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Cytoplasmic γ Actin as a Z-Disc Protein

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To investigate the precise localization of cytoplasmic γ actin in skeletal muscle and the relationship to dystrophin molecules, we designed an antibody against the N-terminal peptide of cytoplasmic γ actin. Western blot analysis using SDS–PAGE and isoelectric focusing (IEF) gel revealed that the antibody reacted only with the actin isoforms having γ motility, confirming that the antibody is specific to the cytoplasmic (nonmuscle) γ actin. Immunohistochemical analysis of the skeletal muscle of the adult mouse revealed a dot-like staining pattern of the antibody in transverse sections and a striated staining pattern in longitudinal sections. The double immunostaining technique revealed the colocalization of cytoplasmic γ actin with α-actinin, implying the localization of the actin on the Z-disc. Contrary to previous findings (1), we did not detect the colocalization of cytochrome oxidase, a mitochondria marker, with this actin.

Key Words: **actinin; cytoplasmic γ actin; Z-disc; mdx; dystrophin.**

Actin is a major component of the cytoskeletal protein of essentially all eukaryotes and is thought to play essential roles in such cell processes as cytokinesis, cell motility and maintenance of cell shape (2). Six actin isoforms have been identified to date: cytoplasmic (nonmuscle) β and γ actins, cardiac muscle α actin, skeletal muscle α actin, smooth muscle α and γ actins. Each of these isoactins has been conserved throughout evolution (3). The homology of the amino acid sequences between these, each of which is encoded by a different gene, are above 90%, with almost all of the differences localized at the N-terminus of the molecules (4).

These six isoactins demonstrate tissue specific expression patterns. Nonmuscle β and γ actins predominate in nonmuscle cells as do smooth muscle α and γ actins in smooth muscle cells, cardiac muscle α in cardiac muscle cells, and skeletal muscle α actin in skeletal muscle cells. Although a huge amount of skeletal muscle α actin exists in the skeletal muscle cells of higher vertebrates and forms myofibrils with other such cell components including myosin, tropomyosin, troponin, neblin and titin, skeletal muscle is also thought to contain a small amount of cytoplasmic γ actin (5). The fact that multiple actin isoforms exist in a cell and that there is a highly conserved amino acid homology among species implies that these actin isoforms function differently in a cell. The localization of γ actin in skeletal muscle has been previously reported, but the results remain unconfirmed as yet due mainly to the specificity of the antibodies used (6, 7). Pardo *et al.* reported the association of γ actin with mitochondrion using affinity-purified polyclonal antibody specific to the actin that has γ motility, although they did not elucidate whether it was cytoplasmic γ and/or smooth muscle γ actin (1, 8). On the other hand, Otey *et al.* reported the cytoplasmic γ actin coexisted in I bands with skeletal α actin in isolated myofibrils when using a peptide antibody specific to cytoplasmic γ, which cross reacted with smooth muscle α and γ actins (9).

In an attempt to resolve these discrepancies and to investigate the precise localization of γ actins, we designed an anti-peptide antibody specific to cytoplasmic γ actin and stained skeletal muscles with this antibody. Contrary to the findings of previous reports, cytoplasmic γ actin staining was observed in the Z-disc under a confocal laser scanning microscope. This is the first report to identify cytoplasmic γ actin as a new member of the Z-disc protein family.

MATERIALS AND METHODS

Preparation of anti γ actin peptide-antibody. The synthetic peptide, Acetyl-Glu-Glu-Glu-Ile-Ala-Ala-Leu-Cys-COOH corresponding to the N-terminal peptide of cytoplasmic γ actin with its C-terminal conjugated with cystein, was purchased from Asahi Techno Glass Corporation (Table 1). The synthetic peptide was conjugated to keyhole limpet hemocyanin (KLH) through the cystein residue at the COOH terminus of the peptide via *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). Then 150 μg of the KLH-MBS-γ actin peptide conjugate (1 mg/ml) was suspended in 700 μl of PBS, pH 7.3 and mixed with 1.5 ml of Freunds' complete adjuvant. A male New Zealand White rabbit purchased from Kitayama Labes Co., Ltd., was immunized subcutaneously. Two weeks after the first

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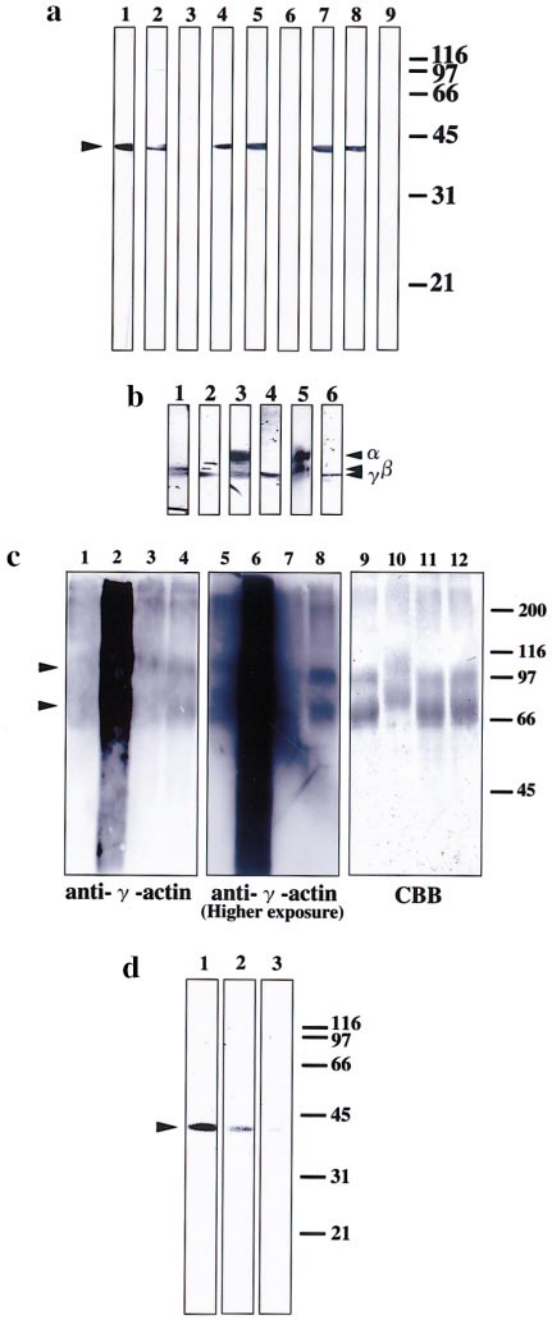

FIG. 1. Polyclonal antibody against N-terminal peptide of cytoplasmic γ actin recognizes only the cytoplasmic γ actin isoform. (a) Western blot analysis using SDS–PAGE. Equal amounts of mouse skeletal muscle (lanes 1–3), brain (lanes 4–6) and cardiac muscle extracts (lanes 7–9) were loaded into each lane. The antibody against synthetic peptide corresponding to the N-terminal peptide of cytoplasmic γ actin recognized a single band at approximately 42 kDa (lanes 1, 4 and 7), identical to the mobility band recognized by anti-pan actin antibody (lanes 2, 5 and 8). No signal was detected by the preimmune serum (lanes 3, 6 and 9). (b) Western blot analysis using IEF gel. Equal amounts of mouse brain (lanes 1 and 2), cardiac muscle extract (lanes 3 and 4) and a mixture of brain and skeletal muscles at the ratio of 5:1 (lanes 5 and 6) were loaded and transferred directly to the membrane. The membranes were stained with anti-pan actin antibody (lanes 1, 3 and 5) or anti-cytoplasmic γ actin antibody (lanes 2, 4 and 6). Note the anti cytoplasmic γ peptide antibody only recognized the actin that has γ motility. (c) Isoform-specific peptide immunoreactivity assay was performed. BSA-MBS (lane 1), BSA-MBS conjugated with N-terminal peptide of cytoplasmic γ (lane 2), α-smooth muscle (lane 3) and γ smooth muscle actin (lane 4) were subjected to Western blot analysis. The membrane was stained with polyclonal antibody against the N-terminal peptide of cytoplasmic γ actin and subsequently exposed to X-ray film (lanes 1–4) at a higher exposure (lanes 5–8). To visualize the samples loaded, the membrane was stained with CBB (lanes 9–12). (d) Primary antiserum was absorbed with 200 μg (lane 2) or 1 mg (lane 3) of synthetic peptide at 4° for 16 h prior to Western blotting. As a control, antibody with no peptide was also used (lane 1). In this assay, the antibody was used at a dilution of 1:5000.

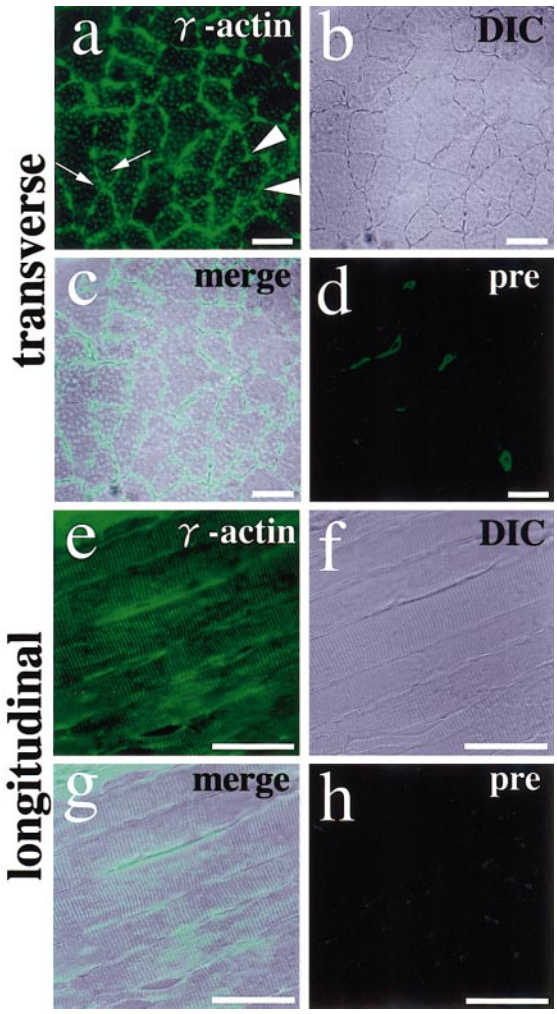

FIG. 2. Localization of cytoplasmic γ actin in mouse skeletal muscles. (a and e) Tibialis anterior muscles were immunostained with the primary antibody against cytoplasmic γ actin. (b and f) Differential interference contrast image corresponding to a and e, respectively. (c and g) Merged image of a and b, e and f, respectively. (d and h) Preimmune serum. (a–d) transverse sections. (e–h) longitudinal sections. Note dot-like staining in the cytoplasm and a striated stronger signal at the periphery of the cell in transverse sections, and zebra-like staining pattern in longitudinal sections. Bars, 100 μm.

injection, the conjugate was dissolved in Freunds' incomplete adjuvant before the rabbit was further immunized. Thereafter, immunization was performed twice at an interval of 1 week. Five weeks after the last injection, the rabbit was anesthetized by an injection of pentobarbital (5 mg/100 g body weight; Abbott Laboratories, North Chicago, IL) and the blood was collected transcardially. The obtained antiserum was added to 0.1% of sodium azide, frozen by liquid nitrogen and preserved at −80°C until needed.

Surgical procedures. Four-week-old male Balb/c mice weighing 25–35 g (SLC Co., Shizuoka, Japan) were anesthetized by an intraperitoneal injection of pentobarbital (5 mg/100 g body weight; Abbott Laboratories, North Chicago, IL) and decapitated using scissors in order to dissect the tibialis anterior, soleus and extensor digitorum longus muscles as quickly as possible. The muscles as well as the brains and cardiac muscles were frozen in liquid nitrogen and used for immunohistochemical analysis, Western blot analysis or direct immunoblotting using electric focusing gel.

Western blot analysis. About 1 g of the skeletal muscles, cardiac muscles or brains were dissected and homogenized in 10 ml of IM-Ac buffer with a protease inhibitor (Complete, Boehringer) using the Polytron homogenizer (Kinematica AG Littau). After the protein concentration was determined by the method of Bradford (10), 3.3 ml of 4× SDS–PAGE sample buffer (80 mg/ml SDS, 20% β-mercaptoethanol, 0.8 mg/ml BPB, 0.25 M Tris–HCl, pH 6.8) was added to the homogenate and boiled at 100°C, for 5 min. Next, 60 μg of protein was separated by SDS–PAGE through a 13% polyacrylamide gel according to the method of Laemmli (11). Protein was transferred to an Immobilon PVDF (polyvinylidene difluoride) transfer membrane (Millipore Corp., MA) by a semi-dry blotting system (Model BE-310, Biocraft, Tokyo, Japan). The membranes were blocked with 5% nonfat dried milk in TBS (20 mM Tris, 150 mM NaCl, pH 7.4) for 30 min and incubated for 1 h at room temperature with anti-cytoplasmic γ actin antibody (1:500) originally prepared or anti-actin antibody (A-2055, Sigma, 1:500) that recognizes the C-terminal of actins and is thus thought to react with all actin isoforms. The membranes were washed four times in 0.02% Tween 20 in TBS, washed once in TBS and then incubated for 1 h with HRP-conjugated swine anti-rabbit immunoglobulins (P0217, Dako Japan Co., Ltd., 1:1000) or alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins secondary antibody (D0306, Dako Japan Co., Ltd., 1:1000). The membranes were washed again as above and visualized using the ECL chemiluminescence system (Amersham Pharmacia Biotech, Japan) or Alkaline Phosphatase Substrate Kit III (SK-5300, Vector Laboratories, Inc.).

Isoform-specific peptide immunoreactivity assay. An isoform-specific peptide immunoreactivity assay was performed according to the method previously reported (12). The synthetic peptides, Acetyl-E-E-E-I-A-A-L-C-COOH, Acetyl-E-E-E-D-S-T-A-L-C-COOH and Acetyl-E-E-E-T-T-A-L-C-COOH corresponding to the N-terminal peptide of cytoplasmic γ, smooth muscle α actin and smooth muscle γ actin with its C-terminal conjugated to cystein, respectively, were purchased from Asahi Techno Glass Corporation (Table 1). The synthetic peptides were conjugated to bovine serum albumin (735086, Boehringer Mannheim Corp., Germany) through the cystein residue at the COOH terminus via *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). About 10 μg of the peptide linked to KLH-MBS was subjected to SDS–PAGE, followed by Western blot analysis using the peptide antibody as mentioned above. For comparison of the proteins applied, another transferred membrane was stained with 0.1% CBB R-250, 7% acetic acid and 50% methanol for several minutes and then destained with 10% acetic acid, 50% methanol.

Direct immunoblotting using IEF gel. Isoactins were separated by the IEF gel method previously described (13) with slight modifications. The cylindrical gel (4% acrylamide, 0.1% bisacrylamide, 9.5 M urea, 2% NP-40, 2% Ampholine (Amersham Pharmacia Biotech, pH 3.5–10:pH 5–7 = 1:4); 150 mm × 2.5 mm inner diameter) was prefocused at 400 V for 1 h with 0.01 M phosphoric acid and 0.02 M sodium hydroxide as the anolyte and catholyte, respectively. Brains and skeletal and cardiac muscle tissues were homogenated in about 5 times the volume of the IEF sample dilution buffer (9.5 M urea, 5% β-mercaptoethanol, 2% NP-40) and centrifuged at 30,000*g* for 30 min. The supernatant was mixed with 1.5 times the volume of IEF addition buffer (9.5 M urea, 5% β-mercaptoethanol, 2% NP-40, 50% glycerol, 2% Ampholine (Amersham Pharmacia Biotech, pH 3.5–10). The mixture was applied to the basic end of the gel and focused at 400 V for 12 h followed by focusing at 800 V for 1 h. After electrophoresis, the gel was immersed in equilibration buffer (5% β-mercaptoethanol, 0.3% sodium dodecyl sulfate, 0.37 M Tris–Cl, pH 8.8) or transfer buffer (5% methanol, 25 mM Tris–Cl, pH 10.4) for 2 h and 30 min. The protein was directly transferred from the tube gel to an Immobilon PVDF transfer membrane (Milipore, Japan) by a semi-dry blotting system. Immunostaining was performed as mentioned above (see Western blot analysis).

Immunohistochemistry. To prepare the samples for immunohistochemistry, the muscles were dissected, frozen in O.C.T. compound by liquid nitrogen, and then 12-μm thick sections were cut using a cryostat. The sections were then attached to glass slides, which had been coated with gelatin, and air-dried.

The primary antibodies against α-actinin (A-7811, monoclonal, Sigma), COX (cytochrome oxidase) (A-6403, monoclonal, Molecular Probes, OR), dystrophin (D-8043, monoclonal, Sigma) and cytoplasmic γ actin originally prepared were used at a dilution of 1:100. After fixation by dipping for 10 min into acetone that had been cooled at −20°C, the sections were blocked for 30 min with 5% nonfat dried milk in TBS, incubated for 1 h with the primary antibody at room temperature, and washed three times in 0.1% Triton X-100 in TBS. The sections were then incubated for 1 h with TRITC-labeled anti-rabbit immunoglobulins secondary antibody (Cat. No. 4010-03, Southern Biotechnology Associates, Inc., 1:100) and/or with FITC-labeled anti-mouse immunoglobulins (N1031, Amersham Pharmacia Biotech, Japan, 1:100). After further washing with 0.1% Triton X-100 in TBS, the sections were mounted in DABCO (1,4-Diazabicyclo-[2.2.2] octane)-PBS (5 mg/ml DABCO, 50% glycerol in PBS, pH 7.3) and observed under a confocal laser scanning microscope (LSM410; Carl Zeiss, Inc., NY). Conventional (without pinhole) or confocal (pinhole size at 20) images were converted to pseudocolor images using Adobe Photoshop version 5.5 (Adobe Systems Inc., CA).

RESULTS

Peptide Antibody Is Highly Specific to Cytoplasmic γ Actin

To elucidate the localization of cytoplasmic γ actin in the skeletal muscle, we attempted to design a novel peptide antibody highly specific to cytoplasmic γ actin, as that previously produced by Otey *et al.* also recognizes smooth muscle α and γ actins (14).

We synthesized the N-terminal peptide of cytoplasmic γ actin and produced the antibody by immunizing a rabbit with the peptide conjugated to keyhole limplet hemocyanine (Table 1). To reduce cross reactivity to the other actin isoforms, we used the N-terminal hepta peptide to produce an antibody with eight amino acid residues less than that used by Otey *et al.* Western blot analysis in combination with SDS–PAGE revealed that this antibody, recognized in all of the tissues, examined a single band of 42-kDa protein (Fig. 1a, lanes 1, 4 and 7) identical in size to the anti-panactin antibody (Fig. 1a, lanes 2, 5 and 8). Given that no other band was

TABLE 1

Comparison of Amino Acid Sequences of N-Terminal Region of Isoactins

Isoform type	Amino acid sequence of N-terminal
Skeletal muscle α	Ac-DEDETTALVCD
Cardiac muscle α	Ac-DDEETTALVCD
Smooth muscle α	Ac-EEEDSTALVCD
Smooth muscle γ	Ac-EEETTALVCD
Cytoplasmic β	Ac-DDDIAALVVD
Cytoplasmic γ	Ac-EEEIAALVID

Note. The peptide sequence underlined twice was synthesized in order to produce the antibody specific to cytoplasmic γ actin. The peptide sequences underlined once were synthesized and conjugated to BSA in order to investigate the specificity of the antibody.

observed in this experiment, we concluded that this antibody is specific to actins.

To investigate the specificity of the antibody to actin isoforms, we next analyzed the antibody by Western blot analysis using IEF gel. The antibody reacted with the actin isoforms having γ motility (Fig. 1b). Brain homogenate contains nonmuscle β and γ actins (Fig. 1b, lane 1) and, accordingly, we designated the antibody to not cross react with β actin (Fig. 1b, lane 2), or, in the same way, with cardiacα or skeletalα actins that are specific for cardiac and skeletal muscle, respectively (Fig. 1b, lanes 3–6). Thus, we concluded that the our novel antibody is specific to nonmuscle γ actin in all of these tissues, but particularly in skeletal muscle.

We next analyzed the cross reactivity of our antibody to the smooth muscle α and γ actins, as the antibody designed by Otey *et al.* that is eight amino acid residues longer is known to recognize both these actin isoforms (14). To compare the cross reactivity, the N-terminal peptide of these three actins was synthesized and conjugated to BSA. The conjugates were then subjected to SDS–PAGE, followed by Western blot analysis with the antibody. Our findings revealed that our novel antibody is highly specific to cytoplasmic γ actin (Fig. 1c, lanes 1–4). Although higher exposure to the film revealed a faint cross reactivity to smooth muscle γ actin (Fig. 1c, lanes 8), no cross reactivity was found with smooth muscle α actin (Fig. 1c, lanes 7). To compare the amount of the protein loaded, another membrane was stained with CBB (Fig. 1c, lanes 9–12). The specificity to the peptide was determined by absorption of the primary antibody with the peptide. The staining of the 42-kDa band was diminished as the concentration of the peptide was increased (Fig. 1d).

These results suggest that the novel antibody described here is highly specific to cytoplasmic γ actin among all the actin isoforms.

Distribution of Cytoplasmic γ Actin in Skeletal Muscle of Adult Mice

To investigate the distribution of cytoplasmic γ actin in skeletal muscle, we immunostained the cryostat sections of the tibialis anterior muscle. Strong signals were detected in transverse sections not in muscle cells (Fig. 5, arrowhead), but rather between them (Fig. 2a, arrowhead) in satellite cells, endothelial cells and/or fibroblasts (discussed below). In addition, a weaker but still strong dot-like signal was detected in the cytoplasm surrounding the muscle cells (Fig. 2a, arrow). Longitudinal sections showed striations of signals in the cytoplasmic region of muscle cells. The interval between striations appears to be in accordance with the length of sarcomeres (compare Fig. 2e with Fig. 2f). These signals were not detected when preimmune serum was used instead of primary antibody (Figs. 2d and 2h). Extensor digitorum longus and soleus muscles were used in addition to that of the tibialis anterior in immunohistochemical analyses. No significant difference in either the pattern or the intensity of the signals of the antibody was observed (data not shown).

Nonpreferential Association of Cytoplasmic γ Actin with Mitochondria

Signals similar to those of cytoplasmic γ actin in skeletal muscle in the present study were reported by Pardo *et al.*, signals they claim are in accordance with mitochondria when observed under a phase contrast microscope using the affinity-purified polyclonal antibody (1). Accordingly, we doubly-stained the skeletal muscle with anti-COX antibody and the antibody against cytoplasmic γ actin so as to investigate the relationship between these molecules (Fig. 3). Contrary to our expectations, the colocalization of signals (Figs. 3c, 3f, 3i and 3l) with only few exceptions (Figs. 3f and 3l, arrowhead) was rarely detected in either conventional or confocal images. Although the reason behind the discrepancies between our results and theirs remain unclear at this time, we speculate that Pardo *et al.*'s antibody may also recognize smooth muscle γ actin, because it is purified with chick gizzard γ actin in which the smooth muscle γ actin is a dominant actin isoform (1).

Colocalization of Cytoplasmic γ Actin with α-Actinin

As cytoplasmic γ actin did not colocalize with mitochondria, we next investigated what kind of cellular structure the γ actin did associate with, in the cytoplasm of skeletal muscle cells. α-actinin is known to exist in Z-discs in skeletal muscle cells. First, we doubly-stained skeletal muscle with anti-cytoplasmic γ actin and with anti-α-actinin. Surprisingly, the strong signal in the cytoplasm was in overall accordance with that of α-actinin on both conventional and confocal

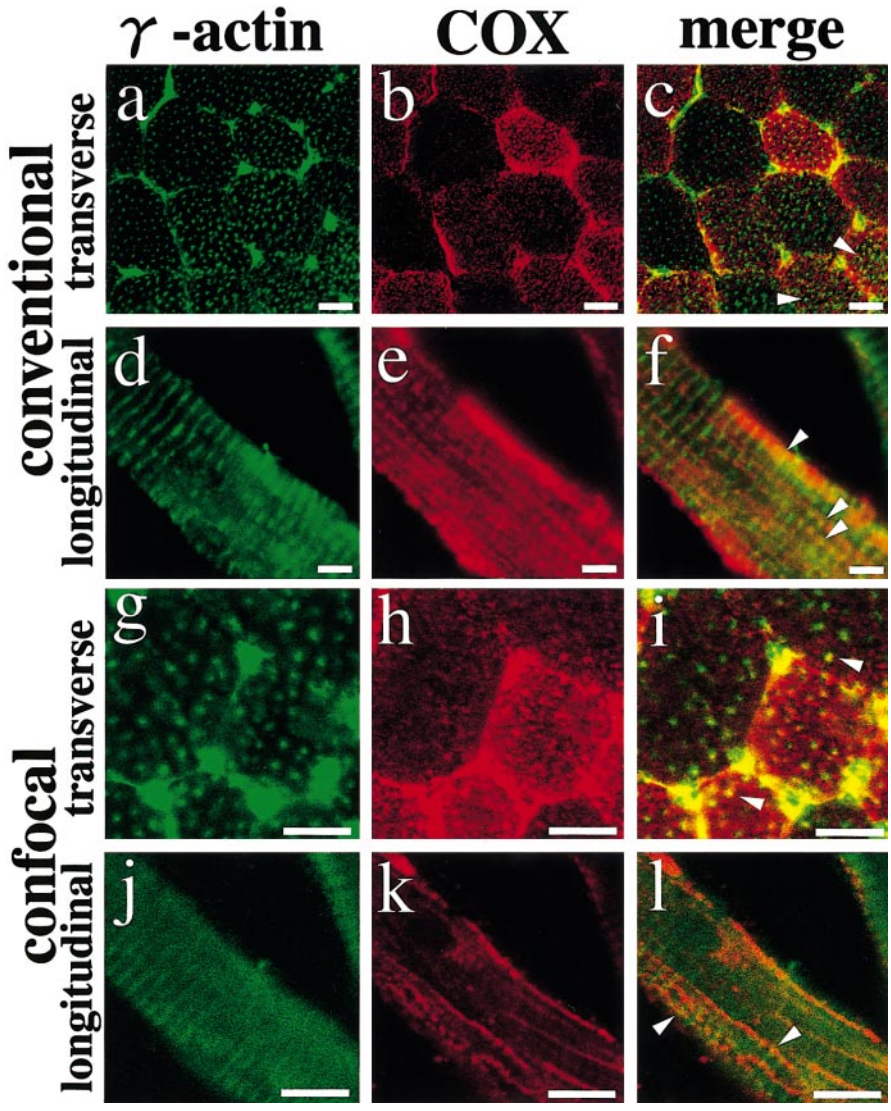

FIG. 3. Cytoplasmic γ actin did not show preferential association with mitochondria. Tibialis anterior muscles were doubly-stained with the primary antibody against cytoplasmic γ actin (a, d, g and j; green) and with the anti-COX antibody (b, e, h and k; red). (c, f, i and l) Merged image of a and b, d and e, g and h, and j and k, respectively. (a–c and g–i) Transverse sections. (d–f and j–l) Longitudinal sections. (a–f) Conventional images. (g–l) Confocal images. Bars, 20 μm.

images of the transverse sections (Figs. 4c and 4k, arrowhead). The conventional microscopy images of the longitudinal sections showed that the striated signal pattern also coincided with that of α-actinin (Fig. 4g, arrowhead). The confocal microscopic images revealed that numerous faint signals localized other than in the Z-disc, which presumably represented actins in thin myofibril filaments (Figs. 4m and 4o, arrow).

Lack of Colocalization of Cytoplasmic γ Actin and Dystrophin

To assess whether the strong signal at the periphery of the muscle cells (Fig. 2a, arrowhead) observed in the present study was derived from the plasma membrane, we stained muscle sections with anti-dystrophin antibody and anti-cytoplasmic γ actin antibody. The majority of the cytoplasmic γ actin signals were not colocalized with the dystrophin signals (Fig. 5c, arrowhead). In addition, no obvious merged (yellow) signals were observed along the plasma membrane of muscle cells (Fig. 3c).

DISCUSSION

Some discrepancies in the distribution of cytoplasmic γ actin appears in the literature. Pardo *et al.* reported that γ actin signals were detected in dot-like structures in the cytoplasmic region of muscle cells,

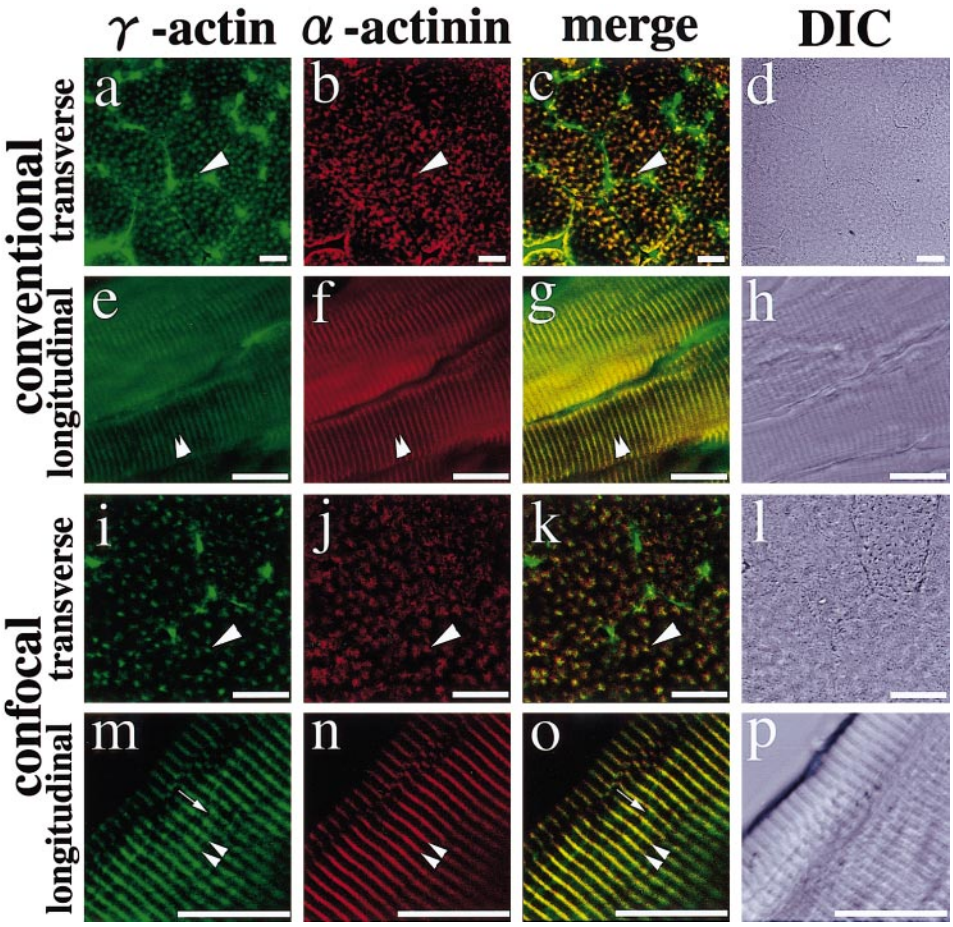

FIG. 4. Cytoplasmic γ actin colocalized with α-actinin in mouse skeletal muscles. Tibialis anterior muscles were doubly-stained with the primary antibody against cytoplasmic γ actin (a, e, i and m; green) and with the anti α-actinin antibody (b, f, j and n; red). (c, g, k and o) Merged image of a and b, e and f, i and j, and m and n, respectively. (d, h, l and p) Differential interference contrast image corresponding to (a–c), (e–g), (i–k) and (m–o), respectively. (a–d and i–l) Transverse sections. (e–h and m–p) Longitudinal sections. (a–h) Conventional images. (i–p) Confocal images. Bars, 20 μm.

and were found to coincide with mitochondria when using an antibody affinity-purified by chick gizzard γ actin (1). On the other hand, it was reported that cytoplasmic γ actin exists in the costamere and internal reticulum of chick muscle (8, 15).

So, we planned to produce a peptide antibody highly specific to cytoplasmic γ actin and investigate the localization of cytoplasmic γ actin in the skeletal muscle. Although Otey *et al.* made a peptide antibody against cytoplasmic γ actin, this antibody also recognizes the smooth muscle α and γ actin (14). To reduce a cross reactivity to these actin isoforms, we used the N-terminal hepta peptide to produce the antibody which is shorter eight amino acid residue than that

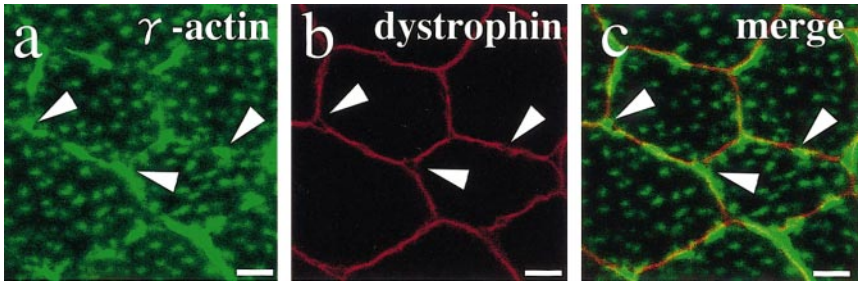

FIG. 5. Cytoplasmic γ actin did not colocalize with dystrophin. Tibialis anterior muscles in transverse sections were doubly-stained with the primary antibody against cytoplasmic γ actin (a; green) and with the anti dystrophin antibody (b; red). (c) Merged image of a and b. Bars, 10 μm.

they used and fortunately we could produce the peptide antibody highly specific to the cytoplasmic γ actin (Fig. 1). By immunohistochemical analysis using this antibody, the strong signals were detected between muscle cells (Fig. 2a, arrowhead). However, these signals found to be not in muscle cells (Fig. 5, arrow). Although these signals could be satellite, endothelial and/or fibroblast cells, the precise identification remained to be elucidated.

On the other hand, the weaker but still strong dot-like signal existed in all around the cytoplasm of muscle cells (Fig. 2a, arrow). As Pardo *et al.* also observed similar signals which they claim to be in accordance with the mitochondria by phase contrast microscope (1), we doubly-stained the muscle with anti-COX antibody. Contrary to the speculation, we could hardly detect the colocalization signals (Fig. 3). Surprisingly, we could detected these signals in precise accordance with α-actinin (Fig. 4). What cause the discrepancies between their results and our ones? Although we could not describe the accurate answer to this question, their antibody may also recognize the smooth muscle γ actin, because their antibody is purified with chick gizzard γ actin in which the smooth muscle γ actin is a dominant isoform of isoactin (1).

Immunoelectron microscopy did not clearly detect the signal on the Z-disc (data not shown). This may be due to the masking of the epitope during the fixation process presumably by the fixation of an other interacting protein. This notion is supported by the failure of immunohistochemical staining to detect intramuscular signals on the Z-disc when we fixed the muscles by transcardiac perfusion with aldehyde fixative prior to immunostaining, although the myofibrils on the surface of muscle were stained (data not shown). However, these intramuscular signals on the Z-disc were detectable on cryostat sections of unfixed, snap-frozen muscles even after fixation on glass slides.

As to the immunohistochemistry, the affinity-purified antibody was also used to get the same results as the antiserum and the signals were diminished by absorbing with the γ actin peptide (data not shown).

So what causes the dispersed distribution of cytoplasmic γ actin on the thin filament. It appears to be random distribution when we consider the immunohistochemical findings (Fig. 4o), possibly reflecting the residual portion of myofibrils that was constructed first by the γ isoform of actins developmentally and later displaced (16).

Craig *et al.* reported that the γ actin isoform exists in the costamere, the regularly spaced region of the cytoplasmic face of the plasma membrane in skeletal muscle (8). In transverse sections, we clearly elucidated that no cytoplasmic γ actin but only dystrophin molecules existed in the subsarcolemmal region, indicating that the actin-binding domain of dystrophin molecules cannot interact with cytoplasmic γ actin. Moreover, the fact that we could not detect any differences between the muscles of 2-week-old mdx and C57BL/10 mice immunostained with anti-cytoplasmic γ actin antibody (data not shown) also supports this notion. Rybakova *et al.* recently reported that the sarcolemma of control mice mechanically peeled with forceps showed the costametric pattern when immunostained with cytoplasmic γ actin antibody, although that of mdx mice did not (15). However, we believe that these findings do not conflict with our result if we speculate, for example, that the Z-disc just beneath the sarcolemma on pealing was not retained at all in mdx mice and that the ladder-like structure in control mice may not have indicated cytoplasmic γ actin in costameres but instead the debris of Z-discs. However, further research is necessary to elucidate this possibility.

While we were unable to confirm the exact role of cytoplasmic γ actin in the Z-disc, the coincidence of cytoplasmic γ actin localization in the Z-disc and the strong signals detected on the Z-disc lead us to speculate that cytoplasmic γ actin has an important function in sarcomeres. However, how the cytoplasmic γ actin accumulates on the Z-disc remains to be elucidated, as does the possibility of cytoplasmic γ actin interacting with the other components such as α-actinin, CapZ, neblin and titin that are known to exist on the Z-disc (17, 18). We intend to continue our investigation into the role of cytoplasmic γ actin in adult skeletal muscle.

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