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COMMENTARY

Skeletal Muscle-specific Calpain, p94

STRUCTURE AND PHYSIOLOGICAL FUNCTION

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ABSTRACT. Recent studies indicate that calpain, a cytosolic Ca^{2+}-dependent protease, constitutes a large family comprising ubiquitous, tissue-specific, and atypical calpains. p94 is a homologue of the catalytic large subunit of calpain, expressed predominantly in skeletal muscle. Recently, p94 has been found to interact with connectin/titin, a muscle elastic protein, and its gene has been identified as being responsible for limb-girdle muscular dystrophy type 2A. The loss of function of a calpain species eventually leads to the activation of proteases including other calpain species responsible for muscle degradation. p94 does not form a complex with the small subunit of calpain (30K), but exists as a homodimer. This, together with other results, led us to consider a novel mechanism for the activation of calpain, a Ca^{2+}-induced subunit rearrangement. BIOCHEM PHARMACOL **56**;4:415–420, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. p94; calpain; connectin/titin; limb-girdle muscular dystrophy type 2A

Calpain, a Ca^{2+}-dependent intracellular cysteine protease, plays important roles in various Ca^{2+}-regulated cellular functions [1, 2]. Calpains can be classified into two types, ubiquitous and tissue specific, on the basis of their modes of expression [3]. Three ubiquitous calpain species, μ-, μ/m-, and m-calpains, are known to require micromolar, micromillimolar, and millimolar levels of Ca^{2+} for their activities, respectively. These species have been used, thus far, in most studies. All are heterodimers, composed of a distinct catalytic large subunit (μCL, μ/mCL, or mCL) and a common regulatory small subunit (30K†). CLs and 30K consist of four (I–IV) and two (V and VI or IV′) domains, respectively. The C-terminal domains of two subunits, IV and VI, are calmodulin-like Ca^{2+}-binding domains. The crystal structures of rat and porcine domains VI, which were clarified recently, indicate that they consist of five EF-hands, instead of the four predicted from their sequences (Fig. 1) [4–6]. Subdomains EF-1 and EF-2 are paired, as are EF-3 and EF-4, and EF-5 is required for dimerization.

As for tissue-specific calpains, seven species, p94 (nCL-1), Lp82, nCL-2, -2′, -3 (CAPN5), CAPN6, and nCL-4, have thus far been identified in mammals [3, 7, 8]. Among them, CAPN6, specific for placenta, lacks enzyme activity because of the absence of the critical active site residues Cys and His. CAPN6 may control the function of other calpain species as a dominant negative regulator. An endogenous inhibitor of calpain, calpastatin, interacts with ubiquitous calpain species through the calmodulin-like domains IV and VI [9, 10]. Therefore, it is likely that nCL-2′, nCL-3 (CAPN5), and CAPN6, which lack a calmodulin-like domain, cannot bind calpastatin; these species may be controlled by other regulators such as CAPN6 (Fig. 1). These species, which have protease domain II but lack the Ca^{2+}-binding domain, are called atypical calpains because their activities are apparently calcium independent, although the sixth EF-hand structure found adjacent to domain II has not been characterized. Among atypical calpains, CAPNs5 and 6 have a domain homologous to the C-terminal domain of the tra3 gene product, which participates in sex determination in *Caenorhabditis elegans*. This suggests that some calpain family members may regulate sex determination in mammals. In lower organisms such as yeast and fungi, some typical and atypical calpain species have been identified, and it is now quite clear that calpain constitutes a large superfamily [11–14]. The physiological functions in mammalian tissues, however, are still obscure. To analyze the physiological function of calpain, mostly tissue-specific calpains and calpain homologues from lower organisms are used together with ordinary typical calpains, because tissue-specific calpain must play some tissue-specific roles, and gene disruption experiments in lower organisms are much easier. In this commentary, our recent results on a skeletal muscle-specific calpain, p94 (experiments being performed to clarify its physiological role in correlation with muscular dystrophy), are summarized.

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† *Abbreviations:* 30K, calpain small subunit; CL, calpain large subunit; nCL, novel calpain large subunit; LGMD, limb-girdle muscular dystrophy; M-is, M-line intervening sequence; and Mex, M-line exon.

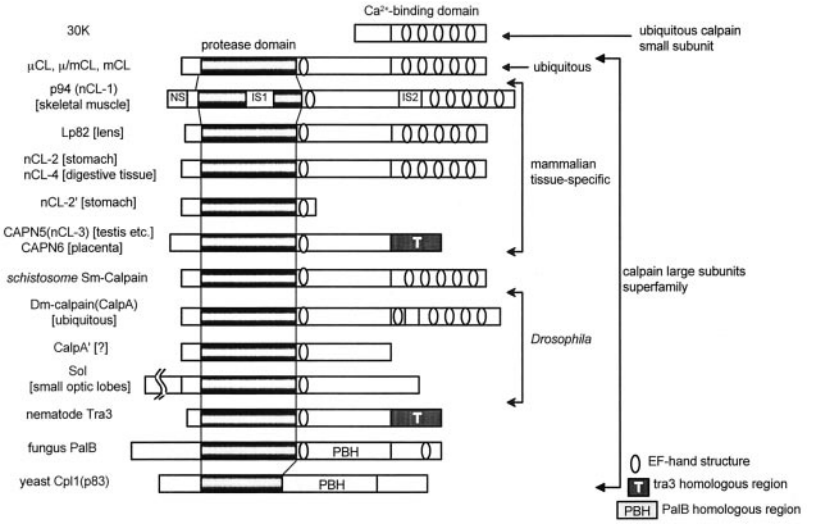

FIG. 1. Schematic structures of the calpain superfamily. p94 and Lp82, nCL-2 and nCL-2′, and CalpA and CalpA′ are generated by alternatively splicing. See text for details.

PROPERTIES OF p94

In 1989, p94 was the first tissue-specific calpain species identified during the cDNA cloning of mammalian ubiquitous CLs from muscle cDNA libraries [7]. Northern blot analysis of various tissues originally suggested that p94 is expressed specifically in skeletal muscle and tongue, and that the amount of mRNA in skeletal muscle is at least 10 times higher than ubiquitous calpain subunit mRNAs. Subsequently, however, p94 was found, albeit in small amounts, in eye, testis, and brain, and the current general concept is that p94 is expressed predominantly rather than specifically in skeletal muscle. Although the basic structure of p94 is similar to those of ubiquitous CLs, it possesses three unique regions: NS in the N-terminus, IS1 in domain II, and IS2 between domains III and IV (Fig. 1). Quite recently, a spliced variant of p94, Lp82, was discovered in rat lens. Lp82 lacks most of these three unique insert regions (Fig. 1). A comparison of the physiological functions of p94 and Lp82 will clarify the role of the three insertion domains. A unique feature of p94 is its rapid autolysis [15]. The half-life time of *in vitro*-translated p94 is less than 30 min, and this autolysis is stopped by replacing the active site Cys with Ser but is not affected by calpain inhibitors, e.g. calpastatin, E-64, or leupeptin [15]. Further, although p94 contains domain IV, the degradation is apparently Ca^{2+} independent. This feature makes the purification of p94 from skeletal muscle very difficult, and delays characterization at the protein level. By using the yeast two-hybrid system, the interaction between p94 and connectin/titin was clarified, and this discovery brought about a breakthrough in p94 research at the protein level.

INTERACTION BETWEEN p94 AND CONNECTIN/TITIN

Because the yeast two-hybrid system is used to analyze protein–protein interactions in the yeast nucleus, we first examined its potential for an interaction-analysis of a cytosolic protease such as calpain [16]. As a result, the interaction between the μ- or m-calpain large subunits and the small subunit was observed, while that between large subunits and calpastatin was not, probably because calpastatin interacts with calpain and inhibits enzymatic activity only in the presence of Ca^{2+}. Thus, Ca^{2+}-independent interactions, rather than Ca^{2+}-dependent interactions, can be analyzed by the yeast two-hybrid system. p94 did not bind to calpastatin or to the small subunit, despite the fact that the sequence of domain IV of p94 is highly similar to sequences in ubiquitous calpains. Instead, some clones coding connectin/titin were identified as p94-binding protein.

Connectin/titin, found as a muscle elastic protein, exists in skeletal and cardiac muscles. It spans more than 1 μm between the Z- and M-lines, and acts by resisting the stretch of relaxed striated muscle [17]. The complete cDNA sequence of 82 kb determined in 1995 reveals that this giant molecule (molecular mass: *ca.* 3000 kDa) consists of at least 244 copies of the immunoglobulin C2 and fibronectin III domains, together with some specialized binding regions and a putative elastic region (PEVK region) (Fig. 2) [18]. Quite recently, the mechanism of connectin/titin elasticity was determined in detail using an optical-tweezers technique [19, 20], showing that the PEVK region extends at moderate stretch, and that connectin/titin detaches from the myosin filament at extreme stretch. In the N2 line region, different splicing products exist for skeletal and cardiac muscles; in the heart, two distinct isoforms having either N2A or N2B exist, while in skeletal muscle, only the N2A isoform is present (Fig. 2).

Connectin/titin has at least two binding sites for p94, one at the N2A region and the other in the M-line intervening sequence 7 (M-is7) at the extreme C-terminus (Fig. 2) [15, 21, 22]. M-is7 is encoded by the M-line exon 5 (Mex5), and this region is also alternatively spliced, resulting in two distinct structures with and without M-is7 [23]. The expression ratio of connectin/titin with and

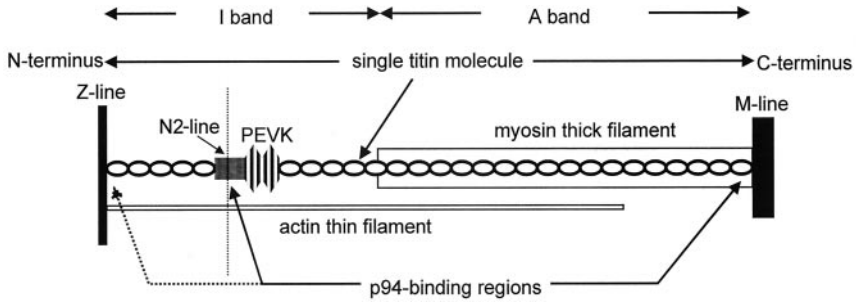

FIG. 2. Interaction between connectin/titin and p94. See text for details.

without M-is7 varies, depending on the type of skeletal muscle tissue; M-is7 (−)-connectin/titin is abundant in fast-twitch muscles of the lower extremity such as the rectus femoris and tibialis anterior, while M-is7 (+)-connectin/titin predominates in psoas and heart. Although the distribution of p94 in various muscle cell types has not been examined in detail, p94 may control the function and/or expression of connectin/titin through the interaction with M-is7. The interaction between p94 and the N2 region requires IS2, whereas that between p94 and M-is7 requires full-length p94. Further, immunostaining of myofibrils with a p94 antibody shows a strong band at the Z-line (Fig. 2). Although the location of p94 at the Z-line has not been analyzed at the molecular level, p94 may bind to the N terminal region of connectin/titin at the Z-line. The physiological function of the interaction between p94 and connectin/titin is not clear; however, if p94 plays an indispensable role in muscular physiology, a heart version must exist, because heart muscle connectin/titin contains all the potential binding sites for p94, i.e. N2A, M-is7, and the Z-line.

CHARACTERIZATION OF p94

Western blot analysis of various muscle fractions with anti-p94 antiserum showed that full-length p94 (molecular mass: 90 kDa) exists in the muscle-insoluble fraction (myofibril fraction), but not in the soluble fraction where ubiquitous calpains exist [24, 25]. A purification procedure was established using an inactive recombinant p94, p94(C129S), which has a Ser in place of the active site Cys-129. During purification, the molecular mass of p94(C129S) was found to be about 180 kDa by gel filtration, indicating that p94 exists as a homodimer. Chicken μ/mCL also forms a homodimer when its cDNA is expressed in insect Sf9 cells [26]. However, when μ/mCL and 30K cDNA are expressed together under similar conditions, μ/mCL and 30K form a heterodimer. Because the μ/mCL homodimer is enzymatically fully active, and since 30K suppresses calcium sensitivity [27], it is reasonable that p94, which does not interact with 30K, is constitutively active as a homodimer.

p94 is extracted stably in the myofibril fraction together with actin-binding proteins by washing the myofibrils with a low-ionic strength solution. However, p94 is rapidly autolyzed during the subsequent purification procedures established for p94(C129S), suggesting that a stabilizing factor(s) for p94 exists in the muscle extract. Full-length p94 is degraded into a 55 kDa fragment by autolysis. The conversion from the full-length (90 kDa) protein to a 55 kDa fragment occurs in at least three steps (90 kDa → 60 kDa → 58 kDa → 55 kDa), and protease inhibitors such as leupeptin and E-64 have no effect on the degradation. p94 binds to the N2 region of connectin/titin through IS2, which is essential for its autolysis [15, 16], but the connectin/titin N2 fragment does not inhibit p94 autolysis. The lack of reversible inhibitors for p94 makes it very difficult to purify p94 by conventional procedures, which are very time consuming. Therefore, p94 was purified from the soluble myofibril fraction by affinity chromatography in a single step [28, 29]. Although the complete purification of p94 remains difficult, full-length p94 can be isolated together with three degradation fragments, 60 kDa, 58 kDa, and 55 kDa in mass. The presence of three autolytic cleavage sites in the p94-specific IS1 region* explains why p94 is unstable in contrast to other ubiquitous calpains. Both p94-specific IS1 and IS2 are important for the autolysis and physiological function of p94. The autolytic cleavage sites in p94 are apparently different from the cleavage sites in ubiquitous calpains, suggesting distinct physiological substrates and roles [30–33].

RELATIONSHIP BETWEEN p94 AND LGMD2A

In 1995, p94 was identified as the gene responsible for LGMD type 2A (LGMD2A) [34–37], which led to intense interest in it. Nine forms of LGMD have been identified and are classified into two groups: two dominant forms (LGMD1A and 1B) and seven recessive forms (LGMD2A–2G) [38–43]; the chromosomal localizations of the defects in all types have been mapped (Fig. 3). Among the nine types, LGMD2C–F are caused by a loss of the sarcoglycan-complex associated with dystrophin in the skeletal muscle membrane. Whereas sarcoglycans in LGMD2A patients are normal, various point mutations and deletions dispersed throughout the entire length of the p94 gene have been reported [44, 45]. Nonsense, splice-site, frameshift, and other mutations are found [46], and some mutants lack the

* Kinbara K, Ishiura S, Tomioka S, Sorimachi H, Jeong SY, Amano S, Kawasaki H, Kolmerer B, Kimura S, Labeit S and Suzuki K, Manuscript submitted for publication.

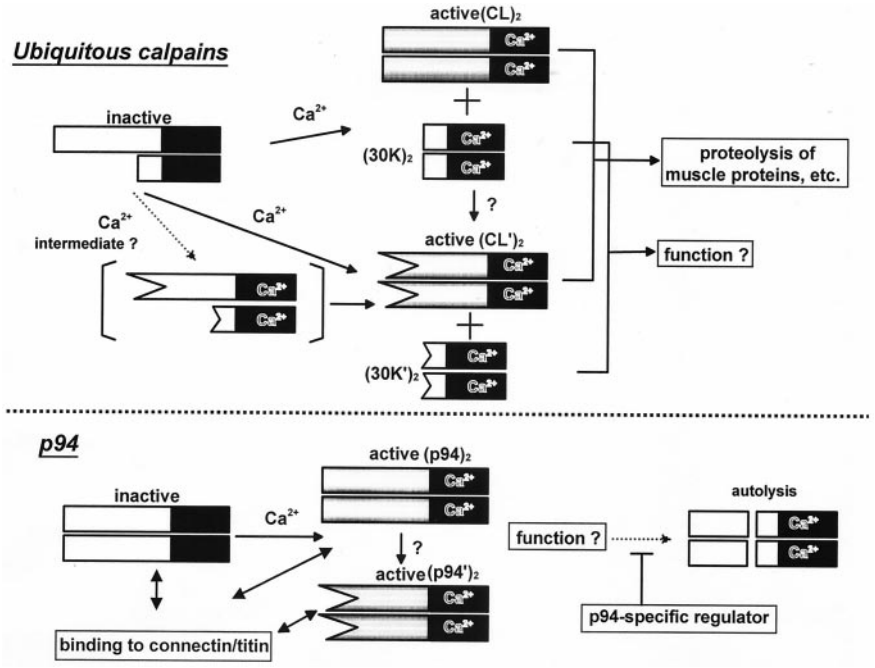

FIG. 3. Ca^{2+}-induced subunit rearrangements of ubiquitous and skeletal muscle-specific calpains.

protease domain of p94, indicating that the loss of p94 function may be a cause of LGMD2A. Various clones with point mutations, identified in patients, were expressed in COS cells, and their autolytic and enzyme activities and connectin/titin-binding abilities were examined. A complete or near complete loss of α-fodrin degradation activity, used as a representative of *in vivo* substrate, is observed in common among these point mutants. The results confirm that aberrant proteolysis is a cause of LGMD2A.† Interestingly, some p94 mutants with a point mutation in domain III show clear Ca^{2+} sensitivity, suggesting that domain III is responsible for the correct formation of domain IV. An identification of the *in vivo* substrate of p94 and an examination of dimer formations in LGMD2A mutants are urgently needed to clarify the etiology of LGMD2A. Interleukin 6 transgenic mice show muscle atrophy similar to that seen in muscular dystrophy patients, and a decrease in p94-mRNA levels and increases in other proteolytic systems including ubiquitous calpains are reported [47–49]. The p94 level correlates inversely with muscle degradation, whereas the ubiquitous calpain proteolytic system shows a positive correlation. This situation seems quite similar to the case in LGMD2A. Thus, an analysis of the signal transduction pathway, starting from the inactivation of p94 and leading to the eventual activation of ubiquitous calpains and other proteases responsible for the degradation of muscle proteins, is an important issue in determining the physiological function of calpain, clarifying the molecular cause of LGMD2A, and developing effective treatments.

† Ono Y, Shimada H, Sorimachi H, Rhichard I, Saido TC, Kinbara K, Sasagawa N, Beckmann JS, Ishiura S and Suzuki K, Manuscript submitted for publication.

CONCLUDING REMARKS

Calpain activity needs to be strictly regulated in cells, because aberrant intracellular proteolytic activity may lead to the disappearance or accumulation of cellular proteins and is toxic to cells. The most important factor in regulating the activity of calpains is Ca^{2+}. Ca^{2+} binds to domains IV and VI and activates calpain by inducing conformational changes that probably lead to the dissociation and reassociation of subunits.

Our model for the Ca^{2+}-induced rearrangement of calpain subunits is summarized in Fig. 3. Ca^{2+} induces the dissociation of ubiquitous calpain into subunits, and the dissociated CL and 30K interact to form homodimers, $(CL)_2$ and $(30K)_2$. $(CL)_2$ has full enzyme activity with a higher Ca^{2+} sensitivity. Thus, $(CL)_2$ appears to be an active calpain species *in vivo*. 30K may have a function independent of proteolysis after dissociation from CL, because some 30K family members identified recently display specific functions by forming homodimers. The dissociation of 30K from CL, which probably occurs at the membrane in the presence of Ca^{2+} and phospholipids, appears to be a critical step for calpain activation *in vivo*. 30K inhibits the protease activity intrinsic to CL by reducing the Ca^{2+} sensitivity, i.e. the rearrangement of calpain subunits in the presence of Ca^{2+} is essential for activation.

N-terminal truncation of calpain is usually observed upon incubation with Ca^{2+} and has been regarded as a means for calpain activation, since truncated calpain shows a higher Ca^{2+} sensitivity. The truncation of CL induces dissociation and thus facilitates activation. The truncated homodimers $(CL')_2$ and $(CL)_2$ have indistinguishable enzymatic properties.

This Ca^{2+}-induced subunit rearrangement provides a novel aspect to the activation mechanism of calpain. In this respect, an analysis of the properties of domains IV and VI, which determine the specific interaction between subunits, with and without Ca^{2+} is essential.

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