Possible functions of p94 in connectin-mediated signaling pathways in skeletal muscle cells

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Short title: p94 signaling pathways

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Key words: p94, connectin/titin, skeletal muscle, muscular dystrophy

Abstract

Calpains are intracellular Ca^{2+}-requiring “modulator proteases”, which modulate cellular functions by limited and specific proteolysis. p94/calpain3, a skeletal-muscle specific calpain, has been one of the representative calpain species which indicates physiological importance of calpain proteolytic system; a defect of proteolytic activity of p94 causes limb girdle muscular dystrophy type2A (LGMD2A, also called “calpainopathy”). Immunohistochemical studies on myofibrils showed that p94 localizes at the Z- and N2-line regions of sarcomeres. It was also identified by the yeast two hybrid studies that p94 binds to the N2A and M-line regions of connectin. Furthermore, genetic studies indicate that p94 is indispensable for skeletal muscles, although its precise functions are still unclear. Interestingly, connectin provides sarcomere not only with elasticity but also with binding sites to various multi-functional proteins such as muscle ankyrin repeat proteins (MARPs), muscle RING finger proteins (MURFs), titin-capping protein (T-cap/telethonin), sarcomeric-α-actinin, p94 etc. Binding sites for these proteins are not randomly placed along connectin but rather accumulated in the Z-, N2-, and/or M-line regions, indicating the existence of “signal complexes” unique to each regions. The concept of these complexes are strongly supported by the facts that mutations of connectin or its binding proteins in these regions severely perturb muscle functions, as in the case of LGMD2A caused by mutations in the p94 gene. Therefore, it is hypothesized that the “signal complexes” in the Z-, N2-, and M-lines modulate muscle cell homeostasis by transducing signals of external stimulations/stresses to trigger appropriate response at various different cellular

events such as protein modification and gene expressions. In this article, we performed detailed immunohistochemical analyses of p94 on isolated single myofibers. Together with recent findings about p94, it is suggested that sarcomeric localization of p94, especially its M-line localization, is affected by the combination of cellular contexts such as contractile status of myofibrils, fiber type compositions, sarcomeric maturation, and the composition of the “signal complexes” in each region.

Introduction

Calpains comprise a large family of intracellular Ca^{2+}-requiring cysteine proteases, which cleave their substrates at limited and specific sites to modulate their functions, thus called “modulator proteases”. In mammals, 14 genes for calpains exist, which can be classified into two groups. One group is expressed in most of the tissues ubiquitously (“ubiquitous calpains”). The conventional calpains, such as μ-calpain and m-calpain, belong to this group (Suzuki *et al*. 2004). The other is predominantly expressed in a specific tissue, such as p94 in skeletal muscle and nCL-2/-2’ in stomach (Sorimachi *et al.* 1993; Sorimachi *et al*. 1995; Hata *et al*. 2001).

Conventional calpain forms a heterodimer composed of the larger catalytic subunit (“μCL (μ-calpain large subunit)” and “mCL” for μ- and m-calpains, respectively) and the smaller regulatory subunit (common for both, “30K” named after its approximate molecular mass). The large subunits are divided into four functional domains (I-IV) (Figure 1, for review, see Goll *et al*. 2003; Suzuki *et al*. 2004). Domain I is autolyzed upon activation, involved in regulation of activity and subunit dissociation. Domain II is the cysteine protease domain, which shows weak similarity to papain and cysteine cathepsins. In the absence of Ca^{2+}, the domain II is further divided into two subdomains (IIa and IIb) to retain inactive “open” structure. Domain III is a Ca^{2+}-binding domain, the 3-dimensional, but not primary, structure of which is similar to that of the C2 domain. The C2 domain was originally identified as a Ca^{2+}-binding motif in protein kinase C and later found in many, mainly Ca^{2+}-regulated, proteins. Domain IV is also a Ca^{2+}-binding domain, which contains 5 EF-hand

Ca^{2+}-binding motifs. In the course of more calpain homologues being identified, it was noticed that many of these molecules possess unique primary structures in combination with calpain protease domain, in which the regions surrounding active site residues, Cys, His, and Asn, are highly conserved (Sorimachi and Suzuki 2001; Suzuki *et al.* 2004).

It has been demonstrated that calpain activity is indispensable for a variety of cellular functions, such as cell differentiation (Cottin *et al.* 1994; Moyen *et al.* 2004), cell cycle (Raynaud *et al.* 2004), cell migration, and cell adhesion (Glading *et al.* 2002; Franco *et al.* 2004). Calpains are also implicated in human diseases, for instances, Alzheimer's disease (Saito *et al.* 1993), type 2 diabetes (Horikawa *et al.* 2000), and muscular dystrophies (Richard *et al.* 1995). These reports indicate that calpains play roles in various tissues in various ways to regulate cellular functions, although the actual physiological roles of each calpain are still unclear.

p94 is one of the most distinct calpain members among calpain family in the following aspects. First, p94 is predominantly expressed in skeletal muscles, with less amounts of its several different splicing variants being expressed in skeletal muscle and non-muscle cells (Ma *et al.* 1998; Welm *et al.* 2002; Kawabata *et al.* 2003; Tullio *et al.* 2003). In sarcomeres, p94 binds to connectin (also called "titin"; in this manuscript we use "connectin" hereafter) at the N2A and M-line regions as originally shown by the yeast two-hybrid assays (Sorimachi *et al.* 1995).

Secondly, p94 contains three unique sequences, namely NS (N-terminal sequence), IS1 (insertion sequence 1), and IS2, which are located at the N-terminus, in

the subdomain IIb, and between domains III and IV, respectively (Figure 1). The NS sequence is rich in Pro, has no similarity to the N-termini of other calpains, and serves as the autolytic sites upon activation (Rey and Davies 2002). The IS1 region is inserted in the N-terminal region of domain IIb (Sorimachi *et al.,* 1993). The IS1 region includes another site of autolysis, and is involved in extremely rapid autolytic activity of p94 (see below). A part of the IS2 region and its periphery are the binding site for the N2A region of connectin (Sorimachi *et al.* 1995). A putative nuclear localization signal sequence is also included in the IS2 region (Sorimachi *et al.* 1993; Sorimachi *et al.* 1995).

Thirdly, Ca^{2+} dependence of activation of p94 is different from those for ubiquitous conventional calpains, μ- and m-calpains. Activation of μ- and m-calpains requires more than 10 and 100 μM of Ca^{2+}, respectively, at minimum *in vitro*. However, it has been shown that p94 autolyzes in the absence of Ca^{2+}, strongly suggesting different regulatory mechanisms for proteolytic activity of p94 (Sorimachi *et al.* 1993).

Fourthly, p94 shows exhaustive rapid autolysis at least *in vitro*. The half life of p94 is less than 10 minutes *in vitro* (Sorimachi *et al.* 1993). Native p94 extracted from skeletal muscles, however, is mainly in unprocessed form immediately after extraction (Kinbara *et al.* 1998). Overexpression of wild type p94 in transgenic mice did not show any obvious phenotype (Spencer *et al.* 2002). These results indicate that p94 proteolytic activity is under a certain control *in vivo* unlike *in vitro*.

Finally, the gene for p94, *CAPN3*, was identified as a gene responsible for

limb girdle muscular dystrophy type 2A (LGMD2A) (Richard *et al.* 1995). Since LGMD2A is caused by a defect of calpain homologue, it is also called “calpainopathy”. It should be noted that LGMD2A describes phenotype, whereas calpainopathy corresponds rather to genotype. Thus, both are used for human, but for animals other than human, “calpainopathy” is preferred to be used. A defect of proper proteolytic functions of p94 is responsible for LGMD2A as revealed by properties of 10 independent missense pathogenic mutants of p94 (Ono *et al.* 1998). Some mutations such as S744G, R769Q, and S606L retained autolytic activity (Ono *et al.* 1998, Jenne *et al.*, 2005), but S744G and R769Q were shown to be unable to proteolyze p94 substrates, indicating that proper recognition and cleavage of substrates, including p94 itself, by p94 are essential for functional skeletal muscle cells. Since then, several roles of p94 in skeletal muscle cells have been suggested. However, it has yet to be clarified as to how, when, and where p94 functions in skeletal muscle.

In addition to being a binding partner for p94, connectin is a unique and important constituent of sarcomeres. Connectin is the largest protein in mammals (molecular weight is more than 3,000 kDa) and is exclusively expressed in striated muscles (Maruyama 1976; Wang *et al.* 1979; Labeit and Kolmerer 1995). A single molecule of connectin extends to a half size of sarcomeres. The N-terminal end of connectin is located in the Z-line and its C-terminal end is located in the M-line.

Several important functions in skeletal muscles are proposed for connectin. One of the classical roles of connectin in striated muscle is to give elasticity to sarcomeres by generating passive tension, which is attributable to the molecular

structures in the I-band connectin (Granzier and Labeit 2004). In brief, the I-band region of connectin is composed of structurally distinct two motifs; tandemly arranged IG (immunoglobulin-like) motifs and the PEVK domain which is rich in Pro, Glu, Val and Lys. Because of its unusual amino acid composition, the PEVK domain has no specific secondary structure, and, belongs to "intrinsically unstructured protein (IUP)" family (Tompa 2002). The molecular basis for generating passive tension is explained as follows; the length of sarcomeres extends, the tandem IG-modules are unfolded followed by extension of the PEVK domains (Granzier and Labeit 2004).

Recently, another aspect of biological functions of connectin has been highlighted, *i.e.*, a platform for various ligands in sarcomeres. Connectin provides with many specific protein binding sites at the Z-line, N2A, and M-line regions, not only for other myofibrillar proteins, such as sarcomeric-α-actinin (s-α-actinin) (Ohtsuka *et al.* 1997; Sorimachi *et al.* 1997), myosin heavy chain (MHC) (Labeit *et al.* 1992; Soteriou *et al.* 1993), myosin-binding protein C (Labeit *et al.* 1992; Soteriou *et al.* 1993), etc. Importantly, there are many non-structural, so-called regulatory proteins that interact with connectin at the Z-line, N2A, and M-line regions. These include muscle ankyrin repeat proteins (MARPs) (Miller *et al.* 2003), muscle-specific RING finger protein-1 (MURF-1) (Centner *et al.* 2001), p94/calpain 3 (Sorimachi *et al.* 1995), T-cap/telethonin (Valle *et al.* 1997; Gregorio *et al.* 1998; Ojima *et al.* 1999), etc. Some of these connectin binding proteins were reported to localize in the nucleus in certain conditions, suggesting a linkage between functions of connectin and nuclear response against cellular circumstances. Furthermore, close to the C-terminus at the M-line

region, connectin has a serine/threonine-kinase domain similar to that of the smooth muscle myosin light chain kinase (MLCK) (Labeit *et al.* 1992; Labeit and Kolmerer 1995; Gregorio *et al.* 1999). Therefore, it is also suggested that connectin exerts its functions through its own enzymatic activity. Together, these properties of connectin prompts an idea that skeletal muscle cells have unique signal complexes which include distinct regions of connectin and proteins binding to these regions as critical components. In fact, mutations of connectin or its binding proteins in these regions severely perturb muscle functions, as in the case of LGMD2A caused by mutations in the p94 gene (Richard *et al.* 1995), tibial muscular dystrophy (TMD) and muscular dystrophy with myositis (MDM) caused by mutations in the M- and N2A-line regions of connectin, respectively (Udd *et al.* 1993; Garvey *et al.* 2002), and LGMD2G caused by T-cap mutations (Moreira *et al.* 2000). The impact of those findings is that those mutations are not readily explained as to their effect on sarcolemmal and/or myofibril structures. In other words, it is suggested that connectin is responsible for skeletal muscle integrity not only as a structural and elastic component but also as a link between sarcolemma and intracellular signal transduction systems. Here, we performed immunohistochemical analyses of p94 using single myofibers. Together with recent findings about p94 from other studies, the nature of p94 localization in sarcomeres was suggested to be dynamic, reflecting local structural change in connectin as well as p94 itself. In this article, we discuss functions of p94 and connectin regarding signal transduction pathways in skeletal muscle, based on our immunohistochemical analyses of p94 and other recent findings about p94.

Material and methods

Single myofibers were prepared as described previously (Bischoff 1986; Ojima *et al.* 2004). In short, dissected extensor digitorum longus (EDL) muscles from C57BL/6 mice were digested with 0.5% type I collagenase (Worthington Biochemical, Lakewood, NJ) at 37^{o}C for 120 min. Separated single myofibers were fixed with 4% paraformaldehyde in PBS for 20 minutes. After washing with 0.5% triton X-100 in PBS, specimens were blocked with 5% goat serum (Vector Laboratory, Burlingame, CA) in PBS for 15 minutes, and then incubated with anti-s-α-actinin antibody (1:1000; EA-53, Sigma Chemical, St. Louis, MO) and antiserum against NS domain of p94 (1:300) (Sorimachi *et al.* 1995) for 16 hours at 4^{o}C. The reactions were detected with secondary antibodies conjugated with Alexa 488 or Alexa 555 (Molecular Probes Inc., Eugene, OR). Double-stained myofibers were analysed on a laser scanning confocal microscopic system (LSM 510; Carl Zeiss, Inc., Germany), which employed a Zeiss Axiovert inverted microscope. Images were recorded and processed with LSM510 imaging software.

Results

After binding of p94 to connectin was originally reported, localization of p94 in the myofibrils have been studied (Sorimachi *et al.* 1995; Baghdiguian *et al.* 1999; Keira *et al.* 2003). Although histochemical analysis using skeletal muscle sections has been successful in showing that p94 is localized in the N2-line region of sarcomeres and in the nuclei, we examined the localization of p94 in single myofibers to obtain further insight into p94 localization. Single fiber staining can potentially give us information about more precise 3-dimensional localization of the protein of interest within individual cells.

Clear staining at the Z-lines where sarcomeric-α-actinin (s-α-actinin) locates (arrows in Figure 2) was observed using anti-pNS antiserum, being consistent with the immuno-staining results shown previously (Sorimachi *et al.* 1995). In addition, anti-pNS antiserum gave weak signals in the region surrounding the Z-lines (arrowheads in Figure 2), which is likely to correspond to p94 binding to the N2A regions of connectin. Since the myofibers used here were not treated with relaxation buffer, sarcomeres observed in our experiments were relatively shortened (generally less than 1.9 μm). Therefore, it was anticipated that the N2A regions of connectin was tangled, *i.e.*, tandem IG motifs were individually folded and packed together not being regularly exposed for antiserum access. This may be one of the reasons why p94 staining in the N2A regions was detected not as clear distinctive lines but as hazy structures.

Yeast two-hybrid studies showed that p94 interacts with the M-line region of

connectin as well as the N2A regions of connectin (Sorimachi *et al.* 1995; Kinbara *et al.* 1997). However, we did not observe p94 at the M-line region in sarcomeres of single fibers prepared in this study. Why p94 was not detected at the M-line regions in sarcomeres? One of the possibilities is that the used muscle fibers might lack the p94 binding site in the M-line connectin, *i.e.*, "is7" region between the last two IG motifs, M9 and M10 (Kinbara *et al.* 1997). The M-line region of connectin is subjected to various alternative splicing and several C-terminal domains, including is7 region, in the M-line region of connectin, were shown to be excluded in fast myofibers (Labeit and Kolmerer 1995). Single myofibers used in this study were prepared from EDL muscles, which are mostly composed of fast myofibers, and, thus, contained little number of is7 region. This could explain the absence of p94 detection in the M-lines. Second possibility is that the contractile status of sarcomeres might account for the change of p94 localization. When skeletal muscles exhibit spontaneous contractions, extension and contraction of sarcomeres might alter the local structure of p94 binding sites in connectin affecting the localization of p94. Therefore, when the sarcomeres are in its contracted status as was discussed above, it might be difficult that p94 binds to the M-line region of connectin, leading to the loss of detection of p94 on the M-lines. It is also possible that the epitope of anti-pNS antiserum, *i.e.*, the N-terminal portion of p94, is buried upon its binding to the M-line region of connectin. The yeast two-hybrid studies indicated that binding of p94 to the M-line connectin requires whole molecule of p94 (Sorimachi *et al.* 1995), suggesting that the structure at the whole molecule level is important for the interaction between p94 and the M-line connectin.

Discussion

Functions of p94 at the N2A region of connectin

Skeletal muscles always undergo physical stresses. In particular, sarcomeric as well as sarcolemmal components receive the mechanical stresses during spontaneous contractions. How can skeletal muscles sense mechanical stresses and prepare themselves to dynamic change in extracellular and intracellular conditions? Recently, it has been proposed that the connectin and its associated protein complexes in sarcomeres are involved in mechanical sensing and response (Granzier and Labeit 2004). In particular, three regions of connectin, the Z-, N2A-, and M-line regions, are found to bind several signaling molecules as well as structural proteins to form "signal complexes" (Figure 3). These signal complexes are considered to be important not only for muscle functions but also for mechanisms of muscle diseases. For, a defect of one of the molecules included in these complexes such as T-cap/telethonin, myotilin, p94, and connectin itself causes various types of muscular dystrophies (Richard *et al.* 1995; Salmikangas *et al.* 1999; Hauser *et al.* 2000; Moreira *et al.* 2000; Hackman *et al.* 2002), and others such as MARPs and MURF1 are upregulated upon muscle atrophies (Bodine *et al.* 2001; Lecker *et al.* 2004; Nikawa *et al.* 2004; Witt *et al.* 2004).

In the N2-line region, the N2A element is one of the splicing variants of connectin, and consists of several immunoglobulin-like (IG) motifs as well as unique sequences in between (Figure 3). p94 is the first molecule that was shown to bind to the N2A region of connectin. p94 interacts with two IG motifs, I82 and I83, in the N2A region, which is proximate to the N-terminus of the PEVK domain (Figure 3B)

(Sorimachi *et al.* 1995).

Muscle ankyrin repeat proteins (MARPs) also participate in the N2A protein complex on connectin. Members of MARPs family include cardiac ankyrin repeat protein (CARP) (Baumeister *et al.* 1997; Kuo *et al.* 1999), diabetes related ankyrin repeat protein (DARP) (Ikeda *et al.* 2003), and ankyrin-repeat domain protein 2 (Ankrd2/Arpp) (Pallavicini *et al.* 2001). Although expression patterns of three MARPS, CARP, DARP, and Ankrd2, are slightly different in striated muscles, they all have an ability to interact with a tyrosine-rich binding motif between two IG motifs, I80 and I81, of connectin, where the N-terminus of the p94 binding site in the N2A region is adjacent (Miller *et al.* 2003; Witt *et al.* 2004).

MARPs contain the nuclear localization signal, and possibly function as transcription regulators. For example, CARP regulates Nkx2.5-dependent muscle gene expression both in cardiac and skeletal muscles during development (Baumeister *et al.* 1997). CARP also forms complex with the transcription factor, Y-box protein 1a (YB-1a), to suppress expression of muscle-specific genes such as myosin heavy chain (MHC) genes (Zou *et al.* 1997). It should be noted that a defect of MHC isoform change was observed in transgenic mice over-expressing a splicing variant of p94 lacking IS1 region (Spencer *et al.* 2002). As p94 also contains potential nuclear localization signal, p94 and MARPs can potentially shuttle between myofibrils and myonuclei. Thus, using the N2A region of connectin as a scaffold, MARPs and p94 may interact with each other to form signal complex, which may transduce signals from myofibril to the nuclei.

Skeletal muscle phenotypes of MDM (muscular dystrophy with myositis) mice represent another indication for the importance of p94 and MARPs in the N2A region. The *mdm* allele contains a retro-transposon insertion that spontaneously occurred within the connectin gene (Garvey *et al.* 2002). This allele causes the expression of a connectin lacking 83 amino acid residues in the N2A region, which includes the p94 binding region. Mouse homozygotes of *mdm*, $Ttn^{mdm/mdm}$, show severe muscular dystrophy phenotypes, and die within 3 months from birth (Garvey *et al.* 2002; Witt *et al.* 2004). Intriguingly, CARP is more abundantly expressed in skeletal muscles of *mdm* mice than in those of wild type mice, whereas yeast two-hybrid assay showed that the N2A region of *mdm*-type connectin could not bind p94 (Witt *et al.*, 2004). These results show a possibility that a loss of the interaction between p94 and the N2A region causes up-regulation of CARP and severe muscle degeneration. Considering the importance of CARP in transcriptional regulation of several muscle genes, it is interesting to speculate that expression of several skeletal muscle genes could be regulated by a balance between p94 and CARP, which may compete for the N2A binding site because of the close proximity of both sites, and/or by proteolytic regulation of CARP by p94. At least, *mdm* mice studies clearly indicate that the N2A region of connectin plays critical roles in maintaining skeletal muscle homeostasis.

Functions of p94 at the M-line region of connectin

The M-line region of connectin is another attractive region when one considers connectin-mediated signal transduction pathways. For, the M-line region of

connectin contains (1) the binding site for MURF-1 (Centner *et al.* 2001), (2) a serine/threonine kinase domain (Labeit and Kolmerer 1995), and (3) another binding site for p94 (Sorimachi *et al.* 1995; Kinbara *et al.* 1997).

MURF-1 interacts with two IG-motifs, A168 and A169, of connectin, which is located at periphery of the M-line (Figure 3C) (Centner *et al.* 2001). Several lines of evidence support that MURF-1 is a multi functional protein involved in skeletal muscle homeostasis. First, exogenously expressed MURF-1 disrupted the assembly of myofibrillar proteins around the M-lines, suggesting that MURF-1 has a critical role in construction of connectin-based M-line structure (McElhinny *et al.* 2002). Secondly, MURF-1 binds to glucocorticoid modulatory element binding protein-1 (GMEB-1), which controls transcription in response to cellular glucocorticoid levels (McElhinny *et al.* 2002), suggesting a role in regulating gene expression. Thirdly, MURF-1 has been shown to function as ubiquitin ligase (Bodine *et al.* 2001), and one of splicing variants of MURF-1 interacts with small ubiquitin related modifier-3 (SUMO-3) (Dai and Liew 2001), suggesting that MURF-1 regulates protein turnover *via* ubiquitin and/or SUMO systems.

Disruption of a part of the connectin gene corresponding to both the binding site for MURF-1 and the kinase domain resulted in embryonic lethality, suggesting that MURF-1 binding and/or connectin kinase activity is essential for muscle development (Gotthardt *et al.* 2003). It is possible that the kinase activity is sterically modulated by association of MURF-1 with connectin. For, the MURF-1 binding site on connectin is IG-motifs, A168 and A169, whereas the kinase domain is located between the next two

IG-motifs, A170 and M1. The p94 binding site in the M-line region of connectin is located between the two IG-motifs, M8 and M10, at the C-terminus of connectin (M9 and a unique sequence called “is7”, see Figure 3C) (Sorimachi *et al.* 1995; Kinbara *et al.* 1997). Since connectin molecules extending from the opposite side of sarcomere overlap with each other in the M-line region, p94 bound to the M-line connectin can contact with MURF-1 binding to another connectin extending in the reverse direction (Figure 3C) (Gregorio *et al.* 1999). Thus, there might be functional interaction between p94 and MURF-1 such as proteolysis of MURF-1 by p94 at least in response to certain signal(s).

In tibial muscular dystrophy (TMD), skeletal muscles of anterior lower legs are preferentially affected and show atrophy (Udd *et al.* 1993). TMD is caused by mutations in the extremely C-terminal IG-motif of connectin, namely, M10, which is located next to the p94 binding site in the M-line connectin. In TMD muscles, the expression of myomesin, which binds to IG-motif M4 of the M-line connectin, was shown to be intact, suggesting that the sarcomeric localization of M4 epitope of connectin is not altered (Hackman *et al.*, 2002). It is, however, found that in TMD skeletal muscles, M8 and M9 regions of M-line connectin, which is N-terminal to the affected M10, could not be immunohistologically detected. Therefore, the mutation in M10 might disrupt the structure of its proximity, and as Hackman and colleagues discussed, binding of p94 to the adjacent M9+is7 region may be compromised. On the other hand, yeast two-hybrid study showed that only M9+is7 region is enough for interacting with p94 (Kinbara *et al.*, 1997). At present, it is unclear whether or not p94

is dissociated from the C-terminal region of connectin in TMD skeletal muscles. As described above, a peripheral region of the M-line connectin contains the kinase domain and the binding sites for MURF-1 and p94, forming a possible signal complex. TMD mutation must perturb functions of this signal complex. Given the facts that the heart muscle is not affected in TMD patients, it is predicted that molecular mechanisms of TMD pathogenesis involves the components of skeletal muscle specific signal complex at the M-line region of connectin. Since p94 is not expressed in mature heart, it is possible that p94 in the M-line region would play a critical role(s).

Possible functions of p94 in the Z-lines

Our immunohistochemical studies showed clear staining of p94 in the Z-lines (Figure1) (Sorimachi *et al.* 1995). The Z-line region of connectin, however, is not responsible for p94 binding (data not shown), or an alternate binding partner for p94 in Z-lines has not yet been identified. Besides p94, the Z-lines contains various protein components including the N-terminal region of connectin, s-α-actinin (Sorimachi *et al.* 1997), T-cap/telethonin (Gregorio *et al.* 1998; Mues *et al.* 1998), myotilin (Salmikangas *et al.* 1999), subunit of K^+-channel (minK) (Furukawa *et al.* 2001), and barbed end of thin filament protein complex, etc. The importance of the Z-line structure has been discussed, *e.g.*, as to that it links myofibrils to sarcolemma through costameres (Evarsti 2003). Mutations in the genes for T-cap/telethonin and myotilin cause limb-girdle muscular dystrophies, more directly suggesting that complex in the Z-line is also essential for proper functions of muscles. Thus, again, it is tempting to imagine a

signal complex specifically formed at the sarcomere Z-line regions where p94 also plays a crucial role in signal transduction pathway linking sarcolemma and the nuclei. So far, p94 is the unique mutual factor identified in the Z-, N2-, and M-line region. It might be, therefore, the case that p94 facilitates coordinated signal transduction by adjusting distinct signal complex in each region.

Conclusion

The concept of “connectin- and p94-mediated stress response pathways” has now become more convincing based on immunohistological data, biochemical analyses as well as genetic studies related to skeletal muscle pathology. Under the normal conditions, in skeletal muscles, p94 binds to connectin where p94 activity may be suppressed to keep a pool of inactive form of p94 in sarcomeres. Detailed evaluation of immunostained single myofibrils suggested that the localization of p94 in the sarcomere Z-, N2A, and M-line regions might be regulated under the muscle conditions such as contractility.

The connectin-bound, quiescent p94 molecules are in close proximity to other connectin binding proteins including MARPs, MURF-1, etc. When skeletal muscles receive mechanical stresses/signals, p94 could dissociate from connectin, transform into an active protease in the myonuclei and other intracellular regions of muscle cells. These activities might prompt appropriate cellular responses, such as gene expressions, in coordinate with other connectin binding proteins. Although much has yet to be elucidated, the impact of recent studies on p94 including this study is that a great change of viewpoints on physiological properties not only of p94 but also of connectin have been introduced. In other words, p94 is not just a protease binding to a sarcomeric protein, and connectin is not just a platform for various protein ligands, but rather, together, these molecules work as unique devices crucially important for muscle homeostasis. p94 has brought about a great change of viewpoints on physiological functions of connectin, and, *vice versa*, so has connectin on those of p94.

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Acknowledgement

We would like to thank Dr Siegfried Labeit and Dr Christian Witt in Universitätsklinikum Mannheim, Dr Carol C. Gregorio in University of Arizona, and Dr Sumiko Kimura in University of Chiba for valuable discussions. 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 JSPS (the Japan Society for the Promotion of Science), a Research Grant (14B-4) for Nervous and Mental Disorders from the Ministry of Health, Labour and Welfare, and “Ground-based Research Announcement for Space Utilization” promoted by the Japan Space Forum. This paper is dedicated to the memory of Prof. Koscak Maruyama. We all miss him.

Figure Legends

Fig. 1. Schematic domain structures of p94 and the conventional calpain (μ-, and m-calpain) large subunits.

I, IIa+IIb, III, and IV represent the four calpain domains. NS, IS1, and IS2 are p94-specific insertion sequences. Domains I, and III are α-helical, and C2-like Ca^{2+}-binding domains, respectively. Domain IV contains 5 EF-hand motifs, also involved in Ca^{2+}-binding. Subdomains IIa and IIb compose the protease domain when activated. The active center of calpains is formed by Cys, His, and Asn indicated in the domain II.

Fig. 2. Low magnification (A-C) and high magnification (D-E) confocal micrographs demonstrating the localization of p94 and sarcomeric-α-actinin (s-α-actinin).

Single myofibers were stained with anti-s-α-actinin antibody (red in A, C, D, and F) and anti-pNS antiserum (green in B, C, E, and F). C and F are superimposed images of A-B and D-E, respectively. Anti-pNS antiserum clearly visualized p94 in Z-lines, where anti-s-α-actinin was positive (arrows). Further, anti-pNS antiserum detected faint patterns around Z-lines (arrowheads). These staining could be p94 in the N2A region of connectin. Since the N2A region of connectin was not stretched enough (approximately, the length of sarcomeres were less than 1.9 μm), epitopes of antiserum may not have aligned in order. Clear M-line staining of p94 was not detected (see text for possible reasons). Bars indicate 20 μm and 5 μm in C and F, respectively.

Fig. 3. A model of p94- and connectin-mediated signal transduction pathways in skeletal muscles.

(A) p94, connectin and associated proteins in a sarcomere

p94 binds to connectin at the N2A and M-line regions based on yeast two hybrid studies and is also localized in the Z-lines shown by immunofluorescent studies (see Figure 2). Muscle ankyrin repeat proteins (MARPs), including cardiac ankyrin repeat protein (CARP), diabetes related repeat protein (DARP), and ankyrin-repeat domain protein 2 (Ankrd2), are able to interact with the N2A region of connectin. MURF-1 is located in the M-line. T-cap, MARPs, myotilin, and s-α-actinin are involved in Z-line protein complexes. Ovals with broken lines indicate each p94- and connectin-mediated signal transduction complexes. Z, N2A, and M indicate Z-lines, N2A- and M-lines, respectively.

(B) Protein complex at the N2A region of connectin

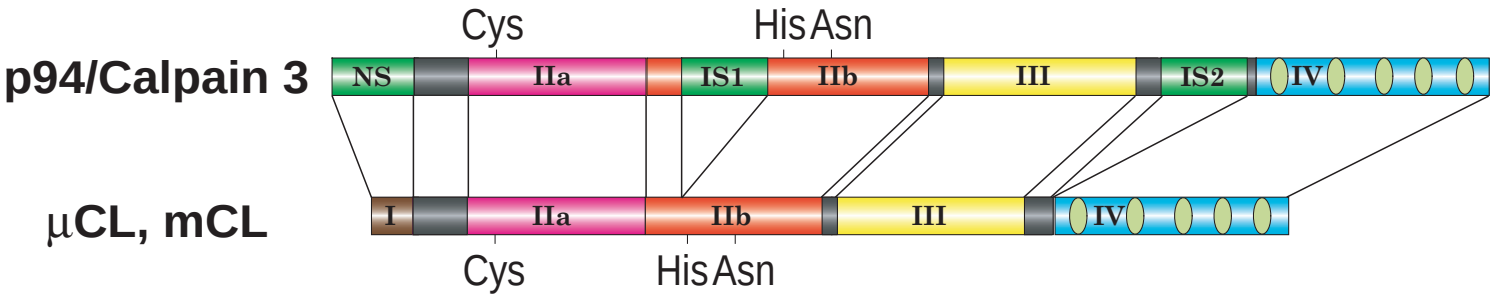
p94 binds to I82/I83 of immunoglobulin-like (IG) domains, which are adjacently located to the N-terminus of the PEVK domain. MARP family interacts with a tyrosine rich binding motif between I80 and I81. Two-headed arrow indicates the deletion site of connectin in *mdm* mice.

(C) Protein complex at the M-line of connectin

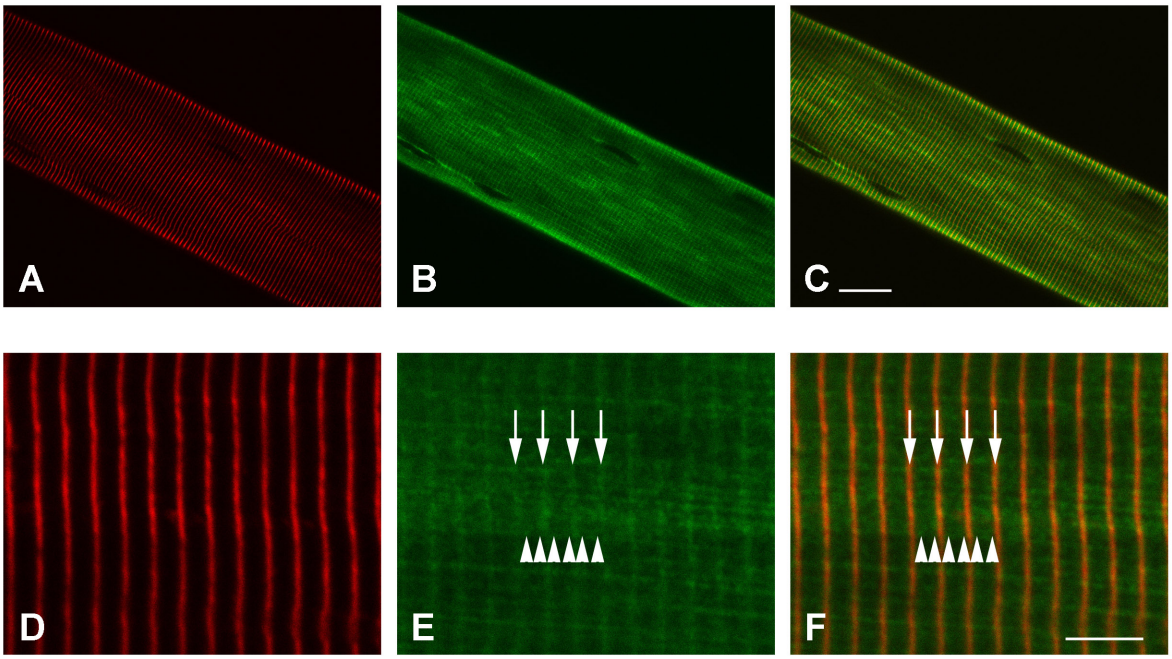
p94 binds to M9+is7 at the periphery of the M-lines. MURF-1 interacts with A168/A169 IG domains of connectin. Connectin molecules run in an antiparallel fashion and intersect at the Centner of the M-line. p94 could interact with MURF-1 and the kinase domain of the other side of connectin at the periphery of M-lines.

Arrows indicate the mutated site in tibial muscular dystrophy (TMD) patients. M10 is the last C-terminal domain of connectin.

Ojima, K. *et al.*, Figure 1



Ojima, K. *et al.*, Figure 2



Ojima, K. *et al.*, Figure 3