Myogenic Stage, Sarcomere Length, and Protease Activity Modulate Localization of Muscle-specific Calpain*

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p94/calpain 3 is a Ca\(^{2+}\)-binding intracellular protease predominantly expressed in skeletal muscle. p94 binds to the N2A and M-line regions of connectin/titin and localizes in the Z-bands. Genetic evidence showing that compromised p94 proteolytic activity leads to muscular dystrophy (limb-girdle muscular dystrophy type 2A) indicates the importance of p94 function in myofibrils. Here we show that a series of p94 splice variants is expressed immediately after muscle differentiation and differentially change localization during myofibrillogenesis. We found that the endogenous N-terminal (but not C-terminal) domain of p94 was not only localized in the Z-bands but also directly bound to sarcomeric \(\alpha\)-actinin. These data suggest the incorporation of proteolytic N-terminal fragments of p94 into the Z-bands. In myofibrils localization of exogenous expressed p94 shifted from the M-line to N2A as the sarcomere lengthens beyond \(-2.6\) and \(2.8\ \mu\text{m}\) for wild-type and protease-inactive p94, respectively. These data demonstrate for the first time that p94 proteolytic activity is involved in responses to muscle conditions, which may explain why p94 inactivation causes limb-girdle muscular dystrophy.

p94/calpain 3 is a member of the calpain family of intracellular Ca\(^{2+}\)-requiring Cys proteases, which cleave substrates at specific and limited sites to modulate their structure and function. In contrast to the conventional \(\mu\)- and m-calpains, which are ubiquitously expressed in almost all cells (1, 2), p94 is predominantly expressed in skeletal muscle with lesser amounts of several differentially spliced variants in skeletal muscle and nonmuscle cells (3). The human p94 gene, \(CAPN3\), was identified as responsible for limb-girdle muscular dystrophy type 2A (or “calpainopathy”) (4). In mice transgenic overexpression of a protease-inactive form of p94, p94:C129S, and p94 gene knock-out caused a mild myopathy and muscular dystrophy, respectively (5–7). Furthermore, a defect in the proper proteolytic activity of p94 is a common feature of limb-girdle muscular dystrophy type 2A pathogenic mutations (8). These findings indicate that p94 protease activity is essential to maintain healthy condition of skeletal muscle. However, the specific physiological functions of p94 as a protease in skeletal muscle remain largely unknown.

Skeletal muscles contain highly organized sarcomere structures that consist of systematically expressed myofibrillar proteins. One of the earliest myofibrillar proteins expressed in vertebrate myogenic cells is connectin/titin (9), which is the largest protein molecule known and is abundantly expressed in striated muscles where it constitutes an intrasarclemic filament (10, 11). During sarcomere assembly, connectin is considered a key molecule for integration of thin filament/Z-band precursors (I-Z-I bodies) and the thick filaments, which are independently assembled in growth tips of elongating myotubes. The N-terminus of connectin is located in both I-Z-I body and mature Z, and the C terminus is in the M-line of the thick filament (12–14). A single molecule of connectin spans half of the sarcomere from Z to M to integrate the Z-band and the thick filaments, maintaining the location of thick filaments between the Z-bands.

Connectin also plays an important role as a scaffold for its specific ligands such as sarcomeric-\(\alpha\)-actinin/\(\alpha\)-actinin 2 (s-\(\alpha\)-actinin)\(^2\) (15, 16), T-cap/telethonin (17), and muscle-specific RING-finger protein-1 (MURF-1) (18) as well as p94 (19). These proteins interact with connectin at three significant regions in the sarcomeres, namely, Z-bands and N2A and M-line regions, where highly organized protein complexes form. p94 directly interacts with connectin at both N2A and M regions (19). Interaction between p94 and connectin at N2A stabilizes p94, which otherwise autolyzes very rapidly; thus, connectin may regulate p94 proteolytic functions (20, 21). p94 has been also detected in Z (19, 22), although a molecular basis for anchoring p94 in Z is unknown. Thus, p94 exists in all three

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2 The abbreviations used are: s-\(\alpha\)-actinin, sarcomeric-\(\alpha\)-actinin/\(\alpha\)-actinin 2 (s-\(\alpha\)-actinin); IS1 and IS2, specific insertion sequences 1 and 2 of p94; MHC, myosin heavy chain; NS, N-terminal specific sequence of p94; p94CS, p94:C129S protease-inactive mutant; PEVK, Pro-, Glu-, Val-, and Lys-rich spring elements of connectin; PM, proliferating presumptive myoblast; YTH, yeast two-hybrid; WT, wild type; RT, reverse transcription.
regions in sarcomeres, suggesting that p94 may contribute to myofibril organization in cooperation with other connectin ligands and/or connectin.

This study aims 1) to characterize the spatiotemporal expression and localization of p94 and its splicing variants during skeletal muscle differentiation in relation to connectin, 2) to identify p94 interacting molecules in the Z-band components, and 3) to clarify the role of the proteolytic activity of p94 in the unique context of muscle cells. Detailed immunohistochemical study using primary cultures of skeletal muscle cells revealed that p94 and its splice variants differ in their expression and localization during maturation of sarcomere structures. It was also demonstrated that correlation between localization of p94 and sarcomere lengths exists, which at least in part involves its protease activity.

EXPERIMENTAL PROCEDURES

Experimental Animals—All procedures used for experimental animals were approved by the Experimental Animal Care and Use Committee in the Tokyo Metropolitan Institute of Medical Science. C57BL/6 mice were purchased from Nihon CLEA Inc.

Preparation of Mouse Skeletal Muscle Cells—Mouse skeletal muscle cells were prepared as previously described (23). Isolated myogenic cells were cultured on Matrigel (BD Bioscience)-coated Labtech chamber slides (Nalge Nunc International Inc.) or 60-mm dishes (Asahi Techno Glass) in growth medium (20% fetal bovine serum and 0.2 mM ascorbic acid (Wako Pure Chemical Industries Ltd.) in Dulbecco’s modified Eagle’s medium). To induce muscle differentiation, the medium was switched to differentiation medium (5% horse serum (Invitrogen) and 0.2 mM ascorbic acid in Dulbecco’s modified Eagle’s medium) for further culture. All media were supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM l-glutamine (Invitrogen).

RT-PCR Analysis—Total RNA was prepared from cultured muscle cells from the quadriceps femoris and the soleus muscles of 15-week-old C57BL/6 mice using TRISOL (Invitrogen) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 5 μg of total RNA using a First Strand Synthesis kit (GE Healthcare). Primers used to detect p94, splicing variants of p94, connectin, and μ-calpain are shown in Table 1. The cycle number for PCR was 25 for all samples.

Western Blot Analysis—Cells were harvested in the presence of the homogenizing buffer (20 mM Tris/Cl (pH 8.0), 0.1 mM EDTA (pH 8.0), 1 mM dithiothreitol, 28 μM E64, 20 μg/ml soybean trypsin inhibitor, and 2 mM phenylmethylsulfonyl fluoride). The amount of protein was quantified with a DC RC protein assay kit (Bio-Rad). Equal amounts of protein from each sample were subjected to SDS-PAGE. Gels were transferred onto Immobilon-P transfer membrane (Millipore Corp.). After blocking, membranes were incubated with antibodies against p94 (anti-pIS2C antibody, 1:1000; further purified anti-pIS2 antibody (21) using the C-terminal part of the pKrich peptide (24), i.e. NH2-VKKKKNKPIIFVC-COOH), embryonic myosin heavy chain (MHC; clone F1.652, 1:50; Developmental Studies Hybridoma Bank, University of Iowa), or desmin (1:1000; Sigma). An affinity-purified rabbit anti-p94:ex15 16 antibody was generated using the KLH-conjugated peptide SVDVPVPREPHT-C, corresponding to the sequence encoded by transcript of exons 14 and 17 of the mouse p94 gene. Subsequently, membranes were incubated with peroxidase-conjugated secondary antibodies (1:10; Nichirei Inc.) followed by visualization of reacted bands using a POD immunostaining kit (Wako Pure Chemical Industries Ltd.).

Immunofluorescent Staining— Cultured cells and muscle tissues were fixed and then stained as previously described (23). For muscle tissues, longitudinal cryostat sections (7 μm) of EDL muscles from 6-month-old C57BL/6 mice were made. Antibodies used in immunofluorescence studies were affinity-purified goat anti-pIS2C antibody (1:300), mouse anti-s-α-actinin antibody (1:1000; Sigma), affinity-purified chicken anti-connectin N2A antibody (1:500 (25)), affinity-purified rabbit anti-connectin M8M9 antibody (1:300 (26)), rabbit anti-p94 NS antibody (1:300 (24)), and affinity-purified rabbit anti-pΔex15/16 antibody (1:300; this study). Alexa-488- or Alexa-555-conjugated secondary antibodies were used (Molecular Probes Inc., Eugene, OR). Nuclei were stained with TOTO3 or 4,6-diamidino-2-phenylindole dihydrochloride (Molecular Probes Inc., Eugene, OR). Specimens were analyzed on a laser scanning confocal microscopic system (LSM510; Carl Zeiss Inc.) that employed a Zeiss Axiovert inverted microscope with a Plan-Apochromat 63× (NA 1.4) lens. Images were recorded and processed with LSM510 imaging software.

Yeast Two-hybrid Screening—The cDNA sequence corresponding to the NS domain of human p94 (nucleotides 307–531 of NM_000070, amino acids 1–75) was cloned into pGBK7 (Clontech). The bait plasmid and a human skeletal muscle cDNA library (HL4010AB, Clontech) were cotransformed into Saccharomyces cerevisiae AH109 according to the manufacturer’s instructions. About 9 X 10⁶ transformants were screened on selection medium plates, SD-LWHA, at 30 °C, yielding 20 grown colonies. Prey plasmids were isolated from these colonies and subjected to DNA sequencing. Three prey

### Table 1

| Primer        | Sense (5'→3')         | Antisense (5'→3')       |
|---------------|------------------------|-------------------------|
| p94 Exon 1–Exon 24 | atcgagctcacggattcATATCCTCCCTTATTAGCTTTCC | GGGCTACACATCACGTGTTG |
| p94 Exon 4–Exon 9   | AACCACCGCAATGAGTTCTGG | TGTCCACAAAGCTCCAGTCC   |
| p94 Exon 4–Exon 17  | AACCACCGCAATGAGTTCTGG | TGTCCACAAAGCTCCAGTCC   |
| p94 Exon 10–Exon 17 | AACCACCGCAATGAGTTCTGG | TGTCCACAAAGCTCCAGTCC   |
| Connectin/titin M9M10 | AAGGTACCAACCACTGCCACGTTTATTGCAA | AAAAGCATTTTTCCAACCCCTTTTGTGCA   |
| μ-Calpain       | GTGTTGGAATACCACATTTTACGAGG | TGGTACACTCTTGAAATCC CTTTCCTTGTTGTTTGGTTTTGG |

* These primers are as same as used in Kawabata et al. (48). The additional unrelated sequences were for cloning purposes.
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**FIGURE 1. Analysis of the expression of p94 and its splicing variants during myogenesis.** A, a schematic domain structure of p94, the antigens for the antibodies, and the exon structures amplified by RT-PCR. NS, NS1, and IS2 are p94-specific insertion sequences. Domains Ia + Ib, IIa, III, and IV are the conserved calpain domains. Most of IS1 and IS2 are encoded by exon 6 and exons 15 and 16, respectively. The expected sizes of the RT-PCR products are shown. FL, full length. B, the expression of embryonic MHC (eMHC) and desmin were analyzed by Western blotting. As skeletal muscle differentiation proceeds, the expression of embryonic MHC relative to desmin decreases. C–F, RT-PCR analysis of p94 transcripts using primer sets for amplification of exons 1–24 (C), 4–9 (D), 10–17 (E), and 4–17 (F). Arrows and open arrowheads correspond to the transcripts with no exons skipped and those with exon skipping, respectively (C–F). Note that the skipping of exon 6 or exons 15 and 16 were not discernible from each other in transcripts covering exons 4–17 (F, open upper arrowhead). G, stable expression of the p94 protein in cell lysates using a p94-specific anti-p94 antibody. Full-length p94 and its proteolytic fragments are indicated by the arrow and closed arrowheads, respectively. The open arrowheads indicate p94 fragments, which could be p94:ex6, 15, and/or 16, respectively. Lanes in panels B–I: H2O, negative control; days 1, 3, 6, 10, and 13, cells differentiated for the indicated number of days; QC, Sol, and TA, quadriceps femoris, soleus, and tibialis anterior muscles, respectively, from a 15-week-old C57BL6 mouse; p94:CS and p94:ex15/16 antibody. The expected sizes of the RT-PCR products are shown.

plasmids were confirmed for their reproducibility, two of which encoded s-α-actinin (corresponding to amino acids 441–568 in ACTN2); the other encoded von Willebrand factor. Because von Willebrand factor is an extracellular protein secreted by endothelial cells (27), we did not further analyze if it indeed interacts with p94 in vivo. Thus, only s-α-actinin was further analyzed as to its interaction with p94.

**cDNA Constructs for Expression in Mammalian Cells**—The cDNA corresponding to full-length human p94, mouse p94 variants lacking exon 6, and/or exons 15 and 16 (p94ΔexX) and their protease-inactive forms, p94: C129S (p94:CS) and p94: ΔexX:CS, were cloned into pcDNA3.1/N-FLAG vector (28). The NS domain of human p94 was cloned into pEGFP-C1 (Clontech). The cDNA for human s-α-actinin 2 (nucleotides 174–2921 of NM_001103, amino acids 1–894) with an N-terminal Myc tag was cloned into pcDNA3.1 (Invitrogen).

**Immunoprecipitation**—Expression vectors for Myc-tagged human s-α-actinin and FLAG-tagged human p94 or green fluorescent protein-tagged human p94 NS were transfected into COS7 cells. Cells were harvested 72 h after electroporation and then homogenized in lysis buffer (0.5% digitonin, 50 mM CsCl, 10 mM triethanolamine, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin (pH 7.8)). Cell lysates were centrifuged at 20,000 × g for 15 min, and the supernatants preincubated with protein G-Sepharose (GE Healthcare) to absorb nonspecific binding proteins. After centrifugation, the supernatants were incubated with anti-Myc antibody (9E10; Developmental Studies Hybridoma Bank), anti-FLAG M2 antibody (Clontech), or anti-green fluorescent protein antibody (IL-6, Clontech). After incubation with protein G-Sepharose for 60 min, which was omitted when ANTI-FLAG affinity gel was used, immunocomplexes were collected by centrifugation. The resin was washed with an excess amount of lysis buffer followed by elution of immunoprecipitated proteins with SDS sample buffer.

**Transfection into Primary Skeletal Muscle Cells**—Transfection was performed using TransIT-LT1 transfection reagent (Mirus Inc.). On the indicated day, cells were fixed and analyzed by immunofluorescent staining. Quantitative comparison of different sarcomeric localization was performed by the following formula: M-line (N2A region)/total number of counted sarcomeres. Sarcomere length was measured as the distance between the centers of adjacent M-lines, which is positive for connectin M8M9, using Zeiss laser scanning con-
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**RESULTS**

**Transcription of the p94 Gene during Myofibrillogenesis**—Expression of p94/calpain 3 and its splicing variants during myofibril development was analyzed by detecting corresponding transcripts in primary mouse skeletal muscle cell culture. Because previous studies have revealed that mouse embryonic muscles express p94 variants without exons 6, 15, and/or 16 (29), the relative abundance of transcripts resulting from those exon-skipping events was examined (Fig. 1, A–F). Differentiation of cultured cells was confirmed by decreased expression of embryonic MHC compared with the expression of desmin (Fig. 1B) and by the formation of sarcomere structures (see below). In proliferating presumptive myoblasts (PM), i.e. cells before the onset of differentiation, p94 and its splicing variants were hardly detected (Fig. 1, C–F, PM). Predominating among the tested p94 splice isoforms, transcripts for full-length p94 (Fig. 1A, FL) became detectable immediately after induction of muscle differentiation and was observed throughout muscle maturation (Fig. 1, C–F, arrows). Splice isoforms lacking exon 6 (Fig. 1A, Δex6) were recognized from days 1–13 (Fig. 1D, open arrowhead). Splice isoforms lacking exons 15 and 16 (Fig. 1A, Δex15/16) were also observed from days 1–13, but the amount relative to the transcripts with exons 15 and 16 (Fig. 1A, ex15/16+) was very low (Fig. 1E, open arrowhead). The expression of variant transcripts without exons 6, 15, and 16 (Fig. 1A, Δex6/15/16) was slightly detected at day 3 (Fig. 1F). All tested adult skeletal muscles only expressed a full-length p94, i.e. no exon-skipping variants were detectable (Fig. 1, C–F, QC and Sol). In contrast, transcripts of the μ-calpain large subunit gene showed constant expression in all samples (Fig. 1G), consistent with its proposed nature as a housekeeping gene (30).

**Changes in the Protein Amounts of p94 Isoforms during Myofibrillogenesis**—Next, the expression of protein products corresponding to the p94 transcripts detected above was examined. No 94-kDa full-length p94 protein was detected in cells cultured under proliferating conditions (Fig. 1H, PM). Immediately after the induction of differentiation, full-length p94 was detected (Fig. 1H, arrow), and the amounts of protein were not down-regulated during myofibrillogenesis. In addition, the amounts of protein in the focal microscopic imaging software. Calculated ratios were resorted according to sarcomere lengths. In cultured cells expressing p94WT, p94CS, p94Δex6:WT, and p94Δex6:CS, 108, 107, 47, and 86 sarcomeres, respectively, were counted.

**FIGURE 2. Expression of transcripts corresponding to the p94 binding sites of connectin in the N2A and M-line regions.** A, a single connectin molecule spans the Z-band to the M-line. The locations of the amplified regions in connectin by RT-PCR are schematically represented in sarcomere structure (bars). The double-headed arrows correspond to the p94 binding sites. The antigens for the anti-connectin N2A and M8M9 antibodies are indicated by brackets. I and M together with the numbers indicate the immunoglobulin motifs in the I-band and the M-line, respectively; PEVK, kinase, and is7 indicate the PEVK region, the kinase domain, and the insertion sequences, respectively. Expression of transcripts corresponding to the p94 binding sites in the N2A region (B) and the M-line (C) was analyzed by RT-PCR. The arrows and the open arrowhead indicate transcripts corresponding to the transcripts with and without the p94 binding region as shown in A. QC and Sol, quadriceps femoris and soleus, respectively.
Localization of p94 during Early Myofibrillogenesis—To clarify when p94 is incorporated into specific positions within myofibrils, localization of p94 in cultured myotubes at different stages was analyzed. At day 3, s-α-actinin was observed as punctuated pattern, i.e. the I-Z-I bodies, in emerging growth tips where de novo synthesized myofibrillar proteins are assembled into sarcomere structures (14, 33) (Fig. 3A, bracket), and Z-band striations were seen in the shaft of the same myotubes (Fig. 3A, arrows). Conversely, using an anti-pIS2C antibody, p94 was detected as small granules in the cytosol of whole myotubes without colocalization with s-α-actinin (Fig. 3B, E, and H).

The relationship between p94 and connectin localization was explored by staining myotubes using two different anti-connectin antibodies. The anti-N2A antibody (antigen map in Fig. 2A) detected grainy signals in the growth tips and striated patterns in the shaft of some myotubes (Fig. 3D, bracket and arrows). An anti-M-line connectin (M8M9, antigen map in Fig. 2A) antibody detected striated signals in the shaft of myotubes but none in the growth tips (Fig. 3G, arrows and bracket). No colocalization of p94 with these two regions of connectin was observed (Fig. 3F, E, and H). At this stage, p94 signals were also detected in myonuclei (Fig. 3B and H). In summary, p94 was not localized in the Z-bands including the I-Z-I bodies, in the N2A regions, or in the M-lines at this stage, whereas s-α-actinin and connectin were already organized in striated patterns.

was expressed at all stages examined (Fig. 2B). The 559-bp transcripts corresponding to the M-line region with is7 (is7+) were also constantly expressed (Fig. 2C). The 256-bp bands encoding is7-lacking molecules (is7−) were faintly detected in cultured cells. Consistent with previous reports on muscle fiber-type-dependent expression of is7 (12); both is7+ and is7− variants were expressed at similar levels in the quadriceps femoris, which predominantly consists of fast twitch myofibers, whereas is7+ predominated in the soleus, composed of both fast and slow myofibers (Fig. 2C). These results indicated that the two p94 binding sites in connectin are almost constantly provided during muscle differentiation.

Cytosolic but Not Sarcomeric Localization of p94—Colocalization of p94 with connectin at the N2A region or the M-line in the late but not in the early stage of myofibrillogenesis. Cultured skeletal muscle cells at day 3 (A–I) and day 10 (J–R) were stained with the anti-pIS2C antibody (B, E, H, K, N, and Q) in combination with one of the antibodies against s-α-actinin (A and J), connectin N2A (D and M), or connectin M8M9 (G and P). Nuclei were stained with TOTO3 (blue, A–I). Merged images are shown at the right (C, F, I, L, O, and R). Brackets indicate the areas of the growth tips. At day 3, s-α-actinin showed stress fiber-like structures in a growth tip and the Z-band striation (arrows) in the shaft of myotubes (A and Q). N2A (D and F) and M-line (G and I) striations were observed in the shaft of myotubes (arrows), p94 was detected in a granular structure in the cytosol and was not colocalized with s-α-actinin and connectin N2A and M-line regions (A–I), p94 signals were also detected in myonuclei (B, C, H, and I). At day 10 myotubes, arrowheads and brackets indicate p94 localization, whereas arrows indicate the localization of s-α-actinin (J and L), connectin N2A (M and O), and connectin M8M9 (P and R). Insets show magnified images (J–R), Bars, 10 μm.

FIGURE 3. Colocalization of p94 with connectin at the N2A region or the M-line in the late but not in the early stage of myofibrillogenesis. Cultured skeletal muscle cells at day 3 (A–I) and day 10 (J–R) were stained with the anti-pIS2C antibody (B, E, H, K, N, and Q) in combination with one of the antibodies against s-α-actinin (A and J), connectin N2A (D and M), or connectin M8M9 (G and P). Nuclei were stained with TOTO3 (blue, A–I). Merged images are shown at the right (C, F, I, L, O, and R). Brackets indicate the areas of the growth tips. At day 3, s-α-actinin showed stress fiber-like structures in a growth tip and the Z-band striation (arrows) in the shaft of myotubes (A and Q). N2A (D and F) and M-line (G and I) striations were observed in the shaft of myotubes (arrows), p94 was detected in a granular structure in the cytosol and was not colocalized with s-α-actinin and connectin N2A and M-line regions (A–I), p94 signals were also detected in myonuclei (B, C, H, and I). At day 10 myotubes, arrowheads and brackets indicate p94 localization, whereas arrows indicate the localization of s-α-actinin (J and L), connectin N2A (M and O), and connectin M8M9 (P and R). Insets show magnified images (J–R), Bars, 10 μm.
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**FIGURE 4. Localization of the p94 isoform p94Δex15/16 in the sarcomere.** In cultured myotubes at day 6, localization of p94Δex15/16 was examined using an anti-pΔex15/16 antibody (B, E, and H) in combination with an anti-s-α-actinin antibody (A, D, and G). Merged images are shown at the right (C, F, and I). D–I are these images at higher magnification. Localization of p94Δex15/16 was observed as thick stripes at the Z-bands (E and F, arrowheads), as dotted lines between the Z-bands (E and F, arrowheads), and doublets around the Z-bands (H and I, closed arrowheads). p94Δex15/16 also accumulated as small particles in the cytosol of the shafts of juvenile myotubes (asterisk). Arrows indicate the Z-band localization of s-α-actinin. In the merged image, nuclei (blue) were stained with 4,6-diamidino-2-phenylindole dihydrochloride (C). Bars, 10 μm.

**p94 Is Targeted to the N2A Regions and the M-lines in the Later Stages of Myofibrillogenesis.—**At day 6 of culture, more than 90% of myotubes showed typical myofibrillar protein localization, with s-α-actinin, connectin N2A, and M8M9, at their proper locations; that is, Z, N2A, and M, respectively. p94 was still detected in the cytosol as granular structures, as seen at day 3, in more than 80% of myotubes, and infrequently, the localization of p94 precisely coincided with the localization of connectin M8M9.

At day 10, embryonic MHC was hardly detected in cultured cells by Western blot analysis (Fig. 1B), indicating that the cells had become almost fully mature myotubes. In these mature myotubes, in addition to the cytosolic distribution, two main types of p94 positive structures were observed. One was M-line striations coinciding with M8M9 signals (Fig. 3, P–R, arrowheads and arrows). The other was in the I-bands, possibly in the N2A regions (Fig. 3, M–O, brackets and arrows). Intensity in the M-lines was stronger than that in the I-band staining. Localization of p94 in Z was not significant (Fig. 3, J–L). These results indicate that p94 is incorporated into myofibrils after the sarcomere structures are fully mature, through association with above mentioned regions of connectin.

**Distinct Localization of p94 Isoforms in Sarcomeres.—**As shown above, expression of p94Δex15/16 was restricted in developing myotubes (see Fig. 1, E, F, and I). Thus, the localization of p94Δex15/16 was examined in cultured myotubes at day 6 (Fig. 4, A–C). The anti-pΔex15/16 antibody captured fuzzy thick striations, which almost overlapped with the s-α-actinin signals at Z (Fig. 4, E and F, brackets) or doublets around Z (Fig. 4, H and I, arrowheads). These data indicate that p94Δex15/16 is probably localized to N2A or somewhere between Z and N2A. In addition, p94Δex15/16 was found in M (Fig. 4E, open arrowheads). Contrary to the anti-pIS2C antibody results, the anti-pΔex15/16 antibody stained N2A/Z more strongly than M. These results suggest that in developing myotubes, not only the expression but also the localization of p94 isoforms are regulated in a way distinct from that for the full-length p94.

The p94 NS Domain Is Localized in the Z-Bands.—As shown previously, p94 was detected in Z, especially by antibodies against the p94 N terminus (19, 22). However, clear Z localization of p94 was not detected in cultured skeletal muscle cells with the anti-pIS2C antibody. When the cells at day 6 of culture were stained using the anti-pNS antibody, which was elicited against the N terminus of p94 (NS region), the Z-band structure was detected in the shaft of relatively mature myotubes (Fig. 5, A–C, arrows and arrowheads), indicating that a p94 fragment containing at least the NS region exists in Z. In contrast, much less if any staining at N2A and M was obtained with this antibody. The anti-pNS antibody also detected small granular structures in the cytosol of myotubes (Fig. 5B), which were not colocalized with s-α-actinin.

The localization of p94 in adult skeletal muscles was also examined with anti-pIS2C and anti-pNS antibodies. Anti-pIS2C antibody preferentially labeled N2A (Fig. 5E, closed arrowheads), leaving Z less intensely stained (asterisk), whereas both Z and N2A were stained with the anti-pNS antibody (Fig. 5H, closed arrowheads and asterisk). In both cases, signal intensity was less in M (Fig. 5, E and H, open arrowheads). In summary, p94 is localized in Z, N2A, and M in both maturing muscle cells and skeletal muscle tissues. It is noteworthy, however, that antibodies raised against distinct regions of p94 showed different propensity for staining in those three regions, suggesting that some proteolytic fragments of p94 are associated with the sarcomere structure.

**s-α-Actinin Binds to p94 through the NS Region.—**To identify p94 binding molecules, yeast two-hybrid (YTH) screening was carried out. A middle region of s-α-actinin (corresponding to amino acids 441–568 in ACTN2) was identified from the human skeletal muscle cDNA library as a binding partner for the p94 NS region. Using a series of truncation mutants of s-α-actinin, it was shown that the original clones identified by the screening encoded the minimal binding site for the p94 NS region, which corresponds to the region spanning the C- and N-terminal halves of the second and third spectrin-like repeats, respectively (Fig. 5).

Because interaction between the full-length molecules of p94 and s-α-actinin was not detected in YTH, coimmunoprecipitation analysis was carried out. Myc-tagged s-α-actinin and FLAG-tagged p94CS, a proteolytically inactive missense (C129S) mutant, were coexpressed in COS7 cells and subjected to immunoprecipitation with either anti-Myc or anti-FLAG antibody. Interaction between p94 and s-α-actinin was demonstrated in both immunoprecipitations (Fig. 6, A and B). The p94...
The NS region fused to green fluorescent protein was also shown to interact with s-α-actinin (Fig. 6, C and D). These results indicate that p94 directly interacts with s-α-actinin via the NS region, which comprises at least a part of the mechanism for its Z localization.

Protease Activity-independent Targeting of p94 to Specific Positions in Myofibrils—It was of interest whether the proteolytic activity and/or the molecular structure of p94 affects its targeting to the specific positions in the sarcomeres. Therefore, FLAG-tagged p94 and its splicing variants with and without proteolytic activity were expressed in cultured skeletal muscle cells to examine their localization. Six days after transfection, cells expressing FLAG-tagged wild-type p94 (p94WT) gave signals one-third to one-tenth less than those observed for cells expressing protease-inactive FLAG-p94:C129S (p94CS). This is possibly due to p94WT disappearing by autolysis more rapidly than p94CS and/or that the excess amount of expressed p94WT is cytotoxic for myotubes. Both p94WT and p94CS were mainly incorporated into the M-lines (Fig. 7, B, E, and H, closed arrowheads). Other p94 variants, such as p94/H9004ex6, p94/H9004ex15/16, and p94/H9004ex6/15/16, with and without the CS mutation showed trends essentially identical to those of p94WT and p94CS. Myotubes expressing excess amount of p94CS and other inactive variants did not show any defect in assembling striated myofibrils. These results indicate that exogenously expressed p94 and its variants are targeted to the same specific position in the myofibrils as is endogenous p94 and that proteolytic activity was not required for the process.

Stretch and Protease Activity Dependence of Myofibrillar Targeting of p94—To compare the behavior of p94WT and p94CS in a more dynamic aspect, the localization of exogenously expressed p94 was evaluated in relation to sarcomere lengths, i.e., the extent of contraction/extension. p94WT and p94CS were preferentially targeted to M in short, contracted sarcomeres.

FIGURE 5. Z-band localization of p94 and its interaction with s-α-actinin at the NS domain. A–C, localization of p94 was examined in cultured myotubes at day 6 using an anti-pNS antibody. p94 colocalized with s-α-actinin at Z (arrowhead, B and C). D–I, in longitudinal cryostat sections of EDL muscles from 6-month-old C57BL/6 mice, p94 was detected with anti-pS2C (E) or anti-pNS (H) antibodies, and Z was detected with an anti-s-α-actinin antibody (D and G, arrowheads). In adult muscles, in contrast to day-6 myotubes, p94 was observed in both N2A and M (E, F, H, and I, closed and open arrowheads, respectively) with both anti-pS2C and anti-pNS antibodies. p94 was also detected in Z using an anti-NS antibody (H and I, *). Bars, 10 μm. J, a schematic for the domain structure of s-α-actinin and its interaction with p94 NS as detected by YTH assay. s-α-Actinin consists of an N-terminal actin binding domain, four spectrin-like repeat domains, and tandem EF-hand motifs in the C-terminal region. The p94 NS binding site in s-α-actinin was located between the centers of the second and third spectrin-like repeat domains (amino acids 441–568). K, ribbon presentation of the s-α-actinin spectrin-repeat region. The two antiparallel subunits of the s-α-actinin spectrin-repeat region are depicted in blue and green. Minimum p94 binding region (amino acids 441–568) is shown in red or pink. Ribbon diagrams were generated using Molfeat Version 2.2 three-dimensional imaging software (FiatLux Inc, Tokyo, Japan).
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In this study the distinctive features of the expression and localization of p94 and its splicing variants during myofibrillogenesis in the primary culture of skeletal muscle cells and a novel interaction between the p94 NS region and s-α-actinin are shown. Furthermore, we have shown that the localization of exogenously expressed p94 changed in correlation with sarcomere length, with p94 proteolytic activity required for this response. This potentially indicates that cellular localization of p94 and possibly of its proteolytic activity is regulated in a stretch-dependent manner.

DISCUSSION

In this study the distinctive features of the expression and localization of p94 and its splicing variants during myofibrillogenesis in the primary culture of skeletal muscle cells and a novel interaction between the p94 NS region and s-α-actinin are shown. Furthermore, we have shown that the localization of exogenously expressed p94 changed in correlation with sarcomere length, with p94 proteolytic activity required for this response. This potentially indicates that cellular localization of p94 and possibly of its proteolytic activity is regulated in a stretch-dependent manner.

Data showing that NS binds to s-α-actinin and connectin, is detected, but p94 is predominantly distributed in the cytosol as small granules. This observation raises at least two questions worthy of further investigation. One is what molecules other than unproteolyzed p94 are contained in these granules, as the signals (detected by both anti-pNS and -pIS2C antibodies) were distinct from the diffuse staining of soluble enzymes. Another is how targeting of p94 to Z, N2A, or M is protected at this developmental stage even if proteosomes are incorporated into sarcomeres; N- and C-terminal fragments are incorporated into sarcomeres (III). Later, p94 isoforms are mainly targeted to Z and N2A, whereas NS- and IS2-containing fragments, respectively, accumulate in M/N2A and Z. (IV). In adult tissues both NS- and IS2-containing p94 is found in N2A, but no p94 isoform was detected in the sarcomeres (IV). Previously, the IS2 epitope was detected at both Z and N2A in adult myofibrils (19), which is different from the results shown here; that is, localization mainly at N2A and faintly at M. This is probably because a more specific, affinity-purified anti-pIS2C antibody was used in this study.

Regulation of p94 in the Cytosol on Its Autolysis and Targeting to Z/N2A/M — p94 targets to Z/N2A/M after the assembly of basic sarcomere components. By day 3 of differentiation of cultured myotubes, clear striation of Z, N2A, and M, containing s-α-actinin and connectin, is detected, but p94 is predominantly distributed in the cytosol as small granules. This observation raises at least two questions worthy of further investigation. One is what molecules other than unproteolyzed p94 are contained in these granules, as the signals (detected by both anti-pNS and -pIS2C antibodies) were distinct from the diffuse staining of soluble enzymes. Another is how targeting of p94 to Z, N2A, or M is protected at this developmental stage even if proteosomes are incorporated into sarcomeres; N- and C-terminal fragments are incorporated into sarcomeres (III). Later, p94 isoforms are mainly targeted to Z and N2A, whereas NS- and IS2-containing fragments, respectively, accumulate in M/N2A and Z. (IV). In adult tissues both NS- and IS2-containing p94 is found in N2A, but no p94 isoform was detected in the sarcomeres (IV). Previously, the IS2 epitope was detected at both Z and N2A in adult myofibrils (19), which is different from the results shown here; that is, localization mainly at N2A and faintly at M. This is probably because a more specific, affinity-purified anti-pIS2C antibody was used in this study.
In the same day 6 culture, exogenously expressed FLAG-p94WT and -p94CS showed almost identical localization, mainly in N2A/M rather than in Z. This suggests that p94 proteolytic activity is not required for p94 targeting and that p94 molecules detected at N2A/M possess both NS and IS2 regions; these are undegraded p94 molecules. It may be possible that the affinity of NS to Z is blocked by the extra FLAG-tag sequence, allowing most of the nonproteolyzed FLAG-p94 to be targeted to N2A/M. Still, there should be a mechanism in the cytosol so that exogenously expressed p94 does not exhaus-tively autolys.

In more mature myofibrils, at day 10 in culture and in adult skeletal muscle, NS and/or IS2 epitopes become detectable in Z, N2A, and M. Thus, it is suggested that mature muscle cells facilitate more efficient targeting of nonproteolyzed p94 to those three sites in the sarcomeres.

Altogether these results suggest the existence of unidentified mole-cules/mechanisms that regulate the activity of p94 in the cytosol. Because p94Δex proteins are expressed in amounts similar to p94 at the early stages of myotube development, it is tempting to consider that the interaction between p94 and p94Δex is one of the mechanisms that regulates p94 in both its protease activity and targeting. Later, in mature muscle cells where sar-comere structures gain more integrity and complexity, other mecha-nisms might allow rapid targeting of p94 to myofibrils and/or suppress its autolysis until being targeted to myofibrils.

Regulated Expression of p94Δex during Muscle Development and Its Relevance—One of the p94 variants, p94Δex15/16, was shown to be transiently expressed and predominantly incorporated into N2A only in immature myotubes but not in adult skeletal muscles. Developmental changes of p94Δex/p94 ratios at Z, N2A, M, and in the cytosol are schematically shown in Fig. 9B. Interestingly, we detected larger amounts of p94Δex at N2A than at M, whereas full-length p94 is predominantly at M. This may indicate that p94Δex has higher affinity to N2A than M at this stage. It is possible that the disappearance of p94Δex from the N2A site enables p94 to gain access to it, which is important for myofibrils to mature fully.

In transgenic mice overexpression of p94Δex6 or p94Δex15, but not full-length p94WT, blocked/delayed maturation of myofibers (34). These variants, which were shown to be less active than p94 especially with regard to autolytic activity (29), could act detrimentally at various developmental stages. Thus, these previous data and our data are in support that both the transient expression and the disappearance of p94 isoforms at the early stages of myogenesis are important for myotube maturation, probably because these isoforms respond differently to the cellular mechanisms regulating p94. The differences in the threshold sarcomere lengths for the shift of localization from M to N2A, 2.6 and 2.2 μm for p94WT and p94Δex6 WT, respectively, could be one indication of these effects.
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Interaction between p94 and s-α-Actinin—By YTH screening, the middle region of s-α-actinin, which encompasses the junction of two consecutive spectrin repeats and binds to the NS region of p94, was identified as the long sought binding partner for p94 in Z. Direct interaction between s-α-actinin and NS as well as full-length p94 was detected in COS7 cells. In yeast, however, the full-length s-α-actinin did not bind to p94, probably because full-length s-α-actinin tightly forms homodimers (35) and/or YTH sometimes shows false negative results. It should be noted that s-α-actinin showed a complicated fashion when binds to p94; i.e. s-α-actinin fragments (amino acids 401–894 and 430–894) did not bind to p94 NS region even though these fragments contain the region able to bind p94 (amino acids 441–568). Similar to our results, it was reported that s-α-actinin showed intricate patterns when interacting with connectin (36). Therefore, although further study is required for determination of the precise p94 binding site in s-α-actinin, minimum p94 binding region so far determined is the C-terminal half of R2 plus the N-terminal half of R3 (Fig. 5K, red or pink region).

s-α-Actinin is one of the major components of Z (37) and has multiple binding molecules, such as α-actin (38), the N-terminal Z-repeat region of connectin (15, 16), myopalladin (39), FATZ1/calsarcin-2/myozenin-1 (40), calsarcin-3/myozenin-3 (41), and cypher/ZASP/Oracle (42). Our finding of interaction between NS and s-α-actinin suggests that p94 also contributes to the integrity of Z. Consistently, previous studies showed that p94 antisense oligonucleotide treatment disrupted Z-band structures and diminished s-α-actinin detection (43). Because the NS but not IS2 epitope was preferentially localized in Z during myofibrillogenesis, the N-terminal proteolyzed fragment(s) of p94 could be important for this aspect.

p94 Localization Is Affected by the Sarcomere Length and Its Proteolytic Activity—The dynamic and distinct localization of exogenously expressed p94 relative to sarcomere length sug-
FIGURE 9. Expression and localization of p94 in the context of myofibril development and sarcomere function. A, developmental changes in the expression and localization of p94 and its isoforms are summarized. I, at the early stage of myofibrillogenesis, the N and C termini of connectin are associated with I-Z-I bodies and thick filaments, respectively. At this stage p94 and its isoforms are distributed in the cytosol (Cyt) as small punctuate structures. II, the sarcomere scaffold is assembled; that is, interdigitating Z, N2A, and M structures are formed. Most of the p94 remains in the cytosol, with some populations of p94 and isoforms targeted to Z and/or N2A/M. III, later, p94 is incorporated into Z, N2A, and M. p94 isoforms tend to be targeted to N2A rather than to M. IV, in adult skeletal muscles, p94 isoforms were absent, and p94 localizes in Z/N2A/M. The structures of p94 and its isoforms are schematically shown; the gray domain might be proteolytically removed. Localization and relative ratios of p94 and its isoforms at specific regions and the signals with each antibody are qualitatively summarized. B, qualitative profiles of p94Δex/p94 ratios in the four positions during myofibrillogenesis as discussed in A is shown. C, a proposed model of p94 translocation in response to over and under stretching of muscle. In the range of normal contraction-stretching conditions, exogenously expressed p94 is located in the M-lines. When sarcomeres are over-stretched, p94 accumulates in the N2A regions, being released from the M-lines and/or recruited from cytosol.
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shown to proceed by “nicking” in NS, IS1, and/or IS2 (21), with the reaction in IS2 regulated by N2A. These proteolytic modifications of p94 itself possibly result in a more “movable” conformation for p94, altering its affinity to connectin and other molecules. Consistent with this idea, exogenously expressed p94 and p94xex6, which show different autolytic profiles (29), were slightly different in their propensity to change localization between M to N2A. At present, one explanation for the sluggish but not absent response of p94CS, devoid of autolytic activity, to sarcomere length changes is that in our cultured myotubes endogenous wild-type p94 can intramolecularly proteolyze p94CS. This is consistent with previous reports that p94 null mice showed more severe phenotypes than did transgenic mice overexpressing p94CS (5, 7). To provide more insight into the physiological functions of p94, what property of p94 is involved in its response to sarcomere conditions, and how it is achieved should be elucidated.

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