Localization of 17-kDa Myosin Light Chain Isoforms in Cultured Aortic Smooth Muscle Cells

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Smooth muscle myosin II contains two 17-kDa essential light chain isoforms (LC17gi and LC17nm) of which the relative contents differ among myosins. To understand the roles of LC17 isoforms in the functions of myosin, we performed an immunofluorescence microscopic examination of their localization in primary cultured cells isolated from rat aortic smooth muscle. To identify the isoforms, rabbit polyclonal antibodies were prepared against C-terminal nonapeptides corresponding to either LC17gi or LC17nm from porcine aortic smooth muscle myosin. These isoforms differ in only 5 amino acid residues within the C-terminal 9 residues. These antibodies specifically recognize each LC17 isoform on urea-PAGE of total rat aortic cell lysates. Immediately after plating, the smooth muscle cells stained heterogenerously with each antibody, indicating differing contents of LC17 isoforms among cells. On double staining 1-2 d cultures with both antibodies, LC17nm was detected diffusely throughout the cytoplasm, whereas LC17gi was concentrated in specific regions such as the cell periphery and the base of cytoplasmic processes. These results support the suggestion that myosin containing LC17gi is essential for force-generation by aortic smooth muscle and that myosin containing LC17nm may play an important role in maintaining smooth muscle tension.

Key words: aortic smooth muscle cells, cultured smooth muscle cell, 17-kDa light chain localization, myosin 17-kDa light chain, myosin light chain isoforms.

Smooth muscle cells exhibit significant differences in their electrophysiological and mechanical properties (1). Recent interest has been directed toward determining whether these differences may be due to contractile protein isoforms (2-5), in addition to the membrane properties of excitation-contraction coupling (1) and contractile regulation (6).

Aortic smooth muscle myosin is a hexameric protein consisting of two each of three subunits, a heavy chain of about 200-kDa, a 20-kDa regulatory light chain (LC20), and a17-kDa essential light chain (LC17). The heavy chain functions mainly as a motor protein, while LC20 acts as a switch of the motor activity. The function of LC17 is, however, less clear.

Mammalian smooth muscle contains myosin isoforms even in adult tissues. Two kinds of myosin heavy chain with molecular masses of 200 and 204 kDa have been detected in several species (7-9). There are also two kinds of LC17 isoform in smooth muscle myosins (2, 10, 11) and their molar ratio depends on the kind of smooth muscle tissue. The ratio of these two kinds of LC17 isoform in porcine aorta myosin is 55:45 (11, 12). These isoforms differ only in 5 amino acid residues within the 9 C-terminal residues of the total of 150 amino acids (13). The sequences of the 9

amino acids are AFVRHILSG in LC17nm and ELVRMVL-NG in LC17gi (13), identical to those of the bovine isoforms LC17b and LC17a, respectively (14). LC17gi thus has an extra negative charge at neutral pH, and the two LC17 isoforms can therefore be separated by urea-PAGE, isoelectric focusing, or ion-exchange chromatography. Three myosin species with different combinations of the two LC17 isoforms per myosin molecule are present in porcine aorta, and the ratio of their occurrence is as follows; LC17nm-LC17nm:LC17nm-LC17gi:LC17gi-LC17gi, is 22:46:32 (12). This ratio is consistent with the random binding of two LC17 isoforms with the myosin heavy chain, suggesting no regulation of the association of expressed LC17 isoforms with the myosin heavy chain. The two LC17 isoforms are expressed from a single gene by an alternative RNA splicing mechanism (15, 16) that is regulated in a tissue-specific manner.

The peptide segments in LC17 with 5 amino acid substitutions between the two isoforms may form a helix corresponding to the H-helix, the final helix in 4 homologous EF-hand structures in LC17 (17), and the polar surface of this helix may face the outside of the myosin molecule (12), an hypothesis based on the crystal structure of chicken subfragment-1 (S1) (18). It is expected, therefore, that the two LC17 isoforms which provide different charges on the myosin surface will exhibit different functions. The ATPase parameters of smooth muscle myosin (4) or S1 (2), and the maximum shortening velocity of chemically skin-

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ned smooth muscle fibers (3) depend on the ratio of the two LC17 isoforms.

A recent kinetic study of permeabilized smooth muscle using flash photolysis of caged ATP indicated that the affinity of MgADP is higher for tonic smooth muscles with a high content of LC17nm than for phasic smooth muscle (5). It has been reported that the content of the LC17 isoform corresponding to LC17gi increases at the end of pregnancy in human and monkey uterine smooth muscles (10). These reports all suggested that the two LC17 isoforms in smooth muscle myosins play different functional roles.

In this study, we used immunofluorescence microscopy using specific antibodies for the two LC17 isoforms to demonstrate that LC17gi concentrates in specific regions in aortic smooth muscle cells in culture, distinct from the diffuse localization of LC17nm throughout the cytoplasm. Our results suggest that LC17gi may contribute to the generation of force necessary for cell shape changes in addition to actin-activated ATPase activity, and also to shortening velocity in aortic smooth muscle.

MATERIALS AND METHODS

Antibody Preparation— The LC17nm C-terminal nonapeptide (AFVRHILSG) was generated by solid-phase synthesis as described previously (19). The corresponding LC17gi nonapeptide (ELVRMVLNG) was purchased from Hokkaido System Science. Rabbit polyclonal antibodies were prepared by standard procedures. Aliquots of 5 mg of each synthetic peptide were added to 1 ml of 5 mg/ml keyhole limpet hemocyanin (KLH) in 0.1 M borate buffer (pH 10), and then 0.5 ml of 0.3% glutaraldehyde was then added. After stirring for 2 h at 25°C, 0.125 ml of 1 M glycine was added to block the unreacted glutaraldehyde, and the mixtures were stirred for a further 30 min. The peptide-KLH conjugate was dialyzed against TBS (150 mM NaCl and 20 mM Tris-HCl, pH 7.5).

Rabbits were immunized with an emulsion of 1 mg of peptide-KLH conjugate and Freund's complete adjuvant, and booster immunizations were administered at 2-week intervals with one-third of the amount of antigen. Blood was collected 7 to 10 days after each booster injection. Antisera were obtained by centrifugation after clot forming at 4°C. Antibodies were affinity purified according to the method of Talian *et al.* (20). LC17 isoforms were separated by urea-PAGE (11) and electrotransferred onto nitrocellulose membranes. After blocking with a blocking buffer (150 mM NaCl, 20 mM Tris-HCl, 0.05% Tween20, and 2% gelatin, pH 7.5), the region blotted with LC17nm or LC17gi was cut out from the membrane, and incubated for 2 to 16 h at room temperature with antiserum diluted 20-fold. The bound antibodies were then eluted with a small volume of 0.2 M Gly-HCl (pH 2.5) and neutralized with 1 M Tris. The eluted antibodies were passed through an LC17 peptide-Sepharose column to remove antibodies that cross-reacted with both isoforms. The samples were concentrated and further purification by Protein A-Sepharose column chromatography. FITC was conjugated with antibody as follows. Nitrocellulose strips with the bound complex of LC17nm and antibody were washed with TBS-Tween and placed in 5 ml carbonate-bicarbonate buffer. Solid FITC was added and the mixtures were stirred for 2 h at 25°C in

the dark. The strips were washed thoroughly with TBS-Tween (TBS containing 0.05% Tween20) and the FITC-conjugated antibodies were eluted. Further purification was performed as described above.

Preparation of Aorta Extracts and Immunoblotting— Aortas were removed from anesthetized rats and washed in cold PBS. Minced aortas (0.1 g) were homogenized with a Polytron (Brinkmann, Westbury, NY) in 500 µl of 2% SDS, 1% 2-mercaptoethanol, and boiled for 10 min. After re-homogenization, the homogenates were microcentrifuged and the supernatants were mixed with equal volumes of SDS-PAGE sample buffer. The samples were separated in SDS-10% polyacrylamide gels. When the myofibrillar extract of a rat aorta was to be subjected to urea-PAGE, the minced aorta was homogenized in three volumes of buffer A (20 mM MOPS-NaOH, 5 mM EDTA, 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, and 2-mercaptoethanol, pH 7.0) using a glass homogenizer and centrifuged. After washing with three volumes of buffer A, the pellet was suspended in three volumes of buffer A containing 0.6 M NaCl. After centrifugation, the supernatant was kept as the myofibrillar extract. Solid urea was added to the extract and PAGE was carried out in the presence of 8 M urea by the method of Perrie and Perry (21).

Immunoblotting was carried out by the semidry electrotransfer over 90 min at 15 V of proteins in the polyacrylamide gels onto nitrocellulose membranes. After blocking with blocking buffer, the membranes were incubated for 1 h at room temperature with antibody diluted 10-fold or antiserum diluted 1,000-fold with blocking buffer. After rinsing with TBS-Tween, the membranes were incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma Chemical, St. Louis, MO, diluted 1:2,000) and visualized with an alkaline phosphatase conjugate substrate kit (Bio-Rad).

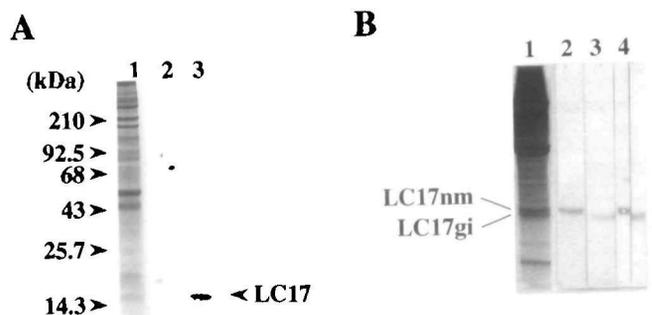*Aortic Smooth Muscle Tissues and Cells—* For the prepa-

Fig. 1. **Immunoblots of myosin LC17 isoforms in extracts of rat aorta.** Panel A, SDS-PAGE patterns of rat aorta extracts. The extracts were electrophoresed in SDS-polyacrylamide gels (10%), and stained with Coomassie Brilliant Blue (lane 1), or transferred onto nitrocellulose membranes and probed with affinity-purified antibody to LC17nm (lane 2) or LC17gi (lane 3) from porcine aorta smooth muscle myosin (arrowhead). Panel B, urea-PAGE patterns of rat aorta extracts from which two LC17 isoforms and LC20 were separated. Lane 1, silver-stained gel. Lanes 2 and 3, probed with anti-LC17nm and anti-LC17gi, respectively. Lane 4 was cut in half; the left side was probed with anti-LC17nm and the right side with anti-LC17gi. Note that the two antibodies recognize only LC17nm and LC17gi.

ration of smooth muscle tissue, anesthetized male Wistar Kyoto rats were perfused with Zamboni's fixative. The aortas were removed and cryoprotected by sinking in 15–30% (w/v) sucrose in PBS and plunging into a slush of liquid nitrogen. Tissue was stored at $-70°C$ for the preparation of frozen sections. Smooth muscle cells were isolated from rat aorta by enzymatic dispersion as described by Chamley *et al.* (*22*). The resulting cells were seeded onto glass coverslips laid in culture dishes and cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS (GIBCO BRL, Gaithersburg, MD) at 37°C in a humidified 5% CO_2 atmosphere.

Immunofluorescence—Frozen aortas were sectioned on a cryostat (CM 3000, Leica, Heidelberg, Germany) at a thickness of 5 μm, and the frozen sections were collected on slides pretreated with gelatin. Cells in culture were fixed at various stages after seeding in 2–4% *p*-formaldehyde dissolved in phosphate buffered saline (PBS) for 10 min. After washing with PBS, they were treated with methanol at $-20°C$ for 7–8 min. Frozen sections and cultured cells on slides were rinsed in PBS and then incubated with a primary antibody against either LC17nm or LC17gi, prepared as described above, at 37°C for 60 min. After washing, the samples were incubated at 37°C for 60 min with FITC-labeled goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) or Texas Red-labeled goat anti-rabbit IgG (Amersham, Tokyo). For double-staining of LC17nm and LC17gi, anti-rabbit LC17nm antibody conjugated with FITC, anti-rabbit LC17gi antibody, and Texas Red-labeled goat anti-rabbit IgG were used. The coverslips were washed with PBS, mounted in glycerol with propyl gallate, and observed with an epifluorescence microscope (Olympus, BX50-FLA, Tokyo).

RESULTS

Preparation and Specificity of Antibodies—The specificity of antibodies raised against synthetic peptides was examined by immunoblotting after SDS-PAGE of the aortic media extract. Two cross-reactive bands appeared corresponding to LC17 isoforms of a nonspecific peptide and an approximately 70-kDa peptide. Therefore, we purified the antibodies further by the procedure of Talian *et al.* (*20*). Figure 1A shows the specificity of the purified antibodies examined against SDS-extract of rat aorta. Only a single band can be seen by immunostaining with either
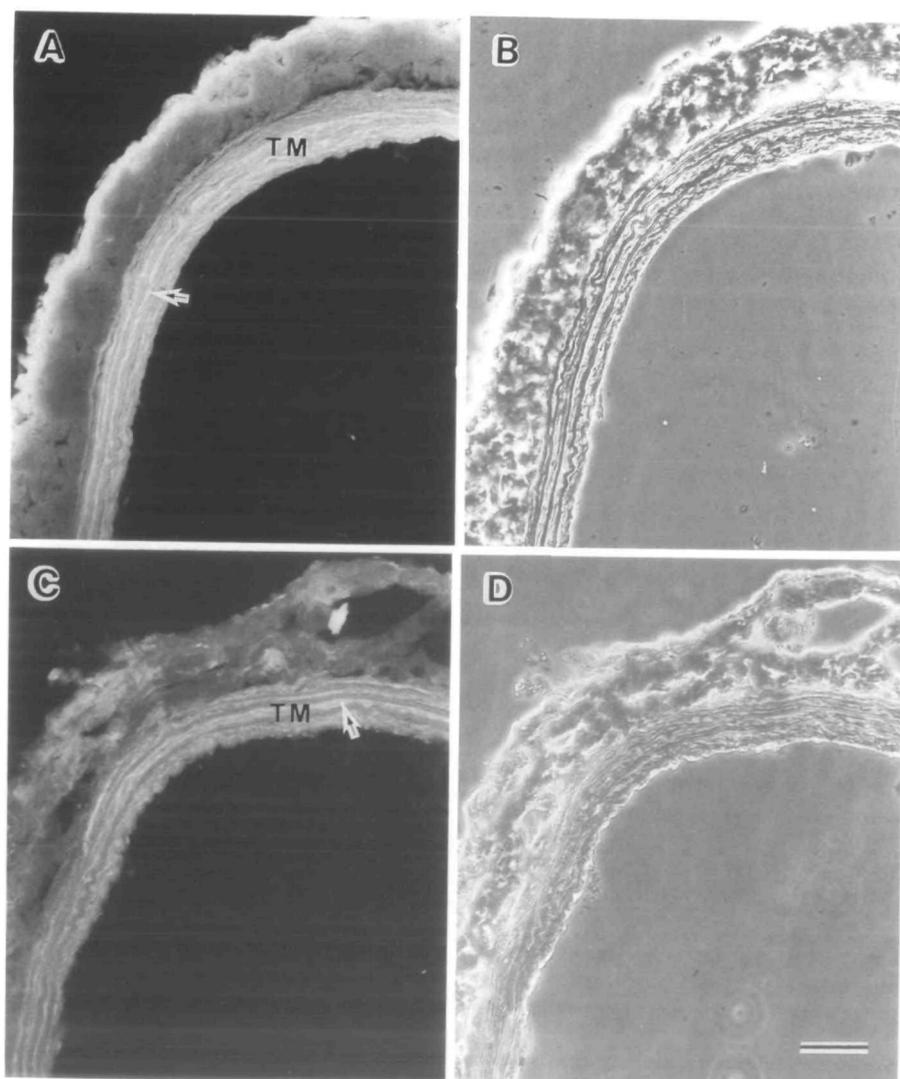
Fig. 2. **Immunofluorescence micrographs of myosin LC17 isoform staining in frozen sections of rat aorta.** A, anti-LC17nm staining; B, phase-contrast micrograph of A; C, anti-LC17gi staining; D, phase-contrast micrograph of C. The tunica media (TM) consists of many concentrically arranged laminae interconnected by elastic fibers (dark lines in phase-contrast micrograph) and smooth muscle cells (arrows); the latter appear strong on antibody staining (A and C). Bar, 50 μm.

anti-LC17nm or anti-LC17gi antibody (Fig. 1A, lane 2, or 3), although numerous protein bands are observed by Coomassie Brilliant Blue staining (Fig. 1A, lane 1). The mobility of this single band is identical to that of the LC17 isoform of purified aortic myosin, indicating that these antibodies are LC17 isoform-specific.

The specificity for the LC17 isoform was tested in 10% urea gels in which the two LC17 isoforms are separated into separate bands, LC17nm and LC17gi (Fig. 1B, lane 1). As shown in lanes 2 and 3 in Fig. 1B, anti-LC17nm and LC17gi recognize only their respective isoforms. There are no other detectable cross-reactive bands, indicating that these antibodies are isoform-specific.

Cell-Specific Distribution of LC17 Isoforms in Aortic Smooth Muscle Cells — The tunica media, the middle arterial layer, of rat aorta consists of elastic laminae interconnected by elastic fibers and smooth muscle. When frozen sections of the rat aorta were examined with anti-LC17nm and anti-LC17gi antibodies, the smooth muscle layers in the laminae were specifically stained by each antibody (Fig. 2). The enzyme-treated tunica media was dispersed into single cells and small clumps. The enzyme-dispersed smooth muscle cells immediately after plating were of various shapes, including ribbon-, spindle-,

ellipsoidal, and round-shapes, which are thought to depend for the most part on the activities of the trypsin and/or collagenase used for dispersal and partly due to mechanical stimuli during the isolation procedures (Fig. 3). The cytoplasm of the cells appears thick and phase dense. When cells cultured for a short time (~6 h) were treated with anti-LC17nm or anti-LC17gi, heterogeneous staining was observed. Only a few cells (about 10%) stained strongly with anti-LC17gi antibody, thus giving rise to a mosaic appearance (Fig. 3, A, B, and C), whereas a few cells (7%) were less intensely stained with anti-LC17nm (Fig. 3D). Staining with anti-LC17gi produces a similar mosaic pattern independent of how the cell samples were prepared (Fig. 3, A, B, and C), while staining of the same cell samples with anti-LC17nm produces similar patterns as shown in Fig. 3D, *i.e.* a weak, diffuse distribution of LC17nm fluorescence in many cells (data not shown). The cells became attached to the glass coverslip during the first 1 day of culture and then began to flatten within 1-2 days. After 2 days in culture, most of the cells showed reduced staining with both antibodies. After further culture (on 4 d), the cells were spindle- and polyhedral-shaped, and no staining was seen with either anti-LC17nm or anti-LC17gi (Fig. 3, E and F, data for anti-LC17nm not shown). This is consis-
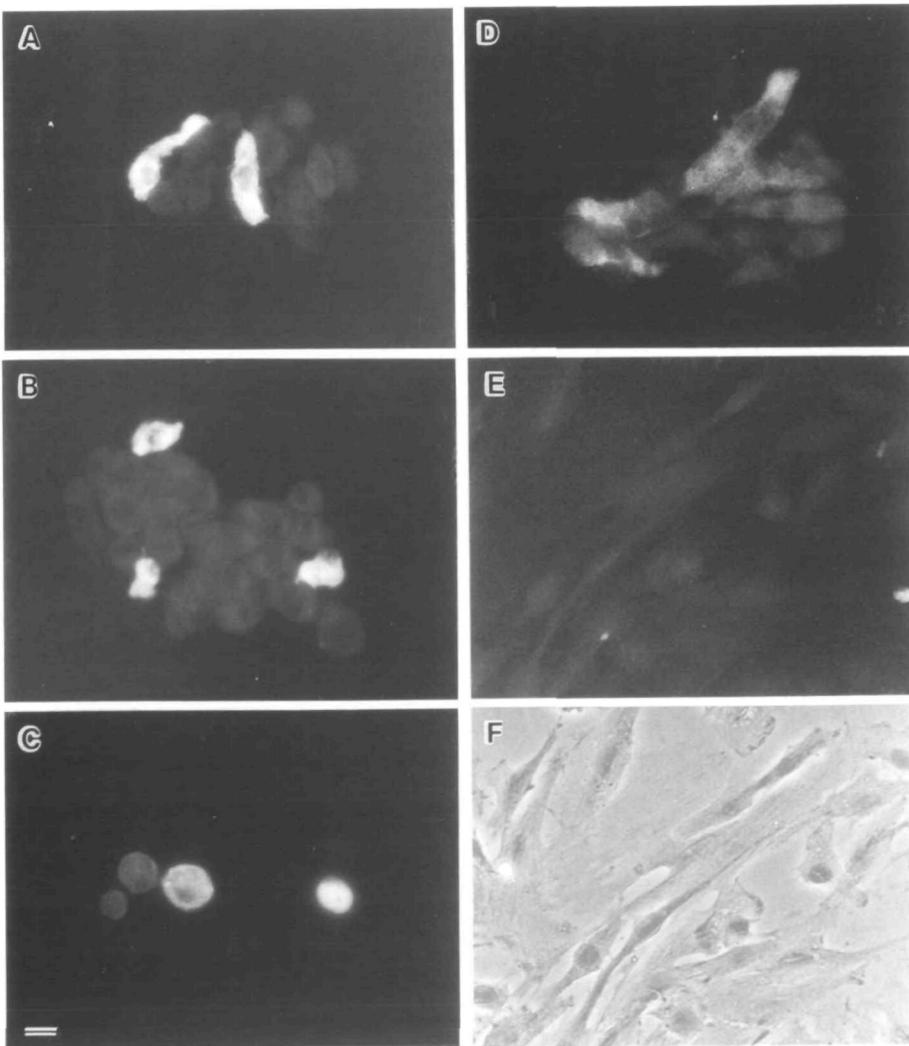

Fig. 3. **Immunofluorescence staining of cultured smooth muscle cells of rat aorta using anti-LC17nm or anti-LC17gi antibody.** Cells were enzymatically dissociated from rat aorta and stained with anti-LC17nm or anti-LC17gi antibody within 6 h after plating (A, B, C, and D), and after 4 days in culture (E and F). A, B, C, E, anti-LC17gi staining; D, anti-LC17nm staining; F, phase-contrast micrograph corresponding to E. The cell shapes were variable for each cell preparation (A, B, C, and D) but the staining patterns were similar.
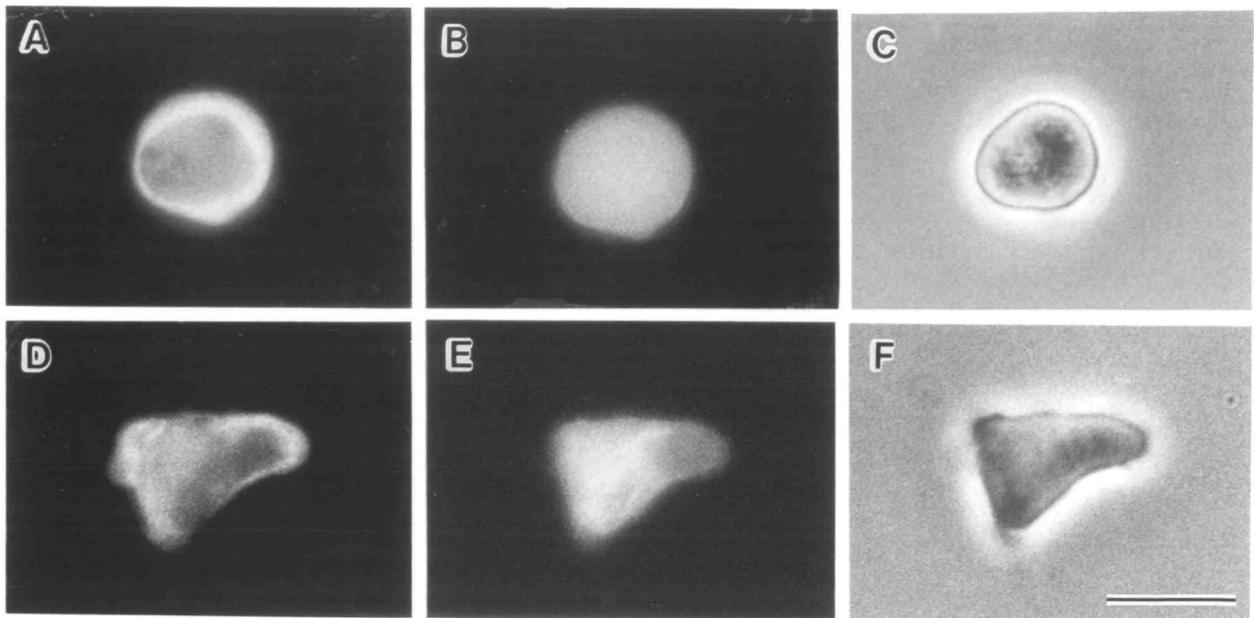
Fig. 4. **Differential localization of myosin LC17 isoforms in smooth muscle cells of rat aorta on day 1 in culture.** (A), (B), (D), and (E), immunofluorescence micrographs of cell stained for LC17gi (A, D), or LC17nm (B, E). (C), phase-contrast image corresponding to (A) and (B). (F), phase-contrast image corresponding to (D) and (E). Bar, 10 μm. The staining patterns for LC17gi and LC17nm are different.

tent with the results of a previous study on cultured smooth muscle cells stained with anti-smooth muscle myosin (23). The mouse fibroblast cell line A31 and bovine endothelial cell line FBHE were not stained by either antibody (data not shown).

Subcellular Localization of LC17 Isoforms with Changes in Cell Shape—After 18-48 h in culture, isolated aortic smooth muscle cells that had attached to the substratum spread, with some cells becoming temporarily round, and then ribbon- or spindle-shaped, the shapes characteristic of cultured aortic smooth muscle cells. Such morphological changes are associated with cell spreading in which myosin is thought to play a key role. To investigate the contributions of the two LC17 isoforms to cell spreading, we examined the intracellular distributions of both isoforms during the spread of aortic smooth muscle cells in culture. We used anti-LC17nm antibody directly conjugated to FITC and anti-rabbit LC17gi antibody stained secondly with Texas Red-labeled goat anti-rabbit IgG because the antibodies to both LC17nm and LC17gi are polyclonal. In cells immediately after plating, labeling was visible throughout the cytoplasm with both antibodies (Fig. 3, A-D). In cell cultured for 1 day, however, a marked difference was observed in the localization of the two LC17 isoforms by double fluorescence staining. The LC17gi isoform appeared to be concentrated specifically at the periphery of the cells (Fig. 4, A and D), in marked contrast to LC17nm which was distributed evenly throughout the cytoplasm (Fig. 4, B and E). When the cells extended thick processes, which appeared dense in phase-contrast microscopy, the regions stained strongly with anti-LC17gi (Fig. 5, A and C). This focal increase in the concentration of the LC17gi isoform occurs not only at bipolar sites but sometimes also at several sites in the peripheral region within the same cells (Fig. 5C). Similar increases were also seen at the base

of and within thin, long extensions, *i.e.* filopodia (Fig. 5, E and G), suggesting that filopodia are generated from these sites. Further extension of cell processes occurs toward the longitudinal axis. Figure 6 shows features of cells from the early stage spreading (Fig. 6A) to the end stage (Fig. 6H). The cells develop a polarized morphology with prominent cell extensions at opposite poles. The extensions taper at an early stage (Fig. 6C, arrow), then spread and became broader at the edges (Fig. 6, C, E, G, and H, arrowhead). Dual staining for both LC17 isoforms (Fig. 6, A and B) shows LC17gi to be enriched around the base of the tapered extensions but not the edges (Fig. 6A), while LC17nm is distributed diffusely throughout the cytoplasm (Fig. 6B). Similar staining patterns were also observed with anti-LC17gi antibody in cells with broader extensions. LC17gi appears enriched at the base of one side of the two extensions relative to the opposite side (Fig. 6, D and F), and is associated with the membrane at the edge of the extensions (Fig. 6F, small arrow). In spread cells, staining with anti-LC17gi produces a punctate staining pattern, with the label occasionally appearing in rows, possibly along tracks of actin (Fig. 6, D and F, small arrows). At the last stage of spreading, LC17gi is enriched on structures corresponding to stress fibers along the longitudinal axis of the cell (Fig. 6H). The punctate LC17gi staining partly reveals the periodic pattern of stress fibers, as observed with other myosins (24), suggesting the localization of myosin with LC17gi within microfilament bundles. LC17gi could not be detected at the edges of extensions (Fig. 6H, arrowhead).

In contrast, spread cells show weak and diffuse staining with anti-LC17nm antibody (data not shown).

DISCUSSION

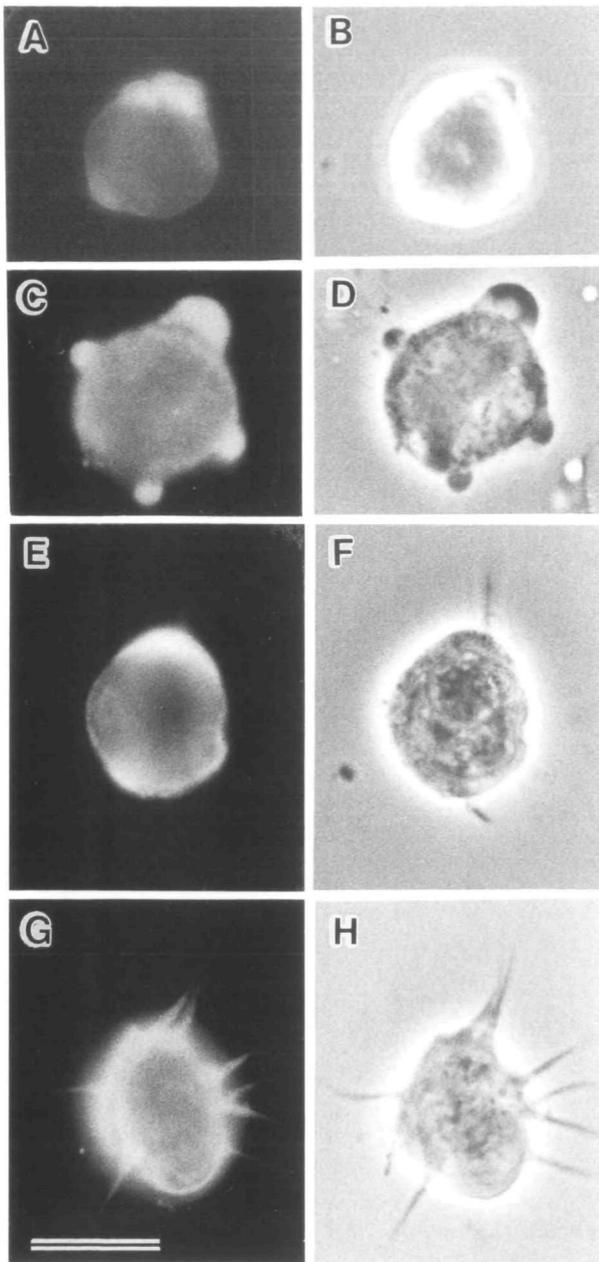Previously, in several lines of study on the physiological

Fig. 5. **Localization of myosin LC17gi in aortic smooth muscle cells with various types of processes on day 1 in culture.** (A), (C), (E), and (G), fluorescent staining. (B), (D), (F), and (H), phase-contrast images corresponding to (A), (C), (E), and (G). Bar, 10 μm.

aortic smooth muscle myosins are composed of two LC17 isoforms. These isoforms in porcine aortic smooth muscle differ in only 5 of the 9 C-terminal amino acids (13). In this study, we prepared antibodies against the C-teminal nonapeptides corresponding to either LC17gi or LC17nm from porcine aorta myosin.

The localization of myosin II in aortic smooth muscle cells in culture has been examined using fluorescence microscopy (22, 23). In this previous study, antimyosin II staining appeared evenly distributed within the cell. In the present study, staining of the cells immediately after plating with both anti-myosin II LC17 antibodies produced similar patterns (Fig. 3). However, the staining patterns are different in motile cells after 18–48 h in culture (Figs. 4 and 5). As shown in Fig. 4, LC17gi concentrates in the peripheral region of the round cells and is less dense at the center of the cells, while the fluorescence indicating LC17nm is diffuse throughout the cell (Fig. 4, B and E). LC17gi translocats in association with changes in cell shape. At early stages of shape change, LC17gi is concentrated near the base of processes (Fig. 5A) or often within thicker processes (Fig. 5C) and filopodia (Fig. 5G), but not at their tips. With further extension of the processes, LC17gi becomes highly concentrated near the nucleus, but is not found at the leading edge (Fig. 6, A, D, F, and H). In more stable regions of the cytoplasm, LC17gi is distributed in both a punctate and continuous manner along actin-cables and stress fibers (Fig. 6, D, F, and H). Although we do not know the mechanism responsible for process extension, these results suggest that the contractile force of myosin II containing LC17gi would be greatest at the LC17gi concentrated regions and could be responsible for the extension of long processes. In migrating *Dictyostelium* (25) and loco-moting fibroblasts (26), myosin II is absent within the initial protrusions where myosin I is concentrated, but is detected in established protrusions in fibroblasts. However, the protrusion of the forward-moving cell edge observed in wound healing, morphogenesis, and postmitotic cell spreading is probably driven by myosin II (27–29). Therefore, it seems reasonable that the myosin II containing LC17gi in aortic smooth muscle cells accumulates in regions necessary for rapid force-generation.

In contrast, the location of LC17nm shows no changes related to cell shape that require myosin-myosin interaction. Although we did not examine differences in filament formation between reconstituted myosins containing LC17nm and LC17gi, the former may have a higher affinity for actin filaments than myosin filaments and thus may colocalize with actin filaments throughout the cytoplasm, which were, are detectable with Rh-phalloidin (data not shown). This is supported by the results of our previous study (4).

In exchange reaction of LC17 isoforms in aortic smooth muscle myosin, the apparent dissociation constant of myosin containing 81% LC17nm for F-actin was 20-fold less than that of myosin containing 23% LC17nm, and the former has a lower actin-activated ATPase activity (4). The present study, therefore, provides important confirmation of our suggestion that LC17nm appears to render aortic myosin suitable for maintaining muscle tension with low energy expenditure.

The relative content of LC17nm in smooth muscles has been reported to be associated with physiological functions

functions of LC17 isoforms of smooth muscle myosin II, we have shown that there are important differences in acto-myosin ATPase activity between the two LC17 isoforms (4). In this study, we focused on the localizations of LC17gi and LC17nm in cultured smooth muscle cell from rat aorta utilizing specific antibodies. Our results indicate that the two LC17 isoforms are also localized differently within cells. The two LC17 isoforms coexist in aortic smooth muscle cells, but LC17gi concentrates at variable regions in association with cell shape, while LC17nm is distributed diffusely throughout the cytoplasm (Figs. 4 and 5).

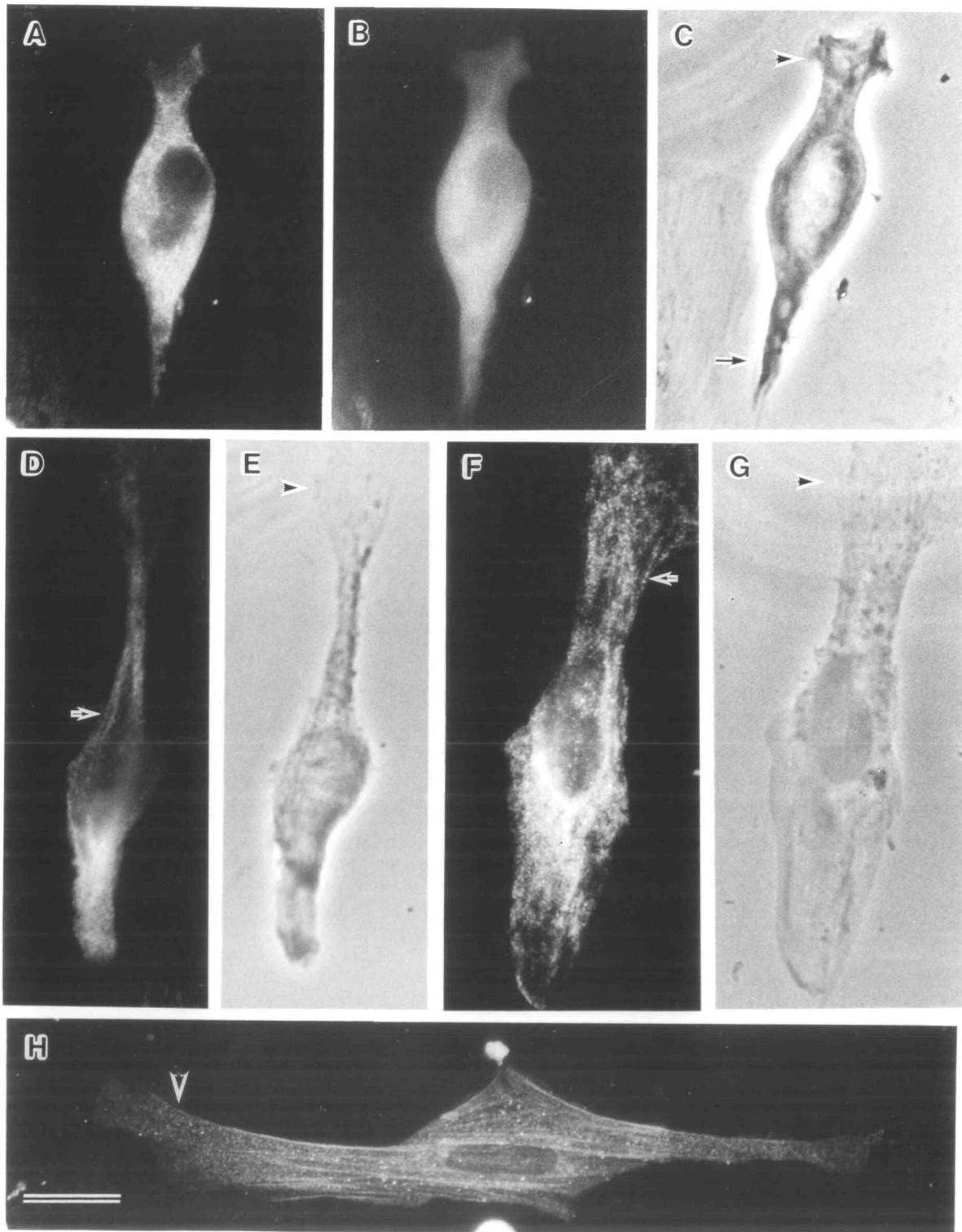We reported previously that porcine, rabbit, and rat

Fig. 6. **Localization of myosin LC17 isoforms in spreading aortic smooth muscle cells on day 2 in culture.** (A) and (B), fluorescence micrographs of cells stained for LC17gi (A) and for LC17nm (B) at an early stage in spreading. (C), phase-contrast micrograph corresponding to (A) and (B). (D) and (F), fluorescence micrographs of a cell developing extensions and stained for LC17gi. Punctate LC17gi staining appears in rows (small arrows). (E) and (G), phase-contrast images corresponding to (D) and (F). (H), fluorescence micrographs of a cell at the end of spreading stained for LC17gi. Myosin LC17gi is visible on stress fibers (arrow). No LC 17gi can be detected at the edges of the extensions (arrowheads). Bar, 10 μm.

such as maximum shortening velocity (3), rate of force development (30), and motility (31). Chicken gizzard smooth muscle contains only LC17gi (32), which endows myosin with a higher ATPase activity and a lower affinity

for F-actin. This may explain why gizzard smooth muscle contracts and relaxes rapidly. On the other hand, both LC17 isoforms are present in aortic smooth muscle, which contains myosin with a lower ATPase activity and slower shortening velocity. These molecular properties explain the observation that aortic smooth muscle does not contract rapidly but rather works to resist blood pressure with persistent tension development.

Both antibodies specifically stain the tunica media in rat aorta (Fig. 2), but the smooth muscle cells isolated from the media are heterogeneously stained: neither isoform is not expressed evenly in all cells but rather predominate in some cells, while staining is very weak, if present at all, in other cells. Thus, the contents of LC17 isoforms differ among cells (Fig. 3). The relative contents of LC17 isoforms vary among species even in the same smooth muscle type. Porcine aortic smooth muscle contains 39% LC17nm and 61% LC17gi, while that of rat contains 66% LC17nm and 34% LC17gi (*11*). It is, therefore, suggested that there are three myosin species in aortic smooth muscle, hetero-dimers of both LC17nm and LC17gi isoforms and homo-dimers containing either isoform alone. Strong staining with each antibody is observed in only ~10% of aortic cells immediately after plating. This indicates that aortic smooth muscle cells contain various amounts of the three myosin species at the LC17 isoform level, and cells containing the highest concentration of LC17 homodimers show the strongest staining. The factors responsible for the differences in the expression of LC17 isoforms among cells are not known at present.

Alterations in the staining intensity of smooth muscle cells can be detected using anti-LC17gi (Fig. 3) or anti-LC17nm (data not shown) during the course of culture. No significant alteration in staining intensity, however, was observed with a monoclonal anti-pan myosin antibody that recognizes an epitope present on the heavy-chain of muscle myosin from skeletal, smooth muscle and non-muscle myosin (data not shown). Myosin persists in cultured cells in significant amounts throughout culture. Thus, one explanation for the disappearance of staining in cultured cells is that the cells express new 17-kDa light chain isoforms with different immunoreactivities other than LC17nm or LC17gi. Further biochemical analyses including immuno-blotting should lead to a more complete understanding of the changes in LC17 isoform expression.

Since it has been indicated that the LC17 isoforms are generated from a single gene by an alternative splicing process (*15*), cytochemical analyses of changes in the expression of the isoforms in aortic smooth muscle tissue in association with differentiation using isoform-specific antibodies should provide a better understanding of the regulatory mechanisms of RNA splicing.

In conclusion, the different subcellular localizations of two LC17 isoforms observed in this study suggest that they may have different functions.

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