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Review

Calpains — An elaborate proteolytic system☆

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ABSTRACT

Calpain is an intracellular Ca^{2+}-dependent cysteine protease (EC 3.4.22.17; Clan CA, family C02). Recent expansion of sequence data across the species definitively shows that calpain has been present throughout evolution; calpains are found in almost all eukaryotes and some bacteria, but not in archaebacteria. Fifteen genes within the human genome encode a calpain-like protease domain. Interestingly, some human calpains, particularly those with non-classical domain structures, are very similar to calpain homologs identified in evolutionarily distant organisms. Three-dimensional structural analyses have helped to identify calpain's unique mechanism of activation; the calpain protease domain comprises two core domains that fuse to form a functional protease only when bound to Ca^{2+} *via* well-conserved amino acids. This finding highlights the mechanistic characteristics shared by the numerous calpain homologs, despite the fact that they have divergent domain structures. In other words, calpains function through the same mechanism but are regulated independently. This article reviews the recent progress in calpain research, focusing on those studies that have helped to elucidate its mechanism of action. This article is part of a Special Issue entitled: Proteolysis 50 years after the discovery of lysosome.

1. Introduction

Of the many different proteolytic enzymes/systems, calpain (EC 3.4.22.17; Clan CA, family C02) is both unique and perplexing. Nowadays, calpain homologs are defined based on the primary sequence characteristics of the calpain-like protease domain (CysPc) (Fig. 1) [1–5]. This criterion has been used to identify various calpain homologs in a wide range of living organisms (Fig. 2). Calpain, first identified in 1964 [6] and known by several different names ever since [7–10], is an evolutionarily well-conserved protease family [11]. More intriguingly, calpain superfamily members are sub-classified into several groups according to their expression profile or domain structure. This is one of the topics covered in this review.

However, the physiological relevance of calpain (particularly with respect to its positive effects) is difficult to define. The involvement of calpain activity in various pathophysiological phenomena has been reported but usually as an aggravating factor [12–18]. Therefore, although it is accepted that the regulation of calpain is critical for cell function, questions remain regarding when and how calpain is activated. Genetic techniques (using both forward and reverse genetics) have expanded our knowledge of the cause-effect relationships between calpain deficiency and defective tissue function [19]. These conditions, called "calpainopathies", provide strong evidence for the physiological importance of calpain. The findings of such phenotype-oriented studies are also summarized in this review.

Another issue that has attracted attention is this: how does activated calpain recognize its substrate? This is directly relevant to calpain-mediated proteolysis, because calpain is different from other major intracellular proteolytic components such as proteasomes [20], autophagy [21] and caspases [22] in one or both of two aspects. First, the action of calpain on its substrate is known as "proteolytic processing" and the product (the proteolyzed substrate) is different from its original unproteolyzed form but is not degraded. In contrast, both proteasomes and autophagy degrade the protein substrate. Second, calpain itself is involved in substrate recognition, whereas proteasomes and autophagy rely on their substrates being "tagged" by other systems, such as ubiquitination and selective/non-selective autophagosome formation, respectively. The caspase system recognizes substrates *via* short aa sequences.

The mechanistic basis of calpain activation is becoming clearer thanks to continuous efforts in resolving its 3-D structure. The 3-D structures of the non-Ca^{2+}-bound and Ca^{2+}-bound forms of m-calpain show that Ca^{2+}-dependency is intrinsic to the CysPc domain [23–28]. In addition, the existence of varied domain structures among calpain homologs raises interesting questions, such as how the combination of

Abbreviations: aa, amino acid(s); C2, C2 domain; C2L, C2 domain-like domain; CAPN, calpain; CysPc, calpain-like cysteine protease sequence motif defined in the conserved domain database at the National Center for Biotechnology Information (cd00044); PEF, penta EF-hand; RMSD, root-mean-square deviation

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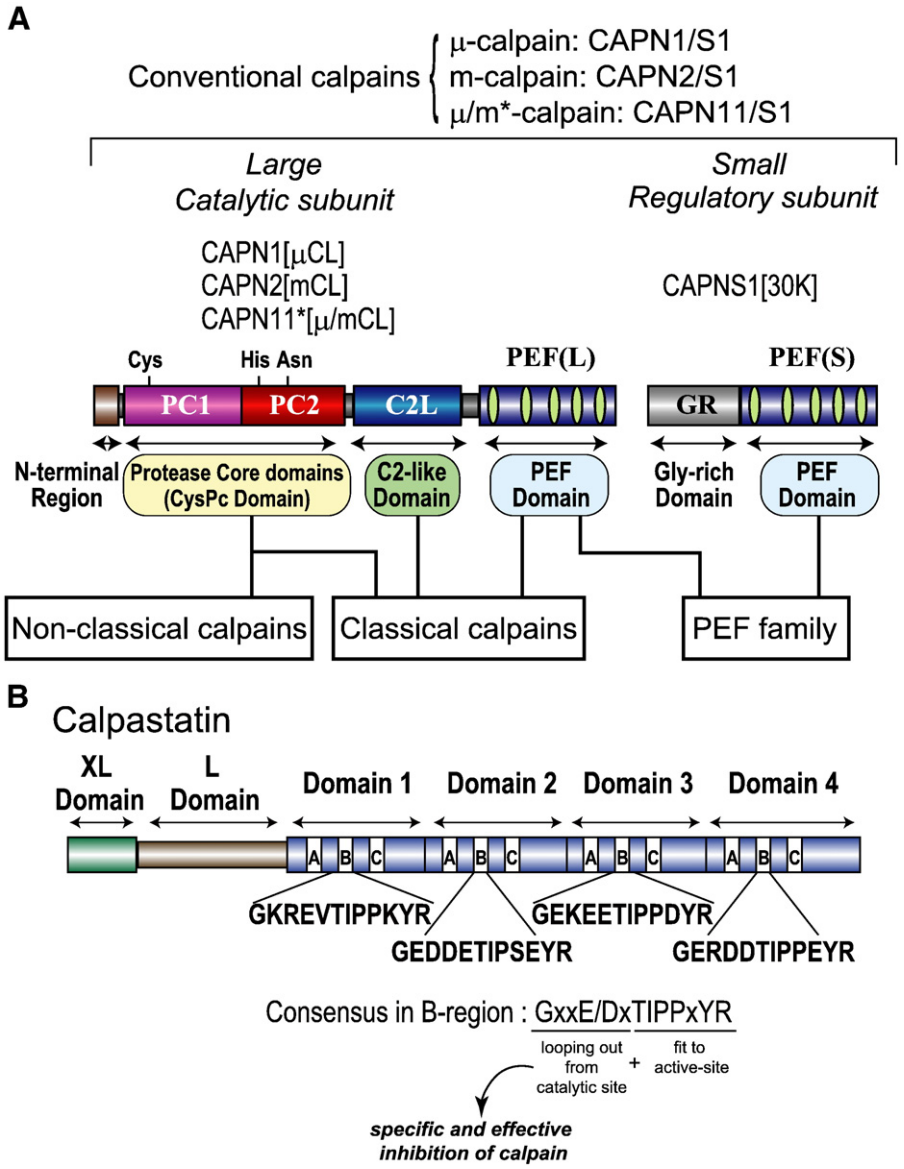

Fig. 1. Schematic structures of calpain superfamily members. A. Conventional calpains are composed of catalytic and small regulatory subunits. Domain structures are defined according to the text. Key structures for sub-classification of calpain related molecules are indicated. N, N-terminal region; PC1/PC2, protease core domains 1/2; C2L, C2-like domain; PEF(L/S), penta EF-hand domain in large(L)/small(S) subunit; GR, Gly-rich domain. *, avian CAPN11 is a catalytic subunit for a chicken conventional calpain designated as μ/m-calpain, *i.e.*, CAPN11/S1. B. Domain structure of the longest isoform of human calpastatin is shown. Four repetitive inhibitory units are labeled as domains 1 to 4; however, they do not have any secondary structures. A-, B- and C-regions in each domain are important for inhibitory activity of calpastatin. The consensus aa sequence in B-region, which interact with the active site of calpain, is shown. Exons encoding XL and L domains are subjected to alternative splicing.

different functional domains modulates the properties of the CysPc domain [4]. Therefore, the last part of this review focuses on the new insights provided by detailed knowledge of calpain structure.

2. What is the calpain system?

2.1. Proposed unified nomenclature for calpains

The general concept underlying calpains was formulated from studies on μ- and m-calpains [29] in mammals and μ/m-calpain in birds [10,30]. These form the foundation of the calpain superfamily, *i.e.,* the conventional calpains. All other calpains are referred to as "unconventional". The structure of calpain is based on that of the conventional calpains. There are, however, several points that prevent the complete systematic classification and citation of calpain-related molecules. Recently, a thorough discussion among experts in the field led to the setting out of agreed principles of definition (Fig. 1A) [5]. What was recommended is to adopt formal nomenclature system for mammalian gene products such as CAPN1, CAPN2, and so forth to distinguish gene products from enzyme entities (="calpain"). Some calpains previously known by independent names are denoted by "the formal name [the old name]" system (*e.g.*, CAPN3[p94] and CAPN8[nCL-2]) in this review. Accordingly, conventional calpain heterodimers are referred to as CAPN1[μCL] or CAPN2[mCL], and CAPNS1[30K] (Table 1, Fig. 1A).

CAPN1[μCL] and CAPN2[mCL] are divided into four domains/regions: the N-terminal anchor helix region, the CysPc protease domain, the C2 domain-like domain (C2L), and the penta-EF-hand domains (PEF(L)) [31]. CAPNS1[30K] contains an N-terminal Gly-rich (GR) domain and a penta-EF-hand domain (PEF(S)). The most important change is that the protease domain (CysPc) now includes two core domains, PC1 and PC2, which faithfully reflect the 3-D structure of the non-Ca^{2+}-bound form of m-calpain (Fig. 1A, see Section 4.1) [23,24]. Names for calpain enzyme entities such as μ-calpain and m-calpain are also proposed to be called

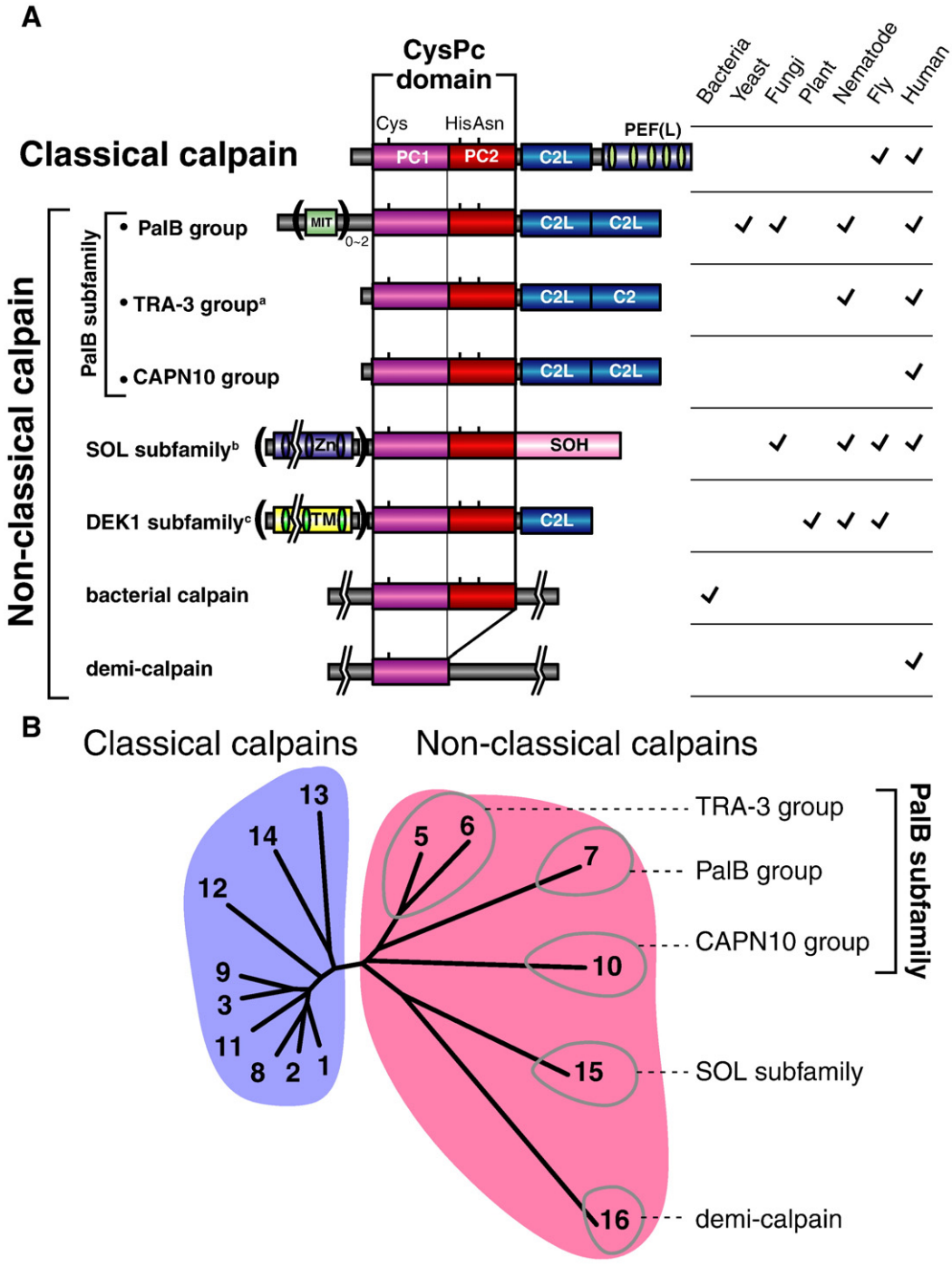

Fig. 2. Structural classification of calpain. A. Calpain homologs have been identified in almost all eukaryotes and in some bacteria. Based on the structural essence, non-classical calpains are further sub-classified. Their distribution in various organisms is also summarized. MIT, microtubule-interacting and trafficking domain, C2, C2-domain; Zn, Zn^{2+}-finger-containing domain; SOH, SOL-homology domain; TM, transmembrane domain. For other symbols, please refer to Fig. 1A legend. [a], in eutherian CAPN6 homologs, the active site cysteine are substituted to other amino acids. [b], the number of Zn^{2+}-finger motifs varies according to the organisms: 0 in some of fungi and nematode homologs, 6 in fly, and 5 in humans. [c], the TM domain is missing in nematode and fly homologs. B. Phylogenetic tree of human calpains. The tree was drawn by the neighbor-joining/bootstrap method using MAFFT v6.240 (http://align.genome.jp/mafft/, strategy: E-INS-i). Each calpain homolog is represented by number, *i.e.*, 1, CAPN1; 2, CAPN2; *etc.* It is noted that classical calpains and non-classical calpains form into two distinct islands, and that non-classical calpains tend to diverge from each other as detected by the length from branching points.

using subunit compositions, *e.g.*, CAPN1/CAPNS1 (CAPN1/S1 for short) for μ-calpain and CAPN2/S1 for m-calpain.

2.2. Overview of calpain homologs

Calpains belong to the papain superfamily of cysteine proteases. To be considered a calpain homolog, it is critical that a protein has aa sequences that are significantly similar to those of the CysPc domains of CAPN1[μCL] and CAPN2[mCL]. According to this criterion, the human genome contains 15 genes that encode calpains (Table 1). A recent survey of the sequence database revealed that calpain genes are present in a wide range of living organisms, including prokaryotes (Fig. 2A) [4]. Interestingly, calpain genes are not so common in lower, primitive organisms. For example, to date, no calpains have been identified in 95% of the known bacterial genomes, including *Escherichia coli* and all archeabacteria. On the other hand, calpain

Table 1
Human genes for calpain and calpain-related molecules.

Gene	Chromosome	Product name[also known as]	Tissue-specificity	Structure[a]	Representative homolog	Splice variant[b]
CAPN1	11q13	CAPN1[μCL]	Ubiquitous	Classical		
CAPN2	1q41-q42	CAPN2[mCL]	None in erythrocytes	Classical		
CAPN3	15q15.1-q21.1	CAPN3[p94]	Skeletal muscle	Classical		+
CAPN5	11q14	CAPN5[hTRA-3]	Ubiquitous	Non-classical	TRA-3 in nematode	
CAPN6	Xq23	CAPN6[CANPX]	Embryonic muscles, placenta	Non-classical	TRA-3 in nematode	
CAPN7	3p24	CAPN7[PalBH]	Ubiquitous	Non-classical	PalB in *Aspergillus*, Rim13/Cpl1 in yeast	+
CAPN8	1q41	CAPN8[nCL-2]	Gastrointestinal tracts	Classical		+
CAPN9	1q42.11-q42.3	CAPN9[nCL-4]	Gastrointestinal tracts	Classical		+
CAPN10	2q37.3	CAPN10	Ubiquitous	Non-classical		+
CAPN11	6p12	CAPN11[c]	Testis	Classical		
CAPN12	19q13.2	CAPN12	Hair follicle	Classical		+
CAPN13	2p22-p21	CAPN13	Ubiquitous	Classical		
CAPN14	2p23.1-p21	CAPN14	Ubiquitous	Classical		
CAPN15 (*SOLH*)	16p13.3	CAPN15[SOLH]	Ubiquitous	Non-classical	SOL in *Drosophila*	
CAPN16 (*C6orf103*)	6q24.3	CAPN16[demi-calpain, C6orf103]	Ubiquitous	Non-classical		
CAPNS1	19q13.1	CAPNS1[30K]	Ubiquitous	N/A		
CAPNS2	16q12.2	CAPNS2	Ubiquitous	N/A		
CAST	5q15	Calpastatin	Ubiquitous	N/A		+

[a] Classical or non-classical indicates that the molecule has or does not have tandem structure of CysPc-C2L-PEF(L), and N/A means not applicable.
[b] + indicates that alternatively spliced variants are generated from the same gene.
[c] Avian CAPN11 is also called μ/mCL.

homologs are found in almost all eukaryotes; however, there are irregular cases such that *Saccharomyces cerevisiae* has a calpain gene, *RIM13*/*CPL1*, while certain other unicellular organisms such as *Encephalitozoon* and *Schizosaccharomycete* do not. Nevertheless, divergent bacterial and yeast calpain sequences suggest that the calpain superfamily represent the oldest branch of the papain superfamily although the origin of calpains during evolution is difficult to determine [11]. From this aspect, comparative analysis of the sequences of calpain homologs from various organisms represents one of dynamic approaches to comprehend this unique protease family [4].

Generally, the calpain homologs are further classified according to two different criteria [4,5,32], which will be summarized in the following Sections, 2.3 and 2.4.

2.3. Structure-oriented classification of calpain superfamily members

The domain structure of the catalytic subunits of the mammalian conventional calpains, CAPN1[μCL] or CAPN2[mCL], is defined as "classical". The classical structure comprises the C2L and PEF domains plus the CysPc domain (Fig. 1A). Accordingly, "non-classical" calpains (some of which are products of alternative splicing from classical calpain genes) exclude C2L and/or PEF domains [33]. Additional domain structures are found within both classical and non-classical calpains. These structures have provided hints with respect to the cellular mechanisms and/or conditions under which specific calpain homologs operate [34–36].

2.3.1. Classical calpains

In humans, nine out of 15 calpain genes encode the classical calpains, CAPN1[μCL], CAPN2[mCL], CAPN3[p94], CAPN8[nCL-2], CAPN9[nCL-4], and CAPN11 to CAPN14 [37]. Most of classical calpains are conserved in vertebrates, among which fish have a duplicate set of these genes [38]. Few invertebrates have genes encoding classical calpains; the blood fluke *Schistosoma mansoni* has four [39], and the fruit fly *Drosophila melanogaster* and the African malaria mosquito *Anopheles Gambiae* each have three [40]. No classical calpain homologs have been found in nematodes [41], trypanosomes [42], plants [43,44], fungi, or yeast [45].

Comparison of the known properties of human classical calpains clearly shows that sequence similarities do not necessarily reflect functional and/or biochemical similarities. For example, of the human classical calpains, only CAPN1[μCL] and CAPN2[mCL] form heterodimers with CAPNS1[30K] *in vivo*. The question is, therefore: what is the prototype calpain? In other words, although studies first identified conventional calpains, they may be quite new in evolutionary terms.

Another interesting point is that orthologous calpain species do not necessarily have similar function. This is exemplified by CAPN11. Avian CAPN11 forms heterodimers with CAPNS1[30K] to form the conventional μ/m-calpain (CAPN11/S1), which is expressed ubiquitously, whereas expression of avian CAPN1 and CAPN2 is markedly low and/or restricted to specific tissues. By contrast, in eutherians (such as humans), CAPN11 is specific to the testis and there is no evidence to suggest its involvement in the formation of CAPN11/S1[μ/m-calpain] [46]. Furthermore, the CAPN11 gene is not present in marsupialia [37]. Another example is the CAPN8[nCL-2] ortholog, xCL-2, in *Xenopus laevis*. Phenotypes caused by the disruption of the *Capn8* gene in mice are specific to gastrointestinal tract [47], but severe developmental defects occur in frogs [48].

2.3.2. Non-classical calpains

Non-classical calpains are further divided into several subfamilies according to structural characteristics (Fig. 2A). In each subfamily, again, structural similarity does not necessarily implicate similar functions. The following subgroups categorize most of eukaryotic non-classical calpains known so far: PalB, SOL, and DEK1 subfamilies (Fig. 2A).

The PalB subfamily is the most evolutionarily conserved. It is found in humans, fungi, yeast, protists, nematodes, and insects except fruit fly, but not in plants [49]. PalB is a calpain homolog first identified in *Emericella nidulans* as the product of the gene responsible for the fungi's adaptive responses to alkaline conditions [50–52]. Studies of PalB homologs in fungi exemplified the power of cutting edge genetic approaches to examine the physiological functions of calpain, which will be described later in this review [53,54]. The general consensus regarding the PalB-type structure is that it contains two tandem C2L/C2 domains at the C-terminus of the CysPc domain. PalB homologs are further divided into three groups by structure (Fig. 2A). In the PalB group and the CAPN10 group, two tandem C2L domains exist and each C2L domain has varying extent of divergence from those in classical calpains. Another variation is the TRA-3 group, which contains one C2L domain and one C2 domain in tandem. TRA-3[CLP-5] was originally identified in nematodes and, later, its vertebrate homolog, CAPN5[hTRA-3], was identified [55,56].

The SOL subfamily is also evolutionarily conserved; orthologs exist in almost all animal species, including humans, insects, nematodes, and green algae. In vertebrates, SOL homologs correspond to CAPN15. SOL homologs are characterized by a specific SOL-homology (SOH) domain at the C-terminus of CysPc, and several Zn^{2+}-finger motifs within the N-terminal domain. For example, *Drosophila* SOL, the product of the gene causing the *small optic lobes* (*sol*) when mutated [57], contains six Zn^{2+}-finger motifs, while human SOL (CAPN15) contains five [58,59]. Thus far, there is no direct evidence implicating Zn^{2+} in the regulation of SOLH; however, it is anticipated that further study of this evolutionarily interesting group will uncover the regulatory mechanisms of CysPc function.

Plant calpains, known as phytocalpains, comprise another interesting branch of the non-classical calpains, the DEK1 subfamily [60–62]. The representative maize DEK1 (defective kernel 1) molecule is involved in aleurone cell development [43]. DEK1 homologs are also found in other plants, including rice plants [63] and *Arabidopsis* [64–66]. These DEK1 homologs commonly contain one C2L domain at the C-terminus and a trans-membrane (TM) domain at the N-terminus. The structure of DEK1 homologs within the animal kingdom, however, is more diverse. For example, *Tetrahymena thermophile* expresses a calpain that has over all similarity to plant DEK1, whereas nematode homologs (CLP-3, -4, -6, and -7) do not contain a TM domain. Although topology of the N-terminal region needs to be determined, the CysPc domain was shown to be located in the cytosol and detached from the cell membrane *via* intramolecular autolytic cleavage [67]. A study using a recombinant DEK1 CysPc domain suggested that DEK1 is, in fact, a Ca^{2+}-dependent cysteine protease [60]. Taken together, these features suggest that the DEK1 may provide a promising line of research into the divergent, but basically conserved, functions of a calpain superfamily.

Because calpain homologs in prokaryotes are rather divergent from one another as well as human calpains, and lack a shared domain structure (apart from the CysPc domain), these calpain homologs are collectively referred to as "bacterial calpain" [4]. There is also an uncharacterized, yet potentially interesting gene product in humans, tentatively named CAPN16 or demi-calpain, due to the fact that it contains only the N-terminal half of CysPc (PC1).

2.4. Classification of calpain family according to expression profile

Of the 15 mammalian calpain genes, six are considered to be tissue-specific according to the expression of their major protein products. The other calpains, including the conventional calpain catalytic subunits, CAPN1[μCL] and CAPN2[mCL], are ubiquitously expressed. It is widely assumed that ubiquitous calpains play a fundamental role in all cells, whereas tissue-specific calpains are involved in more specified cellular functions. In fact, defects in ubiquitous calpains may be lethal, as seen in *Capn2*$^{-/-}$ mice [68], whereas defects in tissue-specific calpains may cause tissue-specific phenotypes such as muscular dystrophy caused by mutations in *CAPN3* [69].

In general, under disease/damaged conditions, such as muscular dystrophy, cardiomyopathy, traumatic ischemia, or lissencephaly, the over-activation of conventional calpains (probably due to compromised intracellular Ca^{2+} homeostasis) has been identified as an exacerbating factor [70]. Thus, the physiological activity of conventional calpains tends to be analyzed with respect to specific inhibition, resulting in the attenuation of pathological symptoms [71]. In contrast, the study of tissue-specific calpains is based upon the assumption that they are purposely expressed within a tissue and are, therefore, involved in the specific functions of that tissue.

The tissue-specific calpains that have been identified to date are: CAPN3[p94] in skeletal muscle [72], CAPN6 in placenta and embryonic striated muscle [73,74], CAPN8[nCL-2] and CAPN9[nCL-4] in the gastrointestinal tract [33], CAPN11 in the testis [46,73], and CAPN12 in hair follicles [75]. As to the structures of the gene promoter regions, which are most likely responsible for the specific expression profiles of each homolog, not much has been clarified. In the case of CAPN3, highly conserved structures containing a TATA-box and several E-boxes have been identified in the immediate upstream regions of human, mouse and rat *CAPN3*/*Capn3* [76], while several other promoter regions that show distinct expression profiles were also identified [77]. The promoter region of *Capn8* contains several GATA boxes, two TATA boxes and some cAMP responsive elements [78]. Interestingly, the human and murine *CAPN8*/*Capn8* gene is located in close proximity to *CAPN2*/*Capn2* on chromosome 1, and their transcripts have overlapping complementary sequences.

2.5. Regulatory components of the calpain system

Besides various calpain homologs, there are two molecules that are essential regulatory components of the calpain system: CAPNS1[30K] and calpastatin. Since the impact of these molecules, either positive or negative, is limited to a few calpains, the question remains as to when these proteins were incorporated into the calpain system during evolution.

2.5.1. Small regulatory subunit of conventional calpains

Like classical calpains, CAPNS1[30K] also contains five EF-hand motifs, referred to as PEF(S) (Fig. 1A). In mammals, the interaction between the fifth EF-hand motif of CAPNS1[30K] and CAPN1[μCL] or CAPN2[mCL] generates heterodimeric conventional calpains [23,24], as expected from the previous biochemical study [79] and the 3-D structure of the CAPNS1[30K] homodimer [80,81]. The N-terminus of CAPNS1[30K] contains hydrophobic Gly-clusters, most of which are autolyzed during the activation of conventional calpains. No particular conformation was identified in this region by 3-D structural analysis and it is tentatively referred to as the Gly-rich (GR) domain.

Biochemical study suggested that CAPNS1[30K] functions as a chaperone-like component for nascent catalytic subunits of the conventional calpains [82]. Accordingly, *Capns1*$^{-/-}$ mice are embryonic lethal, barely surviving beyond E11.5, because both CAPN1[μCL] and CAPN2[mCL] proteins are down-regulated, indicating that CAPNS1[30K] is an absolute requirement for the stability of both conventional calpain catalytic subunits *in vivo* [83,84]. So far, such drastic importance of CAPNS1[30K] has only been observed for CAPN1[μCL] and CAPN2[mCL].

In the human genome, five other genes encode proteins containing a PEF domain besides the classical calpains and CAPNS1[30K] [85]. One of these proteins, CAPNS2[30K-2], is significantly similar to CAPNS1[30K] [86]. At present, the physiological role of this protein is unclear.

2.5.2. Calpastatin — a specific and unique calpain inhibitor

Calpastatin is the only known endogenous specific inhibitor for conventional calpains [87]. Of the calpain homologs so far examined, CAPN8[nCL-2] [88] and CAPN9[nCL-4] [89], but not CAPN3[p94] [90], are also inhibited by calpastatin *in vitro*. Although calpastatin is effectively proteolyzed by CAPN3[p94], the physiological significance of this propensity is not clear.

One calpastatin molecule contains four inhibitor units, each unit inhibiting one calpain molecule with variable efficiency [91–93] (Fig. 1B). Peptide sequences as short as 20 aa derived from these inhibitory units are capable of inhibiting calpain, although at the low efficacy. The sequence of calpastatin is poorly conserved between species and, therefore, the specificity of its effect on calpain is difficult to explain. Recently, the 3-D structure of CAPN2/S1[m-calpain] (co-crystallized with one of the inhibitory units of calpastatin and Ca^{2+}) partly revealed the trick; the structural "softness" of calpastatin, which has been cited as "intrinsically unstructured", enables tight binding to calpain while, at the same time, allowing the adjacent several aa residues to loop out from the active site of calpain, thereby keeps itself from proteolysis [27,28] (Fig. 1B).

3. Calpain function

The importance of proteolytic systems for maintaining cellular function has been increasingly recognized. Calpains, one of the major intracellular proteolytic enzymes, are no exception. The impact of calpain function depends on the nature of the particular protein substrate and the particular conditions investigated [94–100]. In this section, three examples (fungus PalB, mammalian CAPN3[p94], and mammalian CAPN8[nCL-2]/CAPN9[nCL-4]) are discussed (Fig. 3).

3.1. PalB as a key molecule in an environmental adaptation of fungi

E. nidulans is a microorganism that grows over a wide pH range. Therefore, adaptive mechanisms to environmental changes are critical for the life of this unicellular eukaryote. The *pal* (phosphatase mutants: loss in activity at alkaline pH but normal or increased activity at acidic pH) mutants show gene expression profiles suited to acid environments, even under normal/neutral conditions; thus, they are unable to grow under alkaline conditions [50]. PalB, together with other *pal* gene products, is involved in the proteolytic activation of PacC (Fig. 3A). PacC is a key transcription factor that regulates pH-dependent changes in gene expression. The full-length PacC protein has an inactive conformation and is activated by a two-step proteolytic conversion process [101,102]. The first step is pH-dependent and is catalyzed by PalB, while the second is thought to be mediated by the proteasome in a pH-independent, constitutive manner. This pH adaptation system is called the Pal-PacC pathway, in which PalB functions as an important rate-limiting factor, *e.g.*, a modulator protease.

The physiological importance of the Pal–PacC pathway is suggested by the fact that it is well conserved in yeasts, and some components in the pathway have mammalian homologs that are involved in signaling cascades. The PalB orthologs are Rim13[Cpl1] in

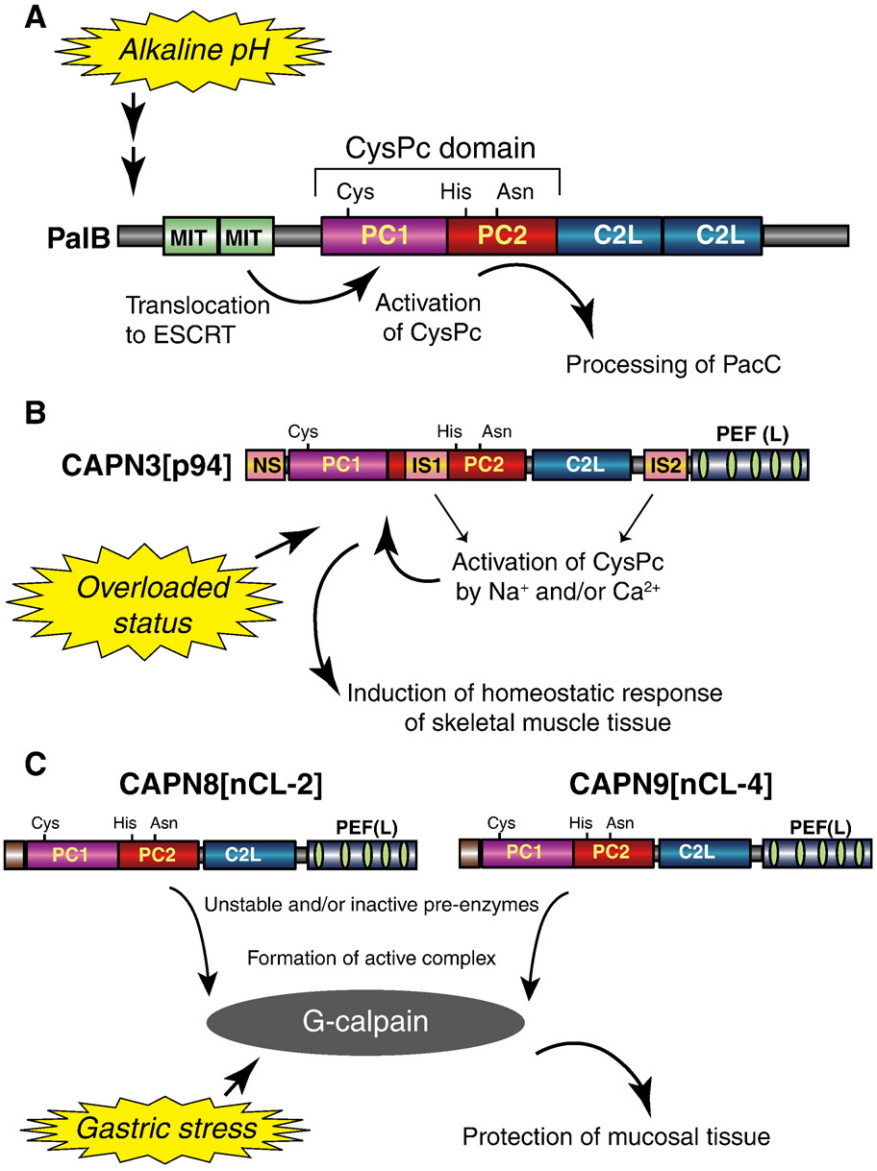

Fig. 3. Examples of calpain's function *in vivo*. A. PalB is involved in the proteolytic activation of PacC, a key transcription factor for the survival of *E. nidulans* under alkaline environment. PalB is translocated to ESCRT-III complex *via* the interaction through its N-terminal tandem domains so that it is activated to process PacC. B. Skeletal muscle-specific CAPN3[p94] is a product of the gene responsible for LGMD2A. The lack of protease activity of CAPN3 causes defective homeostatic response of skeletal muscle when the excessive amount of loads is imposed on the tissue. It is anticipated that characteristics of CAPN3, such as Na^+-dependent activation is critical for its function as a component of homeostatic response of skeletal muscle. C. CAPN8[nCL-2] and CAPN9[nCL-4] form a protease complex designated as CAPN8/9[G-calpain] (G, gastric). When gastric tissue experiences stress, such as an exposure to high dose of ethanol, multiple signaling cascades are induced. One of these cascades is dependent on CAPN8/9[G-calpain] protease activity and necessary for the protection of mucosal tissue.

the yeast and CAPN7[PalBH] in humans. In *Sacchaomycetes*, the Rim (Regulators of *Ime*2) proteins compose a pH adaptive system as well as those for meiosis and sporulation, in which the proteolytic processing of Rim101, a counterpart of PacC, by Rim13[Cpl1] is the critical step [54,103,104]. Unlike PacC, the involvement of proteasome in the activation of Rim101 has not been reported. As mentioned, *Schizosaccharomycetes* have no PalB ortholog, suggesting that molecular components for the signaling pathway regulating tolerance to particular environmental changes have been modified throughout evolution.

Another remarkable feature of these signaling pathways is that they involve membrane trafficking components known as ESCRT (endosomal sorting complex required for transport) and Vps (vacuolar protein sorting) proteins [105,106]. Genetic analyses have identified the functional and hierarchical relationships between the Rim and Vps proteins, along with the relevance of the ESCRT-III subcomplex as a scaffold for Rim13[Cpl1] function [54,107,108]. Some of the physical interactions between ESCRT and Vps proteins are conserved in yeast and mammals [109–112]. The interaction between CAPN7[PalBH] and CHMPs (charged multivesicular body protein) is another example of these conserved interactions. Thus, it is expected that systemic analogy exists between fungi and eukaryotes, even though the situations that necessitate the activation of these systems differ. Further study of this phenomenon would greatly contribute to our knowledge of the physiological functions of CAPN7[PalBH] in mammals.

3.2. CAPN3[p94] and homeostasis in skeletal muscle

The first tissue-specific calpain, CAPN3[p94], was identified in 1989 [72]. CAPN3[p94] is expressed predominantly in skeletal muscle. Structurally, it is categorized as a classical calpain and is approximately 50% identical to CAPN1[μCL] and CAPN2[mCL]. The important characteristic of CAPN3[p94], however, is that it contains three additional insertion regions (known as NS, IS1, and IS2), located, respectively, at the N-terminus, within PC2 of CysPc, and between the C2L and PEF(L) domains. These regions give CAPN3[p94] some unique features.

In 1995, a study involving positional cloning analysis of families from La Reunion Island reported that *CAPN3* mutations were responsible for limb-girdle muscular dystrophy type 2A (LGMD2A), also called calpainopathy, [69]. *Capn3*$^{-/-}$ mice show a human calpainopathy-like phenotype, although milder, indicating that calpainopathy is indeed caused by *CAPN3* defects [113,114]. LGMD2A and *CAPN3* mutations are the only examples of a clear cause–effect relationship between human disease and calpain gene mutations identified so far.

Attempts to identify the specific CAPN3[p94] defects that cause LGMD2A were undertaken using CAPN3[p94] knock-in (*Capn3*$^{CS/CS}$) mice, which express a structurally intact but protease-inactive CAPN3 [p94]:C129S mutant [36,115]. These studies suggested that A) "calpainopathy" is primarily caused by compromised CAPN3[p94] protease activity; B) *Capn3*$^{CS/CS}$ mice show a less severe phenotype than *Capn3*$^{-/-}$ mice; and C) *Capn3*$^{-/-}$ mice, but not *Capn3*$^{CS/CS}$ mice, show reduced Ca^{2+} release from the sarcoplasmic reticulum. Therefore, at least two activities of CAPN3[p94] (proteolytic and non-proteolytic) are involved in its physiological function.

Skeletal muscle comprises the largest tissue mass in the human body and is both a device for motility and a source of metabolic activity and nutrition. Multiple mechanisms protect skeletal muscle from damage and stress, which are almost inevitable in daily life. Obviously, removal of damaged proteins from sarcomere is one important strategy for keeping the quality of myofibrils. There have been discussions about what kind of proteolytic system(s) is (are) suitable for such missions [116,117]. Some of unique properties of CAPN3, such as apparently Ca^{2+}-independent activation [118,119] and interaction with connectin/titin, a gigantic sarcomere protein [120,121], together with the phenotypes of *Capn3*$^{-/-}$ mice [113,114], highlight the possibility that CAPN3 is involved in a homeostatic mechanism of skeletal muscle [114,122] (Fig. 3B). As to how CAPN3 protects skeletal muscle from damaging stimuli, other scenarios are also possible. Analysis of exercise-induced muscle degeneration suggests that another homeostatic mechanism in *Capn3*$^{CS/CS}$ skeletal muscle, namely the adaptive upregulation of muscle ankyrin-repeat protein-2 and heat shock proteins, is compromised [115]. Together, it is suggested that mutations in *CAPN3* disrupt its activity to control multiple aspects of the homeostatic mechanisms within skeletal muscle, resulting in LGMD2A.

The targets of CAPN3[p94] protease activity are of major interest in the field of calpain research. Along with straightforward approach to identify its substrate proteins, the behavior of CAPN3[p94] under various conditions is indicative of its function. In this respect, the correlation between the distribution kinetics of CAPN3[p94] within the sarcomeres and its protease activity may warrant further investigation, as this phenomenon indicates that the protease activity of CAPN3[p94] affects its sensitivity and/or reactivity to conditions within the sarcomere [36,123].

3.3. CAPN8[nCL-2] and CAPN9[nCL-4] complexes as a defense against gastric stimulation

Both CAPN8[nCL-2] and CAPN9[nCL-4] are predominantly expressed in the surface mucus cells (pit cells) of the stomach [124]. Smaller amounts are expressed in goblet cells in the intestines. Based on primary sequence data, both of these calpain homologs are classical calpains but they are located at slightly different positions with respect to the other calpains. Of the mammalian calpains, CAPN8 [nCL-2] is the most similar to CAPN2[mCL] (62% identical), while CAPN9[nCL-4] is equally similar to all other classical calpains. Therefore, these two calpains appear not to be the closest relatives from an evolutionary perspective.

Previous studies of CAPN8[nCL-2] and CAPN9[nCL-4] placed an emphasis on characterizing their similarities and/or differences. These approaches revealed that both calpain homologs are Ca^{2+}-dependent and sensitive to known calpain inhibitors, and that CAPN9[nCL-4], but not CAPN8[nCL-2], requires CAPNS1[30K] for its activity *in vitro* [88].

However, a recent study using *Capn8*$^{-/-}$ and *Capn9*$^{-/-}$ mice showed that neither CAPN8[nCL-2] nor CAPN9[nCL-4] forms a stable complex with CAPNS1[30K] *in vivo* [47]. Rather surprisingly, they form a hybrid complex *in vivo*, termed as gastric calpain (G-calpain, CAPN8/9). The disruption of either *Capn8* or *Capn9* causes the down-regulation of the other molecule, suggesting that each species contributes to the stability of the other. Although trace amounts of CAPN8[nCL-2] or CAPN9[nCL-4] can be detected in the absence of the other, they are not proteolytically active.

Both *Capn8*$^{-/-}$ and *Capn9*$^{-/-}$ mice, which are essentially double knockout mice, show no symptoms under normal conditions but are significantly more susceptible to ethanol-induced gastric ulcers. This phenotype is unchanged in CAPN8[nCL-2] knock-in (*Capn8*$^{CS/CS}$) mice, which express a mutant CAPN8/9[G-calpain] complex that only retains the protease activity of CAPN9[nCL-4]. Therefore, CAPN8/9 [G-calpain], with dual protease activity, is an essential component of stomach tissue that resists gastric stimulation. This is a novel and unique form of active calpain complex. Considering that pit cells specifically function as a disposable barrier, it is assumed that the CAPN8/9[G-calpain] complex plays a role in the sensitivity and/or reactivity of pit cells to imposed stress [47] (Fig. 3C).

Taking advantage of recent improvements in a single nucleotide polymorphism (SNP) database, human *CAPN8* and *CAPN9* were found to contain several aa-substituting SNPs. An *in vitro* expression study showed that the introduction of some of these SNPs compromised the proteolytic activity of CAPN8/9[G-calpain] complex. Apparently, none

of these SNPs is fatal. The important point, however, is whether individuals with particular SNPs develop pathological symptoms such as gastric ulcers and gastritis. It should be mentioned that, in terms of a general health issue, this approach is the first example showing that the investigation of calpain function in transgenic animals can be extended to human disease.

4. How does calpain work?

4.1. Insights into the structural identity of CysPc

The first evidence of calpain structure–function relationships was provided by the primary sequence of chicken CAPN11 [30]. This structure has both a cysteine protease domain and a Ca^{2+}-binding PEF domain, consistent with the preceding biochemical studies on its Ca^{2+}-dependent protease activity. Later, when the Ca^{2+}-free 3-D structure of CAPN2/S1[m-calpain], *i.e.*, a heterodimer of CAPN2[mCL] and CAPNS1[30K], was solved, it was found that the protease domain is split into two halves, and that the active-site residues and potential substrate binding cleft have non-functional conformations [23,24]. Therefore, the calpain protease (CysPc) domain was characterized as two core domain structures, PC1 and PC2 [5], within one protease domain.

Subsequently, structural studies of calpain were directed at deciphering the mechanism underlying Ca^{2+}-binding, which transforms calpain into its active conformation. One of the difficulties in deducing the mechanisms involved in calpain activation by Ca^{2+} was that it did not appear theoretically feasible that Ca^{2+}-binding to the PEF domains would cause conformational changes sufficient to fuse the two core domains into a functional protease domain unless novel Ca^{2+} binding sites were assumed. In fact, the CysPc domain itself has Ca^{2+}-dependent protease activity, although its specific activity is very low [125,126].

So far, 3-D structures of CysPc domains from CAPN1[μCL], CAPN2[mCL], CAPN8[nCL-2], and CAPN9[nCL-4] in the presence and/or absence of Ca^{2+} have been solved, revealing the following: A) These structures are highly similar to one another with a few differences [25,26,127] (Table 2 and Fig. 4A). B) Two Ca^{2+}-binding sites (CBS-1 and -2 in PC1 and PC2, respectively) are present within the CysPc domain [25]. C) In the presence of Ca^{2+}, PC1 and PC2 move closer to each other although the secondary structures are essentially unchanged (except CAPN9[nCL-4]). PC2s of active CAPN1[μCL], CAPN2[mCL], and CAPN8[nCL-2] are rotated and translated by ca. 50° and 6 Å, respectively, when the alignment of PC1s of them and inactive CAPN2[mCL] is fixed [128] (Fig. 4A). D) The structure of the CBS-1 and -2 is unique and there is, so far, only one example showing similarity to CBS-1 (known as the "Ca^{2+}-bowl") although there is no sequence similarity between them. Ca^{2+}-bowl is found in the high-conductance voltage- and Ca^{2+}-activated K^+ (BK or SLO1) channel [129]. E) The active-site cleft of the calpain CysPc domain is rather deep and is narrower than that in papain-like cysteine proteases [130]. Therefore, it is thought that the substrate must be in an extended conformation to fit the cleft which, in turn, explains calpain's preference for digesting inter-domain unstructured regions.

The 3-D structure of the complete CAPN2/S1[m-calpain] molecule co-crystallization with calpastatin and Ca^{2+} finally confirmed that Ca^{2+}-binding to CBS-1 and CBS-2 causes dynamic changes in the relative conformation of PC1 and PC2 [27,28]. Consistent with previous observations made using alternative approaches such as CD spectral analysis and small-angle X-ray scattering analysis, Ca^{2+}-binding to calpain does not change its secondary structure but rather rearranges the domains [131,132]. For example, Ca^{2+}-binding to the PEF domains barely changes the secondary structure but induces displacement of the N-terminal region from the protease core region.

Are the characteristics of the different calpain species explained based on their structure, *i.e.*, .by the comparison of the 3-D structure of their CysPc domains? The answer is "yes" and "no". For example, in the protease domain of CAPN2[mCL] and CAPN8[nCL-2], a Trp residue is projected like a barrier to the active site [26]. This is referred to as a reversible intrinsic inhibitory mechanism and was thought to discriminate CAPN2[mCL] from CAPN1[μCL]. However, the complete 3D structure of CAPN2/S1[m-calpain] does not infer such a mechanism. Therefore, analyzing the domain structures in isolation gives a convenient snapshot but does not necessarily reflect the full nature of the complete molecule, at least in a resting state. In other words, inter-domain, or even inter-molecular, interactions should be taken into account as factors modifying the essentially conserved nature of the CysPc domains. This notion could be extended to deal with the activation and latency mechanisms *in vivo* that must be specialized for each molecule [127].

4.2. Variable parameters involved in calpain activation

Of the mammalian calpains, CAPN1[μCL] and CAPN2[mCL] heterodimerize with CAPNS1[30K] to form functional proteases and the activation mechanisms involved have been identified (see above). However, the functional entities within the other calpain species remain unknown. For example, the mechanism how CAPN8[nCL-2] and CAPN9[nCL-4] cooperate with each other to function as CAPN8/9 [G-calpain] requires further investigation. It is expected that other, less characterized calpains' activation may be subject to redefinition as research progresses. As one of clues to the activation mechanism, unique structures identified in certain calpain species have proved useful. Knowledge about such species-specific activation mechanisms would promote the investigation of physiological functions, and *vice versa*.

Table 2
Similarities of 3D structures of mammalian calpain protease domains. Pairwise alignment of each pair of CysPc domains of calpains indicated was performed using Dali server (http://ekhidna.biocenter.helsinki.fi/dali_lite/) [151]. Upper right and lower left values indicate *Z*-scores (the number of standard deviations; the larger *Z* value means the more similar; $Z = 2$, 5, and 8 correspond to $P = 0.023$, 2.9×10^{-7}, and $<10^{-15}$, respectively) and RMSD (root-mean-square deviation, Å), respectively (three values, X (Y/Z), correspond to when CysPc, PC1, or PC2 domains are compared with each other, respectively). All structures except 1KFU are Ca^{2+}-bound active structures. Note that all active structures except CAPN9 are aligned with RMSD not more than 1.2 Å, and each of PC1 and PC2 of CAPN9 is also well aligned (RMSD ≤ 1.7 Å) although whole CysPc of CAPN9 is not (RMSD > 6 Å) (see also Fig. 4A). Species: 1MDW, rat; all others, human.

CAPN (PDB No.)	CAPN1 (2ARY)	CAPN2 (1MDW)	CAPN8 (2NQA)	CAPN9 (1ZIV)	CAPN2–Ca (1KFU)
CAPN1 (2ARY)		47.2 (31.5/24.8)	46.8 (32.1/25.1)	29.3 (25.6/23.8)	27.8 (25.0/16.4)
CAPN2 (1MDW)	1.0 (1.2/0.7)		46.6 (32.3/25.1)	30.8 (27.3/24.3)	30.8 (25.4/16.5)
CAPN8 (2NQA)	1.0 (1.0/0.6)	1.1 (1.1/0.7)		29.8 (26.8/23.5)	28.1 (25.5/16.3)
CAPN9 (1ZIV)	6.6 (1.7/1.1)	6.6 (1.2/0.9)	6.2 (1.5/1.0)		25.1 (24.2/15.9)
CAPN2–Ca (1KFU)	3.7 (2.1/2.4)	3.8 (1.6/2.3)	3.9 (1.9/2.4)	–* (1.6/2.5)	

[a]Dali failed to compare whole CysPc domains of 1KFU and 1ZIV due to too divergent structures.

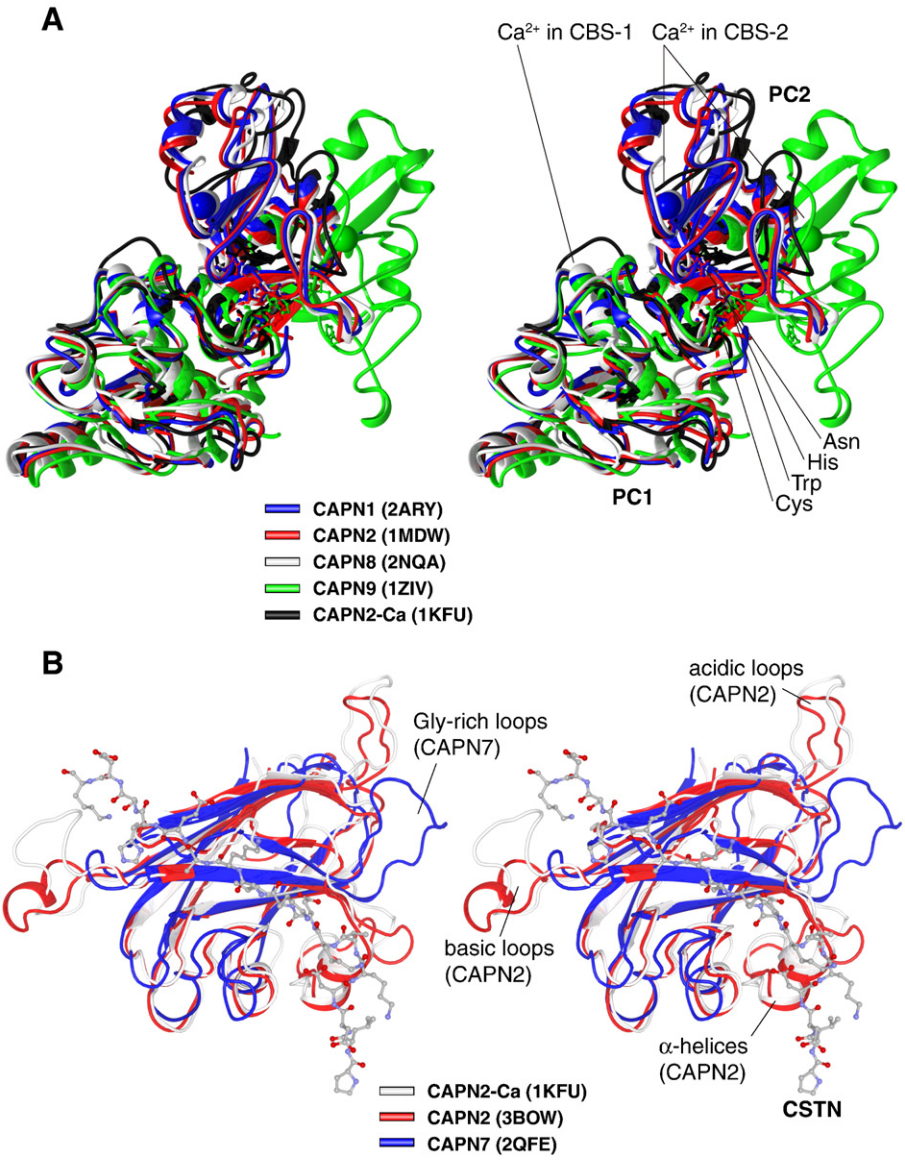

Fig. 4. Comparison of the 3-D structures of the CysPc and C2L domains of mammalian calpains. A. The CysPc domains of active Ca^{2+}-bound CAPN1[μCL] (PDB accession No: 2ARY, blue), CAPN2[mCL] (1MDW, red), CAPN8[nCL-2] (2NQA, white), and CAPN9[nCL-4] (1ZIV, green), and inactive non-Ca^{2+}-bound CAPN2[mCL] (1KFU, black) are aligned in their PC1 domains using MolFeat Ver.4.6 (FiatLux Inc.), and represented as a cross-eye stereo ribbon scheme. Cys, Trp, His, and Asn indicate C115/105/105/97 (for CAPN1[μCL], CAPN2[mCL], CAPN8[nCL-2], and CAPN9[nCL-4], respectively), W116/106/106/98, H272/262/262/254, and N296/286/286/278, respectively, which are active site triad residues and the Trp residue "interfering" them in CAPN2[mCL] and CAPN8[nCL-2]. Whereas active CAPN1[μCL], CAPN2[mCL], and CAPN8[nCL-2] are almost completely aligned, inactive CAPN2[mCL] is moderately aligned, and active CAPN9[nCL-4] is misaligned (see also Table 2). B. Schematic cross-eye stereo ribbon structures of the C2L domains of the active (3BOW, red, a part of co-crystalized calpastatin structure is also shown as ball-stick scheme) and inactive (1KFU, white) forms of CAPN2[mCL] and the distal part of CAPN7[PalBH] (2QFE, blue), superimposed using Dali pairwise comparison (http://ekhidna.biocenter.helsinki.fi/dali_lite/). Both molecules showed fairly good superimposition (RMSD = 2.0 Å), but several loops showed significant differences.

For calpain species whose substrates are not well determined, autolytic activity serves as one indicator of activity. For example, CAPN7[PalBH] expressed in cultured cells as an EGFP-fusion protein shows autolytic activity [35]. Using this fusion construct, it was shown that IST1, a protein that interacts with CAPN7[PalBH], enhances its autolytic activity. This interaction is mediated by the MIT domains within CAPN7[PalBH] and the MIM domains in IST1. This type of interaction is analogous to that observed in proteins involved in the ESCRT system in yeast. IST1 is, in fact, related to ESCRT-III complex proteins. Thus, it is suggested that the MIT domains within CAPN7 [PalBH] form a complex with IST1 to enhance the conversion of CysPc to its active conformation. In *E. nidulans* and *S. cerevisiae*, activation of PalB involves its translocation to the ESCRT-III complex [54,106]. In this respect, the effect of IST1 on the localization and activation of CAPN7[PalBH] is worthy of further investigation, although the physiological target for activated CAPN7[PalBH] remains unknown.

Another example of a unique modification within CysPc is CAPN3 [p94]. CAPN3[p94] autodegrades very quickly, an activity that depends on the specific insertion sequences, IS1 and IS2, located within PC2 and the junction between the C2L and PEF domains, respectively [118]. Recently, it was shown that this autodegradation is Na^+-dependent in the absence of Ca^{2+}, establishing CAPN3[p94] as the first example of an intracellular Na^+-dependent enzyme [119]. Mutation analyses suggest that IS1 and IS2 are required for this Na^+-dependency, and that acidic amino acids located within CBS-1 mediate Na^+-binding to the CysPc of CAPN3[p94]. Some alternative splice variants produced from *CAPN3*/*Capn3* lack IS1 and/or IS2, and show partially attenuated autolytic activity [133]. Taken together, these observations suggest

that CAPN3[p94] has modifying factors for the CysPc domain embedded within its own structure, and these factors are regulated by alternative splicing.

4.3. Hunt for the moment of activation

A compelling question is, even after the active structure of CAPN2/S1[m-calpain] was revealed, how such functional entity is derived *in vivo*. One of the classical unsolved frustrations in the field of calpain is that *in vitro* activation of calpain requires at least μM order of Ca^{2+}, which is almost unavailable *in vivo*. To explain this, the vicinity of plasma membrane is proposed as a favorable spot for the activation of calpain, and *in vitro* experiment has shown that phospholipids, a major component of plasma membrane, lowered Ca^{2+}-concentration required for activating calpain [134–136]. It is also proposed that a limited portion of calpain whose localization coincides with the spot where Ca^{2+}-concentration substantially increases is sufficient for fulfill its function.

To ultimately solve the question, it is essential to detect the moment of calpain activation *in situ*/*in vivo*. For this, several attempts have been successful using specific antibodies for the aa sequence generated de novo in substrate protein and calpain itself [137,138]. Taking an advantage of the 3-D structural knowledge on calpain activation, it would be very useful to develop reagents that label calpain molecules exclusively in active conformation, such as specific antibodies, low-molecular compounds, or substrate analogs.

There are several calpain homologs whose activities have yet to be identified, although their biological/physiological importance has already been demonstrated. For example, CAPN10 is related to a risk factor for non-insulin-dependent (type 2) diabetes [139] and nematode TRA-3[CLP-5], whose human homolog is CAPN5[hTRA-3], is involved in sex determination [55,140]. On the other hand, eutherian CAPN6 is a peculiar case in that it contains natural aa substitutions in the Cys residue of the active site triad (to Lys in humans) [74], and has been shown to be related with microtubule dynamics [141,142]. More intriguingly, CAPN6 in opossum (*Monodelphis domestic*) and chickens contains a complete set of active site residues (Cys–His–Asn), and frogs and fish have three functional TRA-3 homologs. Such diverse molecular nature among calpain homologs does not warrant uniform approach towards their activation mechanism, which might indicate the necessity to throw off a prejudice that calpain is a protease.

4.4. Trials to elucidate the substrate specificity of calpain

It is thought that the conventional calpains, CAPN1/S1[μ-calpain] and CAPN2/S1[m-calpain], have almost indistinguishable substrate specificity. The constraints imposed by the cleft size in the CysPc domain highly conserved among all calpains, they all may prefer to digest inter-domain unstructured regions like the conventional calpains do. On the other hand, novel substrates identified for other calpains are not necessarily good substrates for conventional calpains. For example, calpastatin is a good substrate, not an inhibitor, for CAPN3[p94] [90]. Likewise, β-COP, a subunit of the coatomer complex, is sensitively proteolyzed by CAPN8[nCL-2] but probably less sensitive to conventional calpains [124]. Such distinctions suggest the importance of interfaces between calpain and substrate other than the active-site cleft. Potentially stretchable sequences linking the domain structures within the substrate might not fit into the cleft if the protein does not form a stable complex with calpain [130].

For binding/recognizing substrates, the C2L domain is also important. Active CAPN2/S1[m-calpain] co-crystalized with calpastatin fragment showed that more than 15 aar of calpastatin are in contact with the C2L domain [27,28]. Comparison of the 3D structures of C2L domains of CAPN2[mCL] and CAPN7[PalBH] (the distal C2L) revealed their highly conserved β-sandwich structure (Fig. 4B), regardless of somewhat low aa identity between them (ca. 21%). There are, however, three loops that are distinct between them: the acidic loop (between β2/β3) and the basic loop (β3/β4) in CAPN2[mCL] do not exist in CAPN7[PalBH], whereas the Gly-rich loop (β4/β5) in the latter corresponds to the α-helix structure in the former that points in a different direction, contacting with calpastatin (Fig. 4B). These structural differences may reflect distinct substrate recognition between these two calpains.

Several independent approaches have sought to deduce the rules governing the substrate specificity of calpain [143–145]. One advantage of using inductive methodologies is that such knowledge is useful for the design of specific calpain substrates or probes that can be used to detect active calpains. In parallel with these studies, the aim is to construct a prediction tool by processing the experimental result for machine learning [146]. A draft version can be found at http://calpain.org/ [147]. Such systematic characterization of calpain-mediated proteolysis enables the use of thought-experiments. This approach has also been used to identify the particular characteristics of the caspase system [148–150]. Combining this approach with "ordinary" experiments should prove effective in identifying novel calpain functions.

5. Conclusions and perspectives

This review summarizes both classical and state of the art research in the field of calpain biology. The important findings from biochemical studies now need to be verified *in vivo*. For this purpose, recent technical advances should be incorporated and optimized to reflect the diverse and complex nature of the calpain family. There are still so many exciting aspects of this enigmatic family of proteases that await discovery. The fact that many difficult questions still exist within the field of calpain research should ignite a passion to find the answers. This is the way that calpain research has been, and always will be, carried out.

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