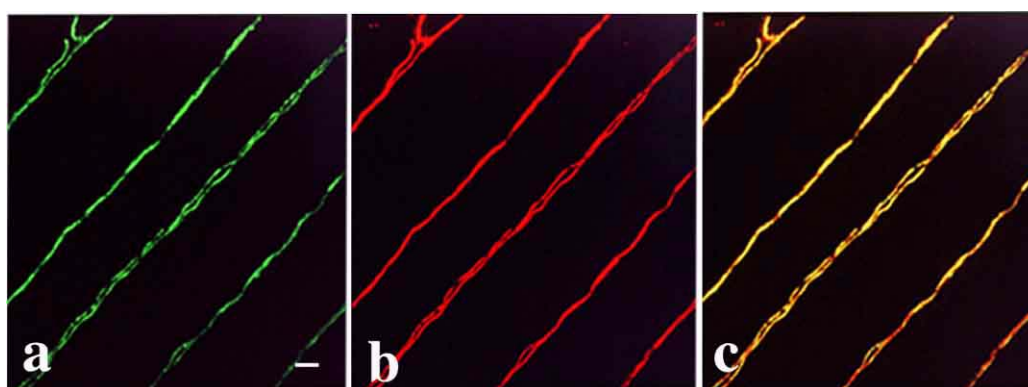
Colocalization of caveolin-3 and dystrophin was examined in the transverse (Fig. 6A) and longitudinal (Figs. 6B and 6C) sections of the ventricular muscle tissue. Both proteins were labeled on the sarcolemma as well as T-tubules (Fig. 6A; arrows) and intercalated discs (Fig. 6C; arrows) in the cardiac muscle cells. The localization of these proteins coincided almost completely (Figs. 6Ac, 6Bc and 6Cc). In the case of uterine smooth muscle cells, caveolin-1 and dystrophin were labeled on the sarcolemma as dots (Figs. 7a and 7b). The merged image shows the colocalization of caveolin-1 and dystrophin (Fig. 7c).

## Discussion

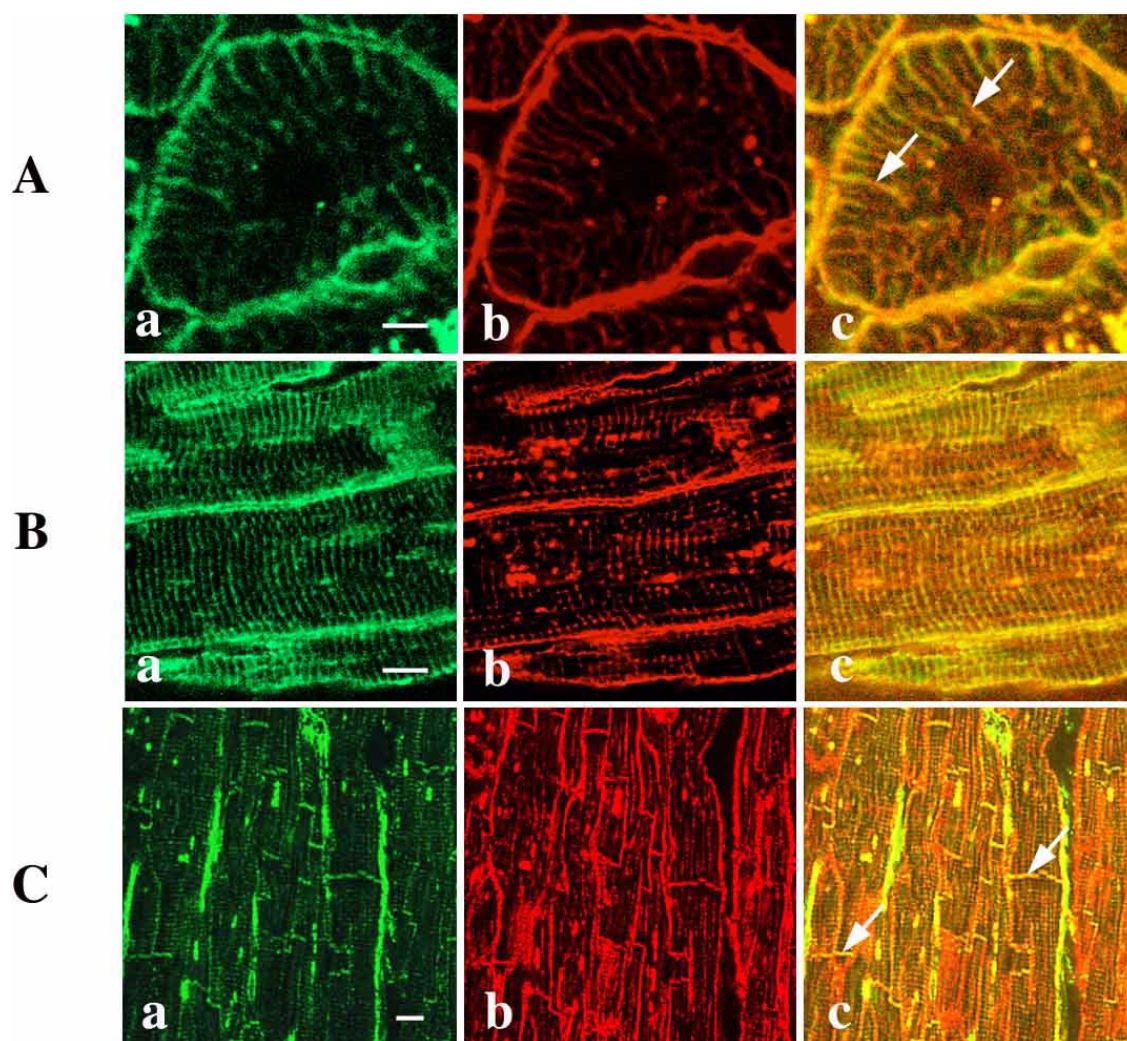
In our immunoblot analysis of the tissue homogenates from skeletal, heart ventricular and uterine muscle, caveolin-1 was clearly detected in all tissue homogenates, and caveolin-3 was detected in skeletal and heart ventricular muscle homogenates (Fig. 1). However, in our immunohistochemical analysis using the same antibodies, skeletal muscle cells were stained only with the anti-caveolin-3 antibody. Small arteries, veins and capillaries in the skeletal muscle tissue were stained with the anti-caveolin-1 antibody but not with the anti-caveolin-3 antibody (Figs. 2c and 2d). Arterial walls are composed of connective tissue and smooth muscle cells and are lined by a single layer of endothelial cells. In capillaries, the walls are composed of endothelial cells and a

basal lamina but not smooth muscle cells. Therefore, caveolin-1 detected in the skeletal muscle tissue homogenate is derived from endothelial cells of blood vessels, and in part, from smooth muscle cells of the small arteries. In regard to the localization of caveolin in smooth muscle cells, North *et al.* (1993) reported its presence on the smooth muscle plasma membrane in the guinea pig taenia coli. Their study was performed before caveolin-3 was identified and no distinction was made between caveolins-1 and -3. Recently, Li *et al.* (2001) found that caveolin-1 but not caveolin-3 was detected in mouse uterus, and both caveolins-1 and -3 were detected in mouse small intestine by immunoblot analysis. We determined by immunoblot and immunohistochemical analyses that smooth muscle cells of the monkey uterus express only caveolin-1 (Fig. 3).

The monkey cardiac muscle cells expressed both caveolins-1 and -3. The plasma membrane, including the intercalated discs and T-tubules, was stained with both anti-caveolins-1 and -3 antibodies (Fig. 4). In the cardiac muscle of adult mice, Parton *et al.* (1997) reported that caveolin-3 was restricted to the caveolae of cardiac muscle cells, whereas caveolin-1 was only detected in capillary endothelia. We further examined an autopsied human cardiac muscle and found the same results as those obtained using the monkey cardiac muscle (data not shown). Therefore, we conclude that, at least in primates, cardiac muscle cells express both caveolins-1 and -3 on the plasma membrane, including the intercalated disc and T-tubule. In the caveolin-3 knockout mice, the skeletal muscle fibers showed dystrophic change,



**Fig. 5.** Confocal laser microscopy of skeletal muscle cells. A longitudinal section of frozen monkey skeletal muscle was examined by double-immunofluorescence staining with rabbit polyclonal anti-caveolin-3 (a) and mouse monoclonal anti-dystrophin (b) antibodies. Note that the merged image (c) shows colocalization of caveolin-3 and dystrophin on the sarcolemma. Bar 10  $\mu$ m.



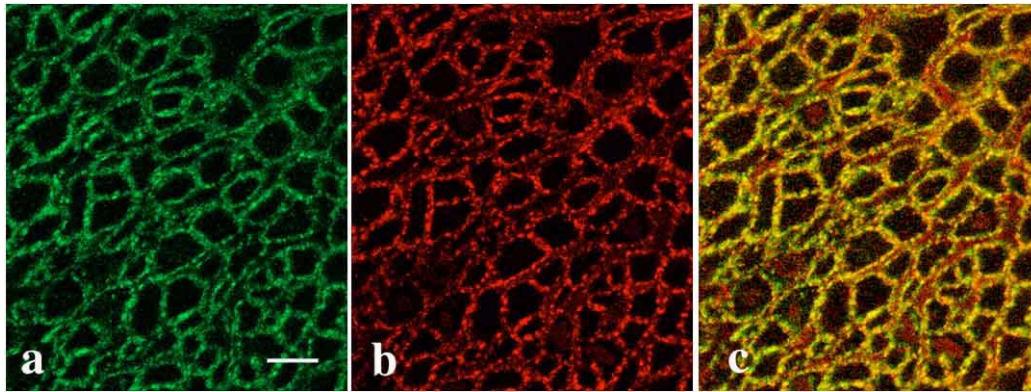
**Fig. 6.** Confocal laser microscopy of cardiac muscle cells. Transverse (A) and longitudinal (B and C) sections of frozen monkey cardiac muscle were examined by double-immunofluorescence staining of caveolin-3 (a) and dystrophin (b) using the same antibodies as those of Figure 5. Note that the merged image (c) shows colocalization of caveolin-3 and dystrophin on the sarcolemma, T-tubules (Ac: arrows) and intercalated discs (Cc: arrows). Bars (Aa) 5  $\mu$ m; (Ba and Ca) 10  $\mu$ m.

albeit mild. However, cardiac muscle cells were not involved, suggesting that caveolin-1 is enough to prevent the dystrophic changes (Hagiwara *et al.*, 2000; Galbiati *et al.*, 2001). In a recent report of caveolin-1 knockout mice, no dystrophic change was described in either cardiac or skeletal muscles (Drab *et al.*, 2001; Razani *et al.*, 2001). In support of our findings, caveolin-1/3 double-knockout mice develop severe cardiomyopathy (Park *et al.*, 2002).

In the rat skeletal muscle cells, Song *et al.* (1996) reported that caveolin-3 and dystrophin are colocalized on the sarcolemma. We immunohistochemically confirmed their results in monkey skeletal muscle cells (Fig. 5) and further observed their colocalization in cardiac muscle cells (Fig. 6). In addition, we found the colocalization of caveolin-3 and dystrophin in T-tubules of cardiac muscle cells. In

smooth muscle cells, colocalization of caveolin and dystrophin was observed by immunohistochemical staining in the guinea pig taenia coli (North *et al.*, 1993) and rat colon (Tanaka *et al.*, 2001). We also showed that dystrophin is colocalized with caveolin-1 in the smooth muscle cells of monkey uterus. Recently, Sotgia *et al.* (2000) reported binding of caveolin-3 to  $\beta$ -dystroglycan. They also reported that transgenic overexpression of caveolin-3 caused down-regulation of dystrophin and  $\beta$ -dystroglycan, leading to the muscular dystrophic phenotype (Galbiati *et al.*, 2000). On the other hand, in caveolin-3 null mice, which also showed mild dystrophic phenotype, dystrophin and  $\beta$ -dystroglycan were found to be, immunohistochemically, at normal levels on the skeletal muscle plasma membrane (Hagiwara *et al.*, 2000; Galbiati *et al.*, 2001). Muscle dystrophic phenotypes





**Fig. 7.** Confocal laser microscopy of smooth muscle cells. A transverse section of frozen monkey uterine smooth muscle was examined by double-immunofluorescence staining with rabbit polyclonal anti-caveolin-1 (a) and mouse monoclonal anti-dystrophin (b) antibodies. Note that the merged image (c) shows colocalization of caveolin-1 and dystrophin on the sarcolemma. Bar 5  $\mu$ m.

resulting from both overexpression and deficiency of caveolin-3 may be caused by different molecular mechanisms. The functional relation of caveolin-3 to dystrophin and its binding complex remains to be clarified.

It is noteworthy that caveolin-3 is present on T-tubules of adult cardiac muscle cells but not on those of adult skeletal muscle cells. Klietsch *et al.* (1993) reported that dystrophin and its binding protein complex are localized to T-tubules in cardiac but not in skeletal muscles. We also confirmed the localization of dystrophin and found that caveolin-3 is present on the T-tubules of adult cardiac muscles. The functional significance of the presence of dystrophin and its binding proteins in the T-tubular membrane of cardiac muscle is as yet unknown. However, several studies have suggested that dystrophin may link to membrane components, such as channels or receptors, in addition to stabilizing the muscle plasma membrane (Blake *et al.*, 2002; Sadeghi *et al.*, 2002). Further studies are needed to elucidate the functional relationship between caveolin-3 and dystrophin in T-tubules.

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