—Review—

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Expanding Members and Roles of the Calpain Superfamily and Their Genetically Modified Animals

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Abstract: Calpains are intracellular Ca^{2+} -dependent cysteine proteases (Clan CA, family C02, EC 3.4.22.17) found in almost all eukaryotes and some bacteria. Calpains display limited proteolytic activity at neutral pH, proteolysing substrates to transform and modulate their structures and activities, and are therefore called “modulator proteases”. The human genome has 15 genes that encode a calpain-like protease domain, generating diverse calpain homologues that possess combinations of several functional domains such as Ca^{2+} -binding domains and Zn-finger domains. The importance of the physiological roles of calpains is reflected in the fact that particular defects in calpain functionality cause a variety of deficiencies in many different organisms, including lethality, muscular dystrophies, lissencephaly, and tumorigenesis. In this review, the unique characteristics of this distinctive protease superfamily are introduced in terms of genetically modified animals, some of which are animal models of calpain deficiency diseases.

Key words: calcium, calpain, gastrointestinal tracts, muscular dystrophy, proteolysis

Introduction

Calpains constitute a unique group of intracellular cysteine proteases found in almost all eukaryotes and a few bacteria (for other reviews see [10, 14, 16, 25, 27, 31, 46, 51, 76, 83, 92, 122, 127, 138, 145, 148, 158, 175, 176, 181]). Calpains are defined as having amino acid sequences significantly similar to that of the protease domain of human μ -calpain (also called calpain-I or μ CANP), the major Ca^{2+} -dependent protease. Apart from the protease domain, calpains contain a wide variety of domains including C2 and C2-like domains, Zn-finger domains and EF-hand containing domains and thus form a superfamily (Clan CA, family C02, EC

3.4.22.17).

Among the best-studied calpains are mammalian μ -calpain and m-calpain (also called calpain-II or mCANP), which are called the “conventional” calpains. The conventional calpains are mainly localized in the cytosol, show ubiquitous expression, and exhibit Ca^{2+} -dependent proteolytic activity at neutral pH. The mode of their action is processing, rather than degrading: calpain proteolyses substrates at one or a limited number of sites to transform and modulate their structures and activities. Therefore, calpain is considered a representative intracellular modulator protease that governs various cellular functions such as signal transduction and cell morphogenesis.

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Calpains belong to the papain superfamily, which is composed of three distinct kingdoms, i.e., the calpain, papain, and bleomycin-hydrolase families [13]. The human genome has 15 genes that encode a calpain-like protease domain (see Fig. 1 and Table 1). These generate diverse types of calpain homologues having combinations of several functional domains such as Ca^{2+} -binding domains [C2 and C2-like domains, and penta-EF-hand (PEF) domains] and transmembrane domains. Furthermore, the recent exponential progress in genome projects has revealed the structures of calpain homologues in various living organisms including insects, nematodes, trypanosomes, plants, fungi, yeasts, and some bacteria (Fig. 1). The importance of the physiological roles of calpains is reflected by the fact that particular defects in calpain functionality have effects in many different organisms, including lethality (e.g., disruption of mouse *Capn2*, which encodes the catalytic subunit of m-calpain) or a variety of deficiencies (e.g., mutations in human *CAPN3*, which encodes the muscle-specific calpain) including muscular dystrophies [133], lissencephaly [177], and tumorigenesis [79] in humans, embryonic lethality in mice [4, 34, 183], impaired neurogenesis in flies [30], lack of definitive sex determination in nematodes [9], defects in aleurone cell development in maize [90], and alkaline/osmotic stress susceptibility in fungi [32] and yeasts [44, 58]. The following sections discuss several disease-related effects of calpains in terms of their animal models, and the divergent physiological roles of calpains are described mainly from the viewpoint of structure-function relationships.

The History and Nomenclature of Calpain

An enzyme corresponding to calpain was first described in 1964 by Guroff in the Journal of Biological Chemistry [49]. After several “re-identifications” [18, 65, 75, 101, 130, 161], calpain, which was called CANP (calcium-activated neutral protease) at that time, was purified to homogeneity by Ishiura *et al.* in 1978 [68]. In 1980, the endogenous specific inhibitor protein named calpastatin was purified by Murachi *et al.* [109]. After intensive biochemical analyses of calpain enzymes, the cDNA for calpain was finally cloned by Ohno *et al.* in

1984 [116]. The cDNA sequence for the catalytic subunit of calpain revealed that calpain is a chimeric molecule consisting of a cysteine protease and a calmodulin-like Ca^{2+} -binding module. In the two decades since this event, hundreds of calpain-related molecules, including calpastatin, have been identified, mainly through the use of cDNA cloning and genome/Expressed-Sequence-Tag (EST) projects (Figs. 1 and 2).

Two major ubiquitous calpains are found in mammals. They are called calpain-I and calpain-II, or μ CANP and mCANP, and the major difference between them is the concentrations required for proteolytic activities, i.e., μM and mM levels of Ca^{2+} *in vitro*, respectively. The dual nomenclature calpains were unified as a hybrid, μ -calpain and m-calpain, in 1991 [157]. As most studies of calpain have actually been studies of μ - and/or m-calpains, these are referred to as the “conventional” calpains. They are both heterodimers and consist of a common small calpain regulatory subunit (CAPNS1, also called “30K”; ca. 28 kDa) and large distinct μ - and m-calpain catalytic subunits (μCL and mCL , respectively; ca. 80 kDa), which have approximately 60% amino acid identity. Today, 15 human calpain genes have been numbered, *CAPN1–3* and *5–16*, as shown in Table 1 and Fig. 1. μCL and mCL correspond to the gene products of *CAPN1* and *CAPN2*, and are now called calpain-1 and calpain-2, respectively. Accordingly, μ -calpain is a heterodimer of CAPNS1 and calpain-1. In this review, to avoid confusion between new and old literature, the original nomenclature is noted after the current names: e.g., calpain-1/ μCL , calpain-2/ mCL , calpain-3/p94, calpain-5/hTRA-3, CAPNS1/30K, etc.

Structure and Function of Conventional Calpains

A number of substrates have been reported for calpains, but few of them have been shown to be *in vivo* substrates, i.e., proteolysis of the protein has physiological significance. For example, protein kinases, phosphatases, phospholipases, cytoskeletal proteins, membrane proteins, cytokines, transcription factors, lens proteins, and calmodulin-binding proteins are just some of the proteins suggested to be *in vivo* substrates. Although casein is not an *in vivo* substrate of calpain, it is

Fig. 1. Schematic structures of calpain superfamily members. Calpain homologues have been identified in almost all eukaryotes, and some bacteria (see also Table 2). Symbols used are: I: the N-terminal regulatory domain; IIa and IIb: the protease subdomains containing the active sites Cys, and His + Asn, respectively; III: the C2-like Ca^{2+} -binding domain; IV and VI: five EF-hand containing Ca^{2+} -binding PEF domain; V: glycine-rich hydrophobic domain; NS, IS1, and IS2: calpain-3-specific sequences; C2: C2 domain found in TRA-3 homologues (also called domain T); MIT: microtubule-interacting and trafficking domain; Zn: Zn-finger motif-containing domain; SOH: SOL subfamily homology domain; DIS: CALPA-specific insertion sequence; TM: transmembrane domain; CSTN: the domain weakly similar to calpastatin.

a very good *in vitro* substrate and is used to assay calpain activity. The complete rules governing substrate specificity remain unclear; however, some preferences for cleavage site sequences have been reported [35, 165] (see also <http://calpain.org>). Calpain is also thought to recognize a wide range of 3D substrate structures. There has been no report to date suggesting significant differences in substrate specificity between μ - and m-calpains.

Calpain has a very specific proteinaceous inhibitor *in vivo* called calpastatin. Calpastatin has four repeats of an inhibitor unit, each of which inhibits one molecule of calpain (Fig. 2A). Both μ - and m-calpains have similar susceptibility to calpastatin. Among other calpain homologues, calpain-8/nCL-2 and calpain-9/nCL-4, but

not calpain-3/p94, are also inhibited by calpastatin [53, 87, 119]. μ - and m-calpains are ubiquitously expressed in vertebrate cells. Thus, their function is considered to be fundamental and essential. Many functions, including the regulation of signal transduction systems [48, 80, 85, 104, 114, 128, 152], cell motility [141, 174], membrane repair [99, 100], and apoptosis [12, 26, 47, 88, 112, 115, 134, 140, 173], have been suggested, although their precise physiological roles remain elusive. Knockout mice for *Capn1*, the gene for calpain-1/ μ CL, show no apparent phenotype [5], while knockout of *Capn2* and *Capn3*, the respective genes for calpain-2/mCL and the regulatory subunit for both μ - and m-calpains, results in embryonic lethality [4, 34, 183], indicating the indispensable roles of conventional calpains. At the same time,

Fig. 2. Schematic structures of human calpains. **A.** Schematic structures of representative products of human genes for calpain homologues (see also Table 1). Green letters indicate tissue/organ-specific calpains. **B.** Classification of mammalian calpains by domain structures. Symbols used are: IQ: a region interactive with calmodulin; L and XL: N-terminal and extended N-terminal regions of calpastatin; see Fig. 1 for other symbols.

these results differentiate between the functionality and/or expression level of μ - and m-calpain, at least at specific developmental stage(s). Intriguingly, *Cast*, the gene for calpastatin, knockout and overexpressing mice showed increased and decreased susceptibility to excitotoxicity induced by kainic acid, strongly suggesting

that the conventional calpains are involved in mitochondrial-mediated apoptotic mechanisms [61, 162].

The catalytic and regulatory subunits of conventional calpains can be divided into four and two domains, respectively (Fig. 2A). The N-terminus of domain I of the large subunit is autolysed upon its initial activation by

Table 1. Human calpain related genes

Gene	Chr.	Name in number	Alias	Protease activity ^{a)}	Domains ^{b)}			Expression	Phenotype(s) of gene deficiency ^{c)}	Note
					C2L	C2	PEF			
Catalytic subunits										
<i>CAPN1</i>	11q13	calpain-1	μ -calpain large subunit (μ CL), μ CANP ^{d)} / calpain-1 large subunit, μ 80K	+	+	-	+	ubiquitous	platelet dysfunction ~ n.s.	μ -calpain is heterodimer with CAPNS1/30K
<i>CAPN2</i>	1q41-q42	calpain-2	m-calpain large subunit (mCL), mCANP/ calpain-II large subunit, m80K	+	+	-	+	ubiquitous except for mammalian erythrocytes	embryonic lethal	m-calpain is heterodimer with CAPNS1/30K
<i>CAPN3</i>	15q15.1-q21.1	calpain-3	p94, calpain 3a, nCL-1	+	+	-	+	+	skeletal muscle	muscular dystrophy
	(not in human)		Lp82, Lp85, etc.	+	+	-	+	+	lens, retina	
	calpain-3b, 3c, ..., 3n		p94; Δ ex15, Tp36, Mp18, Up84, etc.	n.d.	+	-	+	+	ubiquitous	Ups, Tps, and Mps are driven from alternative promoter
<i>CAPN5</i>	11q14	calpain-5	hTRA-3, nCL-3	+	+	+	-	Ubiquitous (abundant in testis, brain)	sudden death ~ n.s.	nematode TRA-3 homologue
<i>CAPN6</i>	Xq23	calpain-6	calpainmodulin, CANPX	-	+	+	-	placenta, embryonic muscles	n.d.	nematode TRA-3 homologue, but no Cys at the active site
<i>CAPN7</i>	3p24	calpain-7	PalBH	n.d.	++	-	-	ubiquitous	n.d.	Aspergillus PalB homologue
<i>CAPN8</i>	1q41	calpain-8	nCL-2	+	+	-	+	stomach	n.d.	forms homooligomer and heterodimer with calpain-9
	calpain-8b		nCL-2'	+	-	-	-	stomach		Ca ²⁺ -dependent
<i>CAPN9</i>	calpain-8c		nCL-2; Δ ex10,17-fs	+	+	-	+/-	stomach		
	1q42.11-q42.3	calpain-9a	nCL-4	+	+	-	+	digestive tracts	n.d.	Heterodimer with calpain-8
	calpain-9b		nCL-4; Δ ex8	+	+	-	+	digestive tracts		
<i>CAPN10</i>	2q37.3	calpain-10	calpain 10a	n.d.	++	-	-	ubiquitous	n.s.	SNP is related to NIDDM
	calpain-10b, 10c, ..., 10h			n.d.	+ or -	-	-			
<i>CAPN11</i>	6p12	calpain-11		n.d.	+	-	-	testis	n.d.	
<i>CAPN12</i>	19q13.2	calpain-12	calpain 12a	n.d.	+	-	+	hair follicle	n.d.	
	calpain-12b, 12c			n.d.	+	-	+/-			
<i>CAPN13</i>	2p22-p21	calpain-13		n.d.	+	-	+	ubiquitous	n.d.	mRNA not detected
<i>CAPN14</i>	2p23.1-p21	calpain-14		n.d.	+	-	+	n.d.	n.d.	
<i>SOLH/CAPN15</i>	16p13.3	calpain-15	SOLH	n.d.	-	-	-	ubiquitous	n.d.	<i>Drosophila</i> SOL homologue
<i>C6ORF103/CAPN16</i>	6q24.3	calpain-16	Demi-calpain	-	-	-	-	ubiquitous	n.d.	contains only domain IIa
Regulatory subunits										
<i>CAPNS1</i>	19q13.1	CAPNS1	CANP/calpain small subunit, 30K, css1	-	-	-	+	ubiquitous	embryonic lethal	regulatory subunit for μ CL and mCL
<i>CAPNS2</i>	16q12.2	CAPNS2	calpain small subunit 2, 30K-2, css2	-	-	-	+	ubiquitous	n.d.	intron less gene, overlap with lysophosphatidylcholine acyltransferase 2 gene (<i>LPCAT2</i>)
Calpain inhibitor										
<i>CAST</i>	5q15	calpastatin	CANP inhibitor	-	-	-	-	ubiquitous	excitotoxicity in neuronal cells ~ n.s.	specific inhibitor for μ - and m-calpains, nCL-2, and nCL-4

^{a)} + indicates that the molecule was experimentally shown to have protease activity, and - means that it does not have active site cysteine, asparagine, and/or histidine residues. ^{b)} + or - indicates that the molecule has or does not have a corresponding domain, respectively, and +/- indicates that it has one or more but less than five EF-hand motifs. ^{c)} Human disease and results of knock-out mice were considered together. ^{d)} Abbreviations: n.d., not yet determined/done; n.s., no significant phenotype; CANP, Ca²⁺-activated neutral protease; (C2L, C2-like domain) nCL, novel calpain large subunit; LGMD2A, limb-girdle muscular dystrophy type 2A; TRA-3, transform genotypic hermaphrodites; PalB, phosphatase mutants; loss in activity at alkaline pH but normal or increased activity at acidic pH; PalBH, PalB homologue; SNP, single nucleotide polymorphism; NIDDM, non-insulin-dependent diabetes mellitus; SOL, small optic lobes; SOLH, SOL homologue.

Fig. 3. Schematic 3D structure of inactive and active m-calpain. Ribbon-type schematic 3D structures of inactive (Ca^{2+} free) and active (Ca^{2+} and calpastatin bound) forms of m-calpain are drawn by FiatLux MolFeat Ver. 4.5 using PDB data, 1KFX and 3DF0, respectively. Dark and light green indicate catalytic (calpain-1/mCL) and regulatory (CAPNS1/30K) subunits, respectively. Red indicates calpastatin bound to active m-calpain with two portions (dotted lines) that are too mobile to determine the 3D structure. The active protease domain (domain II) is formed by fusion of the subdomains IIa and IIb (also called subdomains I and II) upon binding one Ca^{2+} to each of the subdomains. Active site residues, Cys105, His262, and Asn286 (NP_058812), in the protease domain are indicated by the ball-and-stick drawing in yellow. Blue balls (visible in active domains II, IV, and VI) represent Ca^{2+} . Calpastatin reactive site residues bound to domains II and III of m-calpain are shown by a ball-and-stick drawing in red.

Ca^{2+} . This results in a lower requirement for Ca^{2+} [57, 66, 159], a different substrate specificity [111], and the dissociation of both subunits in some cases and not in others [81, 111, 124, 180, 182]. Therefore, autolysis is one of the important regulatory mechanisms of calpain activity and specificity.

Recent 3D structural studies have revealed that in the absence of Ca^{2+} the protease domain (domain II) is divided into two subdomains IIa and IIb (also called subdomains I and II), which are folded into one domain upon Ca^{2+} binding [50, 64, 105, 153] (Fig. 3). This domain is highly conserved in calpain family members, suggesting the functional importance of this domain (Fig. 1). Surprisingly, the protease domain of m-calpain without the other domains showed Ca^{2+} -dependent protease activity [56]. This was explained when the 3D structural studies

of the protease domain in the presence of Ca^{2+} showed that Ca^{2+} bound to domains IIa and IIb [106, 107] (Fig. 3). Thus, the whole calpain molecule mediates Ca^{2+} -dependency as all of the domains IIa, IIb, III, IV, and VI bind at least one Ca^{2+} with varying affinities [106, 107, 166].

The 3D structure of domain III consists of eight anti-parallel β -strands (β -sandwich structure), a structure very similar to TNF- α and the C2-domains found in various Ca^{2+} -regulated proteins such as protein kinase C isoforms and synaptotagmins. Although the primary structures of domain III are highly conserved in calpain homologues, they have no similarity to any other protein including TNF- α and C2-domains. This C2-like domain actually binds Ca^{2+} , and may play an important role in the Ca^{2+} -dependent membrane translocation of calpains

[78, 166].

Domain IV has a 3D structure very similar to that of domain VI of CAPNS1/30K, the regulatory subunit, and each contains five EF-hand motifs. Thus, these domains are referred to as penta EF-hand (PEF) domains [97]. *In vitro* experiments together with 3D structural studies have shown that not all EF-hand motifs bind Ca^{2+} [102, 103]. The fifth EF-hand motif is involved in the dimerization of the catalytic and regulatory subunits [67].

Domain V of the calpain regulatory subunit contains clusters of glycine residues, making it hydrophobic. This domain is thought to interact with plasma membrane and/or membrane proteins through hydrophobic interactions. Most of this domain is autolysed during activation, indicating no involvement of this region in protease activity. In humans, the *CAPNS2* gene encodes a regulatory subunit homologue, whose physiological roles remain unclear [143].

Classifications of the Members of the Calpain Superfamily

Calpain homologues other than the conventional calpains have, in addition to the protease domain, various divergent domains that are not necessarily conserved between other homologues (Figs. 1 and 2). Depending on the molecule, the amino acid sequence identities of the protease domains vary from less than 30% to more than 75%. Several functional domains, presumably originating from independent ancestral genes, are found on N- and/or C-terminal sides of the protease domain. These include C2 and C2-like domains, PEF domains, transmembrane domains, Zn-finger domains, and conserved domains with unknown functions [Small optic lobe (SOL)-homology (SOH) domain, etc.]. These features, together with the organization of mammalian calpain genes, strongly suggest that calpain molecules were generated by the arrangement and restructuring of genes of the ancestral calpain-type cysteine protease with genes encoding other functions.

From a structural viewpoint, vertebrate calpain homologues can be divided into two classes. The first group consists of molecules having domains II, III, and IV (Fig. 2), which correspond to a “typical” structure highly

similar to conventional calpain catalytic subunits. Mammalian typical calpains include calpain-1/ μ CL, calpain-2/mCL, calpain-3/p94, calpain-8/nCL-2, calpain-9/nCL-4, and calpain-11, 12, 13, and 14. Chickens and *Xenopus laevis* have μ /mCL, which shows structure and properties intermediate between those of calpain-1/ μ CL and calpain-2/mCL, and was recently shown to correspond to mammalian calpain-11 from an evolutionary viewpoint [96, 150]. Fish have a duplicate set of most of the 15 mammalian calpains [96]. In invertebrates, only a few typical calpains have been identified thus far. *Drosophila melanogaster* has three typical calpains, CALPA/Dm-calpain, CALPB, and CALPC [36, 41, 69, 163]. *Schistosoma mansoni* and *S. japonicum* also have at least one typical calpain (Sm-calpain and Sj-calpain) [3, 84]. No typical calpain homologues have been found in *Caenorhabditis elegans*, plants, fungi, trypanosomes, or *Saccharomyces cerevisiae*.

The second group (“atypical” calpains) contains various molecules that have the protease domain with extra domain(s) that do not show overall similarity to domains III and IV. These atypical calpains are considered to have modes of action different from those of typical calpains. Atypical calpains include the SOL and PalB subfamilies, and several alternative splicing products of *Capn8* (called nCL-2’ or calpain-8b), *CalpA*, and others. Although the structure is called atypical, PalB subfamily members are conserved in animals, fungi, and yeast, and SOL subfamily members are found in all animals and green algae, whereas typical calpains only exist in vertebrates and *Drosophila*.

In addition to the structural features, mammalian calpains are independently classified into two categories according to their tissue/organ distribution. Calpain-1/ μ CL, calpain-2/mCL, calpain-5/hTRA-3, calpain-7/PalBH, calpain-10, calpain-13, and calpain-15/SOLH are ubiquitously expressed, whereas expressions of calpain-3/p94, calpain-8/nCL-2, calpain-9/nCL-4, calpain-6, 11, and 12 are restricted to specific tissues/organs (Table 1). Ubiquitous calpains are likely to play fundamental roles for all cells, whereas tissue-specific calpains must be involved in specific functions of the tissues in which they are expressed. Therefore, defects in ubiquitous calpains often cause lethality such as in *Capn2* knockout mice [34], while defects in tissue-specific cal-

pains are responsible for tissue-specific diseases such as muscular dystrophy caused by mutations in *CAPN3* [133]. In addition, under various disease conditions such as muscular dystrophies, cardiomyopathies, ischemic disorders, and lissencephaly, conventional calpains tend to be overactivated, probably because of compromised intracellular Ca^{2+} homeostasis caused by the disease, and which often aggravates the disease. Thus, inhibitors for conventional calpains are used to prevent progression of such diseases [1, 7, 11, 15, 62, 129, 151, 156, 177].

Structure and Functions of Calpain Superfamily Members

Skeletal muscle-specific calpain, calpain-3/p94

Calpain-3/p94, the first tissue-specific calpain found in 1989, is a typical calpain approximately 60% identical to calpain-1 and 2, i.e., the large subunits of μ - and m-calpains (Fig. 1) [147]. However, calpain-3/p94 contains three additional p94-characteristic regions, NS, IS1, and IS2 at the N-terminus, in the domain IIb, and between domains III and IV, respectively, which give calpain-3/p94 its unique features as described below.

Calpain-3/p94 is expressed predominantly in skeletal muscle, and the amount expressed is approximately 10 times higher than that of conventional calpains. Calpain-3/p94 possesses several unique properties, the most characteristic one being that calpain-3/p94 protein undergoes extremely rapid autolysis (the half-life *in vitro* is less than 10 min) starting with autoproteolysis in the NS and IS1 regions. This autolysis is dependent on the presence of both the IS1 and the IS2 regions, and inhibitors of μ - and m-calpains such as calpastatin, E-64, and leupeptin have little effect on the autolysis. Surprisingly, this autolysis undergoes Na^+ -dependently even in the absence of Ca^{2+} , showing that calpain-3/p94 is the first example of intracellular Na^+ -dependent enzyme [120]. Furthermore, calpain-3/p94 possesses a nuclear localization signal-like sequence in the IS2 region, and is sometimes localized in the nucleus in addition to the cytosol. Calpain-3/p94 binds specifically to the gigantic muscle protein connectin/titin through the region close to the IS2 region. The protease activity of calpain-3/p94 is almost suppressed *in vivo* as it is bound to the N2A region of connectin/titin, where other important subcel-

lular structures of muscle, such as sarcoplasmic reticulum and T-tubules, are aligned [123].

Some alternative splicing products (e.g., Lp82, Up86) of *Capn3* are expressed in tissues other than muscles [6, 28, 77, 94, 144]. Lp82 shows lens-specific expression and Ca^{2+} -dependent protease activity against βA3 and αB crystallins [42, 95, 113, 169]. The activity is inhibited by E-64, but not by calpastatin. Lp82 is expressed from an alternative promoter in *Capn3*, followed by an alternative exon 1', which lies between exons 1 and 2 of calpain-3/p94 [77]. Interestingly, exon 1' of human *CAPN3* contains an inframe termination codon, resulting in no expression of Lp82 in the human lens [38]. Some other splicing variants are expressed ubiquitously, in testes, or in embryonic skeletal muscles, although the physiological functions of these variants remain unclear [77].

In 1995, mutations in *CAPN3* were shown to be responsible for limb-girdle muscular dystrophy type 2A (LGMD2A, also called calpainopathy) [23, 133]. The positions of the mutation found in the LGMD2A patients were distributed widely within *CAPN3*, with more than half the mutations being missense [19, 24, 33, 131, 132, 135, 137]. No "hot point" was found, making diagnosis of the disease very difficult. *Capn3* knockout mice showed a human calpainopathy-like phenotype though milder than that of humans [82, 134], indicating that calpainopathy is caused by defects in the gene for calpain-3/p94. A primary cause of LGMD2A is a defect in the protease activity, not the structural properties, of calpain-3/p94 [121]. This was confirmed by the demonstration that calpain-3/p94:C129S inactive mutant knock-in mice showed a muscular dystrophy phenotype [117].

Gastrointestinal-specific calpains, calpain-8/nCL-2 and calpain-9/nCL-4

Calpain-8/nCL-2 and its alternative splicing product, nCL-2', are expressed by *Capn8*, with and without, respectively, domains III and IV [149]. They are predominantly expressed in surface mucus cells (called pit cells) in the stomach with lesser amounts in goblet cells in the intestines [54, 149]. Calpain-8/nCL-2 is highly similar to calpain-2/mCL along the whole molecule (ca. 62% identical). Moreover, human (or mouse) *CAPN8*

(or *Capn8*) and *CAPN2* (or *Capn2*) are closely located in chromosome 1q41 (or chromosome 1), and their transcripts have overlap, i.e., complementary sequences [55]. Unlike m-calpain, *Escherichia coli*-expressed recombinant calpain-8/nCL-2 showed Ca^{2+} -dependent caseinolytic activities as a monomer without CAPNS1/30K, and also forms a homooligomer via domain III *in vitro* [53]. *X. laevis* has an orthologue of calpain-8/nCL-2, xCL-2, the disruption of which causes severe developmental defects [20].

Calpain-9/nCL-4 is also a gastrointestinal-specific typical calpain homologue, and was reported to be involved in tumor suppression [93, 179]. It has overall similarity to other typical calpains with similar amino acid sequence identities, suggesting that calpain-9/nCL-4 is the molecule closest to the ancestral calpain species [86]. Calpain-9/nCL-4 requires CAPNS1/30K for its activity *in vitro*, and recombinant human calpain-9/nCL-4+30K showed Ca^{2+} -dependent caseinolytic activity, which was inhibited by calpastatin and other cysteine protease inhibitors, as in the case of conventional calpains [87]. Experiments using *Capn8*^{-/-} and *Capn9*^{-/-} mice, however, showed that neither calpain-8/nCL-2 nor calpain-9/nCL-4 form a complex with CAPNS1/30K, but that they do form a “hybrid” heterodimer with each other [52]. These knockout mouse studies also showed that calpain-8/nCL-2 and calpain-9/nCL-4 are involved in gastric mucosal defense [52].

The PalB subfamily

PalB was first identified in *Emericella* (*Aspergillus*) *nidulans* as a product of the gene responsible for adaptation of fungi to alkaline conditions [32]. Afterwards, its orthologues were identified in *S. cerevisiae* (Cpl1) and in humans (calpain-7/PalBH) [39, 43, 44]. The structural similarity of these three PalB homologues, PalB, Cpl1, and calpain-7/PalBH is revealed in their C-terminus two tandem C2-like domains, which show rather diverged but significant similarity to the domain III of typical calpains [the upstream C2-like domain was once called the PBH (PalB-homology) domain because of its highly divergent sequence] [148]. The PalB subfamily can be defined as calpain species that have two C2 and/or C2-like domains at the C-terminus, and includes calpain-5/hTRA-3, calpain-6, and calpain-10 as well as

calpain-7/PalBH in humans [25].

Yeast *CPL1*, also referred to as *RIM13*, is the only calpain gene in *S. cerevisiae*, and is involved in both the alkaline adaptation and sporulation processes of yeast through its processing activity [44]. Rim101 and the *Aspergillus* orthologue PacC are *in vivo* substrates for Cpl1 and PalB, respectively, and are activated by C-terminal processing [32, 44, 58, 125, 136]. Several other yeasts such as *Candida albicans* and *Pichia pastoris* also have Cpl1 orthologues [89]. Mammals have one orthologue, calpain-7/PalBH, whose physiological functions are elusive, but its involvement in membrane trafficking around endosomes is suggested based on biochemical studies and by analogy with yeasts and fungi [178].

TRA-3 is involved in the sex determination cascade of *C. elegans* [9]. Although enzymatic characterization of the purified enzyme has not been reported, the protease activity of TRA-3 is Ca^{2+} dependent and necessary for female development in XX hermaphrodites through the processing of TRA-2A membrane protein [146]. By contrast, TRA-3 and another *C. elegans* calpain, CLP-1, are involved in neuronal cell necrosis in cooperation with aspartic proteases ASP-3 and ASP-4, which are similar to cathepsins D and E [160]. Surprisingly, a single nucleotide polymorphism (SNP) in *tra-3* was reported to be related to the temperature-size rule of wild-type nematodes [74]. Mammals have two orthologues of TRA-3, calpain-5/hTRA-3, and calpain-6, whose amino acid sequences are more than 30% identical to that of TRA-3 [29, 108, 171]. The C-terminal domain was once called the “T domain”, is conserved in all three molecules, and has weak similarity to the Ca^{2+} -binding C2-domain. Calpain-5/hTRA-3 shows Ca^{2+} -dependent autolytic activity although its substrate has not yet been identified [171]. *Capn5* knockout mice showed that calpain-5/hTRA-3 is expressed in a subset of T cells, but that it is dispensable for development [40]. By contrast, calpain-6 apparently has no active site residues in its amino acid sequence (active site Cys is substituted with Lys), strongly suggesting that calpain-6 has no proteolytic activity. Recently, calpain-6 was reported to be involved in the regulation of microtubule dynamics, which has opened up a new field of calpain study [167].

Calpain-10 was identified by large-scale genetic as-

Table 2. Distribution of calpain homologues (CysPc) in living organisms

Eukaryotes (2204)*	Animals (1,356)	Chordates (951)	
		Hemichordates (2)	
		Echinoderms (16)	
		Arthropods (246)	
		Nematodes (67) [Rhabditida (57), Spirurida (10)]	
		Flatworms (40)	
		Cnidarians (22) [Anthozoans (16), Hydrozoans (6)]	
		Placozoans (12)	
	Fungi (265)	Ascomycetes (207)	Eurotiales (53) [Aspergillus (23), Neosartorya (10), Emericella (8), etc.]
			Saccharomycetales (51) [Saccharomycetaceae (35), Candida (10), etc.]
			Sordariales (29)
			Onygenales (24)
			Helotiales (16)
			Magnaporthales (10)
			Hypocreales (10)
			Pleosporales (10)
			Phyllachorales (4)
		Basidiomycetes (58) [Agaricales (42), Tremellales (11), Ustilaginales (3), Malasseziales (2)]	
	Green Plants (69)	Land plants (35)	Vascular plants (33) [Eudicots (17), Monocots (16)]
			Mosses (2)
		Green algae (34) [Mamiellales (24), Chlamydomonadales (10)]	
	Protists (514)	Kinetoplastids (253)	Leishmania (128)
			Trypanosoma (120)
			Crithidia (5)
		Alveolates (176)	Ciliates (126)
			Apicomplexans (36)
			Perkinsida (14)
		Schizopyrenida (38)	
		Parabasals (Trichomonads) (22)	
		Choanoflagellates (10)	
		Oomycetes (7)	
		Diatoms (4) [Naviculales (2), Thalassiosiraceae (2)]	
		Entamoeba (4)	
Bacteria (94)		Actinobacteria (33)	
		Cyanobacteria (24)	
		Proteobacteria (16)	
		Cytophaga-Flavobacterium-Bacteroides (CFB) (11) [Bacteroidaceae (7), Porphyromonadaceae (4)]	
		Green Nonsulfur (GNS) Bacteria (6)	
		Planctomycetes (3) [Planctomyces (2), Gemmata (1)]	
		NC10 Bacteria (1)	

*Number in parentheses indicates the number of calpain homologues found in that species in the NCBI protein database including duplications. No information was available for 63 entries.

sociation studies of noninsulin-dependent diabetes mellitus (NIDDM, type 2 diabetes) [8, 17, 63]. An SNP in intron 3 of *CAPN10* is related to an increased risk of NIDDM. This SNP may affect transcription levels of *CAPN10* [154], although the relationship between calpain-10 and NIDDM and the physiological role of calpain-10 have so far been elusive [59, 155, 168]. *CAPN10*

generates several alternative splicing products, the longest of which has two C2-like domains moderately and weakly similar to that of domain III. It should be noted that *Capn10* is a candidate responsible gene for the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, a NIDDM animal model [110, 142]. Quantitative trait locus (QTL) analysis using *Capn10*^{-/-}, LG/J, and SM/J

mice showed that *Capn10* is a component of the obesity QTL, *Adip1*, indicating that calpain-10 is involved in obesity in humans and mice [21, 22].

Expanding calpain species

In the NCBI protein database (<http://www.ncbi.nlm.nih.gov/sites>), 2,361 entries for calpain homologues had been made as of April 29, 2010 (searched by CysPc, see Table 2). Among these calpain homologues, 2,204 entries cover almost all eukaryote species whose genome sequences have been decoded. One impressive exception is *Schizosaccharomyces pombe*, despite the existence of *CPL1/RIM13* in all *Saccharomycetales* that have ever been examined. In contrast to the autophagy system, which is found in almost all eukaryotes but not in bacteria and archaea, only 94 entries are found for bacteria, while no calpain species is found in any archaeobacterium. It should be noted that *Leishmania* and *Trypanosoma* have around 20 calpain homologues, whose physiological roles probably involve cell morphogenesis, drug resistance, and stress response mechanisms [37, 45, 118, 170]. Some trypanosome calpains have N-terminal domains weakly similar to calpastatin.

Sol was first identified as a *Drosophila* gene responsible for a defect in neuronal cells (small optic lobes) corresponding to mammalian retina [30]. SOL has several Zn-finger motifs located at the N-terminus followed by the protease domain. Mammals have one orthologue, calpain-15/SOLH, and all other animals including *Leishmania* and *Trypanosoma* and green algae also have orthologues, constituting the SOL subfamily [72, 73]. They share a conserved C-terminal structure called the SOH domain, but the physiological role of SOLH is still unclear.

The first calpain gene reported in plants was the maize *defective kernel 1* gene required for aleurone cell development in the endosperm of maize grains [90]. The primary structure of DEK1 is unique in that it has 21 transmembrane regions located at the N-terminus and a C2-like domain, significantly similar to domain III, located at the C-terminus [172]. Very conserved orthologues (above 70% identity) were found in rice, *Arabidopsis*, barley, grape (pinot noir), eggplant, sugarcane, and loblolly pine [98]. The whole genome sequence of *Arabidopsis* has revealed that DEK1 is the only calpain

homologue in the genome [70, 164]. DEK1 plays a key role in growth regulation in *Arabidopsis*, and although full-length DEK1 protein localizes to membranes, it undergoes intramolecular autolytic cleavage events that release the calpain domain into the cytoplasm, which is sufficient for full complementation of *dek1* mutants [70, 71, 91, 164].

As described above, some of the calpain homologues possess substituted residues in one or more of the very conserved active site residues, Cys, His, and Asn. This nonproteolytic family of calpain homologues includes calpain-6, *Drosophila* CALPC, some of the *C. elegans* homologues, and all of the *Trypanosoma* homologues [37, 41, 160]. These molecules are considered not to have cysteine protease activity. Although the physiological roles of these nonproteolytic subfamily members have yet to be elucidated, some of the reports cited above have shown novel calpain functions other than those of protease [2, 45, 60, 118, 126, 139, 167]. The processes of generation of these calpain species are interesting from an evolutionary viewpoint and the elucidation of their physiological functions will shed light on novel aspects of the calpain superfamily.

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