The importance of conserved amino acid residues in protease sub-domain IIb and the IS2 region in p94 for constitutive autolysis.

Yasuko Ono[a,*], Chikako Hayashi[a,b], Naoko Doi[a,c], Mai Tagami[a] and Hiroyuki Sorimachi[a,c]

[a]*Department of Enzymatic Regulation for Cell Functions (Calpain Project), The Tokyo Metropolitan Institute of Medical Science (Rinshoken), Tokyo, Japan*

[b]*Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, Chiba, Japan*

[c]*CREST, Japan Science and Technology (JST), Saitama, Japan*

*Corresponding author: Yasuko Ono, Department of Enzymatic Regulation for Cell Functions (Calpain Project), The Tokyo Metropolitan Institute of Medical Science (Rinshoken), 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan. Tel.: 81-3-3823-2182; Fax: 81-3-3823-2359; E-mail: yakoono@rinshoken.or.jp

Abstract

p94/calpain 3, a skeletal muscle-specific member of calpain protease family, is characterized by apparent Ca^{2+}-independence during exhaustive autolysis and concomitant proteolysis of non-self substrates. The purpose of our study was to comprehensively profile the structural basis of p94 enabling activation in the cytosol without an extra Ca^{2+}. Ca^{2+}-dependent p94 mutants were screened using “p94-trapping”, which is an application of yeast genetic reporter system called “proteinase-trapping”. Several amino acids were revealed as critical for apparent Ca^{2+}-independent p94 activity. These results highlight the importance of conserved amino acids in domain IIb as well as in the p94-specific IS2 region.

Keywords: calpain; p94/calpain 3; autolytic activity; protease activity; Ca^{2+}-dependence

Abbreviations: AD, activation domain of Gal4; BD, DNA binding domain of Gal4; LGMD2A, limb-girdle muscular dystrophy type 2A; Sf-9, *Spodoptera frugiperda* cells; WT, wild type; CS, C129S protease deficient mutation; DA, D607A missense mutation; IS1 and IS2, p94-specific insertion sequence 1 and 2, respectively; NS, N-terminal sequence unique to p94; CAST-d1, calpastatin domain 1; SD-WHA, synthetic minimal medium with dextrose minus tryptophan, histidine and adenine.

1. Introduction

Calpains (EC 3.4.22.18, clan CA) comprise a unique branch of the cysteine protease superfamily [1-3]. Studies on μ- and m-calpains, or the "conventional" calpains, have shown the importance of Ca^{2+} in regulating calpain activity. Crystal structures of calpain identified Ca^{2+}-binding sites in protease sub-domains IIa and IIb as well as domain IV composed of 5 EF-hand motifs [4-6]. However, there are several calpain paralogues to which the relevance of Ca^{2+} is not clear, as represented by p94/calpain 3, the skeletal muscle-specific member of the calpain family.

The nature of p94 protease activity is largely unknown except that p94 has very rapid and exhaustive autolytic activity that is apparently Ca^{2+}-independent. Further, the interaction of p94 with sarcomeric proteins, such as connectin/titin, is considered one of the *in vivo* mechanisms for regulating p94 localization and stability [7-11]. The importance of p94 function in human skeletal muscle is suggested because mutations in the p94 gene cause limb-girdle muscular dystrophy type 2A (LGMD2A) [12,13]. Importantly, although autolytic activity of p94 demonstrates protease activity, proteolytic activity against non-self substrates would be expected to be most physiologically important [13].

The aim of this study is to clarify the molecular basis for Ca^{2+}-independent p94 protease activity. The yeast "proteinase-trapping system" [14] was modified to screen the p94 missense mutants whose constitutive autolytic activity were transformed to be explicitly Ca^{2+}-dependent [10]. Two groups of amino acids were shown to be essential for the apparent Ca^{2+}-independence of p94 protease activity. One is composed of conserved amino acid residues within IS2, and the another is in domain IIb proximal to those involved in Ca^{2+}-binding [6].

2. Materials and Methods

2.1. General techniques

Standard protocols for manipulating yeast cells, and expression of recombinant proteins in COS7 cells and *Spodoptera frugiperda* (Sf-9) cells were described previously [15]. For detecting p94 expression, COS7 cells were harvested 72 h after electroporation, and sonicated in buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, and 2 mM PMSF). The supernatant obtained by centrifugation at 1,470×*g* for 5 min at 4°C was immediately mixed with Laemmli SDS-PAGE sample buffer followed by heat denaturing at 95°C for 3 min. For the proteins expressed in Sf-9 cells, essentially the same procedures were used except

for centrifugation at 20,630×*g* for 30 min at 4°C. Antibodies used for western blotting include anti-p94-pIS2 [10], anti-p94-pNS [7,10], and RP4CALPAIN3 antibodies (Triple Point Biologics).

2.2. cDNA constructs

Enzymes were purchased from TAKARA BIO, Stratagene, or New England Biolabs. The expression vectors were constructed using human p94/calpain 3 cDNA, pSRD, pFastBac1 (Invitrogen) and pAS2-1C as previously described [10]. Mutations were introduced by PCR using *Pfu*, *Pfu*-Turbo, or ExTaq DNA polymerases, and all DNA sequences were verified [10].

2.3. Screening for p94 mutants defective in autolytic activity

Random mutations were introduced into the sequence for IIb-III-IS2-IV (aar 342-821 of NP_000061) of p94 using primers, 5´-GATGAGGTCCCGTTCAAAGG-3´ and 5´-GGCAATTGGTTGTGAAAGCGC-3´, as described previously [10]. The PCR products (20 μg) were cotransformed with 20 μg of the 9.3 kb *Not*I–*Tth*111I fragment of the BD–p94–AD expression vector into *Saccharomyces cerevisiae* AH109 to cause *in vivo* homologous recombination (Fig. 2C). Transformants selected on the plates, SD–Trp/–His/–Ade (SD–WHA), were further characterized as to their Ca^{2+}/Na^{+}-sensitivities using the plates supplemented with 0.1M or 0.2M $CaCl_2$ (SD–WHA/Ca) or 0.2M NaCl (SD–WHA/Na). Plasmid DNA was isolated from selected colonies, and mutations were identified by DNA sequencing.

2.4. Protease activity assay

Protease activity of p94 was analyzed using the supernatant of Sf-9 cells prepared as described above. For proteolysis of calpastatin, final protein concentrations of cell lysate and calpastatin(CAST)-domain 1 (d1) (TAKARA BIO) were 0.8 μg/μl and 0.1 μg/μl (*ca.*7 μM), respectively, which causes inhibition of the conventional calpains. The reaction was terminated at the indicated times and was analyzed by SDS-PAGE.

3. Results

3.1. Characterization of p94:D607A mutant protease activity

Studies on p94 splicing variants, such as Lp82 and p94Δ [15-18], have implicated that a portion of the IS2 region encoded by an exon 16 is involved in apparently Ca^{2+}-independent rapid autolysis of p94 (Fig.

1A). Accordingly, a short stretch of amino acids in IS2 is highly conserved among p94 from various species. Since acidic amino acids are important for Ca^{2+}-binding, expression of p94:D607A(DA) and p94:E614A in COS7 cells were examined. Both mutants presented Ca^{2+}-dependent autolytic activity, and, p94:DA was slightly more stable than p94:E614A (data not shown).

Therefore, p94:DA was expressed in Sf-9 cells for further studies. p94:WT was detected exclusively as autolyzed fragments (Fig. 1B, lane 1). In contrast, the full-length fragment and autolytic fragments were detected for p94:DA (Fig. 1B, lane 2). Reactivities to the antibodies suggested that p94:DA lacks the N-terminal region of NS, possibly because of autolysis after Ala14 (Fig. 1A, closed arrowhead 1, and Fig. 3B, lane 1). Autolytic activity of p94:DA was Ca^{2+}-dependent (Fig. 1B, lanes 2-5). Under the same conditions, the protease inactive mutant, p94:C129S(CS), demonstrated no autolysis (Fig. 1B, lanes 6-8).

The protease activity of p94:DA against a non-self substrate was examined using d1 of CAST, a specific inhibitor of conventional calpains and a good substrate for p94:WT. The observed MW for CAST-d1 (22 kDa) was higher than calculated (14.2 kDa) (Fig. 1C, arrow). In the presence of Ca^{2+}, p94:DA very efficiently proteolyzed CAST-d1, and CAST-d1 did not inhibit the autolysis of p94:DA (Fig. 1C, lanes 1-5).

These data demonstrate that p94:DA is the first designed missense mutant that recapitulates, albeit Ca^{2+}-dependently, some characteristics of p94:WT protease activity.

3.2. Detection of Ca^{2+}-dependent autolytic activity of p94:DA in "p94-trapping"

In yeast "p94-trapping", p94 autolytic activity reduces transcriptional activation ability of Gal4-p94 hybrid protein, BD-p94-AD, resulting in less growth of yeast cells in selection medium [10] (Fig. 2A, SD-WHA, WT vs CS).

The yeast cells expressing BD-p94:DA-AD was comparable to those expressing BD-p94:CS-AD as to the growth on SD-WHA plates. When the plates were supplemented with either 0.1 M $CaCl_2$ or 0.2 M NaCl, however, the cells expressing BD-p94:DA-AD failed to grow (Fig. 2A, SD-WHA with $CaCl_2$ or NaCl). Exposure of yeast cells to high [NaCl], but not [KCl], causes transient increase of $[Ca^{2+}]_i$ by a mechanism for cellular ionic homeostasis [19,20]. Accordingly, KCl was not significant (Fig. 1C, SD-WHA, KCl).

These observations indicate that "p94-trapping" is useful to discriminate conditionally active p94 mutants like p94:DA from p94:WT or p94:CS based on Ca^{2+}/Na^{+}-sensitivity of yeasts (Fig. 2B).

3.3. Isolation of p94 mutants with Ca^{2+}/Na^{+}-dependent autolytic activity

p94 mutants with autolytic activity similar to that of p94:DA were screened in "p94-trapping" using the sensitivity to Ca^{2+}/Na^{+} of yeasts as an indicator. Random mutations were introduced into IIb-III-IS2-IV domains of BD-p94:WT-AD by PCR and by *in vivo* homologous recombination (Fig. 2C). Since the expression of tandemly linked BD/AD domains is a prerequisite for yeast to grow on SD-WHA plates, nonsense mutations in p94 are excluded, which is advantageous for isolating missense mutants of interest.

Out of 2,796 yeast colonies viable on SD-WHA (*ca*. $2.5x10^5$ transformants screened), 26 colonies were identified as Ca^{2+}/Na^{+}-sensitive. Finally, 18 different p94 mutants were identified in 19 colonies (Table 1, Fig. 2D). Selected plasmids (Table 1, No. 6, 9, 14, 15, 17, and 18) were re-introduced into yeasts to confirm that Ca^{2+}/Na^{+}-sensitivity of the colonies were dependent on the expression of Gal4-p94 mutant proteins.

The results indicated that F604 and V605 as well as rationally anticipated D607 were relevant to p94 autolytic activity (Table 1, mutants 15-18). Another intriguing finding is that mutations found in domain IIb are adjacent to theoretical Ca^{2+}-binding residues [6,21]. This further suggests that protease domain of p94 is activated by a mechanism similar to that of the conventional calpains, and that other regions of p94 facilitate the process spontaneously, *i.e.*, in an apparently Ca^{2+}-independent mechanism [22].

For the clones having two mutations (Table 1, No. 5, 9, 10, 13, and 14), F604L, S423P, V431E, N539D, and I603T are considered primarily responsible, although cooperative effect of two mutations is also possible. The rationale is as follows: (i) only significantly weakened autolytic activity of p94 mutants lets yeasts grow on SD-WHA. Therefore, mutations without suppressive effect on p94 autolysis in COS7 cells, V590M and Y770H, were excluded (Fig. 3A, lanes 4 and 7); (ii) F604L or F604S, respectively, was identified as a responsible single mutation in clone 15 or 16; (iii) mutations in domain IV of p94 tend to enhance p94 autolysis as is shown for Y770H (Fig. 3A, lane 7), S744G and R769Q [13], suggesting the same trend for M792T and S739P; (v) N539 is highly conserved in the calpain superfamily; (iv) I603 is one of well-conserved amino acids encoded by exon 16 (Fig. 1A).

Consistent with above, reduced, but not zero, autolytic activity was observed when p94:S423P or p94:D607N was expressed in COS7 cells (Fig. 3A, lane 3). Both mutants exhibited Ca^{2+}-dependent autolytic activity comparable to that of p94:DA (data not shown).

3.*4. Difference among mutations in domain IIb and IS2*

The impact of mutations identified in domain IIb and IS2 on enzymatic capability of p94 were further analyzed using p94:E393K, p94:F604L, and p94:W360Y. W360 corresponds to W298 and W288 in μCL and mCL, respectively, which undergo a transposition upon activation, thus important for activity [6,23]. The specific activity of m-calpain:W288Y is only 5 % of that of wild type [24], and, consistently, the mutation W360C in p94 is associated with LGMD2A [25].

When expressed in Sf-9 cells, autolysis of p94:W360Y was even more retarded than other mutants were, generating an intermediate autolyzed fragment (Fig. 3B, anti-pIS2, grey arrowhead). Antibody reactivities showed that p94:W360Y retained the intact N-terminus as p94:CS did while other mutants underwent N-terminal autolysis as p94:DA did (Fig. 1A, closed arrowhead 1, Fig. 3B, anti-pNS, closed arrowhead).

All the mutants proteolyzed calpastatin (Fig. 3C, arrows), and autolyzed Ca^{2+}-dependently even with calpastatin (Fig. 3C, closed arrowheads), suggesting that some of p94 unique properties other than apparent Ca^{2+}-independency are intact in these mutants. A preference for autolysis or proteolysis of calpastatin was different for each mutant as follows: p94:F604L was almost identical to p94:DA (Fig. 3C, lanes 13 to 15 vs Fig. 1C, lanes 3 to 5). p94:E393K was less efficient than p94:DA in proteolyzing calpastatin, however, it exhibited most exhaustive autolytic activity among the mutants examined (Fig. 3C, lanes 8 to 10). On the other hand, both activities were significantly retarded in p94:W360Y (Fig. 3C, lanes 3 to 5).

4. Discussion

This study demonstrated that a “p94-trapping” system, a yeast genetic reporter system for monitoring p94 autolytic activity, provided an effective method to screen p94 mutants for conditional, *i.e.*, Ca^{2+}-dependent, autolytic activity. We anticipated that identified mutations suggest amino acids allowing p94 to hold an active conformation within the cellular environment. Some mutations identified in our screening are localized in the same or flanking positions of LGMD2A mutations (Table I). This exemplifies that mutations transforming p94 protease activity to be Ca^{2+}-dependent are not functional *in vivo*. In other words, apparently Ca^{2+}-independent activation of p94 is in fact physiologically essential.

The p94:DA and p94:F604L mutants clearly indicated that the IS2 region is responsible for activating p94 in the absence or in the presence of very low level of Ca^{2+}. It is also possible that mutated amino acid positions within domains IIb to III are important for receiving and filtering changes in IS2 to the whole molecule. In this regard, five of the six identified mutations in domain IIb (V363A, W373R, V379E, L387P,

and E393K; see Figs. 2D and 4A) are adjacent, but not identical, to the Ca^{2+}-chelating residues in μCL and mCL, while Q390 corresponds to V327/R317 in μCL/mCL, whose backbone nitrogen is used for stabilization of a water molecule involved in Ca^{2+}-coordination [6]. Furthermore, all of six residues, at least in part, exist on the surface of the molecule (Fig. 4B) as has been shown for LGMD2A-associated mutations projected to the crystal structure of m-calpain [26]. These results suggest that the domain IIb mutations, including Q390P, very mildly affect the structure of the Ca^{2+}-binding site of p94, and/or that these residues interact with IS2. Further crystallographic studies on the structure of full-length p94 will elucidate the mechanisms how the IS2 region alters the structure of p94, especially the projection of the amino acids conserved between p94 and the conventional calpains, and how autolysis and substrate recognition are coordinated within p94.

The primary advantage of conditionally active p94 missense mutants over spliced variants of p94, such as Lp82 and p94Δ [15,17], as alternatives for p94:WT is that these mutants are expected to retain almost intact p94 structure except for a very local distortion. Identification of p94 mutants using “p94-trapping” under appropriate screening conditions (such as screening with temperature shifts, cDNA libraries, chemicals, etc.) might show how p94 autolytic products interact with cellular components, which appears to be impossible with p94:WT. Studying these p94 mutants would contribute to revealing the regulatory mechanisms of calpain in general and isoform-specific contexts.

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References

[1] Goll, D.E., Thompson, V.F., Li, H., Wei, W. and Cong, J. (2003). The calpain system. Physiol. Rev. 83, 731-801.

[2] Suzuki, K., Hata, S., Kawabata, Y. and Sorimachi, H. (2004). Structure, activation, and biology of calpain. Diabetes 53, S12-18.

[3] Sorimachi, H., Ishiura, S. and Suzuki, K. (1997). Structure and physiological function of calpains. Biochem. J. 328, 721-732.

[4] Hosfield, C.M., Elce, J.S., Davies, P.L. and Jia, Z. (1999). Crystal structure of calpain reveals the structural basis for Ca^{2+}-dependent protease activity and a novel mode of enzyme activation. EMBO J. 18, 6880-6889.

[5] Strobl, S., Fernandez-Catalan, C., Braun, M., Huber, R., Masumoto, H., Nakagawa, K., Irie, A., Sorimachi, H., Bourenkow, G., Bartunik, H., Suzuki, K. and Bode, W. (2000). The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. Proc. Natl. Acad. Sci. U.S.A. 97, 588-592.

[6] Moldoveanu, T., Hosfield, C.M., Lim, D., Elce, J.S., Jia, Z. and Davies, P.L. (2002). A Ca^{2+} switch aligns the active site of calpain. Cell 108, 649-660.

[7] Sorimachi, H., Toyama-Sorimachi, N., Saido, T.C., Kawasaki, H., Sugita, H., Miyasaka, M., Arahata, K., Ishiura, S. and Suzuki, K. (1993). Muscle-specific calpain, p94, is degraded by autolysis immediately after translation, resulting in disappearance from muscle. J. Biol. Chem. 268, 10593-10605.

[8] Sorimachi, H., Kinbara, K., Kimura, S., Takahashi, M., Ishiura, S., Sasagawa, N., Sorimachi, N., Shimada, H., Tagawa, K., Maruyama, K. and Suzuki, K. (1995). Muscle-specific calpain, p94, responsible for limb girdle muscular dystrophy type 2A, associates with connectin through IS2, a p94-specific sequence. J. Biol. Chem. 270, 31158-31162.

[9] Kinbara, K., Sorimachi, H., Ishiura, S. and Suzuki, K. (1997). Muscle-specific calpain, p94, interacts with the extreme C-terminal region of connectin, a unique region flanked by two immunoglobulin C2 motifs. Arch. Biochem. Biophys. 342, 99-107.

[10] Ono, Y., Torii, F., Ojima, K., Doi, N., Yoshioka, K., Kawabata, Y., Labeit, D., Labeit, S., Suzuki, K., Abe, K., Maeda, T. and Sorimachi, H. (2006). Suppressed disassembly of autolyzing p94/CAPN3 by N2A connectin/titin in a genetic reporter system. J. Biol. Chem. 281, 18519-18531.

[11] Ojima, K., Ono, Y., Doi, N., Yoshioka, K., Kawabata, Y., Labeit, S. and Sorimachi, H. (2007). Myogenic stage, sarcomere length, and protease activity modulate localization of muscle-specific calpain. J. Biol. Chem. 282, 14493-1504.

[12] Richard, I., Broux, O., Allamand, V., Fougerousse, F., Chiannilkulchai, N., Bourg, N., Brenguier, L.,

Devaud, C., Pasturaud, P., Roudaut, C., Hillaire, D., Passos-Bueno, M.-R., Zats, M., Tischfield, J.A., Fardeau, M., Jackson, C.E., Cohen, D. and Beckmann, J.S. (1995). Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. Cell 81, 27-40.

[13] Ono, Y., Shimada, H., Sorimachi, H., Richard, I., Saido, T.C., Beckmann, J.S., Ishiura, S. and Suzuki, K. (1998). Functional defects of a muscle-specific calpain, p94, caused by mutations associated with limb-girdle muscular dystrophy type 2A. J. Biol. Chem. 273, 17073-17078.

[14] Dasmahapatra, B., DiDomenico, B., Dwyer, S., Ma, J., Sadowski, I. and Schwartz, J. (1992). A genetic system for studying the activity of a proteolytic enzyme. Proc. Natl. Acad. Sci. U. S. A. 89, 4159-4162.

[15] Ono, Y., Kakinuma, K., Torii, F., Irie, A., Nakagawa, K., Labeit, S., Abe, K., Suzuki, K. and Sorimachi, H. (2004). Possible regulation of the conventional calpain system by skeletal muscle-specific calpain, p94/calpain 3. J. Biol. Chem. 279, 2761-2771.

[16] Ma, H., Shih, M., Hata, I., Fukiage, C., Azuma, M. and Shearer, T.R. (1998). Protein for Lp82 calpain is expressed and enzymatically active in young rat lens. Exp. Eye. Res. 67, 221-229.

[17] Fukiage, C., Nakajima, E., Ma, H., Azuma, M. and Shearer, T.R. (2002). Characterization and regulation of lens-specific calpain Lp82. J. Biol. Chem. 277, 20678-20685.

[18] Herasse, M., Ono, Y., Fougerousse, F., Kimura, E., Stockholm, D., Beley, C., Montarras, D., Pinset, C., Sorimachi, H., Suzuki, K., Beckmann, J.S. and Richard, I. (1999). Expression and functional characteristics of calpain 3 isoforms generated through tissue-specific transcriptional and posttranscriptional events. Mol. Cell. Biol. 19, 4047-4055.

[19] Mendoza, I., Rubio, F., Rodriguez-Navarro, A. and Pardo, J.M. (1994). The protein phosphatase calcineurin is essential for NaCl tolerance of Saccharomyces cerevisiae. J. Biol. Chem. 269, 8792-8796.

[20] Matsumoto, T.K., Ellsmore, A.J., Cessna, S.G., Low, P.S., Pardo, J.M., Bressan, R.A. and Hasegawa, P.M. (2002). An osmotically induced cytosolic Ca^{2+} transient activates calcineurin signaling to mediate ion homeostasis and salt tolerance of Saccharomyces cerevisiae. J. Biol. Chem. 277, 33075-33080.

[21] Moldoveanu, T., Hosfield, C.M., Lim, D., Jia, Z. and Davies, P.L. (2003). Calpain silencing by a reversible intrinsic mechanism. Nat. Struct. Biol. 10, 371-378.

[22] Garcia Diaz, B.E., Gauthier, S. and Davies, P.L. (2006). Ca^{2+} dependency of calpain 3 (p94) activation. Biochemistry 45, 3714-3722.

[23] Moldoveanu, T., Jia, Z. and Davies, P.L. (2004). Calpain activation by cooperative Ca^{2+} binding at two non-EF-hand sites. J. Biol. Chem. 279, 6106-6114.

[24] Arthur, J.S., Gauthier, S. and Elce, J.S. (1995). Active site residues in m-calpain: identification by site-directed mutagenesis. FEBS Lett. 368, 397-400.

[25] Kawai, H., Akaike, M., Kunishige, M., Inui, T., Adachi, K., Kimura, C., Kawajiri, M., Nishida, Y., Endo, I., Kashiwagi, S., Nishino, H., Fujiwara, T., Okuno, S., Roudaut, C., Richard, I., Beckmann, J.S., Miyoshi, K. and Matsumoto, T. (1998). Clinical, pathological, and genetic features of limb-girdle

muscular dystrophy type 2A with new calpain 3 gene mutations in seven patients from three Japanese families. Muscle Nerve 21, 1493-1501.

[26] Jia, Z., Petrounevitch, V., Wong, A., Moldoveanu, T., Davies, P.L., Elce, J.S. and Beckmann, J.S. (2001). Mutations in calpain 3 associated with limb girdle muscular dystrophy: analysis by molecular modeling and by mutation in m-calpain. Biophys J. 80, 2590-2596.

Figure legends

Figure1. Characterization of p94:DA mutant. (A) Schematic structure of p94. Amino acid sequences for the IS2 region encoded by exons 15 and 16 are shown below. Shaded residues are conserved in all 4 species. Closed arrowheads 1-5, reported autolytic sites. Horizontal bars, the regions used as antigens. (B) Autolytic activity of p94:DA. p94:WT, p94:DA or p94:CS were expressed in Sf-9 cells. p94:DA has weak autolytic activity which is enhanced by $CaCl_2$. Closed and open arrowheads, pre-autolyzed and autolyzed fragments of p94, respectively. (C) Proteolysis of calpastatin by p94:DA. In upper gels visualized by CBB-staining, Ca^{2+}-dependent proteolysis of calpastatin domain 1 (CAST-d1) (arrow) was observed when incubated with the lysates of Sf-9 cells expressing p94:DA (lanes 3-5), but not p94:CS (lanes 7-9). Closed arrowhead, the full-length product of p94:DA and p94:CS. Autolytic activity of p94:DA was resistant to CAST-d1.

Figure 2. Screening of conditional p94 mutants in the p94-trapping system. (A) Phenotype of p94:DA in the p94-trapping system. Growth of yeast cells expressing Gal4-p94 hybrid protein (BD-p94-AD) for p94:WT, p94:DA, or p94:CS after 72 h at 30^oC on selection medium plates with supplement as indicated. Autolytic activity results in less growth. D607A caused poor growth on SD-WHA plates supplemented with $CaCl_2$ or NaCl. (B) Relationships of autolytic activity of p94 profiled *in vitro* and in yeast cells. Autolysis of p94:DA (see Fig. 1B) and growth phenotype of yeast cells expressing BD-p94:DA-AD (see Fig.1C), are summarized. (C) Principle of isolating conditionally active p94 mutants by random mutagenesis in "p94-trapping". The growth defect on selection medium supplemented with an excess amount of $CaCl_2$ or NaCl is anticipated to reflect the autolytic degradation of Gal4-p94 hybrid protein induced by an increase of $[Ca^{2+}]_i$ and/or $[Na^+]_i$ in yeast cells. (D) A schematic view of missense mutations of p94 identified by the screening. The positions of mutations are categorized into five groups, highly conserved (top) to diverged (bottom). Arrows and *, respectively, stand for conserved and non-conserved amino acid positions corresponding to those involved in Ca^{2+}-binding of the conventional calpain protease domain.

Figure3. Difference among p94 mutants in autolysis and substrate proteolysis. (A) Expression of selected mutants of p94 in COS7 cells. For p94:S423P and p94:D607N identified by the screening, the pre-autolyzed fragment (closed arrowhead) as well as autolyzed fragments (open arrowheads) were detected as for p94:DA. p94:V590M and p94:Y770H were detected merely as autolyzed fragments as in the case for p94:WT. (B) Heterogeneity of autolysis in NS and IS1 among p94 mutants expressed in Sf-9 cells. The largest fragments (closed arrowhead) for p94:DA, E393K, and F604L lost the reactivity to anti-pNS, whereas those for p94:CS and W360Y retained it. Judging from the size, p94:DA, E393K, and F604L probably autolyzed at the C-terminus of Ala14. Open and gray arrowheads indicate autolyzed fragments generated by the cleavage in IS1 as indicated by closed arrowheads 3 or 4 and 5 in Fig. 1A, respectively. *, non-specific signals. (C) Difference in autolytic and proteolytic activities against CAST-d1 among Ca^{2+}-dependent p94 mutants. The upper and lower images show CAST-d1 proteolysis and p94 autolysis, respectively. The mutation W360Y retarded both p94 autolysis and CAST-d1 proteolysis (lanes 3 to 5), while p94:E393K expressed autolytic activity more preferentially than proteolytic activity against CAST-d1 (lanes 8-10). p94:F604L presented essentially the same trend as p94:DA (lanes 13-15).

Figure 4. Three-dimensional modeling of domain IIb with mutated amino acids. The stereo view models of domain IIb of the rat μ-calpain in the presence of Ca^{2+} (from 1KXR) were drawn in solid ribbon (A) and surface (B) schemes using WebLab Viewer Pro Ver. 3.5 (Molecular Simulations Inc.). (A) Active site residues, Ca^{2+}-binding residues, and the mutated residues indentified in this study are indicated by ball and stick representation in red, blue, and yellow, respectively. The residue shown in black is involved in Ca^{2+}-binding and also identified as mutated in this study. (B) Mutated residues are indicated in yellow. Residue numbers shown in (A) correspond to those for human p94 (NP_000061).

Table 1

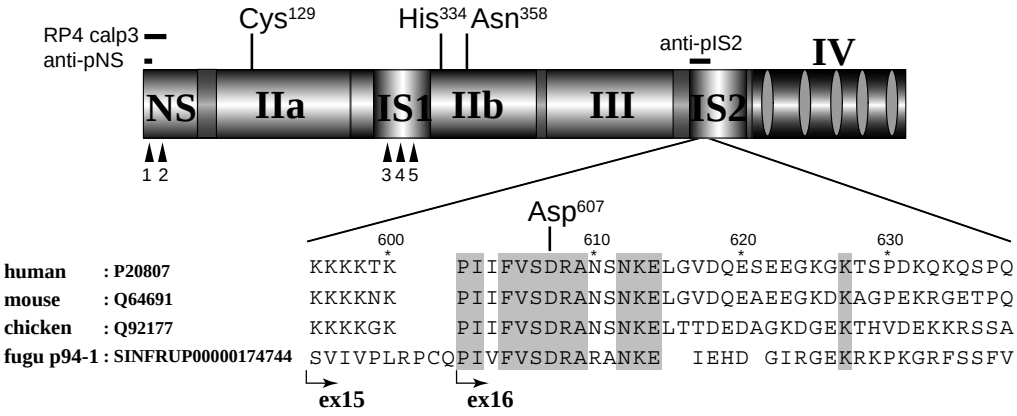
Summary of p94 mutants expressed as BD-p94-AD in colonies with Ca^{2+}/Na^{+} sensitivity

Mutated amino acids were categorized as follows (see Fig.2D); conserved among calpain superfamily (A), mammalian calpains (B), mammalian typical calpains (C), p94 species (P), or diverged (D). Linkers 1 and 2, respectively, represent regions between domains IIb and III and between III and IS2. For double mutants, mutations predicted to be primarily responsible for the phenotype are shown in bold face.

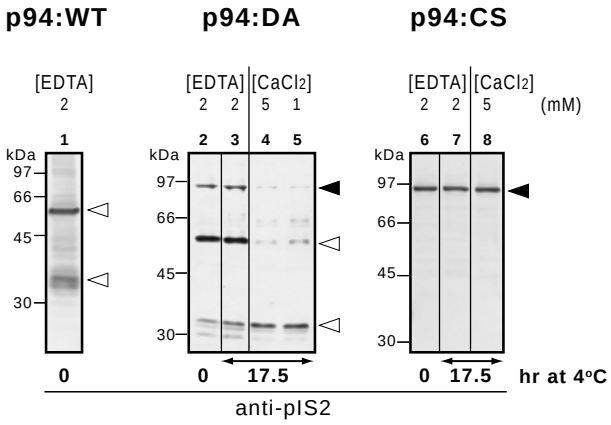
No.	Mutant	Domain(s)	Rank	Number of independent clones identified	LGMD2A mutation in the same (flanking) sites
1	V363A	IIb	C	1	
2	W373R	IIb	D	2	
3	V379E	IIb	C	1	
4	L387P	IIb	B	1	(R386C)
5	Q390P/**F604L**	IIb/**IS2**	D/**P**	1	
6	E393K	IIb	B	1	
7	T417A	linker1	B	1	T417M
8	L421P	linker1	B	1	(A420D)
9	**S423P**/Y770H	**linker1**/IV	**C**/B	1	(R769Q)
10	**V431E**/M792T	**III**/IV	**C**/C	1	V431M, (E791S)
11	L481Q	III	B	1	
12	H519L	III	B	1	
13	**N539D**/S739P	**III**/IV	**A**/C	1	
14	V590M/**I603T**	linker2/**IS2**	D/**P**	1	
15	F604L	IS2	P	1	
16	F604S	IS2	P	1	
17	V605A	IS2	P	1	(S606L)
18	D607N	IS2	P	1	(S606L)
	Total			19	

Figure 1.

A


RP4 calp3
anti-pNS
Cys129
His334 Asn358
anti-pIS2
IV
NS
IIa
IS1
IIb
III
IS2
1 2
3 4 5
Asp607
600
610
620
630
human : P20807 KKKKTK PIIFVSDRANSNKELGVDQESEEGKGKTSPDKQKQSPQ
mouse : Q64691 KKKKNK PIIFVSDRANSNKELGVDQEAEEGKDKAGPEKRGETPQ
chicken : Q92177 KKKKGK PIIFVSDRANSNKELTTDEDAGKDGEKTHVDEKKRSSA
fugu p94-1 : SINFRUP00000174744 SVIVPLRPCQPIVFVSDRARANKE IEHD GIRGEKRKPKGRFSSFV
ex15
ex16

B


p94:WT
p94:DA
p94:CS
[EDTA]
[CaCl2]
(mM)
kDa
97
66
45
30
0
17.5
hr at 4°C
anti-pIS2

C

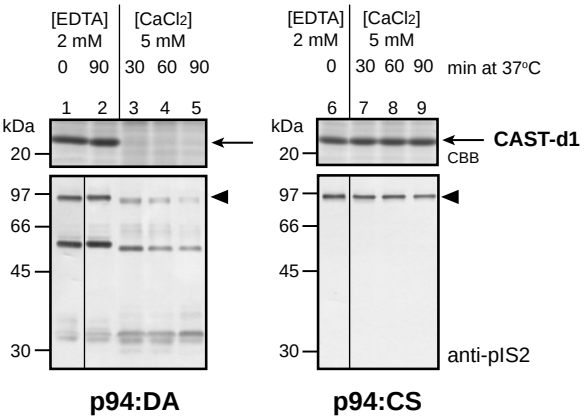
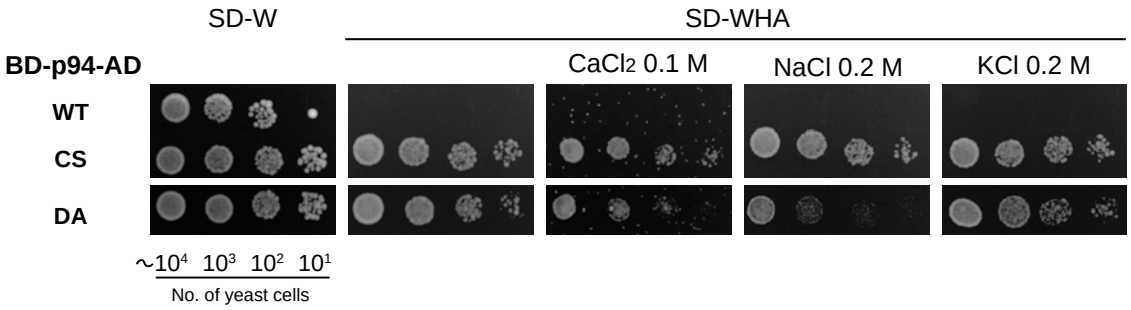

[EDTA]
[CaCl2]
2 mM
5 mM
min at 37°C
kDa
20
97
66
45
30
CAST-d1
CBB
anti-pIS2
p94:DA
p94:CS

Figure 2.

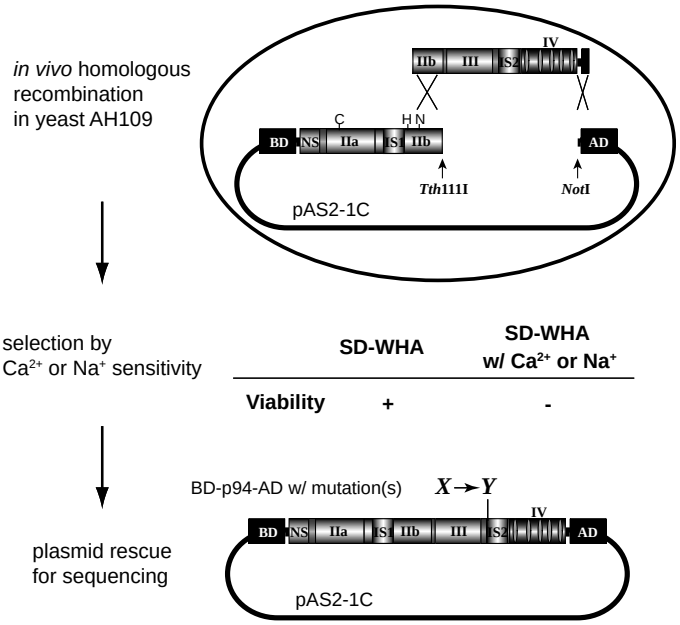
A



B

		autolysis		
		WT	CS	DA
	EDTA	N/A	-	-
	$CaCl_2$	N/A	-	+
growth of yeast cells expressing BD-p94-AD	SD-WHA	-	+	+
	/ $CaCl_2$	-	+	-
	/ NaCl	-	+	-
	/ KCl	-	+	+

C



D

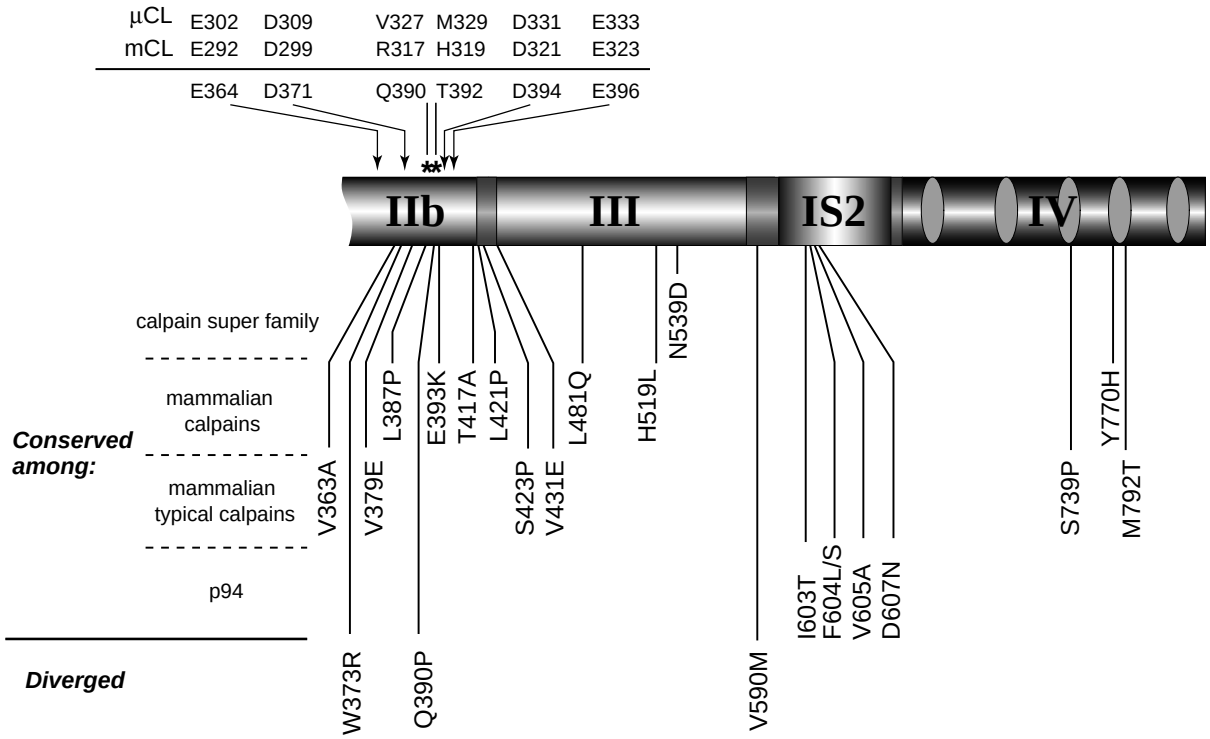
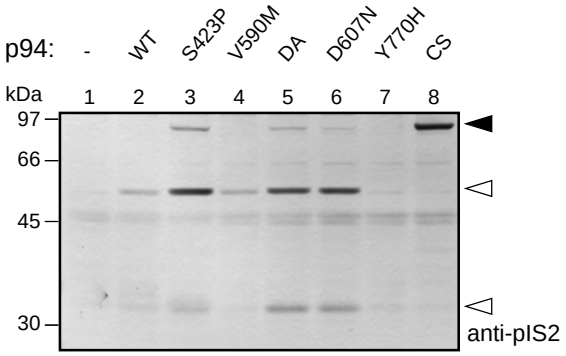
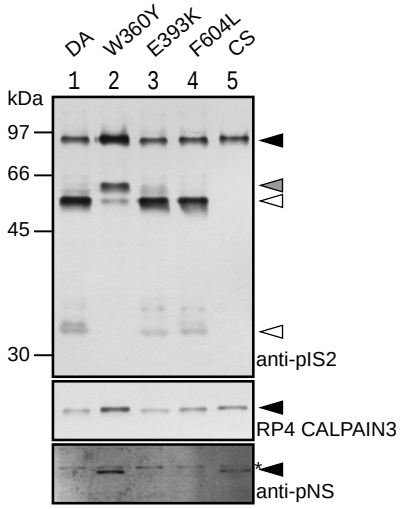


Figure 3.

A


p94: - WT S423P V590M DA D607N Y770H CS
kDa 1 2 3 4 5 6 7 8
97
66
45
30
anti-pIS2

B


DA W360Y E393K F604L CS
1 2 3 4 5
kDa
97
66
45
30
anti-pIS2
RP4 CALPAIN3
anti-pNS

C

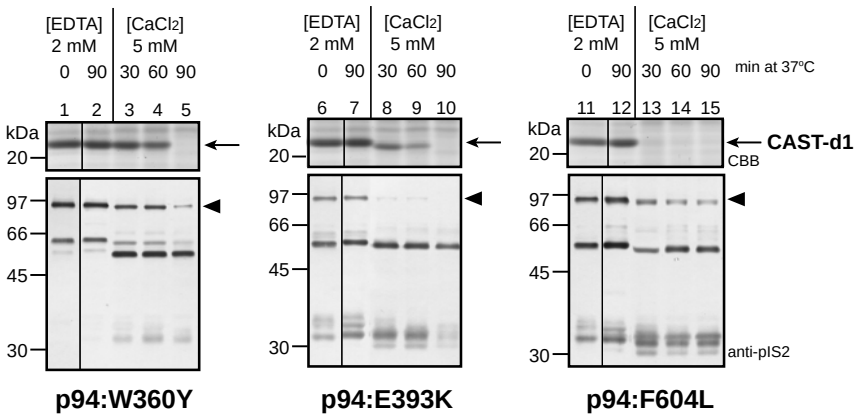
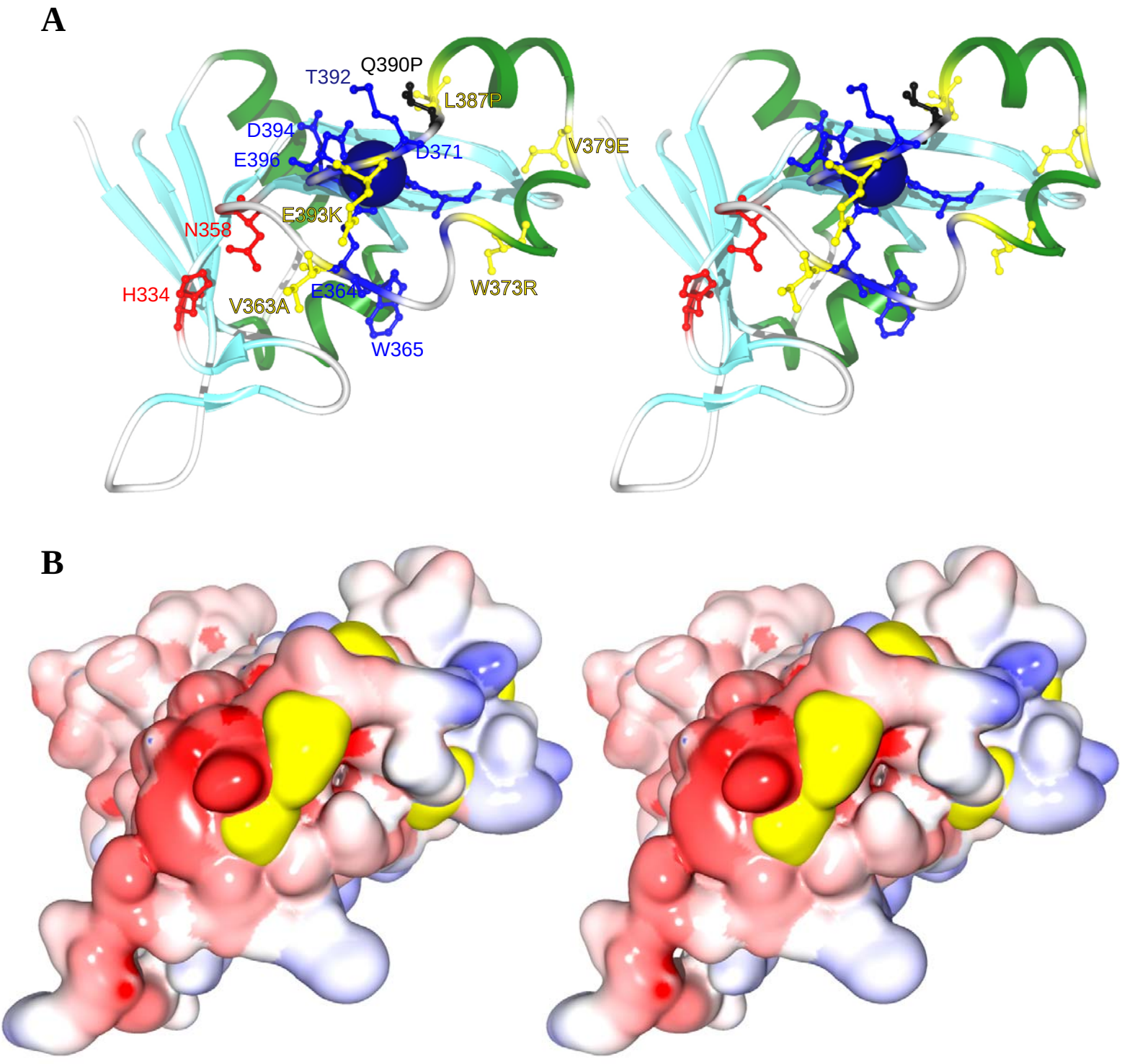

[EDTA] 2 mM
[CaCl2] 5 mM
0 90 30 60 90
min at 37°C
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
kDa
20
97
66
45
30
CAST-d1
CBB
anti-pIS2
p94:W360Y
p94:E393K
p94:F604L

Figure 4.


A
T392
Q390P
L387P
D394
E396
D371
V379E
E393K
N358
H334
V363A
E364
W373R
W365
B