Purification of native p94, a muscle-specific calpain, and characterization of its autolysis

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p94, a skeletal muscle-specific calpain, has attracted much attention because its gene is responsible for limb-girdle muscular dystrophy type 2A. p94, however, has not been characterized at the protein and enzyme levels, owing to its very rapid autolysis. In the present study, a purification procedure for p94 was first established by using a recombinant inactive p94 expressed in COS cells in which the active site cysteine residue was changed to serine [p94(C129S)]. The isolation of native p94 from rabbit skeletal muscle by the established method with conventional procedures was extremely difficult because p94 became highly unstable in a crude extract on the addition of NaCl for separation. Purification of native p94 was possible with an antibody-affinity column but only as an inactive enzyme; p94(C129S) was purified as a homodimer. Characterization of p94, especially autolysis,

was performed with partly purified native p94 and p94(C129S). The autolysis of p94, which consisted at least partly of an intermolecular reaction, proceeded in three consecutive steps; 60 and 58 kDa fragments were produced as intermediates before a stable 55 kDa fragment appeared. Autolysis of p94 was regarded as a degradative step rather than for the activation of the enzyme. All the autolysis cleavage sites were located in the p94-specific insertion sequence 1 region, which explains why p94 is unstable compared with the other calpains. The autolysis sites in p94 clearly showed a different specificity relative to the autolytic and proteolytic cleavage sites of the ubiquitous μ - and m-calpains, in its preference for residues at the P3 to P1' sites, indicating a distinct substrate specificity and function for the muscle enzyme.

INTRODUCTION

Calpain (EC 3.4.22.17), a non-lysosomal Ca^{2+} -activated protease, is assumed to have important roles in various Ca^{2+} -regulated cellular functions in higher animals [1–6]. Calpain homologues have also been found in various eukaryotes including *Caenorhabditis elegans* and yeast [1,7,8]. Among these, p94 [novel calpain large subunit 1 (nCL-1) or calpain 3] is a muscle-specific calpain species with additional domains and properties distinct from those of other calpain members [9,10]. p94 is composed of four domains similar to the ubiquitous calpain large subunits, and has three unique domains, namely NS in the N-terminus, insertion sequence (IS) 1 in the protease domain II, and IS2 between domain III and the Ca^{2+} -binding domain IV (Figure 1) [9]. An important, unique feature of p94 is its rapid autolysis. For this reason, p94 has been little characterized in skeletal muscle, although its mRNA levels are approx. 10-fold higher than those of m- and μ -calpains. The fact that the IS2-deletion mutant does not autolyse suggests that the IS2 region is essential for rapid autolysis [10]. We have recently reported that p94 interacts with connectin/titin [11–13], a giant protein linking myosin filaments from the M-line to the Z disc [14–16]. Connectin has at least two p94-binding regions, the N₂-line region, which is close to the fragile site of α -connectin, and the M-line insertion 7 (M-is7) region in the extreme C-terminus of connectin. Interestingly, both p94-binding regions are alternatively spliced under

physiological conditions, suggesting that p94 might interact differently with alternatively spliced forms of connectin [15,17] and thus might have distinct functions. Truncation of full-length p94 indicates that the interaction between p94 and the N₂ region requires IS2, whereas that between p94 and M-is7 requires full-length p94 [11,12]. The physiological significance of these interactions is under investigation.

p94 was recently found to be the gene responsible for limb-girdle muscular dystrophy (LGMD) type 2A [18]. LGMD is genetically heterogeneous and is classified into two types: two dominant forms (LGMD1A and 2B) and seven recessive forms (LGMD2A–2G) [19–21]. All recessive forms other than type 2A have been reported to be caused by a loss of the sarcoglycan complex in the skeletal muscle membrane. In LGMD2A patients, dystrophin and dystrophin-associated glycoproteins are normal but various point mutations and deletions in the p94 gene have been reported [18,21]. Although the genetic data clearly indicate that the loss of proteolytic or autolytic activity of p94 is the cause of LGMD2A disease, the pathophysiological mechanism that leads to a progressive phenotype and muscle necrosis is obscure at present.

In the present study, as a step towards clarifying the physiological function of p94, we attempted the purification of p94 from skeletal muscle and analysed some of its properties, especially autolysis. The physiological function of this muscle-specific calpain and its relationship to LGMD2A are discussed on the basis of the results obtained.

Abbreviations used: 30K, regulatory 30 kDa subunit of calpains; CL, catalytic large subunit of calpains; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; IS, insertion sequence; LGMD, limb-girdle muscular dystrophy.

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Figure 1 Domain structure of p94

(A) A summary of the function of each domain of p94. (B) Amino acid sequence of domain IV of rat p94, mCL, μ CL and 30K. Residues in white letters on a black background are conserved in all sequences. Five EF-hand structures are underlined. Abbreviation: nCL-1, novel calpain large subunit 1.

EXPERIMENTAL

Materials

Chemical reagents were purchased from Wako Pure Chemicals (Osaka, Japan), Nacalai Tesque (Kyoto, Japan) or Sigma (Tokyo, Japan). Oligodeoxynucleotides were synthesized with a Beckman Oligo 1000 DNA Synthesizer. The N-terminal amino acid sequences of full-length p94 were determined on an Applied Biosystems protein sequencer type 477A/120A and its degraded fragments on a Beckman protein sequencer type LF3000.

Antibodies

The antibodies used to detect p94 were rabbit polyclonal anti-(pK-rich), anti-pNS and anti-pCS antisera, which recognize the N-terminal region of the IS2 region, the N-terminal 18-residue sequence and the C-terminal 12-residue sequence of p94 respectively [10]. The antibody (X105106) against the connectin N₂ region was affinity-purified from a rabbit polyclonal antiserum. In brief, the segment consisting of bp 15307–16258 was amplified from a human N2A-encoding cDNA [15] by PCR and subcloned into a pET16b vector; the peptide was expressed and purified. Raising the antibody and its affinity purification were performed by Eurogenetics (Belgium). Specificity for connectin was evaluated by Western blot and dot-blot analysis (results not shown).

Purification of p94(C129S) expressed in COS cells

Rat p94(C129S) cDNA (140 μ g) in pSRD vector [10,22], in which the active-site Cys-129 had been changed to serine to inactivate the protease activity, and 1.2×10^8 COS cells (20 dishes) were mixed in a cuvette, and transfection was performed by electroporation as described by Chu *et al.* [23] using a Bio-Rad Gene Pulser [23]. Transfected cells were collected after 72 h and disrupted in a French press in buffer A [20 mM Tris/HCl

(pH 7.5)/5 mM EDTA/2 mM dithiothreitol] (1 ml per dish). After centrifugation at 10000 *g* for 30 min, the soluble fraction was filtered through a 0.22 μ m filter (Millipore) and subjected to ion-exchange chromatography on a DEAE-Toyopearl 650S 10 ml column and gel fractionation on a Hi-Load 16/60 Superdex 200 preparative-grade column, followed by chromatography on a Mono-Q (HR10/10) 8 ml column (Pharmacia Biotech) with an FPLC system at 4 °C.

Co-expression of p94 and a connectin fragment in COS cells

A cDNA encoding the N2A region of connectin (pCN₂) was obtained by screening for p94-binding proteins by the two-hybrid system [11], then subcloned into the vector pSRD. cDNA species (5 μ g of each) for pCN₂ and wild-type p94 or p94(C129S) subcloned in pSRD were mixed with 6×10^6 cells, which were then electroporated and disrupted by sonication as described above. The soluble fraction obtained by centrifugation was subjected to SDS/PAGE [10% (w/v) gel] and Western blot analysis.

Preparation of a p94-affinity column

Anti-(pK-rich) antiserum (10 ml) was affinity-purified on a pK-rich peptide affinity column. The affinity-purified anti-(pK-rich) immunoglobulin was immediately dialysed against a coupling solution [0.2 M NaH₂PO₄/0.5 M NaCl (pH 8.3)] for 12 h at 4 °C; it was then bound to a HiTrap N-hydroxysuccinimide-activated affinity column (1 ml) (Pharmacia Biotech) previously washed with 1 mM HCl. After incubation for 30 min at room temperature, the column was washed several times successively with the coupling solution, buffer B [0.5 M ethanolamine/0.5 M NaCl (pH 8.3)] and buffer C [0.1 M acetate/0.5 M NaCl (pH 4)]. The column was incubated for a further 30 min in buffer B and used as a p94-affinity column after additional washing with buffers B and C [24,25].

Figure 2 Purification of p94(C129S) expressed in COS cells

(A) Coomassie Brilliant Blue-stained SDS gel of the p94(C129S) fractions obtained during purification. Lane 1, French press pellet; lane 2, soluble fraction; lane 3, DEAE-Toyopearl column fraction; lane 4, Hi-Load Superdex 200 column fraction; lane 5, Mono-Q column fraction. The arrowhead shows the position of p94(C129S). (B) Elution of p94(C129S) from a Hi-Load Superdex 200 column calibrated with ovalbumin (43 kDa, point A), BSA (67 kDa, point B), aldolase (158 kDa, point C) and catalase (232 kDa, point D). The positions of p94(C129S) and the molecular mass marker proteins are indicated.

Purification of p94 from rabbit skeletal muscle

Fresh rabbit muscle (150 g) was homogenized in 800 ml of 50 mM KCl/1 mM NaHCO₃/5 mM EDTA/5 mM EGTA/0.1 µg/ml *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64)/0.5 µg/ml leupeptin, and the homogenate was centrifuged at 8000 *g* for 5 min to obtain myofibrils as the pellet fraction [11,14]. The combined myofibril fractions obtained from four similar preparations were rapidly washed with 800 ml of 5 mM NaHCO₃ and collected by centrifugation for 15 min at 8000 *g*. The pellet was suspended in 700 ml of 5 mM NaHCO₃/0.1 µg/ml E-64/0.5 µg/ml leupeptin, and the suspension was stirred for 17 h at 4 °C to extract p94 from the myofibrils. The suspension was sedimented by centrifugation for 30 min at 8000 *g*; the supernatant was further centrifuged for 30 min at 10000 *g*. The crude extract (40 ml) was filtered through a 0.22 µm filter, then subjected to chromatography on a DEAE-Toyopearl

Table 1 Purification of recombinant p94(C129S) from COS cells

The starting material was 2.4×10^8 COS cells transfected with p94(C129S) cDNA. The total protein was estimated assuming $A_{280\text{nm}} 1.0 = 1 \text{ mg/ml}$ protein. The quantity of p94(C129S) protein was estimated by densitometric quantification of SDS/PAGE gels.

Purification step	Total protein (mg) (A)	p94(C129S) protein (mg) (B)	Ratio (B/A)	Purification
Crude extract	54.1	1.72	0.0318	1.00
DEAE-Toyopearl	20.7	1.45	0.0700	2.20
Gel filtration	3.39	0.833	0.246	7.74
Mono-Q	0.605	0.539	0.891	28.0

column (10 ml) or a p94-affinity column (2 ml) with an FPLC system at 4 °C.

p94 was separated on a DEAE-Toyopearl column, using 5 bed vol. of a 0–0.5 M NaCl gradient in buffer A. For affinity purification, a p94-affinity column was washed with 5 ml of buffer A with or without 0.5 M NaCl, and p94 was eluted with 0.1 M glycine/HCl buffer (pH 2.5).

Electrophoresis and immunoblotting

Protein samples were subjected to electrophoresis in 10 % (w/v) polyacrylamide gels by the method described previously [10]. The gels were stained with Coomassie Brilliant Blue R250, or immunostained with appropriate antibodies after transfer to a PVDF membrane (Nippon EIDO) as described previously [10]. Peroxidase-conjugated anti-(rabbit IgG) was used as a second antibody, and the immunoreactive bands were detected with a peroxidase immunostain system (Wako Pure Chemicals).

RESULTS

Purification of recombinant inactive p94 and p94(C129S)

Although several attempts have been made to purify p94, full-length p94 has not yet been obtained because of its rapid autolysis. Therefore, to establish a purification procedure for p94, we first tried to purify recombinant p94(C129S) expressed in COS cells.

COS cells expressing p94(C129S) were disrupted with a French press; the soluble fraction obtained by centrifugation was chromatographed on a DEAE-Toyopearl column equilibrated with buffer A. After the column had been washed with buffer A, bound proteins were eluted with a 0–0.5 M linear gradient of NaCl. A peak containing p94(C129S) was found at 0.13–0.17 M NaCl by Western blot analysis with anti-(pK-rich) antiserum (results not shown). The combined peak fractions were collected (lane 3 in Figure 2A), concentrated to 5 ml with Centriprep 10 (Amicon) and applied to a gel-filtration column (Hi-Load 16/60 Superdex 200) equilibrated with buffer D (150 mM NaCl in buffer A) (Figure 2A, lane 4). p94(C129S) was eluted at an elution volume of 56–60 ml, which corresponds to a position with a molecular mass of approx. 180 kDa, suggesting that p94(C129S) is a homodimer (Figure 2B). The pooled gel-filtration fractions were loaded on an anion-exchange Mono-Q column, and eluted with 5 bed vol. of a 0–0.5 M NaCl gradient. p94(C129S), which was eluted at 0.3 M NaCl, was purified completely as determined by SDS/PAGE (Figure 2A, lane 5). The results of the purification procedure are summarized in

Figure 3 Partial purification of native p94 from rabbit muscle by DEAE-Toyopearl chromatography

(A) DEAE-Toyopearl column chromatography of crude rabbit skeletal muscle extract. The arrow shows the elution position of p94. (B, C) SDS/PAGE patterns of various DEAE fractions revealed by Coomassie Brilliant Blue (CBB) staining (B) and immunoblot analysis with anti-(pK-rich) antiserum (C). Molecular masses are indicated at the left.

Figure 4 Affinity purification of p94

SDS/PAGE profiles of p94 fractions obtained during purification on a p94-affinity column. (A, B, C, E) Coomassie Brilliant Blue (CBB) staining; (D, F) Western blot analyses with anti-(pK-rich) antiserum. The column was washed with buffer C without (C, D) or with (E, F) 0.5 M NaCl before the elution of p94 with 0.1 M glycine/HCl, pH 2.5. (A) crude extract of myofibrils loaded on the p94-affinity column; (B) flow-through fraction (unbound proteins); (C, E) three pooled fractions containing p94 in the order of elution; (D, F) Western blot analysis of (C) and (E) with anti-(pK-rich) antiserum. Arrowheads show the full-length and autolysed p94 bands. Molecular masses are indicated at the left.

Table 1. From 2.4×10^8 COS cells, 0.54 mg of p94(C129S) was obtained.

To confirm whether p94(C129S) formed a homodimer, completely purified p94(C129S) was resubjected to a Hi-Load Super-

dex 200 column. As a result, p94(C129S) was eluted at the same volume (60 ml), corresponding to a molecular mass of approx. 180 kDa, suggesting that p94 exists as a homodimer in skeletal muscle.

Figure 5 Degradation of partly purified p94

(A) p94-containing fractions obtained before and after DEAE-Toyopearl column chromatography were incubated at 30 °C for the indicated periods, subjected to SDS/PAGE and immunostained with anti-(pK-rich) antiserum. Bands of approx. 53 kDa in the crude extract indicated by asterisks are not specific, and probably represent an immunoglobulin heavy chain. (B) The connectin N₂ fragment was co-expressed in COS cells with wild-type p94 (lane 1) or p94(C129S) (lane 2), and the cell lysates were examined by Western blot analysis with anti-(pK-rich) and anti-(connectin N₂) antisera. The arrowheads show full-length p94 and the connectin N₂ fragment.

Purification of active p94 from rabbit skeletal muscle

p94 exists stably in the saline-insoluble myofibril fraction [11]. In the present study we found for the first time that p94 is extracted stably together with actin-binding proteins by washing myofibrils with a bicarbonate solution of low ionic strength.

An analysis of the DEAE-purified fractions by staining with Coomassie Brilliant Blue and Western blot analysis with anti-(pK-rich) antiserum is shown in Figure 3. Four immunoreactive bands of 90, 60, 58 and 55 kDa were seen in the first peak at 0.15–0.2 M NaCl. The NaCl concentrations for the elution of native p94 and p94(C129S) were almost the same. A Western blot analysis with anti-NS or anti-CS antisera, which react with the extreme N-terminus or C-terminus of p94 respectively, showed that the 90 kDa band corresponded to full-length p94. Although the molecular mass (90 kDa) was somewhat smaller than that expected from the DNA sequence (94 kDa), the migration position was indistinguishable from that of p94(C129S). N-terminal amino acid sequence analysis of this 90 kDa band showed that the N-terminus was blocked. Three degraded bands (60, 58 and 55 kDa) were detected only with anti-pCS antiserum, whereas the anti-pNS antiserum detected a 31 kDa band (results not shown). Although these results suggested that p94 was degraded during purification, the main peak

Figure 6 Properties of p94 autolysis

(A) Intermolecular autolysis of p94. Purified p94(C129S) was incubated at 30 °C for 30 min with the p94 fraction from the DEAE-Toyopearl column; the autolysis was examined by immunostaining with anti-(pK-rich) antiserum. Lane DE contained the DEAE fraction of p94 alone; the lane labelled 'DE p94(C129S)' contained a mixture of the DEAE fraction and p94(C129S). (B) The effects of Ca²⁺ and EDTA on p94 autolysis. The DEAE fraction containing p94 was incubated for 30 min in the presence of 10 mM Ca²⁺ or EDTA, and a Western blot analysis was conducted with anti-(pK-rich) antiserum. Molecular masses are indicated at the left. The arrowheads show the full-length and autolysed p94 bands.

fractions containing p94 were pooled and concentrated for gel filtration. However, Western blot analysis with p94 antisera detected only the 55 kDa band in the concentrated sample, suggesting that p94 was proteolysed.

Affinity purification of p94

Because native p94 disappears in a very short time, although full-length p94 exists in skeletal muscle extracts, and because the degradation cannot be controlled by various kinds of inhibitor, complete purification of p94 was not possible by conventional purification methods. Therefore we tried to purify p94 in a single step by affinity chromatography as described in the Experimental section. Although various column-washing conditions were examined after the crude extract had been loaded on a p94-affinity column (Figures 4A and 4B), it was impossible to separate many co-existing proteins, for example myosin and actin, from the p94-eluted fraction without the addition of NaCl to the washing buffer (buffer A) (Figures 4C and 4D). p94 was purified completely in the presence of NaCl in high yield (1.50 µg from 150 g), although autolysis occurred, and autolysed 58 and 55 kDa fragments could not be removed (Figures 4E and 4F). Although the pH of the p94-containing fraction was returned to

Figure 7 Sites of autolysis of p94

(A) Peptide sequences determined for affinity-purified rabbit p94 fragments. The corresponding rat sequences deduced from cDNA [9] are shown for comparison because the sequence of rabbit p94 is unknown. (B) A schematic structure of p94 and the amino acid sequences of IS1 are shown in the upper and lower panels respectively. Residues conserved between the three species are shown in white letters on a black background. Arrows indicate the positions of autolysis. RT, HU and CK indicate rat, human and chicken proteins respectively.

neutral by dialysis, the activity of p94 was not recovered under the conditions examined so far.

Degradation of p94

To analyse p94 degradation, a p94-containing fraction (fraction 8 in Figure 3C) was incubated at 30 °C for 30–60 min, subjected to electrophoresis and then analysed with anti-(pK-rich) antiserum. Although p94 was stable in the crude extract at 30 °C as shown in Figure 5(A), the 90, 60 and 58 kDa bands in a fraction obtained from the DEAE-Toyopearl column disappeared rapidly within 5 min and all bands converged on a 55 kDa fragment. The 90 and 60 kDa bands disappeared time-dependently, whereas the signal of the 58 kDa band increased once at 5 min but became undetectable afterward. The 60, 58 and 55 kDa fragments were thus produced from the 90 kDa protein sequentially in this order. The 55 kDa fragment did not degrade further, even after a long period of incubation (more than 12 h at 30 °C), indicating that this fragment has no autolytic activity. When NaCl at the same concentration as that used for the DEAE elution was added to the crude extract in which p94 existed stably, p94 degraded very rapidly. Hence the NaCl concentration affected its autolysis.

To perform a further characterization of p94, an inhibitor was needed that regulated the conversion of 90 kDa to 55 kDa, and the effect of various inhibitors was examined. Inhibitors of μ - and m-calpains such as E-64, leupeptin, *N*-acetyl-Leu-Leu-norleucinal and calpastatin, and other inhibitors (for example aprotinin, *N*-(L-3-*trans*-propylcarbamoyl-oxirane-2-carbonyl)-L-isoleucyl-L-proline, bestatin, chymostatin, phosphoramidon, 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one and 7-amino-1-chloro-3-L-tosylamidoheptan-2-one), had no effect on the autolysis of p94 at concentrations examined (0.1–1 mM) (results not shown). The addition of Hg²⁺ (1 mM) completely stopped the degradation. These results are consistent with those

obtained previously with p94 translated *in vitro* [10]. Taken together, the degradation of p94 occurs because of its intrinsic protease activity, which seems to be clearly distinct from that of ubiquitous calpains.

Because the N₂ fragment of connectin binds specifically to the IS2 region of p94, which is essential for rapid autolysis [10,11], we co-expressed wild-type p94 and the connectin N₂ fragment transiently in COS cells to examine whether this fragment worked as a novel regulator of p94 autolysis. As shown in Figure 5(B), even in the presence of the connectin N₂ region, wild-type p94 autolysed very quickly and disappeared (lane 1). Although it is not known why doublet bands of the connectin N₂ fragment were detected, the expression of wild-type p94 did not hydrolyse connectin fragments, suggesting that the connectin N₂ region is not a substrate for p94.

To confirm that the degradation of p94 purified by DEAE chromatography is caused by its autolysis, purified inactive mutant p94(C129S) expressed in COS cells was mixed with the active p94 fraction. The degradation pattern of p94(C129S) was essentially the same as that of DEAE-purified p94 alone (Figure 6A). Thus the autolysis is at least partly an intermolecular reaction.

p94 has a calmodulin-like Ca²⁺-binding domain similar to domain IV of m- or μ -calpains [9], suggesting that the autolysis of p94 occurs in response to Ca²⁺. We therefore examined the sensitivity of p94 autolysis to Ca²⁺ (Figure 6B). Contrary to our expectation, the presence of 10 mM Ca²⁺ or 10 mM EDTA did not affect autolysis.

Autolysis sites of p94

As mentioned before, the autolysis of p94 occurs in at least three sequential steps producing 60, 58 and finally 55 kDa fragments. Each fragment was affinity-purified and the autolytic cleavage

Table 2 Sites of cleavage by p94 or ubiquitous calpains in various proteins

The cleavage sites of p94 are compared with those of ubiquitous calpains. Arrows indicate the cleavage site. The results in Figure 7 are shown in the upper panel. RT(60), RT(58) and RT(55) are from the rat 60, 58 and 55 kDa fragments, respectively; CK(55) is from the chicken sequence. In the lower panel the sites autolysed by ubiquitous calpain and the cleavage sites of five representative substrates are shown. m, the N-terminal autolysis site of pig m-calpain; μ (78) and μ (76), the amino acid sequences of the 78 and 76 kDa fragments of human μ -calpain. For comparison of substrate specificity, the volumes of residues at each site are shown (taken from [26]).

p94 fragment	P5	P4	P3	P2	P1		P1'	P2'	P3'	P4'	P5'
RT(60)	T	N	M	T	Y		G	T	S	P	S
RT(58)	R	N	M	D	N		S	L	L	R	D
RT(55)	V	P	V	Q	Y		E	T	R	M	A
CK(55)	M	P	M	Q	Y		E	T	R	M	S
Volume ($\text{\AA}^3$)	148 $\pm$ 25	120 $\pm$ 2.9	157 $\pm$ 11	129 $\pm$ 18	175 $\pm$ 38		106 $\pm$ 39	129 $\pm$ 25	151 $\pm$ 41	155 $\pm$ 22	94 $\pm$ 11

Calpain	P5	P4	P3	P2	P1		P1'	P2'	P3'	P4'	P5'
m	A	E	G	L	G		S	H	E	R	A
μ (78)	C	T	G	V	S		A	Q	V	Q	K
μ (76)	A	R	E	L	G		L	G	R	H	E
Fodrin	Q	Q	E	V	Y		G	M	M	P	R
Crystallin	K	P	A	V	T		A	A	P	K	K
Insulin	V	E	A	L	Y		L	V	C	G	E
Dynorphin	G	G	F	L	R		R	I	R	V	G
Vimentin	A	V	R	L	R		S	S	V	P	G
Volume ($\text{\AA}^3$)	111 $\pm$ 37	129 $\pm$ 33	117 $\pm$ 50	157 $\pm$ 14	132 $\pm$ 58		115 $\pm$ 46	126 $\pm$ 41	145 $\pm$ 23	136 $\pm$ 36	125 $\pm$ 48

sites were determined by N-terminal sequencing as shown in Figure 7(A). Because the sequence of the rabbit p94 cDNA is not known, these results were compared with the amino acid sequence of rat p94 [9]. All the peptide sequences of the rabbit fragments showed a high level of identity (more than 80%) to the rat sequence, and all three autolytic sites were identified as being in the IS1 region (Figure 7B). These results suggest that not only IS2 but also IS1 is essential for very rapid autolysis [10]. Because chicken wild-type p94 expressed in baculovirus produced a similar 55 kDa band, the cleavage sites might be conserved between mammalian species. The sites cleaved by p94 were compared with those in ubiquitous calpains (Table 2). All four cleavage sites in p94 had hydrophobic residues at the P3 position, whereas hydrophilic residues existed at the P1 and P2 positions. In addition, small residues seem to be the preferred residues at the P1' site [26]. When the autolytic sites and the cleavage sites of substrates (α -fodrin, α -crystallin B chain, oxidized insulin B chain, dynorphin and vimentin) of ubiquitous μ - and m-calpains are compared, leucine and valine are the predominant residues at the P2 site [27–30], in clear contrast with p94, which requires hydrophilic residues at P2. These results clearly indicate that the substrate specificity of p94 is significantly different from that of ubiquitous calpains, suggesting that their physiological substrates and roles are distinct.

DISCUSSION

After establishing a purification procedure for recombinant inactive p94, we tried to purify native p94 by the same procedure. Although p94 was successfully extracted from skeletal muscle in a stable state, after the first ion-exchange chromatographic step p94 autolysed very rapidly, even in the presence of various calpain inhibitors. Complete purification of p94 was achieved by affinity chromatography. Purified p94, however, was inactive

and contained p94 fragments produced during purification. To obtain enzymically active p94 without degradation fragments, inhibitors or conditions that reversibly inhibit the autolysis are essential. The amount of purified p94 was relatively large, about 1 mg from 1 kg of rabbit skeletal muscle. The results indicate that p94 does not autolyse soon after translation [10] and that a regulator that protects p94 from degradation should exist in skeletal muscle. Furthermore the fact that the autolysis is sensitive to NaCl concentration suggests the dissociation of a regulator from p94 at high concentrations of NaCl.

The fact that p94 shows Ca^{2+} -independent autolytic activity suggests the following two possibilities. First, p94 does not require Ca^{2+} for activity, although it contains a Ca^{2+} -binding domain IV similar to those found in ubiquitous calpains. Secondly, the proteolytic activity of p94 is Ca^{2+} -dependent but the firm binding of Ca^{2+} to p94 makes its Ca^{2+} sensitivity apparently independent of exogenously added Ca^{2+} . We are now studying the binding of Ca^{2+} to p94 and the results will clarify whether or not the proteolytic activity is Ca^{2+} -independent.

It has been postulated that m- and μ -calpains proteolyse α -connectin (molecular mass 3000 kDa) at around the N₂-line region, where p94 binds, and produce smaller β -connectin (1800 kDa) and a 1200 kDa fragment [31]. As described in the Results section, p94 did not proteolyse the connectin N₂ fragment co-expressed with p94. It is thus suggested that p94 protects α -connectin from degradation by ubiquitous calpains rather than degrading it.

All three autolysis sites in p94, as determined from the affinity-purified fragments, were within the IS1 region. Although the possible functions of IS1 have so far received little attention, IS1, together with IS2, is obviously essential for autolysis. This is supported by the fact that autolysis did not occur in an IS1 deletion mutant (results not shown). Furthermore mutations in exon 7, which encodes IS1, are found frequently in the LGMD2A-

affected families on Reunion Island, and the patient phenotype carrying the null mutation of exon7 that arose from aberrant splicing is more severe than that involving mis-sense mutations [21]. These results suggest that a dysfunction of p94 autolysis is important in the development of LGMD2A.

Ubiquitous μ - and m-calpains are heterodimers composed of a catalytic large subunit (CL) and a regulatory 30 kDa subunit (30K). When human μ CL cDNA is expressed in Sf9 cells, it forms a heterodimer in the presence of expressed 30K; however, a homodimer of μ CL, (μ CL)₂, is produced in the absence of 30K [32]. We also found that chicken μ /mCL forms a homodimer, (μ /mCL)₂, when expressed alone in Sf9 cells. The formation of CL homodimers is probably observed in common in various CLs under certain conditions. In this respect, it might not be surprising that recombinant rat p94 expressed in COS cells is purified as a homodimer. Recent X-ray crystallographic analysis of the Ca²⁺-binding domain of 30K (domain IV') has revealed that domain IV' forms a dimer in the presence and the absence of Ca²⁺ through the most C-terminal EF-hand structure, EF-5 (see Figure 1B) [33–35]. Thus 30K and CL are thought to form a dimer through the Ca²⁺-binding domains IV' and IV. Further, as shown in Figure 1(B), p94 contains EF-hand structures with approx. 50% sequence identity to those in μ - and m-calpains. It is highly likely that p94 forms a homodimer in a similar manner. μ - and m-calpains dissociate into subunits in the presence of Ca²⁺; the sensitivity of CL to Ca²⁺ increases on dissociation from 30K [36]. This suggests that 30K functions as an inhibitor by regulating the Ca²⁺ sensitivity of CL, which seems to be an active species *in vivo* [37]. On the basis of these results, our current model for the activation of calpain is as follows: μ - and m-calpains dissociate with Ca²⁺; dissociated CL forms a homodimer and acts as an active enzyme. p94 probably exists as a homodimer *in vivo*. In other words, p94 exists in an active form in contrast with μ - and m-calpains, which exist as an inactive form by forming a complex with 30K. p94 does not interact with 30K but the activity might be regulated by one or more other proteins or factors, as suggested by the stability of p94 in the crude muscle extract. Whether homodimer formation occurs only under extreme conditions in which only the large subunit is expressed, and whether the Ca²⁺-induced dissociation of calpain occurs under physiological conditions, must await further clarification because a recent report argues for Ca²⁺-induced dissociation [38]. Autolysis, which has been discussed as a means of activation of μ - and m-calpains, is apparently not required for the expression of activity of p94. Rather, autolysis degrades p94 into inactive fragments, although very rapid autolysis makes a precise analysis difficult.

In the present study we found some novel molecular features of p94 (Figure 1A). To find conditions or reagents that reversibly inactivate p94 activity is the most important and urgent issue in characterizing p94 more precisely and eventually in clarifying the physiological function of this protease. These results will lead us to understand the molecular mechanism underlying LGMD2A, and at the same time to develop a treatment.

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