Multiple Molecular Interactions Implicate the Connectin/Titin N2A Region as a Modulating Scaffold for p94/Calpain 3 Activity in Skeletal Muscle*1

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p94/calpain 3 is a skeletal muscle-specific Ca2+-regulated cysteine protease (calpain), and genetic loss of p94 protease activity causes muscular dystrophy (calpainopathy). In addition, a small in-frame deletion in the N2A region of connectin/titin that impairs p94-connectin interaction causes a severe muscular dystrophy (mdm) in mice. Since p94 via its interaction with the N2A and M-line regions of connectin becomes part of the connectin filament system that serves as a molecular scaffold for the myofibril, it has been proposed that structural and functional integrity of the N2A-connectin complex is essential for health and maintenance of myocytes. In this study, we have surveyed the interactions made by p94 and connectin N2A inside COS7 cells. This revealed that p94 binds to connectin at multiple sites, including newly identified loci in the N2A and PEVK regions of connectin. Functionally, p94-N2A interactions suppress p94 autolysis and protected connectin from proteolysis. The connectin N2A region also contains a binding site for the muscle ankyrin repeat proteins (MARPs), a protein family involved in the cellular stress responses. MARP2/Ankrd2 competed with p94 for binding to connectin and was also proteolysed by p94. Intriguingly, a connectin N2A fragment with the mdm deletion possessed enhanced resistance to proteases, including p94, and its interaction with MARPs was weakened.

Our data support a model in which MARP2-p94 signaling converges within the N2A connectin segment and the mdm deletion disrupts their coordination. These results also implicate the dynamic nature of connectin molecule as a regulatory scaffold of p94 functions.

Calpain (EC 3.4.22.18, clan CA, family C2) is a family of Ca2+-requiring and papain-like proteases comprising the products of 15 different genes in humans (1–3). Calpain is considered a modulator protease, since it modulates functions of substrates. More than 10 calpain molecules are expressed in skeletal muscle, including p94/calpain 3, a skeletal muscle-specific isoform, as well as the conventional μ- and γ-calpains, whose catalytic subunits are called calpain 1 and 2, respectively. The functions of calpain in skeletal muscle have been investigated regarding pathogenic conditions, such as atrophy and muscular dystrophy (4–6), and the molecular mechanisms involved in myogenesis (7–9).

The physiological relevance of p94 to skeletal muscle integrity is an urgently pursued issue. Defective p94 protease activity by gene mutations causes a muscular dystrophy categorized as calpainopathy (10). The calpainopathy-type muscular dystrophy contrasts with other muscular dystrophies with regard to the mode of calpain involvement. Unlike the conventional calpains that are hyperactivated as a consequence of advanced dystrophic phenotype (i.e. an aberrant increase in intracellular [Ca2+]i) and, in turn, aggravate the symptoms (11), the dystrophic phenotypes of calpainopathy are caused by loss of p94 protease activity from skeletal muscle (12). Phenotypes of transgenic mice in which the p94 protease activity is manipulated in various ways show that the regulated proteolytic action of p94 on target proteins is critical for the maintenance of skeletal muscle functions (13–16). Thus, identification of p94 substrates is a key to clarifying underlying mechanisms.

One of the interesting properties of p94 is its very rapid and exhaustive autolysis in protein expression systems examined so far, whereas native p94 protein can be detected at significant quantities in skeletal muscle, where it constitutes a complex
with other myofibril components, especially connectin/titin (17–21). Therefore, connectin is a candidate that regulates p94 stability and activity. Two distinct regions in connectin, the N2A and C terminus regions, have been identified as p94-binding sites by yeast two-hybrid (YTH) screening (19, 22). These interactions are thought to impede autolytic disassembly of p94, which has been demonstrated for the N2A fragment in a proteinase-trapping assay, a phenomenon we call “p94 trapping” (23, 24). Interestingly, in primary cultures of skeletal muscle cells, p94 translocates from the M-line to the N2A region as myofibrillogenesis proceeds or as the sarcomere lengthens (25). Together, these observations suggest that p94 and connectin function as a complex in skeletal muscle cells.

The importance of connectin as a scaffold for multiple molecular interactions for both the structural and force-generating elements has been recognized (26–29). In fact, the N2A region of connectin appears to be a major site involved in signal transduction in striated muscle tissues. N-terminal to the binding site for p94 in the N2A region of connectin is a specific insertion sequence (“is”), which binds to muscle ankyrin repeat proteins (MARPs) (30) (Fig. 1A). There are three MARP paralogues: MARP1 expressed primarily in cardiac muscle (also referred to as CARP or Ankrd1), MARP2 (Ankrd2 or Arpp) expressed primarily in skeletal muscle, and MARP3 (DARP) expressed in both muscle tissues. MARPs become strongly up-regulated in myocytes under a variety of acute stresses: cardiac injury and hypertrophy (MARP1) (31–33), stretch or denervation (MARP3) (36). MARP1 and -2 are up-regulated significantly, respectively, and MARP3 (DARP) expressed using p94, 10 mM iodoacetamide was also included. The cell lysate was centrifuged at 20,630 × g at 4 °C for 15 min, and

EXPERIMENTAL PROCEDURES

Mouse Experiments—All procedures used for experimental animals were approved by the Experimental Animal Care and Use Committee of the Tokyo Metropolitan Institute of Medical Science.

cDNA Constructs—The cDNAs for human and mouse p94/calpain 3 were subcloned into expression vectors pSRD and pAS2-1 or pAS2-1c for protein expression in mammalian and yeast cells, respectively, as described previously (12, 46). The yeast expression vectors for the full-length and IS2 region of rat p94 were described previously (19). The mouse cDNAs encoding various regions of N2A connectin/titin and full-length MARP1, -2, and -3 were amplified by PCR from mouse skeletal muscle cDNA using PuDNA polymerase (Stratagene, La Jolla, CA). Connectin fragments were expressed as the N-terminally FLAG-tagged proteins using the expression vector pSRD (Table 1). MARP1 with an N-terminal hemagglutinin (HA) or MYC epitope was expressed using pSRD. MARP2 and -3 were expressed using pcDNA3.1 as the N-terminally HA-tagged protein (47). An expression vector, pACT2 (U29899; Clontech, Mountain View, CA) was used to express cloned connectin cDNAs in a YTH assay. p94/D607A was constructed by introducing an Asp^607 → Ala mutation into the human p94 cDNA and inserting this into the XbaI–SacI sites of human p94 cDNA in pFastBac1 (Invitrogen). This mutant p94 was characterized using p94 trapping (23) and used for the biochemical analyses of p94 as described previously (24). cDNA for the FLAG-tagged I80-PEVK connectin fragment was subcloned into pFastBacHTa. Enzymes used for manipulating recombinant DNA were purchased from Takara Bio (Shiga, Japan) or New England Biolabs (Ipswich, MA). Mutations described here were introduced by long PCR using PuDNA Turbo DNA polymerase as described previously (12). Every nucleotide of all of the constructs was verified by DNA sequencing.

Protein Expression in COS7 Cells and Coimmunoprecipitation Assay—COS7 cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum that had been heat-inactivated before use at 56 °C for 30 min. Electroporation was performed using Gene Pulser (BioRad) according to the manufacturer’s instructions. Cells were harvested 72 h after electroporation and lysed by sonication in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl or CsCl, 1 mM EDTA-Na or EDTA-K, pH 8.0, and 1% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM leupeptin, and 1.5 μM aprotinin). For cells expressing p94, 10 mM iodoacetamide was also included. The cell lysate was centrifuged at 20,630 × g at 4 °C for 15 min, and
the supernatant was incubated with anti-FLAG M2 affinity gel (Sigma) according to the manufacturer’s instructions. For detecting the p94-connectin interaction and MARP-connectin interaction, the incubation was carried out for 2 and 4 h, respectively. Immunoprecipitates were collected by centrifugation and were rinsed twice with lysis buffer and then twice with wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl or CsCl), followed by an incubation with 3× FLAG peptide (150 ng/μl in wash buffer) for 30 min at 4 °C. The supernatant was subjected to SDS-PAGE and Western blot analysis.

**Western Blot Analysis**—Proteins were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA). Membranes were probed with appropriate primary antibodies and horseradish peroxidase-coupled secondary antibodies (Nichirei, Tokyo, Japan) followed by visualization using a POD immunostaining kit (Wako, Osaka, Japan) or ECL™ Western blotting detection reagent (GE Healthcare).

**Antibodies**—Antibodies used in this study include anti-FLAG monoclonal antibody (clone M2; Stratagene), anti-HA monoclonal antibody (clone 6E11; Cell Signaling Technology, Danvers, MA), anti-MYC monoclonal antibody (clone 4A6; Invitrogen), anti-slow myosin heavy chain (sMHC) monoclonal antibody (clone NOQ7.5.4D; Sigma), and anti-developmental MHC (dMHC) monoclonal antibody (NCL-MHC; Novocastra, Newcastle upon Tyne, UK). Rabbit polyclonal anti-MARP1 and MARP2 and goat anti-pIS2 antibodies were described before (23, 30). An affinity-purified rabbit anti-TPALKK was generated using the keyhole limpet hemocyanin-conjugated peptide TPALKK-C (aa 8558–8563 in mouse connectin) and used at a 1:10,000 dilution. The antibody was incubated on ice with peptide RAMLKKT-C (aa 14803–14810 of human calpastatin; NP_001035911). The homogenate (total) was fractionated into the soluble fraction (supernatant) and insoluble fraction (pellet) by centrifugation at 20,630 × g for 30 min at 4 °C. An equal amount of protein, 5 μg for each sample, was separated by SDS-PAGE and subjected to Western blot analysis.

**Histology and Immunohistochemistry**—The posterior compartments (gastrocnemius and soleus) of the hind limb skeletal muscles were dissected from 10-week-old Ttn+/−/− (WT) and Ttn<sup>ndm/mndm</sup> mice and frozen in liquid nitrogen-cooled isopentane. Cryosections from each muscle tissue, 20 μm thick per section, were lysed in homogenizing buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA-K, pH 8.0, and 1 mM dithiothreitol) containing protease inhibitors as follows: 1 mM PMSF, 0.1 mM pepstatin A, and 50 μM calpastatin peptide 42 (aa 140–181 of human calpastatin; NP_001035911). The homogenate (total) was fractionated into the soluble fraction (supernatant) and insoluble fraction (pellet) by centrifugation at 20,630 × g for 30 min at 4 °C. Immunofluorescence on cryosections was performed as previously described (25, 30, 44) using antibodies specific for MARP1, MARP2, sMHC, and dMHC in combination with appropriate secondary antibodies conjugated with either Alexa Fluor 488 or 555 (Invitrogen). The nuclei were labeled with 4′,6-diamidino-2-phenylindole contained in a mounting medium (VECTOR-SHIELD mounting medium with 4′,6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA). Sections were analyzed on a laser-scanning confocal microscope (LSM510; Carl Zeiss), and the images were processed using Photoshop CS2 (Adobe Systems, San Jose, CA).

**Expression of Recombinant Proteins in Sf-9 Cells**—Recombinant baculovirus was generated according to the protocol provided by Invitrogen, and the recombinant proteins were expressed as described previously (48). Briefly, *Spodoptera frugiperda* (SF-9) cells were suspended in infection medium containing one-tenth volume of each baculovirus stock solution at a concentration of 1.0 × 10<sup>6</sup> cells/ml, left for 1 h with gentle agitation every 15 min, diluted to a concentration of 1.0 × 10<sup>6</sup> cells/ml, and shaken at 140 rpm at 27 °C for 44–48 h. Preparation of the cell lysate and immunoprecipitation were performed as described above for analyzing proteins expressed in COS7 cells.

**Bacterial Expression and Purification of Recombinant Proteins**—cDNA fragments corresponding to the N2A region of human connectin, 180–183 (nucleotides 15307–16851 in X90569) and human MARP2 (nucleotides 294–1289 in NM_020349) were amplified by PCR from human skeletal muscle cDNA and cloned into the pET vector. Proteins were expressed in *Escherichia coli* BL21(DE3) and purified as described previously (49).

**Proteolytic Assay for Calpains**—One μg of recombinant human MARP2 was incubated with 0.25 μg of recombinant human μ-calpain prepared as described previously (48) in 20 μl of incubation buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA-K, pH 8.0, 1 mM dithiothreitol, 1 mM PMSF, 0.1 mM pepstatin A) with or without 15 mM CaCl<sub>2</sub> at 30 °C for 60 and 90 min. To measure p94 autolysis and proteolysis of connectin fragment activity, the lysate of SF-9 cells expressing p94 and/or the connectin fragment was incubated with 5 mM CaCl<sub>2</sub> at 37 °C. The protein concentration of cell lysates was adjusted to 0.5 μg/μl. For the proteolytic assay of MARP2, recombinant His-MARP2 was added to the lysate of cells expressing p94 at a concentration of 20 ng/μl. The reaction was stopped by the addition of SDS sample buffer, and the sample was subjected to SDS-PAGE followed by silver staining or Western blot analysis. Where indicated, protease inhibitors were added at the following concentrations: 1 mM PMSF, 1 mM leupeptin, 0.1 mM E64c, and 7 μM calpastatin domain I (Takara Bio).

**Peptide Sequencing**—After SDS-PAGE, the proteins were blotted onto Pro-Blot membrane (Applied Biosystems, Foster City, CA) and then visualized by Coomassie Brilliant Blue G-250 staining. The target protein bands were excised and washed three times with an excess amount of 50% (v/v) methanol and then with absolute methanol. The N-terminal sequence was determined using a 491cLC protein sequencer (Applied Biosystems) according to the manufacturer’s instructions.

**YTH Assay—Saccharomyces cerevisiae** strain AH109 was transformed with a series of combinations of expression vectors for various regions of connectin and p94 WT and mutants using
Calpain-Connectin-MARP N2A Complex Modulated by Proteolysis

**A**

**B**

**C**

**D**

**FIGURE 1. Interaction between p94 and the connectin N2A peptide region.** A, schematic structure of the N2A-PEVK connectin/titin region and constructs used in this study. The numbers correspond to aa in the mouse connectin (NP_035782). The in vitro cleavage sites of the 14804 JOURNAL OF BIOLOGICAL CHEMISTRY

The Fast™-Yeast Transformation kit (G-Biosciences/Geno-
tech, St. Louis, MO), according to the manufacturer’s instruc-
tions. Cotransformants were selected on plates with SD
medium that lacked Leu and Trp (SD−LW), and the expression
of reporter genes by growth on plates that also lacked His and Ade
(SD−LW/HA) was measured according to the manufacturer’s instructions
(Clontech).

**RESULTS**

I80-PEVK Connectin/Titin Preferentially Coimmunoprecipitates with Full-length p94 Rather Than Autolyzed Fragments—We surveyed for potential p94-connectin/titin interactions by a coimmunoprecipitation, thus extending our previous YTH studies (19, 22, 44) by a complementary approach.

p94:WT expressed in COS7 cells autolyzes very rapidly at IS1, and only a 55-kDa fragment is dominantly detectable (Fig. 1B, lane 1, anti-FLAG, closed arrowhead; Fig. 1D, arrow 1 (20)). However, when I80-PEVK, I80-PEVK(mdm), or I81–I83 (previously called mCN48 (23)) (Fig. 1A) was expressed with p94:WT, the full-length p94 as well as the autolyzed fragment was detected in coimmunoprecipitates (Fig. 1B, lane 8, 9, or 12, open arrowhead). This suggests that the full-
length p94 binds to the connectin fragment more efficiently than the autolyzed 55-kDa fragment does.

In apparent contrast to our previous conclusion (23), the mdm deletion in a connectin fragment I80-
PEVK did not abolish the interaction with p94 (also con-
firmed when using protease-inactive mutant p94:C129S (p94:CS); data not shown). However, subse-
quent analysis indicated that the I80-PEVK fragment contains in addition to the previously identified p94 binding site in I82–I83 (see Fig. 1A, SpBS (primary p94 binding site)) at least one additional binding site.

**Identification of Novel p94 Binding Sites in the N2A-PEVK Region of Connectin—Coimmunoprecipitation**

**medium that lacked Leu and Trp (SD−LW), and the expression of reporter genes by growth on plates that also lacked His and Ade (SD−LW/HA) was measured according to the manufacturer’s instructions (Clontech).**
Since interaction between p94:WT and I80-I81 was not detectable (Fig. 1B, lane 11), autoylzed fragments appear to be insufficient for mediating interaction with SpBS1 only. It is also possible that SpBS1 only can interact with p94 very weakly and cannot prevent p94 from autolysis.

On the other hand, I82-PEVK, in which PpBS remains intact, did not coimmunoprecipitate either p94:WT or p94:CS, regardless of whether the FLAG tag is N-terminal (Fig. 1, B lane 10) or C-terminal (data not shown). This suggests that the connectin local structure affects its ability to bind to p94.

p94 Interacts with PEVK Connectin through the Proximity of the IS2 Region—Because the interaction between PEVK-N and p94:CS was detectable in the YTH assay, we examined further the binding region for SpBS2 in p94 and compared this with that for PpBS (Fig. 1D). The interaction was complicated but suggested that the SpBS2 binding region overlaps with that for PpBS in the N terminus in the proximity of the IS2 region, with a slight extension toward the C terminus (Fig. 1D, p94:1–600 and p94:1–594). Interestingly, the sequence encoded by exons 15 and 16 of p94 is not necessary for its interaction with SpBS2 if the C-terminal region of p94 is intact (Fig. 1D, p94:ex15’16’). These data can be explained by assuming that SpBS2 binds to an intact structure in the proximity of the p94 IS2 region encoded by exon 14, which requires several extra amino acids at its N and C termini.

The N2A-PEVK Junction of Connectin Has a Site That Is Susceptible to Proteolysis—In the course of the above experiments, some connectin fragments were detected as both full-length and breakdown products even when these were coexpressed with p94:CS (Fig. 1C, lanes 2 and 3, anti-FLAG, closed arrowheads; Table 1). In contrast, corresponding fragments with the mdm deletion (i.e. I80-PEVK(mdmd) and I82-PEVK(mdmd) lacking 83 aa) appeared to be full-length without significant breakdown products (Fig. 1B, lane 3, anti-FLAG; Fig. S1B, lane 2). The same trend was observed when I80-PEVK and I80-PEVK(mdmd) were expressed in SF-9 cells (see Fig. 2C, lanes 1 and 19, anti-FLAG).

The proteolytic site in I82-PEVK was determined to be the N terminus of Ser18934 in the mdm deletion region (Fig. 1A, arrow 1; Fig. S1A). Expression of various fragments encompassing Ser18934 with or without the mdm deletion in COS7 cells suggested that fragments without the mdm deletion are commonly proteolyzed at the same site (Fig. S1C). These results indicate that the N2A-PEVK junction is susceptible to unidentified protease(s) in COS7 and SF-9 cells in the context of fragment structures used in this study and that the mdm deletion confers resistance to this proteolytic attack.

I80-PEVK Connectin Is Proteolyzed by μ-Calpain in the “is” Region—Proteolysis of connectin by calpains, including p94, was reported under several different experimental conditions (16, 50). Thus, we co-incubated purified recombinant proteins of I80–I83 and μ-calpain (Fig. 2A), because p94:WT is not available as a purified protein. Two proteolytic sites in the “is” region, flanking the MARP-binding region of N2A connectin, were revealed (Fig. 2B, arrows b/2 and c/3).

A specific antibody for the N terminus of the proteolyzed fragment b (Fig. 2B), anti-TPALKK, was generated using the corresponding mouse connectin sequence. This antibody reacted with fragment b but not with a, c, or d (data not shown; see “Experimental Procedures”) and was used for further analysis of connectin proteolysis.


table

| Construct | Amino acid residue in NP_035782 | Calculated molecular mass | Observed molecular mass |
|-----------|---------------------------------|--------------------------|-------------------------|
| I80-PEVK  | 8463–9137                       | 80                       | 97, 60*                 |
| I80-PEVK(mdmd) | 8463–8894/8978–9137  | 70                       | 85                      |
| I80–I83Δ | 8463–8895                       | 52                       | 58                      |
| I80–I82  | 8463–8826                       | 43                       | 45, 35                  |
| I80–I81  | 8463–8738                       | 33                       | 33, 26                  |
| I80–is   | 8463–8655                       | 24                       | 26                      |
| I81–I83(mtcpN48) | 8613–8915    | 37                       | 39                      |
| I81–I83/PEVK | 8613–8998               | 46                       | 47, 36                  |
| I81–I83/PEVK(mdmd) | 8613–8894/8978–8998 | 37                       | 42                      |
| I81–I83/I8931term | 8613–8930    | 38                       | 39                      |
| I81–I83/I8951term | 8613–8950     | 41                       | 41                      |
| I82-PEVK | 8739–9137                       | 48                       | 66, 31                  |
| PEVK-N   | 9089–9526                       | 52                       | 59                      |

* An additional 1.4 kDa for the N-terminal FLAG tag is included.
† For proteins expressed in COS7 cells with the N-terminal FLAG tag.
‡ The lower molecular mass corresponds to a proteolytic fragment.


discussion

TABLE 1
Fragments from the mouse connectin N2A-PEVK region used in this study
Calpain-Connectin-MARP N2A Complex Modulated by Proteolysis

**A**

| (a) 69 kDa | (b) 50 kDa | (c) 38 kDa | (d) 17 kDa |
|----------|-----------|-----------|-----------|
| 0.5 µg   | 0.5 µg    | 0.5 µg    | 0.5 µg    |
| 15 mM Ca\(^{2+}\), at 30°C, 90' |

**B**

**C**

| 0' | 0' | 0' | 0' | 0' | 0' | 0' | 0' |
|---|---|---|---|---|---|---|---|
| 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 |

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**FIGURE 2.** **Proteolysis of N2A connectin by calpain.** A, recombinant human connectin fragment, His-I80–I83, and μ-calpain were co-incubated in the presence of Ca\(^{2+}\) with amounts indicated. Arrowhead a and arrows b–d indicate the full-length (69-kDa) and proteolyzed fragments as shown in B. B, proteolytic fragments of I80–I83 are schematically summarized. Protopalytic fragments of I80-PEVK or I80-PEVK(md) were incubated for the indicated times in the presence of 1 mM PMSF and 5 mM CaCl\(_2\) at 37 °C. Note that I80-PEVK, but not I80-PEVK(md), was proteolyzed by endogenous protease(s) in a Ca\(^{2+}\)-dependent manner (anti-FLAG and anti-TPALKK, lanes 1–5), which was accelerated by coexpressing p94:DA (lanes 7–11) or attenuated by p94:CS (lanes 13–17). Proteolysis of I80-PEVK was inhibited in the presence of calpastatin, leupeptin, and E64c (not shown). Arrows e–g and i correspond to fragments labeled in Fig. 2B. *+, nonspecific signals. CBB, Coomassie Brilliant Blue.

Sf-9 protease(s). In contrast, coexpression of p94:CS did not accelerate, but slightly slowed, the proteolysis of I80-PEVK (Fig. 2C, lanes 13–18). It is thus inferred that interaction with p94:CS protects I80-PEVK from the endogenous protease(s), and accordingly, it is likely that p94:DA binds to I80-PEVK and directly proteolyzes it when activated by Ca\(^{2+}\).

Differential Proteolytic Susceptibility of I80-PEVK and I80-PEVK(md)—Judging from patterns of breakdown products, proteolysis of I80-PEVK (Fig. 2, B and C (e)) probably occurs first at the N terminus of Ser\(^{8934}\), generating the 62 kDa fragment (Fig. 2B, arrow 1 and fragment f), followed by that of Ser\(^{8558}\) (arrow 2 and fragment g) and then that of Gln\(^{8646}/\) Thr\(^{8647}\) (arrow 3 and fragment h; ~10 kDa, too small to detect).

On the other hand, I80-PEVK(md) was not proteolyzed as intensively as I80-PEVK was (Fig. 2C, anti-FLAG, lanes 19–23). The presence of coexpressed p94:DA did not significantly enhance the proteolysis, but autolysis of p94:DA with I80-PEVK(md) proceeded more slowly than with I80-PEVK (Fig.
Our results suggest that the “is” region of I80-PEVK becomes further sensitized to proteases, including p94, after a rate-limiting proteolytic step occurred in the junction of I83-PEVK. This model would explain why I80-PEVK(md) is resistant to proteolysis. Additionally, our data suggested that p94:CS protects connectin from proteolysis at sites in the “is” region as well as in the junction of I83-PEVK.

Effect of Connectin Structure on the MARP-Connectin Interaction—We next examined the MARP binding activity of the connectin N2A fragment as another important property of connectin that could be affected by the mdm deletion.

First, each MARP protein had a different I80-PEVK binding activity: MARP1 = MARP3 > MARP2 (Fig. 3A, lanes 2–4, IP, anti-HA). The interaction between connectin and MARP2, a predominant parologue in skeletal muscle, was further weakened by deleting the region I82-PEVK (Fig. 3B, lane 4 versus lane 5) or by introducing the mdm deletion (Fig. 3C, IP, anti-HA, lane 3 versus lane 7). The same trend was observed for the MARP1 (data not shown; Fig. 3C, IP, anti-MYC, lane 2 versus lane 5). Coexpression of MARP1 with MARP2 and I80-PEVK(md), an expected condition in Ttn/mdm/mdm skeletal muscle, decreased the amount of MARP2 (Fig. 3C, IP, anti-HA, lane 6 versus lane 7) but not MARP1 (anti-MYC, lane 6 versus lane 5) coimmunoprecipitated with the connectin fragment.

These results indicate that the region C-terminal to “is” (including those connectin sequences deleted in mdm) affects MARP-connectin interaction, possibly by modulating the structure of “is” surrounded by immunoglobulin domains. Additionally, MARP1 interferes with MARP2 in binding to connectin, both in WT and in mdm.

Distinct Subcellular Distribution of MARP1 and -2 in Striated Muscle—To examine whether the in vitro MARP-connectin interaction observed above corresponds to the in vivo situation, expression of MARP1 and -2 in muscles from WT and Ttn/mdm/mdm mice (n = 3 for each) were compared (Fig. 3D, WT-1–3 and mdm-1–3, respectively). In both WT and Ttn/mdm/mdm, MARP1 was enriched in cardiac muscle in the insoluble myofibrillar bound fraction, consistent with its strong binding to connectin. In skeletal muscle, MARP1 was observed in insoluble fraction only in Ttn/mdm/mdm.

2C, lanes 25–29 versus lanes 7–11). Anti-TPALKK antibody faintly detected a 67 kDa band probably corresponding to fragment i (Fig. 2B), generation of which was slightly enhanced and suppressed by coexpression with p94:DA and p94:CS, respectively (Fig. 2C (i)).

**TABLE 2**

Cellular distribution of MARPs

|         | MARP1 |         | MARP2 |
|---------|-------|---------|-------|
|         | Supernatant | Precipitate | Supernatant | Precipitate |
| Skeletal muscle | WT* | − | +/− | WT* | − | +/− |
|           | mdm | + | + | mdm | + | + |
| Cardiac muscle | WT | − | − | − | − | − |

* Mice homozygous for the wild type allele of Ttn, Ttn**<sup>+/+</sup>*.

* The result of the Western blot analysis in Fig. 3D was summarized quantitatively.

* * Mice homozygous for the mdm allele of Ttn, Ttn<sup>mdm/mdm</sup>.

2C, lanes 25–29 versus lanes 7–11). Anti-TPALKK antibody faintly detected a 67 kDa band probably corresponding to fragment i (Fig. 2B), generation of which was slightly enhanced and suppressed by coexpression with p94:DA and p94:CS, respectively (Fig. 2C (i)).
where MARP1 is up-regulated as previously reported (44). In contrast, MARP2 was detected predominantly in the soluble fraction of skeletal muscle and undetectable in cardiac muscle, suggesting very weak, if not zero, interaction of MARP2 with myofibrils. In Ttnmdm/mdm skeletal muscle, where it was robustly up-regulated, MARP2 was detected slightly in the insoluble fractions as well.

These data show that MARP1 and -2 differ in their cellular distribution in a manner consistent with our in vitro results (i.e. connectin binding activity is MARP1 >= MARP2). It should be noted that these molecular properties are essentially the same both in WT and Ttnmdm/mdm (Table 2), which raises a question as to a relationship between MARP1 and MARP2 under mdm conditions.

Characteristics of MARP1 and -2 in the Mode of Induction in Ttnmdm/mdm Skeletal Muscle—To characterize the expression of MARP1 and -2 in relation to the integrity and the fiber type in Ttnmdm/mdm skeletal muscle, expressions of MARP1, MARP2, dMHC, and sMHC were simultaneously analyzed in situ.

As previously reported, dMHC-positive fibers were absent in WT (data not shown), whereas degeneration/regeneration of muscle fibers was apparent in 10-week-old Ttnmdm/mdm skeletal muscle (Fig. 4, B and D, dMHC), and abnormal morphology, such as clusters of small sized regenerating fibers and a large percentage of fibers with irregular outlines and central nuclei, was apparent (Fig. 4C, HE) (45). In WT, essentially no MARP1-positive fiber was detectable (data not shown), and MARP2 was detectable only at low frequency and signal intensity (Fig. 4A), consistent with the above Western blot data (Fig. 3D). Some MARP2-positive fibers were also positive for sMHC (Fig. 4A, asterisks).

In Ttnmdm/mdm muscle, MARP1-positive fibers were sparsely identified, and significantly more fibers were positive for MARP2, sMHC, and/or dMHC in mdm than in WT (Fig. 4, B–E, and Table 3). MARP1 was not detectable in dMHC-positive fibers (Fig. 4B), suggesting that induction of MARP1 is not significant in regenerating fibers. There was a trend that MARP1-positive fibers were also MARP2-positive and had central nuclei (Fig. 4C, arrow). Expression of MARP2 seemed independent from fiber types specified by MHC (Fig. 4, C and D). Fibers with relatively intense MARP2 signal tended to have central nuclei regardless of their fiber sizes (Fig. 4C, arrowhead). Although frequency was low, MARP1

**TABLE 3**

| Protein | Overall expression in cross-sectional area | Combinations of expression at a muscle fiber level (mdm) |
|---------|------------------------------------------|------------------------------------------------------|
| MARP1   | –                                      | +                                                   |
| MARP2   | +                                      | + + +                                                |
| Slow MHC| + + +                                  | + + +                                                |
| Developmental MHC | + | + |

*a Expression of each protein examined by immunofluorescent study on cross-sections of a posterior segment of hind limbs was categorized based on the positive rate. + + +, >50%; + +, 10–50%; +, 1–10%; –, 0%.

*b Trend in coexpression of proteins in the same muscle fiber from Ttnmdm/mdm muscle is summarized.

**FIGURE 4.** Expression of MARP1 and MARP2 in WT and Ttnmdm/mdm mouse skeletal muscle. MARP1 or -2 (red) and sMHC or dMHC isoform (green) were detected by double-labeled immunofluorescence on sections of posterior parts of hind limbs from WT (A) and Ttnmdm/mdm (B–E) mice using specific antibodies. Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Representative images were selected. A, in WT muscle, fibers faintly positive for MARP2 were identified at a low frequency. Some of these fibers were sMHC-positive (*). B, in the corresponding area of Ttnmdm/mdm muscle, fibers expressing MARP1 were sparsely observed, where central nuclei were also identified (arrowhead). Expression of MARP1 was not detected in fibers expressing dMHC (*). C, two serial sections were stained with anti-MARP1, -MARP2, and -sMHC. Fibers expressing MARP2 and/or sMHC were observed more frequently compared with WT. Expression of MARP1 and -2 is not mutually exclusive and is independent of the muscle fiber types. Examples of combination of MARP1, MARP2, and sMHC expressed in the same fiber were shown by arrows (+, +, –), arrowheads (+, +, –), and asterisks (+, +, +). D, expression of MARP2 was observed both in dMHC-positive (*) and -negative fibers (arrowheads). E, occasionally, MARP1 and MARP2 were localized in or at the periphery of central nuclei in Ttnmdm/mdm myofibers. Bars, 20 μm.
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FIGURE 5. Effects of p94 protease activity on MARP2. A, p94:WT or p94:CS was coexpressed with FLAG-I80-PEVK and/or HA-MARP2 in COS7 cells for a coimmunoprecipitation assay as described in the previous figure legends. Note that coexpression of p94:WT decreased MARP2 in both the cell lysate and coimmunoprecipitate (lanes 3 and 7, anti-HA), whereas p94:CS decreased MARP2 only in the coimmunoprecipitate (lanes 6 and 8, anti-HA). B, in immunoprecipitation, B, the lysate of SF-9 cells expressing p94:DA without (−) or with I80-PEVK or I80-PEVK(mdmd) was incubated with recombinant His-MARP2 in the presence of 5 mM Ca²⁺ and 1 mM PMSF at 37 °C. Autolysis of p94:DA and proteolysis of MARP2 and/or I80-PEVK was inhibited completely by leupeptin and E64c (lanes 5, 10, and 15) but not by calpastatin (lanes 4, 9, and 14). Open and closed arrowheads indicate the full-length and autolyzed fragments of p94, respectively.

FIGURE 6. Effect of proteolysis by μ-calpain on the MARP2-connectin interaction. A, recombinant MARP2 was proteolyzed by recombinant human μ-calpain under the indicated conditions. The open and closed arrowheads indicate μ-calpain and its autolyzed fragment, respectively. B, the N-terminal sequence of MARP2 proteolyzed by μ-calpain was determined to be Arg77 (aa in Q9GZV1) (arrow). NLS, nuclear localization signal; PEST, a region rich in Gin, Ser, and Thr flanked by Pro, which is considered to be susceptibility to calpain proteolysis; ANK, ankyrin repeat motif. C, HA-MARP2Δ1–79 coimmunoprecipitated I80-PEVK as efficiently as the full-length MARP2 did. Open and closed arrows, HA-MARP2 and HA-MARP2Δ1–79, respectively. *, non-specific signals. IP, immunoprecipitation.

and -2 were detected in or at the periphery of central nuclei in Ttn<sup>mdm/mdm</sup> (Fig. 4E). Because of low frequency, correlation between nuclear localization of MARP1/2 and the fiber type or size was not clear.

These observations demonstrate that MARP1 and 2 are not mutually exclusive as to their up-regulation in Ttn<sup>mdm/mdm</sup> muscle. The presence of myofibers in which only MARP2 and/or I80-PEVK were inhibited completely by leupeptin+E64c (lanes 5, 10, and 15) but not by calpastatin (lanes 4, 9, and 14). Open and closed arrowheads indicate the full-length and autolyzed fragments of p94, respectively. 

Relationships among p94, MARP2, and Connectin—Because both p94 and MARP2 show predominant expression in skeletal muscle and interaction with N2A connectin, the relationship between them may be deteriorated by the mdm deletion. 

Less p94:CS and MARP2 coimmunoprecipitated with I80-PEVK when both were coexpressed (Fig. 5A, IP, anti-PI32, lane 8 versus lane 10, anti-HA, lane 8 versus lane 6), indicating that p94 and MARP2 affect their interaction with connectin and with each other. Furthermore, the lesser MARP2 coimmunoprecipitated with connectin when p94:WT was used (Fig. 5A, IP, anti-HA, lanes 6–8). Considering that the amounts of...
Calpain-Connectin-MARP N2A Complex Modulated by Proteolysis

**A**

- MARP-connectin complex
- Various stresses
- Proper proteolytic functions / ON
- Muscle damage

**B**

- Aberrant signal transduction by connectin molecule

**Diagram:**
- MARP2 (N) and MARP2 (C) show the segmentation of functions of connectin.
- Connectin/titin N2A region in WT and mdm shows the effect of deletion.
- MARP2 with MARP1 shows upregulation in the nucleus.
- MARP2 and MARP1 show improper proteolytic functions leading to muscle damage.
immunoprecipitated I80-PEVK were similar regardless of coexpression of p94:WT or CS (Fig. 5A, IP, anti-FLAG, lanes 6–8) and that p94:WT protein existed much less than p94:CS, the observed decrease in coprecipitated MARP2 probably results from the proteolysis of MARP2 by p94:WT during their interactions with connectin.

Thus, proteolysis of MARP2 by p94 and the effect of connectin on it were further examined. First, purified recombinant MARP2 was proteolyzed Ca\(^{2+}\)-dependently in the Sf-9 lysate expressing p94:DA, which was inhibited by leupeptin plus E64c, but not by calpastatin (Fig. 5B, lanes 11–15, anti-MARP2). The proteolysis was not observed in the presence of p94:CS or absence of p94 (Fig. S2C). Therefore, in addition to I80-PEVK, our studies also identified MARP2 as a substrate for p94, at least in vitro.

When the Sf-9 lysate coexpressing p94:DA with I80-PEVK was used, MARP2 was proteolyzed faster than without I80-PEVK (Fig. 5B, lanes 1–3 versus lanes 11–13, anti-MARP2). Since the initial amount of p94:DA decreased when coexpressed with I80-PEVK (Fig. 5B, anti-piPS2, lane 1 versus lane 11), it was predicted that I80-PEVK, as a scaffold, expedites proteolysis of MARP2 by p94:DA. Results obtained with I80-PEVK/mdm or I80-PEVK did not differ (Fig. 5B, lanes 6–8), indicating that I80-PEVK/mdm, although resistant to proteolysis itself, does not perturb proteolysis of MARP2 by p94.

MARP2 Is Proteolyzed by \(\mu\)-Calpain at Its N-terminal Region—

The proteolytic site in MARP2 cleaved by \(\mu\)-calpain corresponded to Arg\(^{77}\) (Fig. 6, A (closed arrow, lanes 7 and 8) and B). Previously, two connectin-binding sites in MARP2 were determined: 24–42 and 188–205 (Fig. 6B, bidirectional arrows) (30). MARP2\(^{1–79}\), however, interacted with I80-PEVK as efficiently as the full-length MARP2 (Fig. 6C, lanes 2 and 3, IP, anti-FLAG), whereas the N-terminal MARP2 fragment, MARP2\(^{(1–76)}\)-enhanced green fluorescent protein, did not show detectable connectin binding (data not shown). This suggests that the second ankyrin motif is sufficient for MARP2-connectin interaction under the conditions used and that properties of MARP2 other than connectin binding are modified by proteolysis.

**DISCUSSION**

In this study, we characterized the relationships among p94, MARPs, and N2A connectin in the WT and mdm contexts. We found that 1) novel binding sites for p94 exist in the N-terminal (SpBS1) and C-terminal (SpBS2) regions adjacent to the previously identified site (PpBS); 2) preautolytic full-length p94 preferentially interacts with N2A connectin; 3) proteases, including p94 and the conventional calpains, proteolyze the N2A region of connectin at several sites (Fig. 7A, closed arrowheads), which is compromised in the fragments with the mdm deletion (Fig. 7B, open arrowheads); 4) the efficiency of binding between the connectin “is” region and MARPs is also affected by the mdm deletion.

Considering the huge molecular size of connectin, characteristics of the subfragment might provide only limited functional insights into whole connectin molecules in vivo. However, our in vitro results demonstrate for the N2A region of connectin a propensity to undergo dynamic changes imposed intramolecularly by the local molecular structures. Identification of N2A connectin and MARP2 as possible p94 substrates implicates that the N2A region of connectin serves as a versatile scaffold for p94, which stabilizes p94 and facilitates proteolysis of MARP2 by p94.

**Localization of p94 Activity and Targets**—Identification of sarcomeric proteins, such as \(\alpha\)-actinin and connectin, as p94 binding partners emphasizes the importance of these interactions to the regulation of p94, especially its stability (19, 21, 23, 25), and that p94 is also a component for signal transduction inherent in skeletal muscle structure. So far, the physiological relevance of p94 localization to the sarcomere has been unclear. Our results present three possibilities, which may be interrelated.

First, more efficient binding of the full-length p94 than its autolyzed fragment to N2A connectin can be inferred as a molecular mechanism for condensing protease activity in the proximity of p94 substrates (i.e. connectin itself and MARP2) and for releasing a remnant of enzyme after completion of p94 functions in a given context (Fig. 7A).

Second, the protection effect of p94:CS against proteolysis of N2A connectin may result from alteration of connectin local structures by p94 binding so that connectin is not proteolyzed spontaneously and/or randomly by yet unknown proteases, one of the candidates for which is conventional calpain.

Third, the negative effect of p94:CS on the MARP2-connectin interaction could alternatively control the extent of proteolysis of MARP2 by p94 as well as its functions. One of the novel p94 binding sites of connectin (e.g. SpBS1) may directly compete with MARP binding to “is”. Furthermore, the presence of several p94–connectin interactions in I80-PEVK may trigger structural changes that affect the efficiency of MARP binding.
Calpain-Connectin-MARP N2A Complex Modulated by Proteolysis

Activity of the p94-Connectin Complex in Ttn<sup>mdm/mdm</sup> Skeletal Muscle—The effect of the mdm deletion on p94 stabilities was apparent in conflict with previous results. Decrease of p94 in Ttn<sup>mdm/mdm</sup> skeletal muscle (43) corroborates the idea that the mdm deletion abrogates p94-connectin interaction required for p94 regulation. In contrast, I80-PEVK<sup>(mdm)</sup> was able to coprecipitate p94WT (Fig. 1B), and autolysis of p94:DA coexpressed with I80-PEVK<sup>(mdm)</sup> was retarded (Fig. 2C). One interpretation is that I80-PEVK<sup>(mdm)</sup> can bind p94 at SpB8s but is distinct from WT not only in its resistance to proteolysis but also in that it hinders certain aspects of p94 protease activity.

In this context, we hypothesize that the p94-connectin N2A complex represents one of functional p94 protease units, p94 as a catalytic subunit and connectin as a regulatory subunit. The observation that gait deficits found in Ttn<sup>mdm/+</sup> mice were restored by p94 overexpression indicates that the mdm deletion does not produce dominant phenotypes but causes a decrease in p94 activity (52).

Structure of Connectin Fragments Affecting Its Susceptibility to Proteolysis—The physiological relevance of connectin proteolysis previously reported (53) remains unknown to date. In cardiac myocytes, doxorubicin treatment causes degradation of connectin by m-calpain to produce a proteolyzed fragment designated T2 (54). The major proteolytic sites of connectin were suggested to be in the elastic I-band region, where N2A and its alternative splicing isoform, N2B, are located, and the adjacent PEVK region (55). μ-calpain is also reported to interact with connectin and generate proteolyzed fragments encompassing N1 (the C-terminal to Z-line) and N2A regions (56).

Proteolysis by p94 of connectin fragments corresponding to the Z-line, PEVK, and M-line regions has also been reported (16, 50). In this study, p94 was shown to target the same proteolytic site in N2A connectin as μ-calpain. Because this proteolytic site resides in “is”, the binding site for MARP2 and for p94 itself, our results provide a decisive basis for further investigation on the effect of p94 on the MARP2-connectin interaction.

We have shown that the mdm deletion caused protection from proteolysis in the region of the mdm deletion and, rather unexpectedly, within “is”. These results indicate that proteolysis of N2A connectin proceeds step-by-step, changing its structure accordingly (i.e. proteolytic sites within the “is” region are exposed only after the proteolysis between I83 and PEVK). One should, thus, consider that structures of connectin fragments used in experiments may affect the results as to the identification of proteolytic sites.

The protease(s) responsible for spontaneous proteolysis of N2A connectin in COS7 cells has not been identified; no inhibitory effect was evident for several different protease inhibitors, including leupeptin, ALLNAl, E-64d, NH<sub>4</sub>Cl, Ac-YVAD-chloromethylketone, or benzoyloxycarbonyl-1-aspart-1-yl-[(2,6-dichlorobenzyloxy)methane (data not shown).

Relationships between MARPs and Connectin with or without the mdm Deletion—Genetic studies using mice lacking MARPs suggested that MARP paralogues have functional redundancy as both structural components and signaling molecules (57). Our study, however, showed that MARPs are different in their connectin-binding activity and, accordingly, in cellular distribution. MARP1 and -2 were shown to be differently induced under the mdm condition. This is consistent with previous studies indicating that MARP1 and -2 respond differentially depending on the quantity and/or quality of “stress” (58). Multiple cellular events should control MARP-connectin interaction in muscles (59), and whether MARPs interact with connectin as a monomer, dimer, or complex with other proteins is one of the critical future issues to be investigated (49).

Studies on fiber type specificity of MARPs showed that high level expression of MARP1 is often associated with small regenerating fibers, which are labeled by embryonic MHC (corresponding to dMHC in this study), in Duchenne muscular dystrophy but not in other muscular dystrophies (60). In Ttn<sup>mdm/</sup><sup>mdm</sup> skeletal muscle, the expression of MARP1 was not detected in dMHC-positive fibers, whereas MARP2 was detected in fibers positive for sMHC or dMHC or negative for both. The same trend of MARP2 was reported in amyotrophic lateral sclerosis (38).

Altogether, these observations suggest that the molecular mechanisms underlying the induction of MARP1 and -2 in Ttn<sup>mdm/</sup><sup>mdm</sup> are distinct and that these mechanisms are different from those in other muscular dystrophy conditions. In other words, different primary causes (e.g. deletion in dystrophin or connectin) of the symptom collectively described as “dystrophy” distinguish the molecular mechanism of each symptom.

Consistent with a short life span (10–12 weeks) of Ttn<sup>mdm/</sup><sup>mdm</sup> mice, skeletal muscle from 10-week-old mice examined in this study presented advanced dystrophic symptoms. The expression trend of MARP1 and -2 observed here may be related to a stage of disease progression. One of the directions for future study is comparative analysis of the time course for expression of MARP1 and -2 in relation to muscle fiber types and sizes.

Regulated Proteolysis as a Modulator for Muscle Functions—Proteolysis of connectin in the “is” region by calpains, including p94, is predicted to release the connectin-MARP complex from the entire connectin molecule, which may desensitize the connectin-MARP interaction to respond to a stretch signal transmitted as structural changes in connectin. Since a release of the MARP2 N-terminal part upon proteolysis by μ-calpain retained binding of MARP2 to connectin, the interaction of MARP2 with molecules other than connectin could be affected. In this regard, previously reported interactions of MARP2 with YB-1 or myopalladin (42, 59), which is mediated by the N-terminal region of MARP2, represent good candidates.

As a condition where the proteolysis of sarcomeric components described above bears biological significance, the maintenance and remodeling of once established muscle tissues should be considered. This process must require coordinated events, including proteolytic dismissal of damaged proteins and reorganization of newly synthesized proteins (61–63). Together with reported functions of muscle proteins, our data suggest that the N2A complex comprising connectin, MARP2, and p94 capable of playing a key role in such a scenario.

This protease machinery is allowed to be dynamic and sensitive accordingly to cellular context because of unique proper-
ties of connectin, including its huge molecular size, intramolecular effects on its interaction with MARPs and on susceptibility to proteolysis, and the selective recruitment of p94 activity. Moreover, the proposed functions of MARPs in accommodating muscles to stress by regulating gene expression indicates a link between the N2A complex and the nucleus. Recent proteomic approaches also indicate that p94 is involved in the regulation of metabolism and protein synthesis (64, 65). The interlocking behavior of connectin with multiple activities of skeletal muscle would assure a balanced projection of p94 activating behavior on molecules of diverging functions. Perturbing this by, for example, the mdm deletion should lead to serious dysfunction of muscles.

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