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Muscle-specific Calpain, p94, Responsible for Limb Girdle Muscular Dystrophy Type 2A, Associates with Connectin through IS2, a p94-specific Sequence*

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p94, a muscle-specific member of calpain family, is unique in that it undergoes rapid and exhaustive autolysis with a half-life of less than 1 h resulting in its disappearance from muscle. Recently, p94 was shown to be responsible for limb girdle muscular dystrophy type 2A. To elucidate the muscular proteolytic system mediated by p94 and to solve the mystery of its unusually rapid autolysis, we searched for p94-binding proteins by the two-hybrid system. Although calpain small subunit plays a crucial role for regulation of ubiquitous calpains, it did not associate with p94. After a screening of skeletal muscle library, connectin (or titin), a gigantic filamentous protein spanning the M- to Z-lines of muscle sarcomere, was found to bind to p94 through a p94-specific region, IS2. The connectin-insoluble fraction of washed myofibrils contained full-length intact p94, suggesting that connectin regulates p94 activity.

Proteolysis in cytosol is a key reaction to modulate various intracellular protein functions such as signal transduction, protein turnover, and cell structure. Calpain, Ca^{2+}-dependent cysteine proteinase (EC 3.4.22.17), is one of the major intracellular proteinases known as interacting various protein kinases, transcription factors, and cytoskeletal proteins (1–5). Ubiquitous μ- and m-calpains, dimers of a large catalytic (μCL[1] (6) and mCL (7), respectively) and small regulatory (30K (8)) subunit. Recently, we found that the ubiquitous calpain large subunit monomer can express full proteolytic activity, and that 30K dissociates from the large subunit upon activation by Ca^{2+} (9, 10). In other words, 30K together with calpastatin, a specific proteinaceous inhibitor for calpain, play pivotal roles in regulation of calpain activity.

p94 is a muscle-specific member of the calpain large subunit family, and distinct not only from the other members but also from other proteases in that it autolyzes very rapidly and extensively leading to almost complete disappearance right after translation even in the presence of EGTA and leupeptin as observed *in vitro* (11, 12). Ubiquitous calpains as well as many other proteases also undergo autolysis at the NH_2 terminus, but only to a limited extent. Quite recently, p94 was identified as responsible for limb girdle muscular dystrophy type 2A (LGMD2A), the first demonstration of the involvement of an enzyme in muscle dystrophy (13). To elucidate physiological meaning of this exhaustive autolysis and the molecular mechanism connecting LGMD2A and p94 function, it is important to clarify the substrates of p94 and the manner to regulate the proteolytic activity of p94.

30K is the first candidate to bind to and regulate p94, since the calmodulin-like Ca^{2+}-binding domains of μCL and mCL (see Fig. 2), which are the binding sites for 30K, are highly homologous to p94 (5). Analysis of p94 at the protein level, however, is very difficult because of the extremely rapid autolysis, and, thus, we examined using the yeast two-hybrid system (14). As a result, 30K was revealed not to bind to p94, and, instead, connectin, striated muscle-specific giant filamentous protein, was shown to associate with p94.

EXPERIMENTAL PROCEDURES

Binding Assay and Screening by the Yeast Two-hybrid System—Wild type of rat p94, human μCL and mCL, rabbit calpastatin and 30K, and active site Cys to Ser mutants of rat p94 (p94:C129S) (12) and human μCL (μCL:C115S) were constructed into pGBT9 or pGAD424 vector DNAs (MATCHMAKER™ Two-Hybrid System: Clontech) for protein expression. These plasmids were co-expressed in *Saccharomyces cerevisiae* HF7c and/or SFY526 strains as described by the manufacturer. β-Galactosidase activity of SFY526 cells was measured using *o*-nitrophenyl galactoside as described by the manufacturer.

For screening, p94:C129S were co-expressed in *S. cerevisiae* HF7c with approximately 2×10^6 of human skeletal muscle cDNA library in pGAD424 vector (Clontech). The plasmids were rescued from colonies grown on −(His, Leu, Trp) plates, sequenced, and retransformed with p94:C129S to confirm positive binding.

p94Δ1 to -5 were constructed by digestion of p94:C129S with *Nco*I + *Sal*I, *Pst*I, *Bal*I + *Sal*I, *Kpn*I + *Sal*I, and *Eco*RI following self-ligation, respectively. p94-IS2 was made by digestion of p94Δ3 with *Eco*RI following self-ligation. These constructs were co-expressed in HF7c, and the growth on −His plates were examined.

Anti-peptide Antisera—The anti-peptide antisera against the following peptides were used: pNS (NH_2-terminal sequence peptide) corresponding to the NH_2-terminal 18-amino acid sequence of p94 (12); pK-rich (Lys-rich peptide) corresponding to the NH_2-terminal Lys-rich sequence of the IS2 region (12); pDM1 corresponding to myotonin protein kinase (MtPK) amino acid residues 281–296 (15).

Fractionation of Skeletal Muscle—Rabbit skeletal muscle was homogenized in 50 mM KCl, 1 mM $NaHCO_3$, 5 mM EGTA and washed 5

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[1] The abbreviations used are: μCL, μ-calpain large subunit; mCL, m-calpain large subunit; LGMD2A, limb girdle muscular dystrophy type 2A; MtPK, myotonin protein kinase.

TABLE I

Interaction between calpain large subunits and calpastatin or small subunit

mCL, μCL, μCL:C115S; p94, and p94:C129S were co-expressed with calpastatin or 30K in SFY526 and HF7c yeast cells. The β-galactosidase activity in 5 independent colonies of SFY526 cells were measured using *O*-nitrophenyl galactoside as described under "Experimental Procedures," and the average and the standard deviation are indicated. Growth on −(H,L,W) plates were examined for HF7c cells, and the ratio of the number of colonies on −(H,L,W) plates to −(L,W) plates were calculated. p53 and SV40 large T, which were contained in the kit, were also examined for positive control.

In pGBT9	In pGAD424	β-Galactosidase activity	S.D.	Growth on −(H,L,W)	−(H,L,W)/−(L,W)
		units × 10^{-2}			%
mCL		1.15	0.13	−	0.0
μCL		1.33	0.17	−	0.0
μCL:C115S	Calpastatin	1.21	0.05	−	0.0
p94		1.41	0.06	−	0.0
p94:C129S		1.77	0.13	−	0.0
mCL		**1.97**[a]	0.26	+	8.6
μCL		**16.7**[a]	3.4	+	17.1
μCL:C115S	30K	**22.9**[a]	5.7	+	18.5
nCL-2′		1.36	0.09	−	0.0
p94		1.29	0.09	−	0.0
p94:C129S		1.29	0.11	−	0.0
p53	SV40 large T	**129**[a]	4.6	+	

[a] Significant at 0.1% significance by *t*-test compared with nCL-2′.

times with the same buffer. The sedimented myofibrillar fraction (fraction 1) was washed 4 times with 5 mM $NaHCO_3$, twice with 30 mM NaCl, and once with 0.1 M $NaPO_4$ buffer (pH 6.6). The remaining insoluble fraction was extracted with 0.2 M $NaPO_4$ buffer (pH 7.0) (fraction 2). These fractions were subjected to 4–15% SDS-polyacrylamide gradient gel electrophoresis and blotted onto a nitrocellulose membrane. Western blot was performed using anti-pNS and anti-pK-rich antisera as described previously (12).

Immunofluorescence Staining—Freshly excised rabbit myofibrils were fixed with 10% formalin containing 50 mM KCl, 10 mM EGTA, 3 mM ATP, 10 mM phosphate buffer (pH 7.5), and 0.5% Triton X-100. Myofibrils were treated with anti-pK-rich, or anti-pNS antisera, or normal rabbit serum, then treated with fluorescein isothiocyanate-labeled anti-rabbit IgG (Cappel, West Chester, PA) and observed by a Leits ORTHO-LUX-2 fluorescence microscope as described previously (16).

Expression in COS Cells—The cDNA for MtPK (15), p94, and p94:C129S (12) were constructed into pSRD vectors and expressed in COS cells by electroporation as described previously (12). Cells were collected, lysed by SDS-sample buffer, and separated in SDS-polyacrylamide gel electrophoresis. Western blot was performed using anti-DM1 antiserum (15), anti-pK-rich antiserum (12), and anti-human calpastatin antiserum (CSL5–10) purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan).

RESULTS AND DISCUSSION

First, association between calpain large subunits and 30K or calpastatin was examined using the yeast two-hybrid system. μCL, μCL:C115S, and mCL showed the expected binding activities to 30K (both β-galactosidase activity in SFY526 and −His viability in HF7c) in the yeast two-hybrid system (Table I). nCL-2′, which lacks calmodulin-like Ca^{2+}-binding domain identified as the 30K-binding site, did not bind to 30K (17). These results are consistent with the previous observations. However, both p94 and p94:C129S did not bind to 30K, implying the existence of other regulatory molecule(s) for p94 than 30K. On the other hand, calpastatin did not bind to any of the large subunits examined, indicating that Ca^{2+} concentration is rather low in the yeast two-hybrid system since calpastatin can only react with calpain in the presence of Ca^{2+}.

To identify a regulatory factor controlling p94 autolysis, a human skeletal muscle cDNA library was screened by the two-hybrid system using p94:C129S whose autolytic activity is completely suppressed (12). 32 out of 71 isolated clones were finally identified as positive, and all encoded various parts of connectin (also called titin), a giant elastic protein (approximately 3,000 kDa) in striated muscle consisting mainly of fibronectin type III and immunoglobulin C2 (IgC2) globular domains (18, 19). As shown in Fig. 1*A*, 31 out of 32 clones encoded identical amino acid sequence to the COOH-terminal parts of human cardiac connectin (20). The positions of these cDNAs indicate that the COOH-terminal 2 repeats of the IgC2 motif and/or the interdomain insertion sequence, "is7," are essential for p94 binding. Since the COOH terminus of connectin is also believed to interact with M-line proteins such as myomesin and M-protein (21, 22), this result suggests that these proteins may be substrates of p94.

The sequence of one clone, pCNT-N_2, showed significant similarity to a chicken connectin cDNA, Cn3B (23), which encodes the region around the N_2-line of connectin (total similarity of the deduced amino acid sequence = 78%, see Fig. 1*B*). As shown in Fig. 1, *A* and *B*, pCNT-N_2 encodes 3 repeats of the IgC2-like motif. The N_2-line, approximately 2,000 kDa upstream from the COOH terminus, is considered to be the site where full-length connectin (α-connectin or titin I) tends to split into a 1,200-kDa NH_2-terminal fragment and the remaining protein (β-connectin or titin II) (18). Although the physiological meaning of this split has yet to be established, calpain is reported to be involved (24, 25). Our results, therefore, strongly suggest that p94 is responsible for the connectin splitting. Furthermore, the sequence just downstream of the p94-binding region of connectin is similar to calpastatin (23, 26) (see Fig. 1*A*), suggesting that this domain regulates p94 activity. Amino acid sequence comparison of the IgC2 motifs among the p94-binding sites revealed no significant consensus sequence (Fig. 1*B*).

The binding activity of pCNT-N_2 to p94 was much stronger than that of COOH-terminal clones (data not shown). This is in contrast to the fact that the ratio of the number of the obtained clones is 1 to 31 compared to COOH-terminal clones. However, the RNA content, including the position of pCNT-N_2 approximately 60 kb upstream from the 3′-end, is thought to be very low since the cDNA library was constructed using poly(A)$^+$-purified RNA. Thus, pCNT-N_2 was used mainly for further analyses. Binding between p94 and the N_2-line region of connectin was confirmed *in vitro* using *in vitro*-translated pCNT-N_2, and the specificity of pCNT-N_2 was examined using the two-hybrid system, showing that μCL and mCL do not bind to this clone (data not shown).

To identify the binding site of p94, truncation mutants of p94 were analyzed. As shown in Fig. 2, truncated mutants Δ3 to Δ5, but not Δ1 and Δ2, possessed the binding activity to pCNT-N_2. Furthermore, p94-IS2, which comprises almost exclusively the IS2 region of p94, was confirmed to bind, indicating that IS2, a

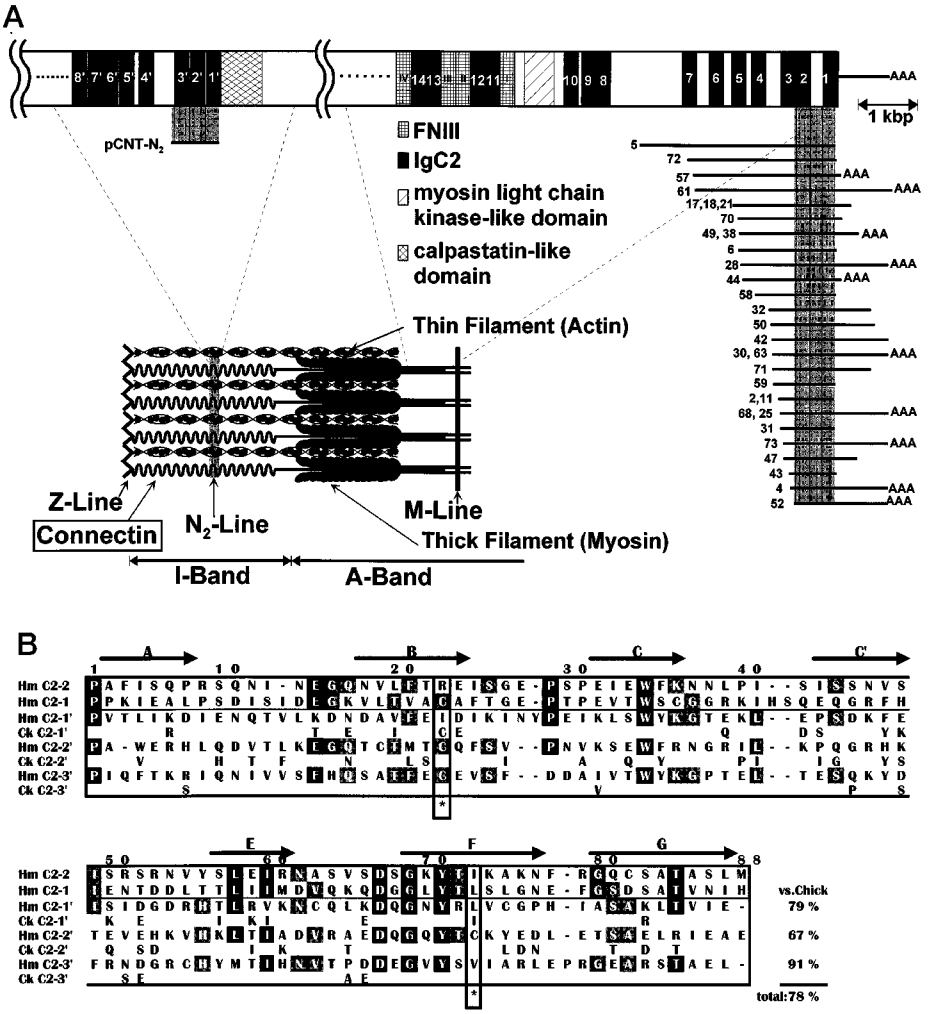

FIG. 1. **Isolated connectin clones that bind to p94.** *A*, positions of isolated connectin clones. At the *lower left* is a schematic illustration of half of a skeletal muscle sarcomere. At the *top* is the structure of two connectin cDNA regions (20, 23) whose relative positions in the sarcomere are indicated by *broken lines*. *Thick lines* under the connectin cDNA structure show the positions of clones obtained from the two-hybrid screening of a human skeletal muscle cDNA library. *AAA* indicates a poly(A)$^+$ tail. *Numbers* in repeating motifs are arbitrarily assigned from the COOH terminus in this paper and do not match other papers (20, 23). *Shaded regions* are the minimal binding sites to p94. *B*, sequence of IgC2 motif repeats where connectin binds to p94. Amino acid sequences of 1, 2, and 1′ to 3′ of IgC2 motifs in *A* were aligned to maximize similarity. Numbers correspond to human C2–2. Conserved residues among 4 and above, or 3 and above for human sequences, are indicated by *reversed* or *shaded-reversed letters*, respectively. The conserved Cys involved in a disulfide bond in immunoglobulins is identified with an *asterisk*; its companion Cys is missing in the connectin IgC2 motifs. *Arrows labeled A–G* denote 7 β-sheet structures observed in immunoglobulin. Percent values at the *right* indicate similarity to the corresponding chicken motif. Chicken sequences Ck C2–1′ to -3′ were taken from Ref. 23.

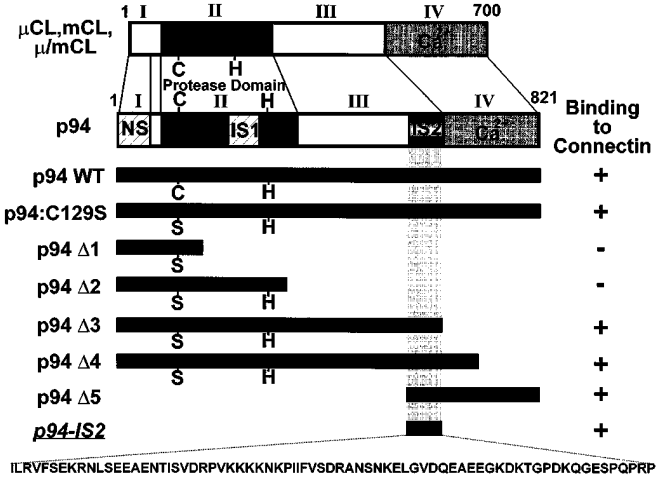

FIG. 2. **IS2 region of p94 binds to pCNT-N_2.** The *upper two boxes* show schematic structures of conventional calpain large subunits for μCL, μ/mCL, and mCL, and p94, where Ca^{2+}, NS, IS1, and IS2 are the Ca^{2+}-binding domain, and three p94-specific insertion regions, respectively (32). Binding to pCNT-N_2 is qualitatively indicated at the *right*. The *shaded region* is the minimum binding site whose amino acid sequence is shown at the *bottom*.

region specific to p94 (5) (see Fig. 2), is the N_2-line binding site. IS2, which shows no similarity to currently available sequences, contains a nucleus translocation signal-like sequence, P*X*KKKK*X*KP (11, 12). Connectin binding and nucleus translocation are apparently inconsistent, but it is possible that a fragment of p94 moves from connectin to the nucleus after autolysis.

Due to its very rapid autolysis, p94 was not detected in muscle fractions in the previous chromatographic studies of the muscle-soluble fraction (12, 27). Based on the current data, an attempt was made to identify the p94 protein during the purification of connectin. Myofibril fractions (Fig. 3, *lane 1*) contained full-length p94 (94 kDa) detected by both anti-pNS and -pK-rich antisera at the position identical with COS-expressed p94 and p94:C129S (*lanes 3K*, *4K*, and *4N*) (12). This fraction contained connectin. Since the half-life of p94 is less than 1 h, the p94 detected here must be stabilized, presumably by binding to connectin. Upon solubilization of connectin in high ionic strength buffer, part of p94 undergoes limited autolysis yielding a 41-kDa fragment detected by anti-pNS (*lane 2N*), which also appeared weakly in the first fraction (*lane 1N*), and 53- and 55-kDa fragments (53K and 55K, respectively) detected by anti-pK-rich antisera (*lane 2K*), suggesting that p94 split into protease domain (NH_2-terminal 41K) and the remaining domain (COOH-terminal 53K) possibly functioning independently after the autolysis. Among them, 53K coincides with the largest degradation fragment of COS-expressed p94 (12) (*lane 3K*), indicating that this fragment is slightly more stable than others. Since connectin is very large, cohesive, and insoluble in ordinary buffers, usual biochemical strategies, such as immunoprecipitation and affinity purification, are ineffective (18).

To examine p94-connectin binding *in vivo*, myofibrils were directly stained by anti-pK-rich antiserum. As shown in Fig. 4*B*, anti-pK-rich antiserum gave signals at the Z-lines and at

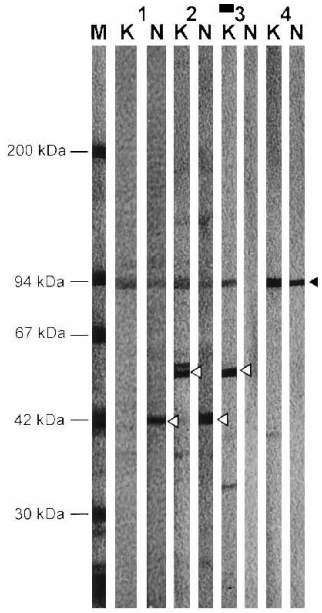

FIG. 3. **Identification of p94 in skeletal muscle.** p94 was detected by anti-pNS (*lanes N*) and anti-pK-rich (*lanes K*), respectively. *Lanes 1* and *2* correspond to fractions 1 and 2, respectively, and lanes 3 and 4 contain COS-expressed wild type p94 and p94:C129S, respectively. *Closed* and *open triangles* indicate full-length and degraded p94, respectively. Molecular mass markers are indicated in *lane M*.

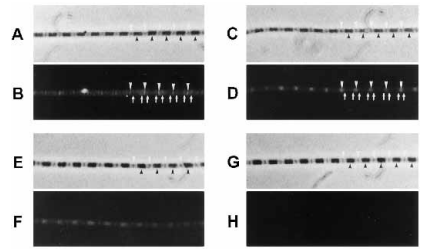

FIG. 4. **Immunofluorescence staining of p94 in myofibrils.** *A*, *C*, *E*, and *G* are phase contrast images, which were stained with anti-pK-rich antiserum (*B*), anti-pNS antiserum (*D*), normal rabbit serum (*F*), and no serum (a second antibody only) (*H*), respectively. *White* and *black arrowheads* indicate positions of the Z- and M-lines, respectively, and *white arrows* in *B* and *D* show positions of the N_2-lines. *Bar*, 5 μm.

positions between the Z- and M-lines, which are approximately 60% from the M-line toward the Z-line and roughly correspond to the N_2-line. The latter signal was, however, not strong, and was not clearly stained by anti-pNS antiserum (Fig. 4*D*), possibly because of very low quantity and stability of p94. Since binding of the COOH-terminal region of connectin to p94 was much weaker, this binding could not be detected. On the other hand, the Z-lines, where the NH_2 terminus of connectin locates, were stained with both anti-pK-rich and anti-pNS antisera. The reason why cDNA clones corresponding to this region could not be obtained is most probably ascribed to the rareness of this part of cDNA in the library. This point must be examined further by isolating cDNA for the NH_2-terminal region of connectin. A possibility that anti-pK-rich antiserum cross-reacted with protein(s) other than p94 cannot be ruled out at present.

Quite recently, mutations in the p94 gene are shown to cause LGMD2A, the first case in which an enzyme is responsible for muscle dystrophy (13, 28). Other muscle dystrophies arise from defects in structural proteins such as dystrophin, merosin, and adhalin (29). Therefore, the molecular mechanisms of LGMD2A and other dystrophies must differ. Since LGMD2A is a recessive disorder and some reported mutations lacked the protease domain of p94 (13), a loss-of-function mechanism is

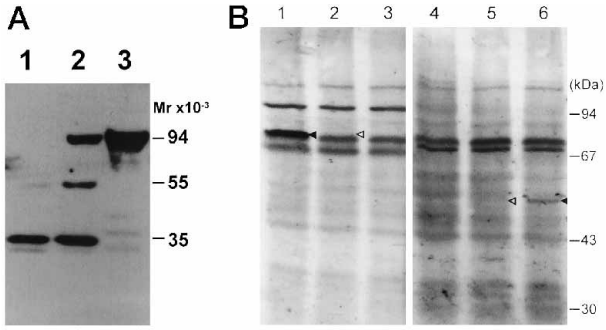

FIG. 5. **Proteolysis by p94 overexpression.** *A*, wild type p94-proteolyzed p94:C129S. Wild type p94 (*lane 1*), p94:C129S (*lane 3*), or both (*lane 2*) were expressed in COS cells and analyzed by immunoblotting using anti-pK-rich antiserum. Each lane contains the same number of cells which were transfected by the same amount (15 μg) of each expression vector DNA, *i.e. lane 2* contained a total of 30 μg of DNA. *B*, myotonin protein kinase was proteolyzed by p94 overexpression. Myotonin protein kinase (MtPK, *lanes 1* and *4*), p94 (*lanes 3* and *6*), or both (*lanes 2* and *5*) were expressed in COS cells and analyzed by immunoblotting using either anti-MtPK (*lanes 1–3*) or anti-pK-rich (*lanes 4–6*) antisera. Expressed (*closed triangles*) or absent (*open triangles*) bands of MtPK and the 53-kDa fragment of p94 are indicated.

presumed. To elucidate the mechanism of LGMD2A, identification of the substrate(s) and regulator of p94 is crucial, although the two-hybrid system could not detect substrates. Thus, various available proteins were investigated for their ability to act as substrates for p94. First of all, as shown in Fig. 5*A*, p94:C129S is remarkably degraded by co-expression of wild type p94, indicating that p94 itself is also a good substrate for p94, *i.e.* intermolecular autolysis of p94 occurs. When myotonin protein kinase (MtPK), responsible for myotonic dystrophy (15, 30), is co-expressed with p94 in COS cells, MtPK disappears as shown in Fig. 5*B*, *lanes 1* and *2*. This is consistent with a previous observation that various protein kinases such as protein kinase C and myosin light chain kinase are good calpain substrates (3, 31). At the same time, the p94 band, only the 55-kDa fragment of which can be observed due to autolysis, disappeared (*lanes 5* and *6*), suggesting that a p94 substrate promotes the down-regulation of p94 itself. Further analyses using the myoblast expression system and mutant mice are now in progress to study the detailed molecular mechanism and the physiological meaning of the interaction between p94, connectin, and the substrates.

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Muscle-specific Calpain, p94, Responsible for Limb Girdle Muscular Dystrophy Type 2A, Associates with Connectin through IS2, a p94-specific Sequence

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