Possible Regulation of the Conventional Calpain System by Skeletal Muscle-specific Calpain, p94/Calpain 3*

Received for publication, August 8, 2003, and in revised form, October 1, 2003
Published, JBC Papers in Press, November 1, 2003, DOI 10.1074/jbc.M308789200

Yasuko Ono‡§, Kazumi Kakinuma‡§, Fukuyo Torii‡, Akihiro Irie‡, Kazuhiro Nakagawa‡§, Siegfried Labeit‡, Keiko Abe‡, Koichi Suzuki**‡‡‡, and Hiroyuki Sorimachi‡§

From the ‡Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-8657, S-CREST, Japan Science and Technology, Kawaguchi 332-0012, Japan, Institut für Anästhesiologie und Operative Intensivmedizin, Universitätsklinikum Mannheim, Mannheim 68167, Germany, and **Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan

p94 (also called calpain 3) is the skeletal muscle-specific calpain and is considered to be a “modulator protease” in various cellular processes. Analysis of p94 at the protein level is an urgent issue because the loss of p94 protease activity causes limb-girdle muscular dystrophy type 2A. In this study, we enzymatically characterized one alternatively spliced variant of p94, p94exons 6–15–16 (p94Δ), which lacks two of the p94-specific insertion sequences. In contrast to p94, which has hardly been studied enzymatically due to its rapid, thorough, and apparently Ca2+-independent autolytic activity, p94Δ was stably expressed in COS and insect cells. p94Δ showed Ca2+-dependent caseinolytic and autolytic activities and an inhibitor spectrum similar to those of the conventional calpains. However, calpastatin did not inhibit p94Δ and is a substrate for p94Δ, which is consistent with the properties of p94, presenting p94 as a possible regulator of the conventional calpain system. We also established a semi-quantitative fluorescence resonance energy transfer assay using the calpastatin sequence specifically to measure p94 activity. This method detects the activity of COS-expressed p94 and p94Δ, suggesting that it has potential to evaluate p94 activity in vivo and in the diagnosis of limb-girdle muscular dystrophy type 2A.

Calpain (EC 3.4.22.17, clan CA, family C2) is a Ca2+-requiring cysteine protease representing one of the most important families of the cysteine proteases (1–9). To date, various molecules showing significant similarity to the calpain protease domain have been identified in almost all kinds of living organisms and constitute the “calpain superfamily” (6). Two representative members, μ- and m-calpains, the so-called “conventional” calpains, are ubiquitously expressed and have been well characterized. These two calpains consist of a distinct larger catalytic subunit containing a protease domain (μ- or m-calpain large subunit, abbreviated as μCL or mCL, respectively) and a common smaller regulatory subunit (abbreviated as 30K according to its molecular weight). On the basis of amino acid similarities, the large and small subunits have been described as consisting of four and two domains, respectively, which agrees with the recently resolved three-dimensional structure of m-calpain (10, 11) (Fig. 1A).

Conventional calpain has a specific endogenous proteinase inhibitor, calpastatin (12). Calpastatin contains four repetitive inhibitory units, each of which inhibits equimolar amounts of conventional calpain. The conserved reactive site interacts with the calpain protease domain, whereas the flanking α-helical regions bind to domains IV and VI of the large and small subunits, respectively. Synthetic oligopeptides (see Fig. 4D) corresponding to the calpastatin-reactive site specifically inhibit conventional calpain efficiently, although their inhibitory activity is weaker than that of the full-length inhibitory unit.

The primary structure of p94 (also called calpain 3) is very similar to those of μCL and mCL throughout the entire molecule, beyond the p94-specific sequences NS, IS1, and IS2 (13). Previous studies have revealed several unique characteristics of p94 that diverge greatly from those of the conventional calpains. For instance, 1) p94 undergoes very rapid, thorough, and apparently Ca2+-independent autolysis in solution (half-life in vitro is less than 10 min) (14); 2) inhibitors of the conventional calpains, including calpastatin, EDTA, and EGTA, have no effect on p94 autolysis (14); 3) the gene for p94 produces several alternatively spliced products (15, 16); and 4) p94 associates with the N2 line region and the C terminus of connectin/titin, the gigantic filamentous molecule essential for myofibrils (17, 18); for a review of connectin/titin, see Refs.

* This work was supported in part by a grant-in-aid for scientific research on priority areas (cell cycle) from the Ministry of Education, Science, Sports and Culture, a grant-in-aid for scientific research and research fellowships for young scientists from the Japan Society for the Promotion of Science, Research Grant 14B-4 for Nervous and Mental Disorders from the Ministry of Health, Labor and Welfare, the Deutsche Forschungsgemeinschaft Grant La668/7-1, and “Ground-based Research Announcement for Space Utilization” promoted by the Japan Space Forum. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Glyco-Chain Functions Laboratory, Frontier Research System, Riken, Wako 351-0198, Japan.
‡ Present address: New Frontiers Research Laboratories, Toray industries Inc., Kamakura 248-8555, Japan.
§§ To whom correspondence should be addressed: Laboratory of Biological Function, Dept. of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, the University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. Tel./Fax: 81-3-5841-8118; E-mail: ahsori@mail.ecc.u-tokyo.ac.jp.

1 The abbreviations used are: μCL, μ-calpain large subunit; mCL, m-calpain large subunit; p94Δ, p94exons 6–15–16; LGMD2A, limb-girdle muscular dystrophy type 2A; CBB, Coomassie Brilliant Blue; 2-ME, 2-mercaptoethanol; BSA, bovine serum albumin; CSTN, calpastatin; ECFP, enhanced cyan fluorescence protein; EYFP, enhanced yellow fluorescence protein; FRET, fluorescence resonance energy transfer; PMSF, phenylmethylsulfonyl fluoride; AEBSP, 4-[2-aminoethy]-benzenesulfonyl fluoride; Tricine, N-2-hydroxy-1,1-bis(hydroxy-methyl)ethylglycine; HSP, heat shock proteins; Z, benzoyloxy carbonyl.

This paper is available on line at http://www.jbc.org
19–21). The predominant expression of p94 in skeletal muscle, where its mRNA levels are ~10 times higher than those of µCL and mCL, indicates the physiological importance of p94 in that tissue (13). Consistent with this, a defect in the p94 gene causes limb-girdle muscular dystrophy type 2A (LGMD2A), suggesting that p94 functions are indispensable for proper muscle functions (22). Several studies, including ours, indicate that the loss of substrate processing activity, but not hyperactivation or a defect in the structural properties of p94, causes LGMD2A (16, 23–26). Therefore, it has become an urgent issue to determine the in vivo substrates of p94 to gain insight into the physiological functions of p94 and its relationships to molecular mechanisms of LGMD2A (27).

However, the autolytic activity of p94 has hampered the study of the p94 protein. Therefore, we have focused on identifying the conditions that will allow us to analyze the proteolytic activity of p94. We have observed previously that deletion of either the IS1 or IS2 region, which is mainly encoded by exon 6 or exons 15 + 16, respectively, prevents the rapid autolysis of p94 (14). In accord with this, we have recently shown that alternatively spliced variants of p94 that lack either exon 6, exons 15 + 16, or all of these are detected as non-autolyzed forms, unlike the full-length p94, when they are recombinantly expressed in COS cells (16).

In this study, we chose one of the splicing variants, p94:exons 6′-15′-16′ (abbreviated as p94∆ in this paper, see Fig. 1A), which is produced from a transcript lacking exons 6, 15, and 16 and therefore lacks most of the p94-specific insertion sequences, IS1 and IS2, for in vitro enzymatic characterization on the basis of its stability and expression efficiency. The enzymatic properties of p94∆ were compared with those of p94, and µ- and m-calpain.

We thus identified several novel enzymatic properties of p94. Notably, neither p94 nor p94∆ is inhibited by calpastatin, and moreover, both hydrolyze calpastatin. These results led us to predict that p94 participates in the regulation of the conventional calpain-calpastatin system in skeletal muscle. We used these findings to establish a calpastatin-based fluorescence assay specifically to measure p94 protease activity, showing a possibility of application of this assay as a diagnostic tool for LGMD2A.

**EXPERIMENTAL PROCEDURES**

**Materials**—Enzymes used for manipulating recombinant DNA were purchased from Takara Shuzo Inc. (Kyoto, Japan). The cDNAs for mouse p94/calpain 3 and p94:exons 6′-15′-16′ (p94∆) (16), and Spodoptera frugiperda cells (SF-9) were kindly provided by Dr. Muriel Herasse (Généthon, Evry, France) and Dr. Takeshi Nishino (Nippon Medical School, Tokyo, Japan), respectively. A baculovirus expression vector, pFastBac1, culture media, and transfection reagent were purchased from Invitrogen. Mammalian expression vectors, pECPF-N1 and pEYFP-N1, were purchased from Clontech. Casein was purchased from Merck. Protease inhibitors were purchased from Calbiochem, Peptide Institute Inc. (Osaka, Japan), or Sigma. Other chemicals were obtained from Nacalai Tesque Inc. (Kyoto, Japan) or Wako Pure Chemicals (Osaka, Japan).

p94-specific anti-pNS (antigen, PTVISPTVAPRTGAEPRS, at the N terminus) and anti-pIS2 (identical to anti-pK-rich antigen, NTISVDIRPVKKKKNHPFV, overlapping with the IS2 region) antisera have been described previously (14). Monoclonal antibodies 2C4 and 12A2, which recognize N terminus and the region around the catalytic residue Asn-358 of p94, respectively, were purchased from NovoCastra (Newcastle, UK). Monoclonal antibodies that recognize µCL and mCL were purchased from Biomol and Chemicon, respectively. Recombinant domain 1 of human calpastatin protein and the peptides corresponding to its reactive site were purchased from Takara Shuzo (7316 and SP007) and Sigma (C9181).

**Recombinant Proteins Expressed in Insect Cells**—The cDNA for p94∆ or p94:C129S, an active-site mutant, was inserted into the pFastBac1 vector using appropriate restriction enzyme sites. Recombinant baculovirus was generated according to the manufacturer's instructions. SF-9 cells were cultured and infected as described previously (28). The cells were then shaken vigorously at 27 °C for 50 h at a density of 1 × 10⁶ cells/ml and collected by centrifugation at 600 × g for 5 min at 4 °C. Recombinant proteins were purified following the procedures described previously (28), with minor modifications as follows. The harvested cells were washed twice with ice-cold phosphate-buffered saline. The pellets were suspended in buffer A (20 mM Tris-Cl (pH 7.5), 5 mM EDTA, 10 mM 2-mercaptoethanol (2-ME) containing 0.1 mM leukopetin, and the cells were homogenized with a French press (SLM Aminco). The cell lysate was then ultracentrifuged at 150,000 × g for 40 min at 2 °C. Recombinant proteins were purified from the supernatant by the AKTA system (Amersham Biosciences) and placed in a cold chamber, using successive rounds of column chromatography: DEAE-Toyopearl (Tosoh, Tokyo, Japan), DiAcamoQ (Amersham Biosciences), HiLoad 16/60 Superdex-200, and MonoQ. Fractions containing recombinant proteins were determined by Western blotting analysis and caseinolytic assays (for p94∆). Recombinant µ- and m-calpains were prepared in the manner described in Ref 28.

**Protease Activity Assay—Caseinolytic activity of p94∆ was measured essentially according to Ref. 30. In brief, the composition of the standard assay solution was 100 mM Tris-Cl (pH 7.5), 25 mM 2-ME, 10 mM CaCl₂, 10 mM EDTA, and 2-ME for p94∆ was replaced by 0.1 M Tris-Cl for p94. The reaction was carried out by incubating either p94∆ (3 µg), µ-calpain (0.2 µg), or m-calpain (0.4 µg) in 50 µl of the standard assay solution containing 3 mg/ml casein under the conditions indicated. The reaction was stopped by the addition of 150 µl of 7 µl (w/v) trichloroacetic acid. The tubes were incubated on ice for 20 min and centrifuged at 15,000 × g for 10 min, and the A₂₈₀ of the supernatant was measured. An increase in A₂₈₀ of 1.0 in 1 h was defined as 1 unit of caseinolytic activity.**

The effect of Ca²⁺ on p94∆ was determined under the assay conditions described above, varying either the concentration of Ca²⁺, temperature, or pH. Tris acetate buffer was used instead of Tris-Cl buffer to measure the effect of pH. Protease inhibitors were added to the standard assay solutions at the concentrations indicated. The reactions were carried out at 37 °C for 45 min, and the caseinolytic activity was determined as described. The effect of each inhibitor was evaluated relative to the inhibitory effect of 10 mM EDTA set to 100.

To determine the substrate hydrolyzing activity of p94∆, various proteins were incubated with p94∆ in the standard assay solution. Hydrolysis of the proteins was examined by SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining or Western blotting.

In the proteolytic assay using Y-C-CSTN as substrate, 2 µg of Y-C-CSTN was incubated with p94∆ in the presence of 15 mM Ca²⁺ for 0–45 min at 37 °C in a solution containing 0.1 µl Tris-Cl (pH 7.5) and 10 mM 2-ME in a volume of less than 20 µl. The reaction was stopped by the addition of 0.5 ml of 1 × EDTA (pH 8.0) or an equal volume of 2× SDS sample buffer. The fluorescence of the samples was measured by excitation at 434 nm (10 nm bandwidth), and the emission spectra were collected from 450 to 600 nm using an RF-1500 spectrofluorophotometer (Shimadzu, Kyoto, Japan). The ratio of fluorescence at 477 nm to 527 nm was used as a proteolytic activity, which is related to the dose-dependent changes in fluorescent signals, 0–2 µg of p94∆ was incubated with Y-C-CSTN (0.6 µg) in a buffer consisting of 28 µM Ca²⁺, 1 mg/ml bovine serum albumin (BSA), 0.1 mg/ml calpastatin (CSTN) domain 1, 20 mM 2-ME, and 100 mM Tris-Cl (pH 7.5) in a total volume of 0.3 ml for 5–45 min.

**Cell Culture and Assays—**COS-7 cells were maintained and transfected with each plasmid construct as described previously (24). The cell lysate was prepared by sonicating harvested cells in a solution consisting of 50 mM Tris-Cl (pH 7.5), 25 mM 2-ME, 10 mM EDTA, and the protease inhibitors 10 µM Z-D-CH2-DCB, 10 µM TVAD-CMK, 1 µM tourist of rabbit calpastatin (calpain), calpastatin, and moreover, both hydrolyze calpastatin. These results led us to predict that p94 participates in the regulation of the conventional calpain-calpastatin system in skeletal muscle. We used these findings to establish a calpastatin-based fluorescence assay specifically to measure p94 protease activity, showing a possibility of application of this assay as a diagnostic tool for LGMD2A.
Fig. 1. Schematic structure of p94: C129S, p94Δ, and purified proteins. A, schematic structure of p94:C129S and p94Δ. NS, IS1, and IS2 are specific sequences found in p94 but not in the conventional (α- and m-) calpain large subunits (μCL and mCL). Domains I, III, and V are α-helical, C2-like Ca2+-binding, and Gly-rich hydrophobic domains, respectively. Domains IV and VI contain five EF-hand motifs and are very similar to protease domain when activated. Apart in the inactive state, compose the protease domain when activated. Thick horizontal bars indicate the positions of epitopes for the antibodies used in this study. Note that a part of the epitope for anti-pIS2 is retained in p94Δ. Arrows [1] to [4] indicate the proteolytic sites determined in this study. B, purified p94: C129S and p94Δ used in this study. Lane 1, molecular weight marker; lanes 2 and 3, p94:C129S (5 μg) and p94Δ (5 μg), respectively, in the final MonoQ fractions (open triangles) were separated electrophoretically and stained with CBB.
Fig. 2. Characterization of p94Δ caseinolytic activity. A, temperature and incubation time dependence of activity. Activities were standardized with the value at 37 °C for 30 min taken as equal to 1. B, temperature dependence of activity with 20 min of incubation. Closed circles, open lozenges, and open squares represent p94Δ, μ-calpain, and m-calpain, respectively. Activities were standardized as the maximum value.
Regulation of Conventional Calpains by p94/Calpain 3

Table 1: Effects of various protease inhibitors on p94Δ, μ-, and m-calpain

| Name                          | Concentration | p94Δ | μ-calpain | m-calpain |
|-------------------------------|---------------|------|-----------|-----------|
| EDTA                          | 10 mM         | 100  | 100       | 100       |
| Leupeptin                     | 1 mM          | 102  | 101       | 100       |
| E-64c                         | 0.1 mM        | 86.7 | 98.7      | 97.8      |
| Aprotinin                     | 10 μM         | 87.0 | 91.2      | 90.7      |
| Chymostatin                   | 1 mM          | 85.1 | 90.7      | 84.1      |
| Calpain (ZLNLal)              | 10 μM         | 76.1 | 98.7      | 98.1      |
| E-64c                         | 1 μM          | 19.0 | 88.9      | 87.6      |
| TPCK                          | 1 mM          | 65.2 | 57.4      | 73.1      |
| AllNaL (MG-101, calpain inhibitor 1) | 10 μM | 64.1 | 90.1      | 88.7      |

PAGE indicated that p94Δ degrades casein in a manner similar to the caseinolysis by the conventional calpains (data not shown). The specific activity of p94Δ determined by caseinolytic assay was 9.0 units/mg, which is ∼1/80 of the value for recombinant human m-calpain determined with the same expression and purification systems (28, 32, 33). However, because most of the purified p94Δ underwent N-terminal autolysis as described above, we concluded that very low specific caseinolytic activity is one of the enzymatic properties of p94Δ and does not result from partial denaturation or aggregation of the protein.

Enzymatic Properties of p94Δ: Effects of Temperature, pH, Ca2+ Concentration, and Protease Inhibitors—The caseinolytic activity of p94Δ was examined while varying either temperature, pH, or Ca2+ concentration and compared with the corresponding parameters for recombinant μ- and m-calpains. Fig. 2A shows the temperature dependence of p94Δ caseinolytic activity. The initial rate of p94Δ activity increased with temperature. In the range of temperatures examined here, the activity of p94Δ increased persistently with time up to 60 min. The optimal temperature for 20 min of incubation of p94Δ was about 42 °C, which contrasts with the decrease in activity observed for both μ- and m-calpains at temperatures above 25 °C (Fig. 2B). Although p94Δ shows maximum activity at around pH 7.5, as do other calpains, p94Δ maintains its activity even at pH 10 (Fig. 2C). Ca2+ dependence showed that a pCa for half-maximal activity for p94Δ was 2.9, which is lower than those of μ- and m-calpains (3.7 and 3.3, respectively) (Fig. 2D).

The effects of various inhibitors on the activity of p94Δ and μ- and m-calpains were investigated (Table 1). p94Δ has a spectrum of inhibitors very similar to those of μ- and m-calpains, except calpastatin. The calpastatin fragment inhibited μ- and m-calpains completely at molar ratios of almost 1:1, whereas p94Δ was not inhibited by a 100-fold molar excess of the calpastatin fragment or calpastatin-related peptides (Fig. 2E). In fact, calpastatin was shown to be a substrate for p94Δ (see below). Because the IS1 region is a target for p94 autolysis (31, 34), we examined the possibility that synthetic peptides corresponding to IS1 would have a competitive inhibitory effect on p94Δ. However, none of the peptides inhibited the caseinolytic activity of p94Δ, even at a molar ratio of 100:1 (data not shown).

Autolytic Activity of p94Δ—p94Δ is much more stable than full-length wild-type p94. However, as described above, limited autolysis at the N terminus of p94Δ occurs even in the presence of EDTA at 2 °C. Therefore, the autolytic activity of p94Δ was analyzed in more detail. p94Δ, already lacking the N-terminal 33 amino acids of p94, underwent further autolysis in the presence of Ca2+. As shown in Fig. 2, F and G, this second phase autolysis generated an 83-kDa fragment very rapidly (t1/2 = 5 min at 30 °C and 20 min at 0 °C). No change in caseinolytic activity or Ca2+ dependence was caused by preincubation of p94Δ for 5, 10, 20, or 60 min at 30 °C (data not shown). The N-terminal sequence of the 83-kDa fragment was also Ala-34, indicating that the second autolysis occurs at the C terminus of p94Δ (data not shown).

To characterize further the autolytic activity of p94Δ relative to that of full-length wild-type p94, p94:C129S, a good “intramolecular autolytic” substrate for p94, was proteolized by p94Δ (24). Co-incubation of p94:C129S (1 μg) and p94Δ (0.1 μg) resulted in the production of four major fragments with approximate molecular masses of 93, 91, 58, and 33 kDa, as shown in Fig. 2H. Interestingly, none of these fragments was recognized by anti-pNS antiserum, and the full-length 94-kDa protein detected by anti-pNS antisera decreased rapidly in the reaction. The N-terminal residue of the 93-kDa fragment (Fig. 2H (a)) was Ala-15 (Fig. 1A, arrow [1]), whereas the N-terminal residues of the 91- and 33-kDa fragments (Fig. 2H (b and d), respectively) were identical to that of N-terminally autolysed p94Δ (Ala-34, Fig. 1A, arrow [2]). These results indicate that p94Δ efficiently hydrolyzes the N terminus of p94:C129S, as well as that of p94 itself. The N-terminal 58-kDa fragment (Fig. 2H (c)) was Glu-323 (Fig. 1A, arrow [3]), which corresponds to one of the previously determined autolytic sites in native p94 (31). The 33-kDa fragment was not detected by either the monoclonal antibody 12A2 or anti-pL82 antisera. These results indicate that p94:C129S is hydrolyzed by p94Δ first at the N terminus and then in the region of IS1, which generates 58- and 33-kDa fragments corresponding to the C- and N-terminal parts of the 91-kDa fragment, respectively.

Substrates for p94Δ—Previously, we found that p94 causes a decrease in a 60-kDa protein in vivo, when expressed in COS cells (14). Peptide sequencing revealed that it corresponds to heat shock protein 60 (HSP60). Consistent with this, an in vitro assay showed that p94Δ proteolyses HSP60 (Fig. 3A). More-
**FIG. 3.** *In vitro* proteolysis of possible substrates of p94Δ. A, HSP60 degraded proteolytically by p94Δ or m-calpain. HSP60 (0.6 µg) was incubated with 0.3 µg of p94Δ or 0.04 µg of m-calpain in the presence of 10 mM CaCl₂ (+Ca²⁺) or 20 mM EDTA (+E) for 0–120 min and visualized by CBB staining. B, schematic structure of the N2A region of connectin/titin and the binding site for p94. Part of the N2A region of connectin/titin is shown, and the bidirectional arrows indicate the regions corresponding to Ig80–81 and Ig82–83. Ig80-Ig83 are motifs with similarity to immunoglobulin superfamily (I-line region subset); is is the intervening region between Ig80 and Ig81; and PEVK is the region rich in Pro, Glu, Val, and Lys. The vertical arrow indicates one of the m-calpain-susceptible proteolytic sites in connectin/titin. CN48 is the original N2A clone isolated by yeast two-hybrid screening using p94 as bait (17), and CN48-Δ1 to -Δ6 were generated from CN48. Each construct was co-transformed with bait plasmid bearing p94 into AH109, and binding was evaluated by growth on SD-LWA plates and β-galactosidase activity (shown in the right column). aar, amino acid region; N-term, N terminus; C-term, C terminus. C, proteolysis of N2A connectin/titin fragments by p94Δ, μ-calpain, or m-calpain. Ig80–81 (1 µg upper) or Ig82–83 (1 µg lower) connectin/titin N2A recombinant peptide was incubated with 2, 1, 0.5, 0.2, or 0.1 µg (left to right in each panel) of p94Δ (left), m-calpain (middle), or μ-calpain (right) in the presence of 15 mM CaCl₂ for 60 min at 37 °C for p94Δ, or for 20 min at 30 °C for m- and μ-calpains. The lanes labeled +E contain 2 µg of p94Δ (left), m-calpain (middle), or μ-calpain (right) incubated with each peptide in the presence of 20 mM EDTA where no autolytic degradation occurred. Closed triangles indicate uncleaved connectin/titin peptide, Ig80–81 or Ig82–83. Protein fragments were visualized by CBB staining. D, m-calpain:C105S degraded proteolytically by p94Δ, p94Δ (2 µg) and m-calpain: C105S (1.5 µg) were co-incubated for 0–120 min in the presence of 15 mM CaCl₂, 20 mM EDTA (+E), or 15 mM CaCl₂ and 0.7 µg of calpastatin domain 1 (+CSTN). Samples were subjected to SDS-PAGE, and proteins were detected by CBB staining or Western blotting using anti-human m-calpain or anti-pIS2 antisera.
FIG. 4. 

**A.** Proteolysis of calpastatin by p94Δ. A, processes of calpastatin proteolysis by p94Δ determined by reversed-phase column chromatography. Calpastatin domain 1 (30 μg) and p94Δ (1 μg) were co-incubated in the presence of 15 mM CaCl₂ for 0–120 min at 37 °C. The reaction was stopped at each time point by adding 1 ml of 0.1% trifluoroacetic acid, and the products were subjected to C18 reversed-phase column chromatography with a 10–50% acetonitrile gradient. Closed triangles in the 20-min digestion represent fractions of peptides degraded at the most susceptible sites in calpastatin, judged by the earliest appearance of these peaks. Lowercase letters on open triangles in the 60-min digestion correspond to the fractions labeled in A. T and U stand for total protein before separation by C18 reversed-phase column chromatography and undigested calpastatin domain 1, respectively. C, comparison of sites of calpastatin cleavage by p94Δ. All six N-terminal sequences identified from the digestion of calpastatin domain 1 (i–vi) and one from the digestion of calpastatin peptide C9181 (vii) are shown. The autolytic sites at the N terminus (N-1 and -2) and in the IS1 region (IS1-1 to -3) are also shown for comparison (31). The vertical arrow indicates the proteolytic site. Proline residues are reversed, D, alignment of human and rabbit calpastatin inhibitory domains 1–4 and positions of the proteolytic sites for p94Δ. Amino acid sequences around the inhibitory reactive site (indicated by the bidirectional arrow) are shown, and highly and moderately conserved residues are reversed and shaded, respectively. The open triangle indicates the position of the caspase proteolytic site (36). Thick lines indicate the sequence corresponding to the commercially available calpastatin peptides, C9181 and SP007, used in this study. Vertical arrows with numbers correspond to the sequence labels in C.
Fig. 5. Assay for p94Δ utilizing calpastatin and FRET of fluorescence proteins. A, schematic structure of p94Δ-specific substrate (Y-C-CSTN) used in this study. CSTN represents residues 157–255 of rabbit calpastatin (GenBank™/EMBL/DDBJ accession number A26615). B, steps in substrate processing by p94Δ. Y-C-CSTN (2 µg) was incubated alone (–p94Δ) or with 1.5 µg of p94Δ (+p94Δ) in a buffer composed of 15 mM Ca²⁺, 1 mg/ml BSA, 0.1 mg/ml CSTN, 10 mM 2-ME, and 100 mM Tris-Cl (pH 7.5) in a volume of 15 µl for 0–45 min at 37 °C, CBB staining. C, changes in the fluorescence spectrum of the substrate during proteolytic digestion by p94Δ. Reactions in B (+p94Δ) were terminated by the
over, HSP60 is proteolyzed by m-calpain much more rapidly than by p94Δ, suggesting that HSP60 is also a possible in vitro target of the conventional calpains. Although HSP60 is highly conserved from bacteria to eukaryotes (~50% primary sequence identity between human and E. coli proteins), the E. coli HSP60 homologue, GroEL, is very poorly hydrolyzed by p94Δ. Its co-chaperonin, GroES, and combinations of GroEL and GroES with or without ATP were examined, but none was proteolyzed by p94Δ (data not shown).

Several other proteins were incubated with p94Δ to test whether they are substrates. Two different N2A fragments of connectin/titin, IgI80–81 and IgI82–83, were hydrolyzed by p94Δ as well as μ- and m-calpains. The connectin constructs IgI80–81 and IgI82–83 contain the two N-terminal and the two C-terminal Ig motifs of the N2A region, respectively. IgI82–83 includes a p94-binding site for connectin (Fig. 3B) (17). IgI80–81 was more rapidly degraded than IgI82–83 by p94Δ and μ- and m-calpains, as shown in Fig. 3C. N-terminal sequencing of the degradation fragments of IgI80–81 revealed that one of the proteolytic sites susceptible to calpain is on the N terminus of Gly-9434 (GenBankTM accession number NP_775085; ii and in Fig. 4, C and D), which are detectable fragments already appearing after 20 min of digestion (Fig. 4A, closed triangles). These sites both have Pro at the P3 position and a small amino acid (Ala and Gly) at the P1’ position. Moreover, five of six cleavage sites in the calpastatin domain 1 fragment have two Pro residues between P2′ and P3′. These features may reflect the proteolytic preference of p94Δ. One of the autolytic sites in p94Δ/C129S and p94Δ, the N terminus of Ala-15, also has Pro at the P3’ position and a small amino acid (Ala) at the P1’ position (Fig. 4C, N-1). However, there are no further significant similarities found between the amino acid sequences surrounding the cleavage sites in calpastatin and those for autolytic sites in p94Δ/C129S and p94Δ (Fig. 4C, N-2 and IS1-1–3) (31).

To test whether p94Δ proteolytically degrades short peptides, a 27-mer peptide corresponding to the reactive site of calpastatin (Sigma C9181; Fig. 4D) was incubated with p94Δ. Although the efficiency of cleavage was very low (less than 5% after 120 min of incubation), the peptide was cleaved on the N terminus of Lys-99 (Fig. 4, C and D, vii). The site has Pro residues at the P1 and P2 positions but not in the P’ positions, suggesting that short peptides are cleaved by p94Δ differently from the cleavage of calpastatin domain 1 fragment, which is a much better substrate for p94Δ.

Assay System for p94 Utilizing Calpastatin as Substrate—As observed above, calpastatin is the best substrate for p94Δ among the protein substrates examined in this study except for p94Δ/C129S. Therefore, we developed an assay for p94 using the calpastatin fragment and fluorescent resonance energy transfer (FRET). When the purified substrate, Y-C-CSTN (Fig. 5A), was incubated with p94Δ, it was rapidly degraded into several fragments (Fig. 5B). Before the substrate was cleaved by p94Δ, the fluorescence spectrum of Y-C-CSTN excited at 434 nm, the CFP excitation wavelength, showed a peak at 527 nm, which is an emission wavelength of YFP, indicating that YFP is excited by the CFP emission by FRET (Fig. 5C, 0 min). The emission spectrum of Y-C-CSTN excited at 434 nm was measured during the proteolytic process. A time-dependent increase in CFP emission at 476 nm and a corresponding decrease in YFP emission at 527 nm was observed, demonstrating that the proteolysis of Y-C-CSTN by p94Δ can be monitored as the change in the emission spectrum caused by the loss of FRET between those two fluorescent units (Fig. 5C). Various amounts of p94Δ were tested for Y-C-CSTN digestion, and the ratio of YFP emission to CFP emission, 476:527, was plotted for each time point (Fig. 5D). The increase in the fluorescence ratio 476:527 was time- and dose-dependent, and the activity of as little as 250 ng of p94Δ could be assayed with an incubation of addition of 0.5 ml of 50 mM EDTA (pH 8.0), and the fluorescence emission spectra from 450 to 600 nm excited by 434 nm were scanned. D, dose-dependent fluorescent signals in this assay system. p94Δ (0–2 μg) was incubated with 0.6 μg of Y-C-CSTN under the same conditions as in B, but in the presence of 28 mM Ca2++. Reactions were stopped by adding 0.5 ml of 50 mM EDTA (pH 8.0), and the fluorescence emission spectrum from 450 to 600 nm excited by 434 nm was scanned. D, dose-dependent fluorescent signals in this assay system.
Regulation of Conventional Calpains by p94/Calpain 3

45 min. µ-calpain produced no increase in the fluorescence ratio nor proteolysis of Y-C-CSTN over this period of time or in this range of protein concentrations (data not shown), suggesting that Y-C-CSTN is a p94Δ-specific substrate but not for the conventional calpains under our assay condition.

Activity of COS-expressed Full-length Wild-type p94 Detected by Y-C-CSTN—To test whether the assay described above can detect the proteolytic activity of full-length wild-type p94, as well as the activity of p94Δ, among 100 other co-occurring proteases in vitro, the proteolytic activity of p94 expressed in COS-7 cells was measured using Y-C-CSTN as substrate. COS cells are known to express considerable amounts of m-calpain and other cellular proteases, such as caspasps. Ca²⁺-dependent proteolysis of Y-C-CSTN was detected in the lysates of cells expressing full-length wild-type p94 (Fig. 5E, f.l.p94) or p94Δ (Fig. 5E, p94Δ) in the presence of protease inhibitors for major proteases including conventional calpains, caspasps, and serine proteases. On the other hand, the lysates of cells expressing protease-inactive full-length p94:C129S (Fig. 5E, CS) or µ-calpain (Fig. 5E, µ-calpain) showed no activity above background levels, i.e. above the value determined for cells transfected with empty vector, regardless of sufficient amount of the proteins detected (Fig. 5F, CS and µ-calpain). These results clearly indicate that our assay system specifically detects and distinguishes the protease activity of p94 in the presence of interfering proteases.

To our surprise, full-length wild-type p94 showed Ca²⁺-dependent calpastatinolytic activity (Fig. 5E, f.l.p94). The autolysis of recombinantly expressed p94, however, was Ca²⁺-independent (Fig. 5F, f.l.p94; gray arrowhead) as shown previously (14), which is consistent with the fact that most COS-expressed p94 had already disappeared at harvest. These results indicate that the autolysis of p94 does not require Ca²⁺, whereas calpastatinolysis occurs in a Ca²⁺-dependent manner, suggesting different substrate specificities for p94 in the absence and presence of Ca²⁺.

DISCUSSION

In this study, the enzymatic properties of one of the alternatively spliced variants of p94/calpain 3 were examined, to understand better the unique characteristics of p94 and their relevance to its physiological functions. Isolating significant amounts of the proteins is a prerequisite for in vitro enzymatic studies, and this has been almost impossible because of the very rapid autolytic activity of p94 (31). On the other hand, some natural splice variants of p94 expressed in skeletal muscle are somewhat stable. After several different isoforms were examined, p94Δ, which lacks both IS1 and IS2, was the most promising variant for large scale preparation. The structure of p94Δ is similar to that of µCL and mCL, except that it has the NS. It shows proteolytic characteristics similar to those of µ- and m-calpains, such as Ca²⁺ dependence, insofar as IS1 and IS2 are involved in Ca²⁺-independent protease and autolytic activity, a hallmark of p94Δ.

However, there are several traits distinguishing p94Δ from the conventional calpains as follows: 1) p94Δ shows specific caseinolytic activity much lower than that of the conventional calpains; 2) the optimal temperature for p94Δ activity is higher (−42 °C) than that of the conventional calpains (25–30 °C); and 3) p94Δ has a lower pCa for half-maximal activity than µ- or m-calpains. At present, the possibility cannot be excluded that these properties originate from the lack of IS1 or IS2. It should be emphasized, however, that many features of p94Δ are shared with full-length p94 as follows: neither is inhibited by but proteolytically degrades calpastatin; both exhibit proteolytic activity without 30K; both bind connectin/titin at the N2A and M line regions (16); both cut p94:C129S at identical sites; and both undergo autolysis in the presence of EDTA. Therefore, it is conceivable that the properties of p94Δ described above reflect the nature of p94 and are related to the functions of p94 in the context of the physiology of skeletal muscle.

Stable and Active without 30K—The co-expression of 30K is required for recombinant µ- and m-calpains and nCL-4 in the same SF-9/baculovirus system (28, 32, 33, 37, 38). However, proteolytically active p94Δ and inactive p94:C129S were isolated in the soluble and stable fraction without 30K. Furthermore, no interaction between p94Δ and 30K was detected in the yeast two-hybrid system, as was also the case for p94 (17, 16). These data strongly suggest that p94Δ and p94 do not form heterodimers with 30K. It awaits further analyses on the tertiary structures of p94Δ and p94 to determine whether they form homodimers which was predicted from the elution positions of p94:C129S and p94Δ on the gel filtration column. (see Ref. 31 and data not shown).

Autolytic Activity of p94 and p94Δ—Our previous results on the in vitro translation of p94 indicated that the autolysis of p94 proceeds in the presence of excess EDTA (14), which was also observed for the N-terminal autolysis of p94Δ. Branca et al. (39), however, reported Ca²⁺-dependent proteolytic fragmentation of recombinant p94. They isolated a substantial amount of full-length wild-type p94 using the SF-9/baculovirus system. In our hands, however, p94:C129S was expressed abundantly and stably and was successfully purified almost to homogeneity using the same SF-9/baculovirus system (Fig. 1B), whereas wild-type p94 expressed in the same system was detected only as a 55-kDa autolyzed fragment (data not shown). At present, no clear explanation is available for these discrepancies, but p94 may show Ca²⁺-dependent autolysis under certain conditions. Indeed, Ca²⁺-dependent hydrolytic activity was detected for full-length wild-type p94 as well as for p94Δ using a calpastatin-based FRET substrate in this study.

Hydrolysis of p94:C129S by p94Δ, but not by µ-calpain, strongly suggests that the substrate specificities of p94Δ and p94 are the same. In addition to the autolytic site in IS1 (31), p94Δ hydrolyzes p94:C129S at the N termini of Ala-15 and Ala-34. In this study, one of the intramolecular autolytic sites of p94Δ was also demonstrated to be Ala-34. Ca²⁺-independent autolysis at the N terminus of Ala-34 generates a rather stable autolytic fragment of p94Δ, but further autolysis occurred during the storage of p94Δ for over a year (data not shown). Recently, Rey and Davies (34) reported that one of the autolytic sites in a recombinant protein corresponding to the protease domain of p94 was Ala-15, which is consistent with our result. They also reported Ala-45 and Thr-316 as autolytic sites, which were not identified in our experiments. It can be reasoned that the lack of domain III and regions thereafter in their construct exposed sites potentially susceptible to autolytic cleavage. Previously, we have observed that the Ca²⁺-independent autolytic activity of recombinant p94:exon6 was much weaker than that of full-length p94, resulting in the stabilization of p94:exon6 (16). Therefore, it is inferred that the lack of exon 6, which constitutes most of IS1, contributes to the stability of p94Δ by abrogating the pivotal autolytic site. Rey and Davies (34) also reported Ca²⁺-dependent autolysis of their protein, which can be ascribed to the lack of IS2 based on our previous observations (16). Very recently, Fukiage et al. (40) reported the qualitative characterization of Lac82, another alternative splice variant of p94, which is specifically expressed in the lens. They showed that the lack of IS2 does not effectively stabilize the product when NS is replaced by AX1, a lens-specific N-terminal sequence (41). Considering these data

---

2 Y. Ono, K. Kakinuma, F. Torii, A. Irie, K. Nakagawa, S. Labeit, K. Abe, K. Suzuki, and H. Sorimachi, unpublished data.
together, we conclude that NS, IS1, IS2, and AX1, unique sequences specific to CAPN3 proteins, have independent functions and that different combinations of them confer specific characters, e.g. different autolytic activities, upon each splice variant.

p94-specific Assay towards a Diagnosis of LGMD2A—Because the loss of the substrate processing activity of p94 causes LGMD2A (24), we have focused on identifying its substrates. One of the major problems we faced was how to distinguish the activity of p94 from that of other proteases, including the conventional calpains, in a physiological context. The enzymatic properties determined for p94A in this study showed that there are conditions favorable for p94 but not for the conventional calpains, such as high temperature and the presence of calpastatin. Consequently, we established a specific assay system for p94 and demonstrated its ability to identify COS-7-expressed p94, as well as p94A.

In theory, 0.3 mg of muscle, which corresponds to about three 10-μm cryostat slices of a muscle biopsy, contains at least 300 ng of p94 (42). Our system can assay as little as 250 ng of p94 (42). The activity of the COS-7-expressed p94 and p94A detected in this study corresponds to a minimum of ~10 ng of protein. Therefore, we anticipate that the activity of endogenous p94 in tissues (biopsy samples) will be measurable using this assay and that this methodology will be applicable to the diagnosis of LGMD2A by screening for the loss of p94 activity.

Calpain Network and LGMD2A—The apparent Ca\(^{2+}\)-independent proteolysis of p94:C129S and calpastatin is p94-specific, which is not observed for the conventional calpains (17). m-calpain:C105S was also degraded proteolytically by p94A in a Ca\(^{2+}\)-dependent manner, although p94:C129S was not degraded by m-calpain. These results imply, for the first time, that p94 has a certain role in regulating the conventional calpains directly and indirectly by proteolytic degradation of the calpains and calpastatin, respectively.

It has been reported that there are several biological contexts where several different protease systems keep cross-talking. For example, calpain and caspase proteolytic systems function where several different protease systems keep cross-talking. For example, calpain and caspase proteolytic systems function where several different protease systems keep cross-talking.