The protease core of the muscle-specific calpain, p94, undergoes Ca^{2+}-dependent intramolecular autolysis

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Abstract Limb girdle muscular dystrophy type 2A is linked to a skeletal muscle-specific calpain isoform known as p94. Isolation of the intact 94-kDa enzyme has been difficult to achieve due to its rapid autolysis, and uncertainty has arisen over its Ca^{2+}-dependence for activity. We have expressed a C-terminally truncated form of the enzyme that comprises the protease core (domains I and II) along with its insertion sequence, IS1, and N-terminal leader sequence, NS. This 47-kDa p94I-II minicalpain was stable during purification. In the presence of Ca^{2+}, p94I-II cleaved itself within the NS and IS1 sequences. Mapping of the autolysis sites showed that NS and IS1 have the potential to be removed without damage to the protease core. Ca^{2+}-dependent autolysis must be an intramolecular event because the inactive p94I-II C129S mutant was not cleaved by incubation with wild-type p94I-II. In addition, the rate of autolysis of p94I-II was independent of the concentration of the enzyme.

Key words: Autoproteolysis; Calcium; Capn3; Cysteine proteinase; Muscular dystrophy; Propeptide cleavage

1. Introduction

The calpains are cytosolic Ca^{2+}-dependent cysteine proteases that have been implicated in a wide variety of intracellular processes linked to Ca^{2+} signaling [1]. Through limited proteolysis of their substrates calpains play an important role in signal transduction [2], cell cycle regulation [3], cell differentiation [4] and apoptosis [5]. Calpains have been found in organisms ranging from mammals to bacteria, and genome sequencing has shown that multiple isoforms are present in *Drosophila melanogaster*, *Caenorhabditis elegans* and humans [6].

The two ubiquitous mammalian calpains, μ- and m-calpain, each form a heterodimer of the large subunit (products of the *capn*1 and *capn*2 genes, respectively) with a common 30-kDa regulatory subunit (product of the *capn*4 gene) [2,7]. The crystal structure of m-calpain in the absence of Ca^{2+} was recently solved, and it redefined the presence and organisation of four distinct domains in the large subunit [8,9]. Domains I and II together comprise a papain-like cysteine protease core. Domain III is structurally similar to a Ca^{2+} and phospholipid-binding C2 domain, and domain IV, which is similar to domain VI of the regulatory subunit, has a penta-EF-hand structure.

The first of the tissue-specific calpains, calpain 3 (CAPN3 or p94), was described in skeletal muscle [10] and was genetically linked to limb girdle muscular dystrophy type 2A (LGMD2A) [11]. Accordingly, mutations in the gene (*capn*3) that encodes p94 have been found in patients suffering from LGMD2A and result in a decrease or loss of enzymatic activity [11–13], suggesting the importance of p94 in normal muscle physiology. p94 has 54% amino acid sequence identity to the μ-calpain large subunit, and is thought to have the same domain structure but without a requirement for the 30-kDa regulatory subunit [10,14]. The larger size of p94 is due to three extra sequences, an N-terminal extension (NS), and two insertion sequences: IS1 in domain II, and IS2 between domains III and IV. These sequences are not found in the other calpain isoforms nor do they have obvious homologues in the current protein database. They presumably contribute to p94's unique properties and physiological function. For example, IS2 contains a lysine-rich putative nuclear localisation signal and p94 has been found in both the nucleus and the cytoplasm [15]. As well, p94 binds to connectin/titin through its IS2 region, although the NS and IS1 regions may also contribute to this interaction [13,16].

Attempts to analyse p94 at the protein level have been stymied due to the rapid autolysis of p94 in vitro. It has a half-life of less than 20 min, and typical calpain inhibitors such as the μ- and m-calpain endogenous inhibitor calpastatin, and the cysteine protease inhibitors E-64 and leupeptin, fail to inhibit the autolytic activity of p94 [15]. There is even disagreement about p94's dependence on Ca^{2+} for activity. It was previously shown that p94 rapidly autolysed in the presence of EDTA [17], while another report indicated that p94 was stable in EGTA [18]. The presence of a Ca^{2+}-binding penta-EF-hand domain IV and the postulated Ca^{2+}-binding C_2-like DIII [19] in p94 suggests that p94 binds Ca^{2+}. Indeed, Ca^{2+} binding to p94 was directly demonstrated by Branca et al. [18], using a gel overlay method. However, in μ-calpain these Ca^{2+}-binding domains are not directly involved in realignment of the active site for catalysis. This is the role of two novel Ca^{2+}-binding sites in protease domains I and II [20]. Sequence alignments indicate that the key Ca^{2+}-liganding amino acids of these new sites are conserved in the protease

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Abbreviations: IPTG, isopropyl-1-thio-β-D-galactopyranoside; IS, insertion sequence; LGMD2A, limb girdle muscular dystrophy type 2A; NS, N-terminal sequence

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core of p94 and should be functional. In light of these results we have expressed and characterised this region of p94 (domains I and II, p94I-II) in an attempt to clarify the Ca^{2+}-dependence of p94. We report here that p94I-II is a Ca^{2+}-dependent cysteine protease with many properties similar to those of the full-length enzyme, and that it undergoes intramolecular cleavage within its N-terminal NS and IS1 insertion sequences close to the junctions with domains I and II.

2. Materials and methods

2.1. Cloning of the protease region of p94 and site-directed mutagenesis

The cDNA encoding the protease region of p94 was obtained by polymerase chain reaction amplification of reverse transcripts (RT-PCR) of total RNA from human skeletal muscle (Stratagene) using reverse transcriptase (Gibco) and *Pwo* polymerase (Roche). The construct starts at the N terminus of p94 and extends to the end of domain II (residue 419). The cDNA was cloned into the *Nde*I and *Xho*I sites of the pET24d expression vector (Novagen), which incorporates a C-terminal histidine tag onto the construct. To make an inactive mutant of p94I-II, the active site Cys (C129) was replaced by Ser using the PCR method and 5′-GCGAGAAACCAGC-TGTCCCCTAGCTC-3′ as the mutagenic primer [21].

2.2. Protein expression and purification

Plasmid encoding active or inactive (C129S) p94I-II was transfected into *Escherichia coli* BL21(DE3) (Novagen) by electroporation. LB media (100 ml) was inoculated from a single colony and grown overnight at 37°C under kanamycin selection before seeding 4 L of LB/kanamycin broth, which was then grown to an OD_{600} of 0.8 to 1.0 at 20°C. Protein expression was induced with 0.4 mM IPTG for 5 h. The cells were collected, resuspended in 50 ml of lysis buffer (25 mM Tris–HCl, 5 mM EDTA, 1 mM β-mercaptoethanol (pH 7.8)), and lysed through sonication. The soluble fraction was applied to 200 ml of DEAE-Sephacel resin. Protein was eluted with a 0–1 M NaCl salt gradient in lysis buffer, and fractions containing p94I-II, as determined by the presence of a prominent band at 47 kDa on SDS–PAGE, were pooled and applied to a 10-ml Ni^{2+}-chelating agarose column (Qiagen). The column was washed with 20 mM imidazole (pH 7.6), and batch-eluted with 250 mM imidazole (pH 7.6). The pooled fractions containing p94I-II were dialysed overnight into lysis buffer to remove imidazole. p94I-II was further purified by Q-Sepharose FPLC. Bound p94I-II was eluted by a 0–1 M NaCl gradient in lysis buffer. The absorbance profile was measured at 280 nm and selected fractions were analysed by SDS–PAGE. The purified protein was concentrated in a Biomax 30K concentrator (Millipore), diluted in 10 mM Tris–HCl, 10 mM DTT and 5 mM EDTA (pH 7.8) to a final protein concentration of 20 mg/ml, aliquotted into 20 μl volumes, and flash frozen in liquid N_2 for storage. The inactive p94I-II C129S mutant was purified in a similar manner.

2.3. Expression and purification of ^{35}S-L-Cys-labelled active or inactive p94I-II

E. coli transformed with plasmid encoding wild-type or inactive C129S p94I-II were grown in M9 salts minimal media (50 mM Na_2HPO_4, 20 mM KH_2PO_4, 8 mM NaCl, 18 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM thiamine) at 30°C, under kanamycin selection, to an OD_{600} of 1.0. Radio-labelled protein expression commenced with the addition of 0.4 mM IPTG and 500 μCi of ^{35}S-L-Cys. The protein was purified as previously described.

2.4. Enzyme activity

Autoproteolytic activity was measured by incubating recombinant p94I-II (200 μg) in 10 mM Tris–HCl (pH 7.6), 10 mM DTT with 10 mM $CaCl_2$ at 20°C in a final volume of 500 μl. Aliquots were removed at the times indicated, and the reaction stopped with SDS sample buffer (NEB). The digestion products were analysed by 10% SDS–PAGE gel. A control assay consisting of p94I-II incubated with 10 mM EDTA (pH 8.0) in place of $CaCl_2$ was performed in the same fashion.

2.5. Discrimination between inter- and intramolecular autolysis

Unlabelled inactive mutant p94I-II (C129S) (4 μM final concentration) was mixed in a 2:1 molar ratio with ^{35}S-labelled active p94I-II (2 μM final concentration). The constructs were incubated in 10 mM Tris–HCl (pH 7.6), 10 mM DTT, and 10 mM $CaCl_2$ at 37°C for 24 h. Aliquots were removed at various time points into SDS sample buffer, and digestion products were visualised by 10% SDS–PAGE and autoradiography. In other experiments, unlabelled active and labelled inactive preparations of p94I-II were mixed in the same 2:1 molar ratio, incubated and analysed as above.

2.6. The rate of p94I-II autolysis as a function of concentration

p94I-II (5 μg) was allowed to autolyse at room temperature for 4 h in different volumes (8, 40, 200 and 1000 μl) of 10 mM NH_4HCO_3, 10 mM DTT and 1 mM $CaCl_2$. The reaction was stopped by the addition of 5 mM EDTA, pH 8.0. The solutions were frozen and lyophilised overnight. The resulting dry residue was resuspended in SDS sample buffer (30 μl) and the autolysis fragments visualised by SDS–PAGE. Two control assays were performed, one where the sample (8 μl volume) was not lyophilised, and another where 10 mM Tris–HCl (pH 7.6) replaced 10 mM NH_4HCO_3 (pH 8.0).

2.7. Peptide sequencing

The fragments generated from p94I-II autolysis in the presence of Ca^{2+} were separated by SDS–PAGE on a Tris–Tricine 10% polyacrylamide gel, and electroblotted onto PVDF membranes of 0.1-μm-pore size (Millipore) at 0.5 A for 1 h. Cleavage points were determined by automated N-terminal Edman degradation sequencing of major bands using a Hewlett Packard G1005A protein solid-phase sequencer with on-line PTH analysis.

3. Results

3.1. Expression and purification of recombinant p94I-II

The protease core of p94 was relatively soluble during expression, with approximately half of the expressed protein appearing in the supernatant fraction of the lysed *E. coli*. The soluble p94I-II fraction was stable throughout its purification and concentration. It co-eluted with a variety of other *E. coli* proteins from the DEAE resin, but was $>90\%$ pure after batch-elution from the Ni^{2+}-chelating agarose column (not shown). It eluted from Q-Sepharose FPLC at 0.2 M NaCl, and was $>95\%$ pure as indicated by SDS–PAGE (Fig. 1C, lane 0 m). A yield of 15 mg was obtained from a 4-l culture.

3.2. p94I-II autolysis is Ca^{2+}-dependent

When p94I-II was incubated in the presence of 10 mM $CaCl_2$ the 47-kDa protein was cleaved internally. Upon SDS–PAGE analysis, two major fragments of approximately 30 and 15 kDa (bands 1a and 1b in Fig. 1B) were seen. Small quantities of these fragments appeared immediately after the addition of $CaCl_2$ (even in the time it took to withdraw the zero time sample) and increased in intensity over time. The half-life of p94I-II was approximately 20 min as judged by the disappearance of half of the 47-kDa starting material at this time point, and very little p94I-II remained intact after 4 h. Around this time, additional cleavage products (bands 2a and 2b in Fig. 1B) began to accumulate and were the dominant products after 24 to 48 h. No breakdown products were observed over the same time frame when p94I-II was incubated with chelating agent (10 mM EDTA) in place of 10 mM $CaCl_2$ (Fig. 1C).

The N-terminal sequences of the initial breakdown products 1a and 1b, were AEPRS and GTSPS, respectively (Table 1). These correspond to autolysis sites near the N-terminal ends of the NS and IS1 insertion sequences after amino acids 14 and 274, respectively (Fig. 1A). The later cleavage products, 2a and 2b, began with the sequences AIISR and TIIPV, re-

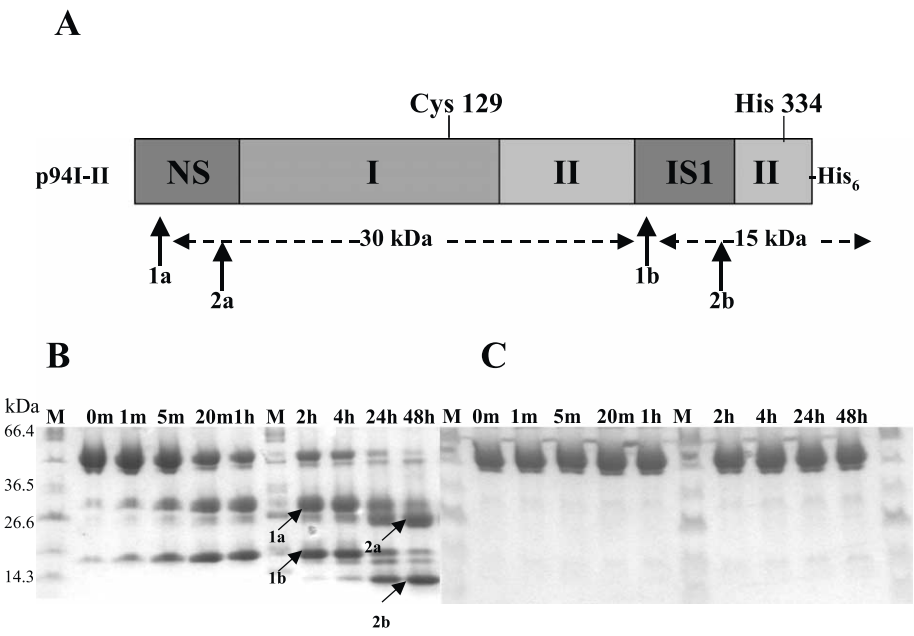

Fig. 1. Ca^{2+}-dependence of p94I-II autolysis. A: Map of p94I-II showing its domain structure and the location of initial (1a, 1b) and subsequent (2a, 2b) autolytic cleavage sites. B: SDS–PAGE analysis of p94I-II autolysis. Lanes are marked with the time of incubation, with m referring to minutes. The initial 30-kDa and 15-kDa cleavage products are indicated in the 2-h time point by arrows 1a and 1b, respectively. The cleavage products produced later in the autolysis are marked in the 48-h time point by arrows 2a and 2b. N-terminal Edman degradation sequencing determined the cleavage points to be within the unique insertion sequences of p94I-II (A and Table 1). M represents molecular weight markers. C: SDS–PAGE analysis of p94I-II incubated in the presence of excess EDTA.

spectively. These correspond to autolysis sites near the C-terminal ends of the NS and IS1 insertion sequences after amino acids 44 and 315, respectively. The cleavage sequences are distinct. All four sequences have a Pro and/or Gly near the cleavage point, and there is a preference for a small residue (Ala, Gly or Thr) at the P1′ position. Three of them have a Thr at the P2 position, and the fourth has a Tyr.

3.3. p94I-II does not proteolyse inactive p94I-II C129S

Cys 129 is the catalytically active residue in the active site and corresponds to Cys 105 in m-calpain and Cys 115 in μ-calpain. When this residue was converted to Ser, p94I-II C129S did not produce cleavage products and was indefinitely stable in the presence of Ca^{2+} (not shown). This indicates that the p94I-II breakdown products (Fig. 1B) were produced by autolysis and not by trace amounts of a metal ion-dependent protease contaminant.

When active and inactive p94I-II were combined in different ratios the autolysis was incomplete as judged by SDS–PAGE. For example, when 10% of the mixture was p94I-II, only ~10% was digested, and 90% remained as the 47-kDa starting material (not shown). This was consistent with a failure of the active enzyme to cleave the inactive C129S mutant in an intermolecular process. To confirm and illustrate this result, the active enzyme was uniformly labelled with ^{35}S-Cys and incubated with unlabelled C129S mutant in a 1:2 molar ratio (Fig. 2A). After 24 h, approximately one third of the starting material had been broken down to the 30-kDa and sub-15-kDa digestion products as judged by Coomassie blue staining, and two-thirds remained undegraded (Fig. 2B). The autoradiograph of this gel showed that all of the labelled protein was broken down by the 5-h time point (Fig. 2C).

In the converse experiment, where radio-labelled inactive p94I-II was incubated with the unlabelled active construct in the same manner (Fig. 2D), the starting amount of inactive protein (one-third of the total loaded onto the gel) remained unchanged as determined by the autoradiograph (Fig. 2F). The autolysis products are clearly visible on the SDS–PAGE by Coomassie blue staining, but did not appear on the autoradiograph. This indicates that they belong to the unlabelled active protein and not to the labelled inactive p94 I-II (Fig. 2E,F).

3.4. p94I-II autolysis is concentration independent

Intramolecular reactions have been reported to follow zero order kinetic rate law; that is, their reaction rates are independent of the concentration of the reactant [22]. If p94I-II cleavage is an intramolecular reaction, then the rate of autolysis should be independent of p94I-II concentration. When p94I-II was incubated in 1 mM $CaCl_2$ over a 125-fold concentration range (from 0.005 mg/ml to 0.625 mg/ml) there was no change in the ratio of the products (30 and 15 kDa) and reactant (47 kDa) after 4 h, as judged by the relative intensity of staining of the protein bands (Fig. 3). Recovery of the most

Table 1
Autolytic cleavage sites in p94I-II

p94I-II fragment	P5	P4	P3	P2	P1	P1′	P2′	P3′	P4′	P5′
1a	A	**P**	R	T	A	A	E	**P**	R	S
1b	T	N	M	T	Y	**G**	T	S	**P**	S
2a	S	**G**	I	Y	S	A	I	I	S	R
2b	E	R	**P**	T	R	T	I	I	**P**	V

P′ positions were determined by N-terminal Edman degradation sequencing of cleavage products generated after 2 h (1a, 1b) and 48 h (2a, 2b) of autolysis. The locations of 1a, 1b, 2a, 2b in p94I-II are indicated in Fig. 1A. Pro and Gly are shown in bold.

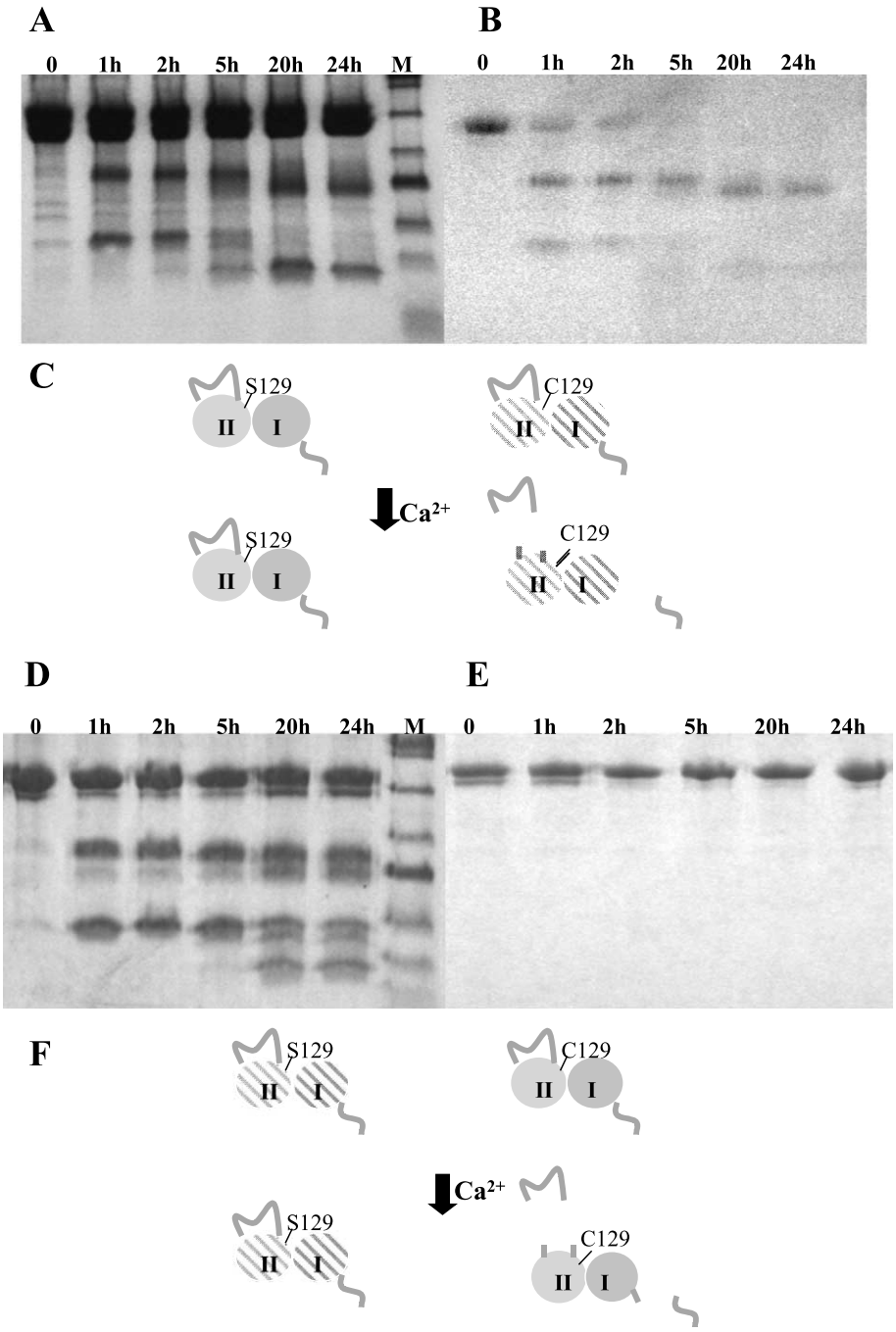

Fig. 2. p94I-II cannot proteolyse the inactive mutant p94I-II C129S. A: SDS–PAGE analysis of the proteolysis of non-labelled inactive mutant p94I-II C129S by labelled p94I-II C129 in 10 mM $CaCl_2$ at a 1:2 molar ratio of active: inactive enzyme. M represents molecular weight markers. B: An autoradiograph of the gel, developed for 7 days. C: Model showing radio-labelled p94I-II (striped, with NS and IS1 regions shown in dark grey) undergoing autolysis of the NS and IS1 in the presence of Ca^{2+} while the unlabelled inactive mutant (S129; domain I and II in medium and light grey, respectively) remains uncleaved. D: Reversal of A, where the inactive mutant S129 was radio-labelled and incubated with the active construct in the same manner. E: An autoradiograph of the SDS–PAGE gel from D. F: Model showing the radio-labelled inactive p94I-II C129S (striped) is not cleaved at its NS or IS1 regions during incubation with unlabelled active p94I-II (grey) in the presence of Ca^{2+}.

dilute digest after lyophilisation was incomplete, but there was equivalent loss of all three major bands. Again, there was no digestion in the presence of excess EDTA. The change of buffer from Tris–HCl (pH 7.6) to NH_4HCO_3 (pH 8.0) did not change the reaction rate or products, and neither did lyophilisation.

4. Discussion

Expression of the protease core of p94 (p94I-II) has facilitated characterisation of some of its biochemical properties that are relevant to the whole enzyme. The uncertainty about the Ca^{2+}-dependence of p94 has been exacerbated by the dif-

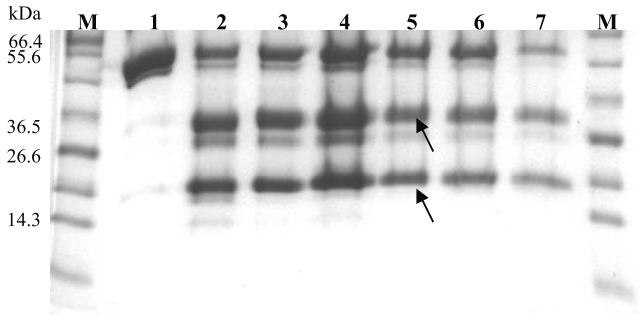

Fig. 3. p94I-II autolysis is independent of enzyme concentration. SDS–PAGE analysis of p94I-II autolysis at varying enzyme concentrations. The ratio of autolytic bands (shown by arrows) to the starting 47-kDa protein is the same for all dilutions. Lanes 1–3 contained the non-lyophilised control samples (5 μg) incubated in 5 mM EDTA, 10 mM Tris–HCl (pH 7.6), and 10 mM NH_4HCO_3, respectively. Lanes 4–7 contained lyophilised samples of 5 μg of protein previously incubated in 8, 40, 200, and 1000 μl volumes, respectively. Lane M was loaded with molecular weight markers.

ficulty in expressing the whole enzyme in a heterologous system, and by its rapid autolysis during purification [15,17,18,23]. p94I-II is stable in the presence of EDTA, and this has enabled us to assay its Ca^{2+}-dependence for autolytic activity. Our results clearly show that the protease core of p94 autolyses in the presence of Ca^{2+}, and that the reaction is blocked by an excess of the divalent cation chelator, EDTA. This is consistent with the discovery of two novel Ca^{2+}-binding sites in the protease core of μ-calpain, one in domain I and the other in domain II, that appear to act cooperatively in aligning the active site of the enzyme [20]. Sequence comparisons of several calpain protease core regions indicate that the residues that use their side chains to coordinate Ca^{2+} ions in μI-II are well conserved. They (D120, E199, E353, D360 and D382) are present in p94 along with Arg118 and Glu384 that form the double salt-bridge pair, which is thought to be a key element of the cooperativity mechanism. Our observation that p94I-II requires Ca^{2+} for autolytic activity confirms this prediction and is in line with the observation of Branca et al. that p94 activity is dependent on Ca^{2+} and can be abolished by chelating agents [18].

That autoproteolysis of p94I-II is an intramolecular reaction is based on two lines of evidence: p94I-II seems completely unable to cut an active site mutant (C129S) in an intermolecular reaction, and the autolysis of the initial cleavage points (1a and 1b) is independent of concentration. Concentration dependence of the rate of autolysis has been used previously in attempts to differentiate between inter- and intramolecular autolysis for the ubiquitous calpains [22,24,26,27]. No definitive conclusions have been made regarding m- and μ-calpain autolysis. Some reports suggest that only intramolecular autolysis occurs [22,26,28], other conflicting results indicate that the autolysis is intermolecular [29], or includes both types of reactions [30]. Here, we have used a novel combination of site-specific mutation and radio-labelling to address the autolysis issue in p94. The evidence seems unequivocal that p94I-II autolysis at the first cleavage points follows a Ca^{2+}- dependent intramolecular reaction pathway with no apparent intermolecular autolysis. We see no evidence that autolysis at the later cleavage points (2a and 2b) is intermolecular but cannot rule out the possibility. The inability of the active mutant to cleave inactive p94I-II even after 24 h suggests that this second autolysis event is also intramolecular, or may be dependent upon initial intramolecular cleavage at the first autolytic site.

Of the four major autolytic sites detected in p94I-II, two reside in NS and two in IS1. Fragment 1b, the first major cleavage product (in IS1) has the same N-terminal sequence as the 60-kDa autolytic cleavage product of rabbit p94, which stretches from IS1 to the C terminus [17]. Additional cleavages within IS1 were noted by Kinbara et al. [17] for rabbit and rat p94, but none of them corresponded to the late cleavage product of p94I-II (2b). Federici et al. [23] reported cleavage of recombinant p94 in NS after residue 30, which is internal to the earliest cleavage site in p94I-II [23]. (Federici et al. [23] also reported cleavage of recombinant p94 just inside domain III after residue 412, which would generate a natural product not unlike the p94I-II studied here). When these cleavage sequences are compared, little in the way of a consensus emerges. This is consistent with the general observations about calpain substrate specificity in that the heterodimeric calpains m- and μ- cleave flexible regions with relatively little preference for a specific amino acid sequence [1,2,7]. The NS and IS1 insertion sequences are quite rich in Pro and Gly and are possibly less well-structured than the other regions of the enzyme. In addition, a model of p94 based on the m-calpain crystal structure displays the unique insertion sequences as protruding from the globular protein, thus making them susceptible to proteolysis [25].

It is significant that the autolysis sites in p94I-II are confined to the NS and IS1 regions and that after extensive cleavage both segments are cut near each end without any damage to the underlying domains I and II. Autolysis at both the N- and C-terminal ends of the IS1 region suggests that IS1 may be an internal peptide that needs to be removed in response to Ca^{2+} signaling. In effect, IS1 may be an internal pro-segment. In many of the cysteine proteases activation involves the autolytic removal of a pro-peptide to generate the active protease [31]. A deletion mutant of p94 from which IS1 was removed had increased stability comparable to m-calpain [32]. Conversely, addition of IS1 to Lp82 (the splicing variant of p94 that does not contain IS1 or IS2) created an unstable enzyme that lacked proteolytic activity on exogenous substrates [33]. This indicates that IS1 is at least partially responsible for the instability of the enzyme. IS1 might provide an additional regulatory step in p94 activation whereby the IS1 region remains in the active site, preventing substrate binding for proteolysis until its release by autolysis.

Once again the removal of extraneous domains from a calpain to form a 'mini-calpain' [20] has greatly facilitated its characterisation. In conclusion, we have unequivocally demonstrated that the protease core of the skeletal muscle-specific calpain, p94, is activated by Ca^{2+} and completely inhibited by EDTA. Our results also suggest that NS and IS1 are flexible in the presence of Ca^{2+} and are close enough to the active site to be hydrolysed in an intramolecular reaction.

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