Newly identified exons encoding novel variants of p94/calpain 3 are expressed ubiquitously and overlap the α-glucosidase C gene

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Abstract There are two classes of an intracellular 'modulator protease', calpain: ubiquitous and tissue-specific. p94/calpain 3 is an example of the latter, predominantly expressed in muscle. A defect in the p94 gene causes muscular dystrophy. Here we report that human and mouse p94 genes have a possible novel alternative promoter expressing p94 variants in all tissues examined including human lens epithelial cells. The possible promoter region and the following novel exons overlap the 3′ region of the neutral α-glucosidase C gene. Unlike p94, the novel p94 variants expressed in COS7 cells do not undergo rapid autolysis, suggesting basic functions different from p94.

Key words: Calpain; Alternative promoter; Ubiquitous expression; Proteolytic processing

1. Introduction

Calpain is a Ca^{2+}-regulated intracellular 'modulator protease', which modulates cellular functions by limited and specific proteolytic processing activity [1–5]. Calpain homologs have been identified in most living organisms, and constitute the 'calpain superfamily' [2]. Two representative well-characterized members, the μ- and m-calpains, or 'conventional' calpains, are ubiquitously expressed, and play important and essential roles in cellular functions. The μ- and m-calpains are heterodimers consisting of a unique larger catalytic subunit (μCL and mCL, respectively), and a common smaller regulatory subunit (30K).

Recent crystal structural analyses of calpain [6,7] have demonstrated that mCL is composed of four functional domains (see Fig. 1A), i.e. the N-terminal anchoring α-helix domain (domain I), important for the regulation of activity and subunit dissociation [8]; the catalytic domain (II), which is divided into two subdomains in the absence of Ca^{2+}; the C2-like domain (III); and the 5-EF-hand (or penta-EF-hand [PEF]) domain (IV).

The discovery of p94 (also called calpain 3) in 1989 [9] led to the concept that calpains are divided into two classes: ubiquitous and tissue-specific. p94 is the first and typical example of the latter, and is predominantly expressed in skeletal muscle. The whole primary structure of p94 is similar to those of μCL and mCL, except for the p94-specific sequences, NS, IS1, and IS2 (see Fig. 1A) [9]. p94 has the following unique characteristics, distinct from the other proteases, as well as those of the conventional calpains: (i) p94 undergoes very rapid, exhaustive, and apparently Ca^{2+}-independent autolysis (half-life in vitro is less than 10 min) [10]; (ii) inhibitors of the conventional calpains, including calpastatin and ethylenediamine tetraacetic acid (EDTA), have no effect on p94 autolysis [10]; (iii) p94 associates with connectin (titin), the gigantic filamentous molecule essential for myofibrils [11,12]; and (iv) a defect in the human p94 gene, *CAPN3*, is responsible for limb-girdle muscular dystrophy type 2A (LGMD2A), indicating that p94 is indispensable for proper muscle function [13]. Several studies indicate that the loss of substrate-processing activity of p94, but not hyperactivation or a defect in the structural properties, causes LGMD2A [14–17].

Mouse *Capn3* has an alternative promoter, which drives a lens-specific splicing variant of p94, Lp82 [18]. Lp82 proteolyses crystallins, and is involved in the molecular mechanisms of rodent cataract formation [18,19]. It should be noted that human *CAPN3* has lost this variant during the course of evolution [20].

Recently, we found that cyclin A is proteolysed to the more stable fragment in the embryonic liver and other myeloid progenitor cells [21]. Northern and Western blot analyses suggest the involvement of p94 or its splice variants, which are expressed at very low levels. Although mRNA for p94 is detected predominantly in skeletal muscle, there are several reports showing small amounts of p94 transcripts in brain, lymphocytes, and spleen [22–24]. Therefore, we performed an extensive search for p94-related molecules in non-muscle cells to elucidate the functions of *CAPN3* in tissues other than skeletal muscle. To our surprise, the newly identified molecules utilize a novel alternative promoter of *CAPN3*/*Capn3*, which drives ubiquitous expression through a novel initiation exon, and not the authentic NS-encoding exon 1.

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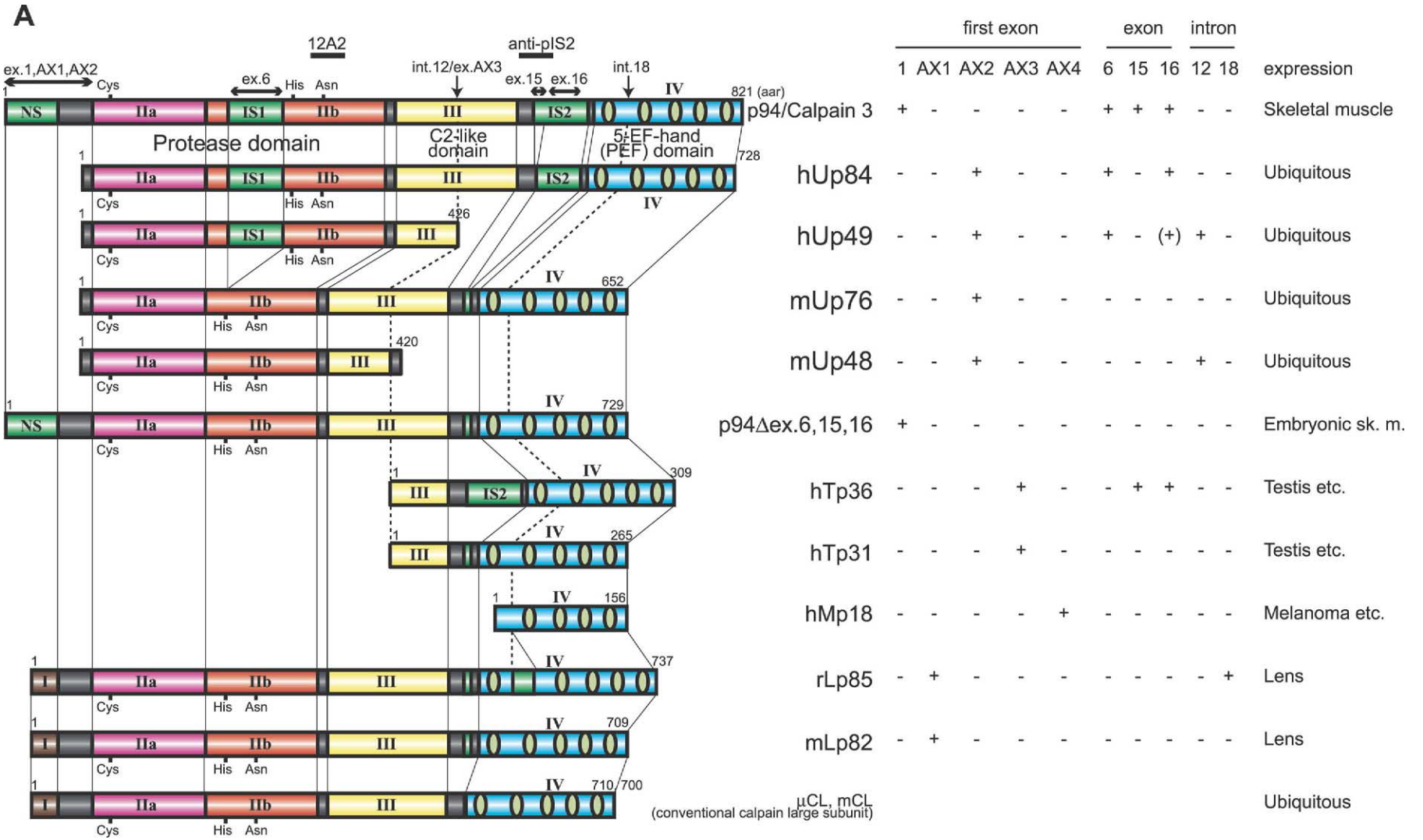

Fig. 1. Schematic structures of p94-related molecules and their gene organizations. A: Schematic structures and the exon usage. Positions of the alternatively spliced exons and the recognition sites of antibodies used in this study are indicated by lines with arrow heads and thick lines, respectively, on the p94 structure at the top. Mouse mLp82, rat rLp85, human hTp36, hTp31, and hMp18 are from the database (AAC61764, AAC15423, BC003169, BG530497, and BC004883, respectively). B: Exon splicing schemes. Domain structures are indicated by different colors. Arrows with numbers in the parentheses indicate positions of primers used in this study (see Table 1). C: Gene organization of the 3′ part of *GANC*/*Ganc* and the 5′ part of *CAPN3*/*Capn3*. The last four exons of *GANC*/*Ganc* and the first 10/9 exons of *CAPN3*/*Capn3* are shown with splicing patterns below. Red, dark blue, or magenta exons following the promoter boxes labeled 'UP', 'SkmP', or 'LP' are driven ubiquitously, skeletal muscle specifically, or lens specifically, respectively. Lengths of exons and introns are shown above or under them. Gray and other boxes correspond to coding and non-coding regions, respectively. D: Amino acid sequences of the N-termini of calpains. Secondary structures determined for human m-calpain [7] are shown below. Closed and open triangles indicate positions of introns inserted and the active site Cys, respectively. Residues conserved among at least five species are inverted.

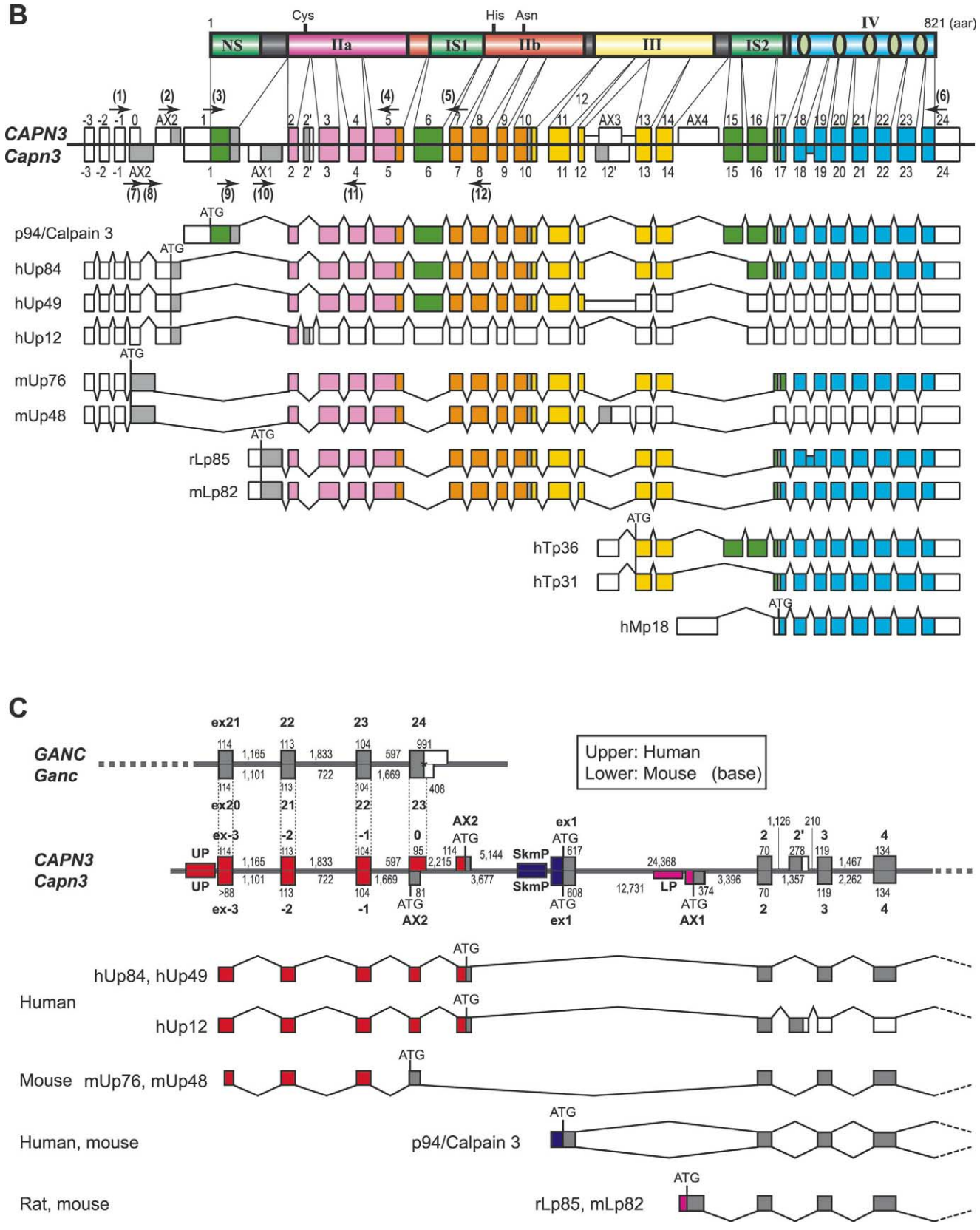

Fig. 1 (*Continued*).

2. Materials and methods

2.1. Polymerase chain reaction (PCR) and primers

PCR was performed using *Pfu* (Stratagene Inc.) or Ex*Taq* (Takara Shuzo Inc.) DNA polymerases, and the human cDNA panels (K1426-1, K1428 and K1420-1; Clontech Inc.) or first strand cDNAs synthesized from mouse eye and human lens epithelial cell SRA01/04 [25] as templates. Primers used were listed in Table 1. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining.

2.2. Construction of expression vectors for p94-related molecules

hUp84 and hUp49 cDNA fragments were amplified from human spleen and resting $CD8^+$ cDNAs (Clontech Inc.), respectively, by PCR using *Pfu* DNA polymerase and primers hUpS2 and hp94Aex24. mUp76/48 cDNA fragments were isolated from mouse spleen cDNA using primers mUpS1 and hp94Aex24. These fragments were ligated into pcDNA3.1-N-FLAG vector (a kind gift from Dr. Tatsuya Maeda, University of Tokyo) to express the proteins with an N-terminal FLAG tag.

2.3. Cell culture and assays

COS7 cell culture, transfection, and detection of expressed proteins by Western blotting were performed as previously described [10,17].

3. Results and discussion

3.1. Identification of a novel isoform of mouse p94

Previously, we had observed shorter transcripts of the p94 gene in chicken spleen [24] and mouse erythroleukemia cells (data not shown), in agreement with recent reports of non-muscle expression of p94-related molecules [22,23]. To identify the p94-related molecules in these cells, 5′ rapid amplification of cDNA ends was performed using a primer corresponding to the protease domain of p94. One mouse cDNA fragment was amplified that had a sequence corresponding to the protease domain, but the 5′ upstream sequence differed from that of the first exon of either p94 or Lp82 (see Fig. 1B). A database search revealed that this upstream sequence is identical to the 3′ part of the neutral α-glucosidase C gene (*Ganc*) [26]. The *Ganc* gene exists ca. 3.4 kb upstream from the first exon of p94 (Fig. 1C). The 5′ sequence of the newly identified p94-related molecule (mUp76, where U stands for 'ubiquitous', and p76 indicates its deduced molecular mass) corresponds to all or part of the last four exons, exons 20–23, of *Ganc* (Fig. 1C). The exon–intron junctions of these four exons coincide with those of *Ganc*. The fourth junction, between the fourth and fifth exons of mUp76, i.e. novel exon AX2 and p94 exon 2, which are 20 786 bp apart, also conforms to the GT-AG rule. The open reading frame of mUp76 in exon AX2, however, is distinct from that of exon 23 of *Ganc* (Fig. 1C).

3.2. Identification of a human orthologue of mUp76 and other splice variants

To exclude the possibility of artificial amplification of a 'chimeric' cDNA of *Capn3* and *Ganc*, an expressed sequence tag (EST) database search was performed. The EST clone AK097401 from human testis was identified, which has a structure similar to that of the 5′ part of mouse mUp76 (hUp49 in Fig. 1). Furthermore, using the human *GANC* sequence, a human orthologue of mUp76 was isolated from spleen cDNA by PCR using primers hUpS1 and p94Aex7 (Table 1, (1) and (5) in Fig. 1B).

Several other alternative splicing variants were further isolated, and are summarized in Fig. 1B. hUp12 uses a novel exon (exon 2′) between exons 2 and 3 of p94, which results in the production of a very short polypeptide of ca. 12 kDa. Intron 12 of p94 is unspliced in hUp49, which, therefore, lacks the C-terminal half of domain III and regions thereafter. mUp48 contains a sequence from a novel exon (exon 12′) between exons 12 and 13 of p94, which results in termination within domain III with a short unique C-terminal sequence. hUp84 lacks exon 15 of p94, whereas mUp76 lacks exons 6, 15, and 16.

In summary, a novel promoter region ('UP' in Fig. 1C) for human and mouse *CAPN3*/*Capn3* should exist in the 3′ part of *GANC*/*Ganc*, generating p94-like proteases (mUp76 and hUp84) and their truncated forms (mUp48, hUp49, and hUp12) with IS1 and/or all or a part of IS2 (Fig. 1B, deposited in DDBJ under accession numbers AB117940–AB117944). These possible novel promoter regions of both mouse and human contain consensus sequences for the nuclear factor of activated T-cells (NF-AT, GGAAAA) and CCAAT enhancer binding protein α (C/EBPα, TTGCNN-AA). The presence of the C/EBPα binding site suggests that the novel p94 promoters are under the control of C/EBPα, and may explain why C/EBPα knockout mice did not show p94-like cyclin A processing activity [21].

Overlapping genes are rather rare, if not non-existent, in organisms with large genomes like mammals [27,28]. We have previously reported an overlap between the mCL and nCL-2/-2′ genes, *CAPN2* and *CAPN8*, in head-to-head orientation [29]. At present, there is no evidence of a relationship between *CAPN3*/*Capn3* and *GANC*/*Ganc*, but it is possible that both genes affect one another's transcription, at least in part.

3.3. Structure of novel isoforms of p94

The newly identified p94 isoforms have truncated N-terminal sequences compared with other calpains (Fig. 1D). They are not only short but lack the N-terminal 'WK(R/K)P' motif,

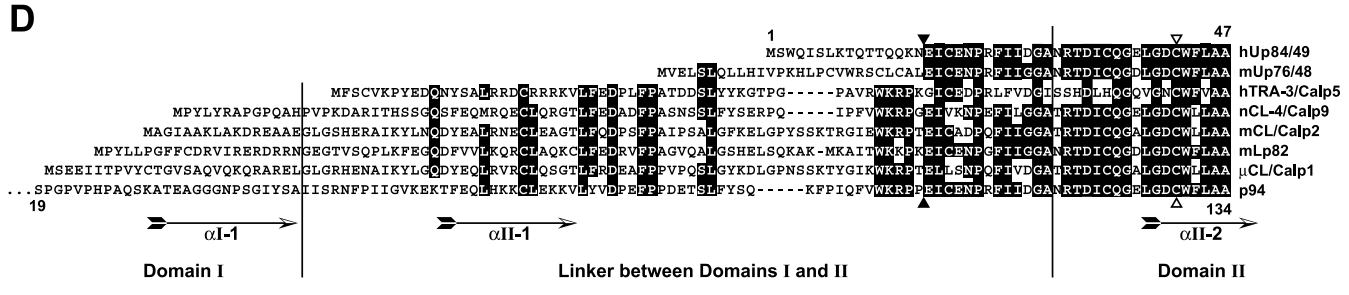

Fig. 1 (*Continued*).

Table 1
Primers used in this study

Specificity		Name	Sequence (5′→3′)	Position in Fig. 1B
Human	Up[a]	hUpS1	CATCTTCTGTGACTACCCAC	(1)
Human	Up	hUpS2	GGAATGAGTTGGCAAATCAG	(2)
Human	p94	hp94Sex1	CCATCATTAGCCGCAATTTTCC	(3)
Human	Up/p94	hp94Aex5	CCTGTGAAGTCCTCCATGGC	(4)
Human	Up/p94	hp94Aex7	TATAAGCTTCTCATCCAGCCCCGTGAC	(5)
Human/mouse	Up/p94	hp94Aex24	GGACTAGTGGTTCAGGCATACATGGT	(6)
Mouse	Up	mUpS1	GGATCCGTAGAGCTCAGCCTGCAGCTT	(7)
Mouse	Up	mUpS2	ATTGTGCCGAAACATCTGCC	(8)
Mouse	p94	mp94Sex1	CGATCATCGGTGTGAAAGAG	(9)
Mouse	Lp82	mLp82S1	AGTGCCTCTTTGAAGACCGT	(10)
Mouse	Up/p94/Lp82	mp94Aex4	CAGACAGTCGTCAATGACCA	(11)
Mouse	Up/p94/Lp82	mp94Aex8	GCACCAACTTCACCTTCTCA	(12)
Human/mouse	μCL	μCLS1	CGTGACTTCTTCCTGGCCAA	–
Human/mouse	μCL	μCLA1	AGGATGTTGAACTCCACCAG	–

[a]Up stands for novel isoforms of p94, hUp84/49/12 for human and mUp76/48 for mouse.

highly conserved among calpain homologs [2]. The linker region connecting domains I and II is rather divergent among calpains, has no secondary structure except for one short α-helix (Fig. 1D, αII-1), and does not exist in the three-dimensional structure of papain, which is very similar to that of the calpain protease domain. Therefore, this region may be involved in a function specific to each calpain homolog. In this sense, the divergent N-termini of hUp84 and mUp76 imply specific functions for these molecules.

In addition to the isoforms described above, the database also contains several interesting cDNA sequences. As shown in Fig. 1A and B, another novel initiation exon (AX3) exists between exons 12 and 13 in ESTs mainly from human testis (hTp36 and hTp31). AX3 is slightly different from exon 12′ found in mUp48. hTp36 and hTp31 have a structure complementary to that of hUp49. Previously, we have observed a significant departure from Mendelian transmission of the Capn3$^{-/-}$ allele [15]. It is possible that these testis-expressing p94 isoforms, as well as mUp76 and mUp48, are involved in this phenomenon. hMp18, which is strongly expressed in melanoma cells (28 EST sequences were found in melanoma cDNA), possesses another alternative initiation exon (AX4) between exons 14 and 15. hMp18 contains only four and a half EF-hand motifs. ALG-2, which has five EF-hands, was reported to have anti-apoptotic functions when expressed in cancer cells [30], suggesting a similar role for hMp18.

3.4. Expression profile of novel isoforms of p94

The promoter of *CAPN3*/*Capn3* that produces p94 ('SkmP' in Fig. 1C) is virtually skeletal muscle-specific [31], and that producing Lp82 ('LP') is lens-specific [32]. Therefore, we examined the expression profiles of the newly identified variants by PCR using primers corresponding to exons −2 and 5 (Table 1, Fig. 1B). As shown in Fig. 2A, D and H, expression was detected in all human tissues examined, as is the case for ubiquitously expressed μCL (Fig. 2C, F and H). Mouse cDNAs gave essentially the same results (data not shown). These suggest basic and essential physiological roles of novel isoforms of p94, like the conventional calpains. In mouse eye, mUp76/48 was hardly detectable (Fig. 2G, lane 1), and, instead, Lp82 was abundantly detected (lane 3) as previously reported [32]. On the other hand, human lens epithelial cells, SRA01/04 [25], in which Lp82 does not exist [20], express hUp84/49 (Fig. 2H, lane 1). It is, therefore, suggested that, analogously to an involvement of Lp82 in rodent cataract formation, hUp84 and/or hUp49 play a role in human cataract formation. These results indicate that the possible novel promoter acts ubiquitously, unlike the promoters for p94 and Lp82. Qualitatively, the levels of expression were much lower than that of μCL. However, resting immune cells (Fig. 2A, lanes 9–12) showed relatively abundant expression.

The location of *GANC*/*CAPN3* (15q15.1) is implicated in diabetes [33]. It is likely that the functions of p94 in skeletal muscle are indirectly involved in the molecular mechanisms of diabetes. Alternatively, ubiquitous hUp84/49/12 may play direct roles in diabetes by, for example, modulating adipose cell functions, which is also assumed for calpain 10 [34].

During preparation of this manuscript, De Tullio et al. reported that circulating mononuclear blood cells express a p94 isoform that lacks IS1 but contains NS and IS2 [23]. Thus, the expression of NS-containing isoforms was also examined. As shown in Fig. 2B, E and H, most tissues express NS-containing isoforms except activated B- and T-cells (lanes 13–16), which is consistent with their report [23].

3.5. Expression of novel isoforms of p94 in COS7 cells

Next, to test whether the novel p94 isoforms are translated into protein, they were expressed in COS7 cells. Western blot analysis detected hUp84, mUp76, hUp49, and mUp48 at the expected positions. Although the expressed protein amounts were much lower than p94:C129S (data not shown), the same amounts were detected for both wild type and the active site Cys to Ser mutants (Fig. 3). Therefore, we concluded that these isoforms do not undergo quick autolysis, unlike muscle type p94 [10]. p94 without exon 15, which is identical to hUp84 except for NS, showed considerable Ca^{2+}-independent autolytic activity [14], indicating that the substitution of NS with AX2 stabilizes p94 against autolysis. Our findings argue that the novel p94 isoforms, with structures and expression profiles divergent from those of the original p94, should be recognized as an important part of the ubiquitous calpain system.

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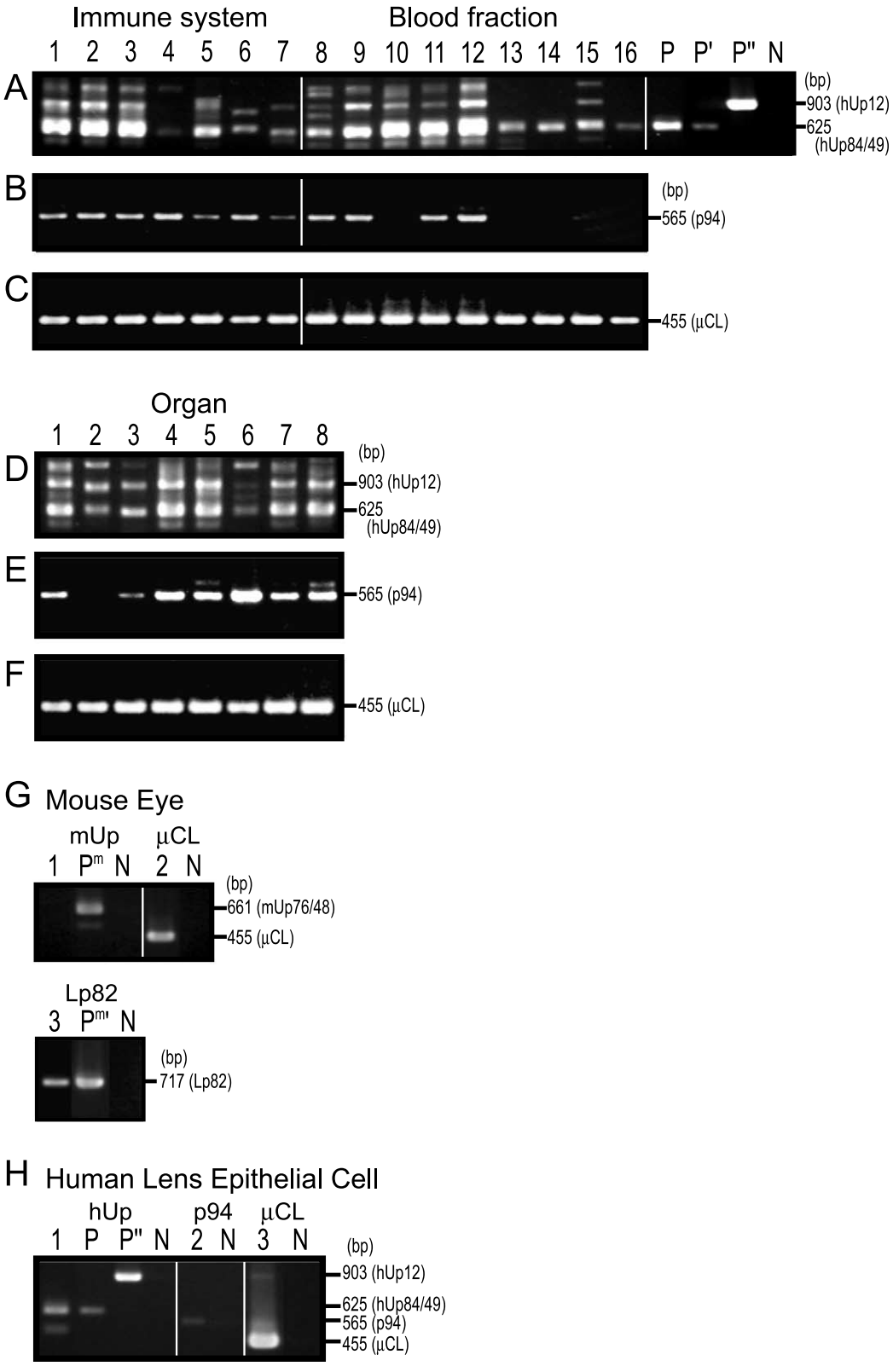
Immune system
Blood fraction
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 P P' P'' N
A
(bp)
903 (hUp12)
625
(hUp84/49)
B
(bp)
565 (p94)
C
455 (μCL)
Organ
1 2 3 4 5 6 7 8
D
(bp)
903 (hUp12)
625
(hUp84/49)
E
565 (p94)
F
455 (μCL)
G Mouse Eye
mUp μCL
1 Pm N 2 N
(bp)
661 (mUp76/48)
455 (μCL)
Lp82
3 Pm' N
(bp)
717 (Lp82)
H Human Lens Epithelial Cell
hUp p94 μCL
1 P P'' N 2 N 3 N
(bp)
903 (hUp12)
625 (hUp84/49)
565 (p94)
455 (μCL)

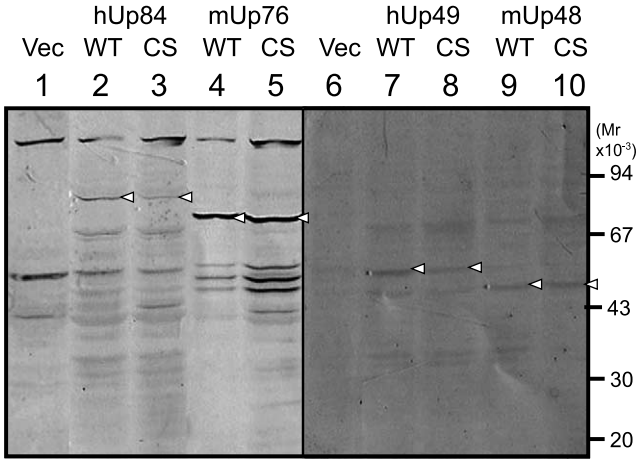

Fig. 3. Novel p94 isoforms are stable when expressed in COS7 cells. Wild type (lanes 2, 4, 7, and 9) or Cys→Ser mutant (C42S for hUp84/49 (lanes 3 and 5) and C53S for mUp76/48 (lanes 8 and 10)) of hUp84 (lanes 2 and 3), mUp76 (lanes 4 and 5), hUp49 (lanes 7 and 8), or mUp48 (lanes 9 and 10) were expressed in COS7 cells, and detected by p94-specific anti-pIS2 antiserum (lanes 1–5), which recognizes the peptide sequence between domains III and IV (NTISVDRPVKKKKNKPIIFV), or monoclonal antibody 12A2 (Novacastra Lab. Ltd.) (lanes 6–10), which recognizes the peptide sequence around active site Asn in domain IIb (RLRNPWGQVEWNGSWS) (see Fig. 1A). Open arrowheads indicate expressed proteins. Lanes 1 and 6, lysates of cells transfected with pcDNA3.1-N-FLAG vector.

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Fig. 2. Expression of novel isoforms of p94 detected by reverse transcription (RT)-PCR. PCR was performed using primers (1) and (4) (see Table 1 and Fig. 1B) specific to hUp84/49/12 (A and D, all lanes, and H, lane 1); (3) and (4) specific to human p94 (B and E, all lanes, and H, lane 2); μCLS1 and μCLA1 specific to human/mouse μCL (C and F, all lanes, G, lane 2, and H, lane 3); (8) and (11) specific for mouse mUp76/48 (G, lane 1); and (10) and (12) specific to mouse Lp82 (G, lane 3). In A, D and H, 903-bp and 625-bp bands correspond to hUp12 with exon 2′ and pUp84/49 without exon 2′, respectively. Other bands also correspond to p94 variants, judging from Southern blot analyses with p94 cDNA as a probe (data not shown), although the precise structures have not yet been determined. Template cDNAs used for PCR are as follows: Lanes for A–C: 1, spleen; 2, lymph node; 3, thymus; 4, tonsil; 5, leukocyte, peripheral blood; 6, bone marrow; 7, fetal liver; 8, mononuclear cells (monocytes, T-, and B-cells); 9, resting $CD8^+$ (suppressor and cytotoxic T-cells); 10, resting $CD4^+$ (helper and inducer T-cells); 11, resting $CD14^+$ (monocytes); 12, resting $CD19^+$ (B-cells); 13, activated $CD19^+$; 14, activated mononuclear cells; 15, activated $CD4^+$; 16, activated $CD8^+$. Lanes for D–F: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas. Lanes for G: 1–3, mouse eye. Lanes for H: 1–3, human lens epithelial cell SRA01/04 [25]. Lanes labeled by letters starting with P and N indicate positive and negative control reactions, respectively, for their immediate left lanes using 1 fg and 0.1 fg of cDNA for hUp84 (P and P′, respectively), 1 fg of cDNA for hUp12 (P″), mUp76 (P^m), and mouse Lp82 ($P^{m\prime}$), and sterile water (N) as templates.

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