J. Biochem. **133**, 659–664 (2003)
DOI: 10.1093/jb/mvg084

Localization of Calpain 3 in Human Skeletal Muscle and Its Alteration in Limb-Girdle Muscular Dystrophy 2A Muscle

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Received February 3, 2003; accepted March 5, 2003

Calpain 3/p94, the skeletal muscle-specific isoform of the calpain large subunit family, is a protein product of the gene responsible for limb-girdle muscular dystrophy type 2A (LGMD2A). Through yeast two-hybrid experiments, calpain 3 has been shown to bind to titin in myofibrils [Sorimachi *et al.* (1995) *J. Biol. Chem.* 270, 31158–31162]. However, because of extensive autolysis activity, calpain 3 localization in skeletal muscle has been undefined. In this study, we generated a polyclonal antibody against an N-terminal 98-amino-acid calpain 3 fragment, which is not homologous to the corresponding regions of other conventional calpains. This antibody stained myofibrils with a unique repeated doublet-pattern. Confocal microscopic observation with marker antibodies confirmed that calpain 3 is localized in the N2 region of myofibrils. Furthermore, using this antibody, we examined the localization of calpain 3 in LGMD2A muscles.

Key words: calpain 3, localization, muscular dystrophy, polyclonal antibody, sarcomere.

Abbreviations: GST, glutathione-S-transferase; LGMD, limb-girdle muscular dystrophy; *mdm*, muscular dystrophy in myositis.

Limb-girdle muscular dystrophy (LGMD) comprises a genetically heterogeneous group of progressive disorders. LGMD2A is an autosomal recessive disorder with a certain pattern of atrophy and weakness of truncal and proximal limb muscles, an elevated serum creatine kinase level and dystrophic changes in muscle biopsy specimens (*1*). The causative gene for LGMD2A encodes calpain 3, also called p94, a skeletal-muscle specific calcium-activated neutral proteinase (*2*). A defect in this muscle proteinase as a cause of muscular dystrophy strongly suggests a pathomechanism different from those due to defects in sarcolemmal structural proteins, such as dystrophin and sarcoglycans located on the sarcolemma.

Calpains constitute a family of calcium-activated neutral proteinases, and are distributed in almost all tissues and cells. Ubiquitous calpain isoforms, μ- and m-calpains, are major intracellular proteinases that participate in several events (*3*). Calpain 3 is a muscle-specific component and distinct from other calpains with regard to its calcium-independence, and its rapid and extensive autolysis (*4*). Calpain 3 consists of four domains conserved in conventional calpains with additional unique insertions of three regions, NS, IS1, and IS2. An alternative splicing isoform lacking these three unique insertions is expressed in the lens (*5*).

Previously, Sorimachi *et al*. found, using a yeast two-hybrid system, that calpain 3 binds to the N2 and M-line regions of titin, a gigantic molecule spanning the M- and Z-lines of sarcomeres on myofibrils (*6*). This binding is thought to stabilize calpain 3 to prevent its autolysis, and its dissociation from titin is thought to lead to local activation of this enzyme in myofibrils. Nevertheless, there has been no clear description of the localization of calpain 3 in skeletal muscle. Baghdiguian *et al*. reported myofibrillar and nuclear localization of calpain 3 in human skeletal muscle, but its exact position in myofibrils was not shown (*7*). Therefore, the *in vivo* binding of calpain 3 to titin has also not been confirmed. Here, we analyzed the subcellular localization of calpain 3 in normal and LGMD2A muscles.

MATERIALS AND METHODS

Muscle Tissue Specimens—Informed consent was obtained from all subjects. The specimens were obtained by biopsy from biceps brachii muscles and then frozen in liquid nitrogen-cooled isopentane immediately after the biopsy.

Production of Calpain 3 Polyclonal Antibody PAbcpn3-1—A polyclonal rabbit antibody for calpain 3 was raised against a fusion protein composed of the N-terminal 98-amino-acid fragment of calpain 3 with glutathione-S-transferase (GST). A cDNA fragment comprising nucleotide numbers 1 to 294 was amplified by PCR from the full-length calpain 3 cDNA, a kind gift from Dr. Sorimachi of the University of Tokyo. The amplified fragment was inserted into the *Eco*RI–*Sal*I site of pGEX 4T-1 (Amersham Pharmacia Biotech). Protein expression and

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immunization of rabbits were performed as previously described (*8*). The antibody was purified by affinity chromatography with antigen-conjugated and GST-immobilized resin.

Western Blot Analysis of Human Skeletal Muscle—Cryosections of human skeletal muscles were dissolved by heating in 50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2.5% 2-mercaptoethanol. Equal amounts of protein were separated by Laemmli's SDS-PAGE on 10% polyacrylamide gels and then transferred to polyvinyl-difluoride membranes. The membranes were incubated with antibody PAbcpn3-1 (1:2,500 dilution), calpain 3 monoclonal antibody calp3d/2C4 (1:100), or calp3d/12A2 (Novocastra Laboratories) (1:20) at room temperature for 1 h. Subsequent incubation with peroxidase-conjugated goat anti-rabbit IgG (TAGO Immunologicals) or anti-mouse IgG (H+L) (Immunotech) was performed at room temperature for 30 min. For detection of immunoreactive bands, an ECL detection kit (Amersham Pharmacia Biotech) was used.

Preparation of Myonuclei—Nuclei were prepared from the limb muscles of female six-month-old Balb/c mice by the method described by Chang-Gyu and Jonathan (*9*). The purity of the nuclei was confirmed by fluorescence microscopy with DAPI and phalloidin staining. The nuclei were dissolved by heating in SDS-PAGE sample buffer and used for a Western blot. A polyclonal lamin C antibody was used at a dilution of 1:2000 (*10*). All animal handling procedures were performed in accordance with a protocol approved by the National Institute of Neuroscience, NCNP, Japan.

Immunofluorescence Staining of Calpain 3—Cryosections of human skeletal muscles of 6-μm thickness were fixed in cold acetone, and then air-dried for 10 min. The sections were incubated with the PAbcpn3-1 antibody at 1:50 dilution, and then an anti-titin T11 antibody (Sigma), which specifically recognizes the A-I junction region of the titin molecule (*11*), at 1:100 and an anti–α-actinin EA-53 antibody (Sigma) at 1:2,500 as markers for sarcomeric localization. As secondary antibodies, fluorescein isothiocyanate–conjugated goat anti-rabbit $F(ab)_2$ (Jackson ImmunoResearch Laboratories) and Alexa Fluor568 goat anti–mouse IgG (Molecular Probes) were used. Fluorescent sections were observed under a Leica TCS SP confocal laser microscope (Leica) or a Zeiss Axiophot2 microscope (Zeiss).

Preparation of Glycerinated Myofibrils—Human glycerinated myofibrils were prepared from biopsy specimens of histologically normal skeletal muscle as described (*12*).

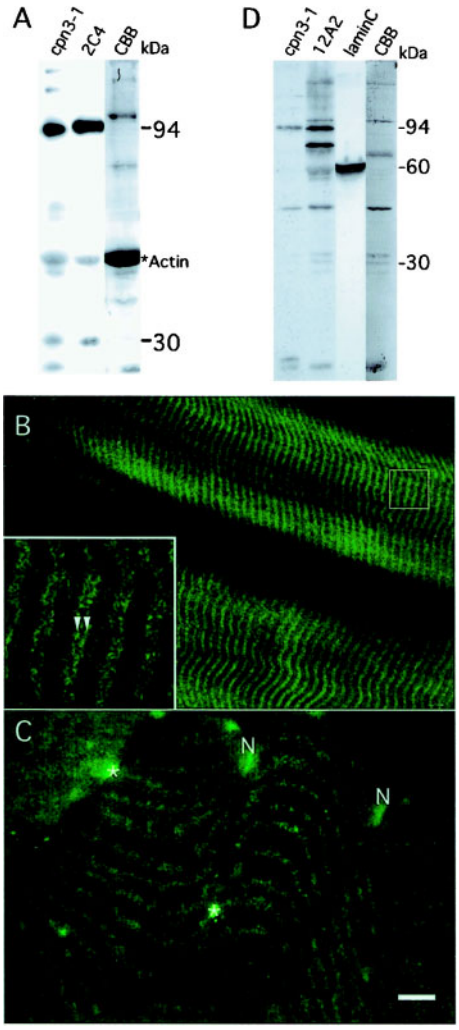

Fig. 1. **Characterization of newly raised polyclonal anti-calpain 3 antibody.** A: Western blotting of a human skeletal muscle homogenate with PAbcpn3-1 (cpn3-1) and MAb2C4 (2C4) antibodies. CBB, protein staining with Coomassie Brilliant Blue. B and C, immunostaining of calpain 3 with Pabcpn3-1 in human skeletal muscles, longitudinal section (B) and cross section (C). In B, the lower left inset is a magnified photograph of the boxed area. The strong staining shows a doublet pattern (arrowheads) at regular intervals along the long axis of myofibrils. In C, the staining shows a "contour-like" pattern in the cytosol and a dispersed pattern in nuclei. N, myonuclei. Asterisks (*) denote endogenous fluorescence of lipofuscin (*19*). The bar denotes 10 μm. D. Western blotting of mouse limb skeletal myonuclei with PAbcpn3-1 (cpn3-1) and MAB12A2 (12A2) antibodies. Lane "lamin C" shows strong staining of isolated myonuclei with an anti-lamin C antibody.

RESULTS

Characterization of the Newly Raised Polyclonal Anti–Calpain 3 Antibody—Calpain 3 is the skeletal muscle–specific homologue of conventional μ- and m-calpains. They exhibit homology: 45% between calpain 3 and μ-calpain, and 43% between calpain 3 and m-calpain at the amino acid level (*4*). However, calpain 3 has three unique sequences NS, IS1 and IS2 in the N-terminal borders between domains I and II, and domains III and IV, respectively (*4*). To produce a polyclonal antibody specific to calpain 3, we chose the N-terminal 98 amino acid sequence, which exhibits no homology to either m- or μ-calpain, subcloned the corresponding nucleotide fragment, and produced the fusion protein in *E. coli*. A polyclonal antibody (PAbcpn3-1) was raised using this protein as an antigen in a rabbit. Western blot analysis of a human skeletal muscle homogenate showed that PAbcpn3-1 detects two bands at 94 kDa and 30 kDa (cpn3-1 in Fig. 1A), which are also detected by a commercially available anti-calpain 3 monoclonal antibody (Mab2C4) that recognizes the N-terminal 20 amino acids (2C4 in Fig. 1A). The 30-kDa band has been described as a degradation product of calpain 3 (*13*). Several minor

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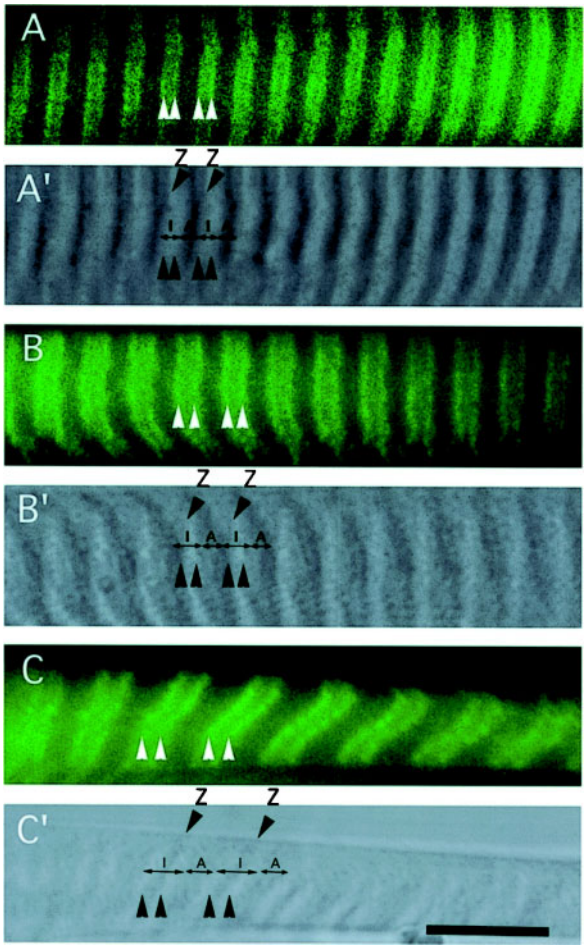

Fig. 2. **Subcellular localization of calpain 3 on glycerinated myofibrils.** The figures show the localization of calpain 3 (A, B and C), with Pabcpn3-1 in phase-contrast images (A′, B′ and C′), on myofibrils with different sarcomere lengths. The strong staining shows a doublet pattern (white arrowheads) close to the Z-lines in I-bands. The intervals within and between doublets changed with the length of the sarcomere in each fiber. Z, Z-line; I, I-band; A, A-band. The bar denotes 10 μm.

bands were faintly detected in the high molecular mass region with PAbcpn3-1. The 45-kDa band faintly detectable in both lanes is probably due to non-specific binding of the antibodies to large amounts of actin (Fig. 1A, lane CBB).

Subcellular Localization of Calpain 3—Cryosections of human skeletal muscle were fixed and immunostained with PAbcpn3-1 by means of a series of conventional methods. In a longitudinal section, a doublet pattern was highlighted at regular intervals along the long axis of myofibrils (Fig. 1B). In a cross section, the fluorescence was distributed in a "contour-like" pattern within myofibers (Fig. 1C). These patterns reflect the sarcomeric localization of calpain 3. In addition, myonuclei were strongly stained in a cross section, as previously reported by Baghdiguian *et al.* (*7*). We confirmed this nuclear localization of calpain 3 by co-staining with an anti-emerin antibody (Fig. 4a and a′). The intensity of calpain 3 staining varied among the nuclei. No difference in the intensity or position of the staining was seen when a thiol-proteinase inhibitor, p-chloro-mercuribenzenesulfonate, was used (data not shown).

The distribution of calpain 3 in myonuclei was also confirmed using isolated mouse myonuclei. Fig. 1D shows Western blot images of homogenates of purified mouse myonuclei with PAbcpn3-1 and a commercially available anti-calpain 3 monoclonal antibody, which recognize the IS1 region (MAB12A2). The quality of the isolated myonuclei was verified by the strong staining of lamin C (Fig. 1A, lane lamin C). All antibodies for calpain 3 clearly stained the 94-kDa band (Fig. 1A, lanes cpn3-1 and 12A2), but the monoclonal antibodies stained extra bands other than the 94-kDa band.

To further characterize the localization of calpain 3 in myofibrils, we also immunostained glycerinated myofibers with PAbcpn3-1 (Fig. 2, A–C). Intense fluorescence was seen on both sides of Z-lines in I-bands close to A–I junctions at regular intervals (Fig. 2, A–C, arrowheads), as can be seen in the longitudinal section of skeletal muscle in Fig. 1B. The distance within the doublets as well as the interval between the doublets varied with the extension of muscle fibers, as shown by phase contrast images (Fig. 2, A′–C′). These immunostaining patterns are similar to those of the N2 epitope of titin (*11*), suggesting that calpain 3 is distributed via binding to titin. Although a yeast two-hybrid experiment showed interaction between calpain 3 and the M-line region of titin (*6*), no immunoreactivity was observed at the M-lines. Moreover, no difference in fluorescent intensity was observed between type 1 and type 2 fibers, as far as we examined with antibodies against slow- and fast-type myosin heavy chains (data not shown).

To determine the exact position of calpain 3 on myofibrils, we performed double immunostaining using PAbcpn3-1 with an antibody to α-actinin, a protein localized on the Z-line, and PAbcpn3-1 with an antibody, T11, that specifically recognizes the A–I junction region of the titin molecule (Fig. 3A) (*11*), followed by confocal laser microscopy. α-Actinin was detected in the middle of each calpain 3 doublet (Fig. 3, B–D), while the T11 antibody highlighted areas outside of each calpain 3 doublet (Fig. 3, E–G), supporting the N2-localization of calpain 3 indicated by yeast two-hybrid experiments (*6*).

Change of the Calpain 3 Localization in LGMD2A Patient Muscles—We examined the localization of calpain 3 in LGMD2A muscles. It is known that some LGMD2A patients show a complete loss of calpain 3, while others retain amounts of calpain 3 comparable to those in normal controls, as judged on Western blot analysis. Patient 1 carries a homozygous Arg461Cys missense mutation (*14*). On Western blotting, the 94-kDa component was detected, but had decreased to less than half the amount with PAbcpn3-1, Mab2C4 and Mab12A2 (lanes cpn3-1, 2C4 and 12A2, respectively in Fig. 4B) compared to in a normal control (Fig. 4A). The myofibrils in this patient were stained differently in intensity with PAbcpn3-1 among myofibers. Some myonuclei were also weakly stained with PAbcpn3-1 (Fig. 4, b and b′). Patient 2 carries a homozygous splice-site mutation (Intron11+1G>T) in the CAPN3 gene, causing the in-frame skipping of the mRNA region corresponding to amino acids 476–508. Western blot showed the almost complete loss of calpain 3 with all antibodies (Fig. 4C).

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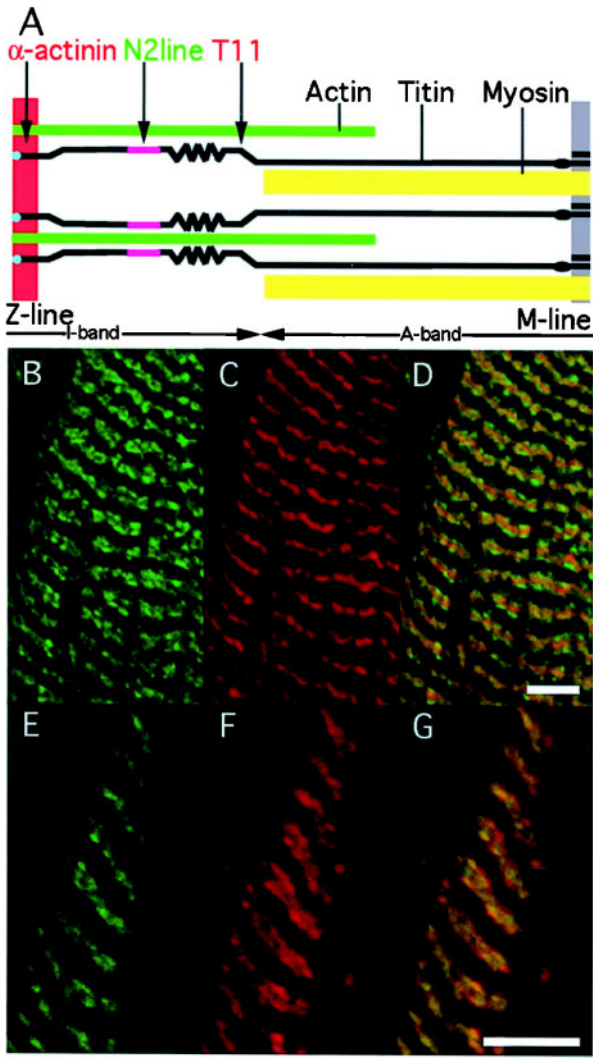

Fig. 3. **Double staining of calpain 3 and marker proteins observed on confocal laser microscopy.** A: Schematic representation of the sarcomeric architecture in skeletal muscle and the positions of epitopes for marker antibodies (red). The epitope of the anti-titin T11 antibody (Sigma), which specifically recognizes the A–I junction region of the titin molecule [11], was used. Immunofluorescence images of calpain 3 with PAbcpn3-1 (B, green) and α-actinin (C, red), and a merged image (D), and calpain 3 (E, green) and the T11 epitope of titin (F) (red), and a merged image(G), in longitudinal sections of skeletal muscle. The bar denotes 5 μm.

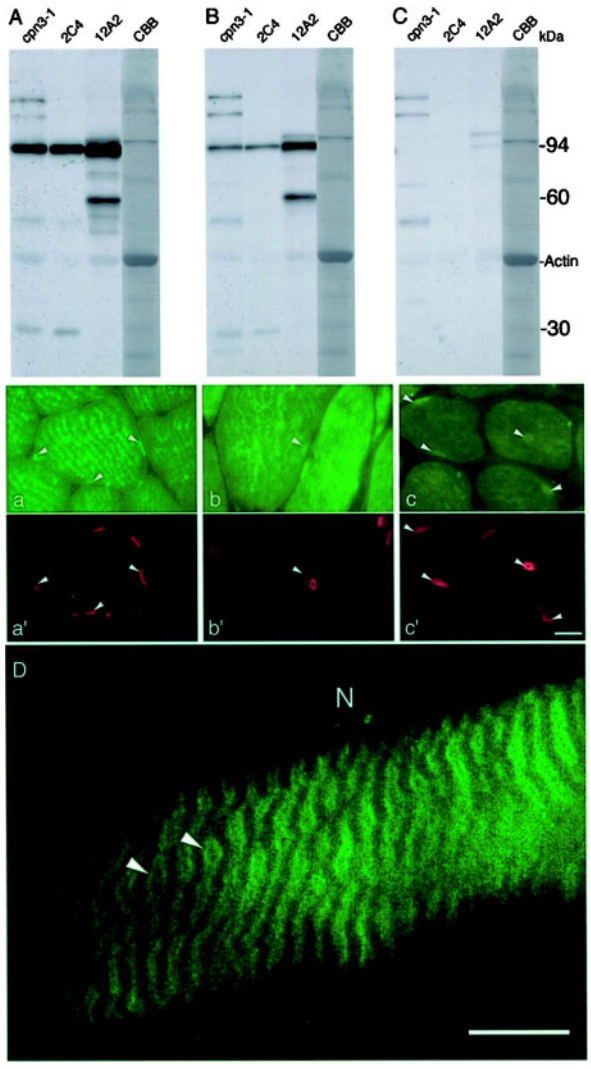

Fig. 4. **Calpain 3 localization in LGMD2A patient muscles.** Western blot analysis of LGMD2A muscle lysates from a normal individual (A), patient 1 (B), and patient 2 (C) with PAbcpn3-1 (cpn3-1), Mab2C4 (2C4), and MAb12A2 (12A2). In C, a specific 60kDa-degradation product was also detected with MAb12A2. CBB, protein staining with Coomassie Brilliant Blue. Immunofluorescence images of muscles from the normal individual (a and a′), patient 1 (b and b′), and patient 2 (c and c′) with PAbcpn3-1(a–c) and emerin antibody (a′–c′). In the muscle from patient 2 (D), the distance within doublets was extended (arrowheads). Intensive staining in myonucleous was also observed (shown in N). The bar denotes 10 μm.

The muscle in this patient showed no staining in myofibrils but faint staining in some myonuclei with PAbcpn3-1 (Fig. 4, c and c′). The calpain 3 localization in myofibers of patient 1 was analyzed in a longitudinal section (Fig. 4D). The distance within the doublet pattern of calpain 3 signals was variable (arrowheads), indicating the sarcomeric structure was partly disorganized.

DISCUSSION

We produced an antibody against a recombinant protein containing the N-terminal fragment of calpain 3. This antibody specifically recognized the whole 94-kDa molecule and its 30-kDa degraded product on Western blotting. It did not cross-react with any other members of the calpain family expressed in skeletal muscles. We detected calpain 3 in the N2 region of myofibrils but not on the M-line, although Sorimachi et al. previously showed that calpain 3 binds to N2 and M-line fragments of titin in yeast two-hybrid experiments (*6*). One possible explanation is that the PAbcpn3-1 antibody can not reach the epitope due to steric hindrance by the N-terminal region of calpain 3 (*15*). Alternatively, calpain 3 may have dissociated from the M-line region of titin during the fixation or staining process. In fact, Sorimachi et al. reported that the binding of calpain 3 to the M-line region of titin is much weaker than that to the N2 region of titin (*6*). Further studies are necessary to clarify whether or not cal-

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pain 3 is present in M-lines. Calpain 3 was also detected in myonuclei, but the staining intensity varied among the nuclei. For this reason, we proposed two possibilities. One explanation is that calpain 3 is expressed along with the differentiation in myogenesis. In satellite cells, calpain 3 will not be expressed. This may cause the variety of calpain 3 staining among nuclei. The alternative explanation is that calpain 3 in some nuclei will be degraded during the staining process.

Recently, it has been reported that a mutation in titin gene causes the muscular dystrophy in myositis (mdm) mice (*16*). In these animals, an approximately 2.4-kb insertion a 5′-transcated LINE-1 retrotransposon in the titin gene results in an 83 amino acid deletion from the N2A region. Haravuori *et al*. reported the almost complete absence of calpain 3 in mdm mice muscles (*17*). These reports suggest the importance of the presence of calpain 3 in the N2A region of the titin molecule. We observed that the mutated protein product was retained at an exact position in one LGMD patient (Fig. 4B), although the sarcomeric architecture was partly disrupted, further suggesting the importance of a functional calpain 3 molecule bound to the N2 region of titin.

Calpain 3 is not absent in all cases of LGMD2A, and normal amounts of mutated calpain 3 were observed in Western blots of some LGMD2A patients (*18*). In this study, we demonstrated the calpain 3 localization in myofibrils and myonuclei *in vivo*. Dissociation of calpain 3 retention in myofibrils and myonuclei was observed in some LGMD2A muscles. Calpain 3 localization in myonuclei as well as in myofibrils was not affected in a LGMD2A patient with a homozygous Arg461Cys missense mutation, while calpain 3 was only absent in myofibrils in a patient with a deletion of amino acids 476–508. Sorimachi *et al.* reported that the IS2 region is shared by the titin binding site and the nuclear localization signal (*4*). To retain calpain 3 at the N2 region of titin, not only the IS2 sequence but also a correct conformation around the IS2 region may be needed. Baghdiguian et al. proposed that calpain 3 is anchored to titin in an inactive reservoir form, and may shuttle between the sarcoplasm and the nucleus (*7*). Our results suggest that the localization of calpain 3 in nuclei may be independent of that in myofibrils. Further analyses to clarify the relationship between the intracellular localization and function of the calpain 3 molecule will help us to better understand the pathomechanism of LGMD2A.

The authors would like to thank Dr. H. Sorimachi (The University of Tokyo) for the generous gift of human calpain 3 cDNA. They also thank Dr. M. Hirano (Columbia University) for reviewing the manuscript. This work was partly supported by the Uehara Memorial Foundation.

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