Chapter 454

Calpain-1/μ-Calpain

Databanks

MEROPS name: calpain-1
MEROPS classification: clan CA, family C2, subfamily C2A, peptidase C02.001
IUBMB: EC 3.4.22.52 (BRENDA)
Tertiary structure: Available
Species distribution: superclass Tetrapoda
Reference sequence from: *Homo sapiens* (UniProt: P07384)

Name and History

Calpains are intracellular, Ca^{2+}-dependent cysteine proteases that are widely distributed and show regulated activity at neutral pH. μ-Calpain is one of the two major calpains (the other is m-calpain; see Chapter 455) ubiquitously found in mammalian cells, and is composed of a catalytic subunit (previously called μCL for short, and now CAPN1 – see below). Ca^{2+}-dependent neutral protease activity was first described in rat brain by Guroff in 1964 [1], and later in the same year, Meyer *et al*. described a kinase-activating factor (KAF) in skeletal muscle [2] that was identified as a Ca^{2+}-dependent protease by Huston & Krebs in 1968 [3]. In 1972, calpain was re-identified by Goll and his colleagues [4] as a calcium-activated sarcoplasmic factor (CaSF) that hydrolyzes Z-lines, and again in 1977 by Nishizuka's group, as a protein kinase C-activating factor [5]. Finally, in 1978, calpain, which was called CANP (**c**alcium-**a**ctivated **n**eutral **p**rotease) at the time, was purified to homogeneity by Ishiura *et al*. [6]. Both names, calpain and CANP, were used for several years, but they were unified as calpain (and calpastatin for its inhibitor) by Suzuki in 1991 [7]. The term calpain was derived from **cal**cium ion-dependent pa**pain**-like cysteine protease by Murachi [8].

After the first report of cDNA cloning in 1984 [9], cDNA and genomic cloning studies exploded, and thousands of calpains and related molecules were identified. All of these amino acid sequences can be extracted by searching for 'CysPC' in the conserved domain database of NCBI [10] from various living organisms, including all animals, plants, fungi, yeast, and even some bacteria. Calpains belong to the papain fold of cysteine proteases, residing in family C2 and have weak similarity to papains and cysteine cathepsins, although these similarities are significantly lower than those between calpains. Evolutionarily, calpain may be the oldest branch of the papain fold [10]. Thus, it is reasonable to define calpains mainly by their sequence in relation to the protease domain of human μ-calpain catalytic subunit (replacing μ-calpain with m-calpain in this definition would give the same result). According to this definition, humans have 15 genes that encode calpain catalytic subunits, which are now called ***CAPNn*** ($n = 1$, 2, 3, and 5∼16), short for **ca**l**pain**, and their gene products are correspondingly called

CAPNn ($n = 1$, 2, 3, and 5∼16) (previously called ***calpain-n***, but the formal nomenclature CAPNn will be used in calpain sections; see below and Table 454.1) [11]. There are two genes, *CAPNS1* and *CAPNS2*, for **cal**pa**i**n **s**mall regulatory subunit, and one, *CAST*, for **cal**pa**st**atin. Other species, *Schistosoma mansoni*, *Caenorhabditis elegans* (an endogenous specific inhibitor protein for calpain), *Anopheles gambiae*, *Drosophila melanogaster*, *Arabidopsis thaliana*, *Emericella* (*Aspergillus) nidulans*, and *Saccharomyces cerevisiae* have 7, 14, 7, 4, 1, 2, and 1 calpain genes, respectively (see also Chapter 459) [12]. No calpain gene is found in *Encephalitozoon* or *Schizosaccharomyces pombe*. In prokaryotes, 53 calpains from 42 bacteria have been found among the 914 completely sequenced microbial genomes in the database. Each of the bacterial species has 1−4 calpain genes. However, most of the genome-sequenced bacteria, including *Escherichia coli* and all of the archeabacteria, have no calpain gene.

The most extensively studied calpains are the major ubiquitous mammalian μ- and m-calpains, and the major ubiquitous calpain in chicken, μ/m-calpain. These were called 'conventional' calpains in the tradition of protein kinase C (PKC) nomenclature (cPKC, nPKC, and aPKC for conventional, novel, and atypical PKC, respectively) [13]; all others are termed 'unconventional' calpains. The chicken μ/m-calpain has properties intermediate between μ- and m-calpains [6,14,15]. Its catalytic subunit is an ortholog of mammalian CAPN11 (see below) [16]. Human calpains are divided into two groups according to their domain structures (Figure 454.1): those with a domain structure identical to the catalytic subunits of conventional calpains are called 'classical' calpains; the others are called 'non-classical' calpains (see Chapter 459 for details). In addition, mammalian calpains are also classified into two groups according to their expression patterns: the ubiquitous calpains and the tissue-specific calpains. μ-calpain is a conventional calpain, a classical calpain, and a ubiquitous calpain (see also Chapters 456−459).

As the names suggest, μ- and m-calpains are activated *in vitro* by micromolar and millimolar Ca^{2+} concentrations, respectively. (These molecules were also called calpain I and calpain II, previously.) μ- and m-calpains are heterodimers consisting of a common, smaller calpain regulatory subunit (CAPNS1, also called 30 K) and a distinct, larger catalytic calpain subunit (CAPN1 or CAPN2, also called μCL and mCL, respectively), which are *ca.* 60% identical in their protein sequences. To avoid confusion, these enzymes are now proposed to be called by their subunit composition, CAPN1/S1 and CAPN2/S1, respectively [17]. As described above, many calpain homologs exist across as well as within species. Thus, the account of the calpains in the present volume is divided among five chapters (454−459), for μ-calpain [the complex of CAPN1/μCL and CAPNS1/30 K], m-calpain [complex of CAPN2/mCL and CAPNS1/30 K], skeletal muscle-specific calpain [CAPN3, also called p94], gastrointestinal-tract-specific calpains [CAPN8, also called nCL-2; CAPN9, also called nCL-4; CAPN10/calpain 10] and other calpains, respectively. The present chapter also includes a general introduction to all the calpains.

As the above history indicates, the products of human calpain genes were given a variety of names at the time of their discovery, but it is now becoming popular that they should be numbered systematically corresponding to their gene names in much the same way as the caspases. For example, μCL and p94 would be called calpain-1 and calpain-3, respectively. The use of this nomenclature is somewhat complicated and misleading by the fact that the name 'calpain' was originally defined for the active heterodimeric enzymes, *i.e.* the μ- and m-calpains, whereas calpain-1 (or μCL) and calpain-2 (or mCL) are subunits of these enzymes and have no enzymatic activity without CAPNS1/30 K under normal conditions. Therefore, to clarify our descriptions of mammalian calpains, the formal gene product names (CAPN1, CAPN2, etc.) are used in the calpain chapters, and the old names are noted after these names: *e.g.* CAPN1/μCL and CAPN3/p94 (see Table 454.1).

Activity and Specificity

Calpains proteolyze most substrates at a very limited number of cleavage sites, cleaving them mainly at interdomain unstructured regions; oligopeptides are generally poor substrates. Casein is an exceptional substrate for calpains, in that it is exhaustively proteolyzed by them. Therefore, casein is a popular substrate for *in vitro* assays of calpains, in which it is used in its natural or denatured form, with or without modification with various chromophores, fluorescent reagents, or isotopes. Calpain purified from mammalian or chicken skeletal muscle by standard methods (described later) has a specific activity of several hundred units per milligram protein, where one unit corresponds to an increase of 1.0 absorbance unit at 280 nm per hour under standard assay conditions. When one unit of calpain is incubated in 0.5 ml of 3 mg/ml casein, 0.1 M Tris-HCl (pH 7.5), 25 mM 2-mercaptoethanol, and the appropriate concentration of $CaCl_2$ to activate each calpain species (0.5 mM and 5 mM for μ- and m-calpains, respectively) at 30°C for 20 min, the acid-soluble supernatant produced by adding 0.5 ml of 10% TCA and centrifugation shows an increase of 0.33 absorbance unit at 280 nm. Calpain activity is dependent on Ca^{2+}, with half maximal activity for μ- and m-calpain occurring with around 50 μM and 0.5 mM Ca^{2+}, respectively.

Although oligopeptides are generally not good substrates for calpains, some oligopeptidyl fluorescent substrates are

TABLE 454.1 Human calpain genes and their representative products

Gene	Chromosome location	Phenotype of gene deficiency in mice	Recommended representative gene product name	Aliases	Classical (c) or non-classical (n) calpain	Expression	Active site (Cys, His, Asn)[a]	C2L	C2	Domains PEF[b]
CAPN1	11q13	platelet dysfunction	**CAPN1**	μ-calpain large subunit (μCL), calpain-1, μCANP/calpain-I large subunit, μ80K	c	ubiquitous	+, +, +	+	–	+
CAPN2	1q41-q42	embryonic lethal	**CAPN2**	m-calpain large subunit (mCL), calpain-2, mCANP/calpain-II large subunit, m80K	c	ubiquitous (except erythrocytes)	+, +, +	+	–	+
CAPN3	15q15.1- q21.1	muscular dystrophy	**CAPN3**	p94, calpain-3, calpain-3a, nCL-1	c	skeletal muscle	+, +, +	+	–	+
CAPN5	11q14	sudden death?	**CAPN5**	hTRA-3, calpain-5, nCL-3	n	abundant in testis and brain	+, +, +	+	+	–
CAPN6	Xq23	n.r.*	**CAPN6**	calpain-6, calpamodulin, CANPX	n	embryonic muscles, placenta, some culture cells	–, +, +	+	+	–
CAPN7	3p24	n.r.	**CAPN7**	PalBH, calpain-7	n	ubiquitous	+, +, +	++	–	–
CAPN8	1q41	stress-induced gastric ulcer	**CAPN8**	nCL-2, calpain-8, calpain-8a	c	gastrointestinal tracts	+, +, +	+	–	+
CAPN9	1q42.11- q42.3	stress-induced gastric ulcer	**CAPN9**	nCL-4, calpain-9, calpain-9a	c	gastrointestinal tracts	+, +, +	+	–	+
CAPN10	2q37.3	no significant phenotype	**CAPN10**	calpain-10, calpain-10a (exon 8 is skipped)	n	ubiquitous	+, +, +	++	–	–
CAPN11	6p12	n.r.	**CAPN11**	calpain-11	c	testis	+, +, +	+	–	–
CAPN12	19q13.2	n.r.	**CAPN12**	calpain-11, calpain-12a, calpain-12A	c	hair follicle	+, +, +	+	–	+
CAPN13	2p22-p21	n.r.	**CAPN13**	calpain-13	c	ubiquitous	+, +, +	+	–	+
CAPN14	2p23.1-p21	n.r.	**CAPN14**	calpain-14	c	ubiquitous	+, +, +	+	–	+

(*Continued*)

TABLE 454.1 (Continued)

Gene	Chromosome location	Phenotype of gene deficiency in mice	Recommended representative gene product name	Aliases	Classical (c) or non-classical (n) calpain	Expression	Active site (Cys, His, Asn)[a]	C2L	C2	Domains PEF[b]
CAPN15/ SOLH	16p13.3	n.r.	**CAPN15**	SOLH, calpain-15	n	ubiquitous	+, +, +	–	–	–
CAPN16/ C6orf103	6q24.3	n.r.	**CAPN16**	Demi-calpain, calpain-16, C6orf103	n	ubiquitous	+, –, –	–	–	–
CAPNS1	19q13	embryonic lethal	**CAPNS1**	CANP/calpain small subunit, 30K, ccs1	–	ubiquitous	–, –, –	–	–	+
CAPNS2	16q13	n.r.	**CAPNS2**	30K-2, ccs2	–	ubiquitous	–, –, –	–	–	+
CAST	5q15-21	excitotoxicity	**calpastatin**	CANP/calpain inhibitor	–	ubiquitous	–, –, –	–	–	–

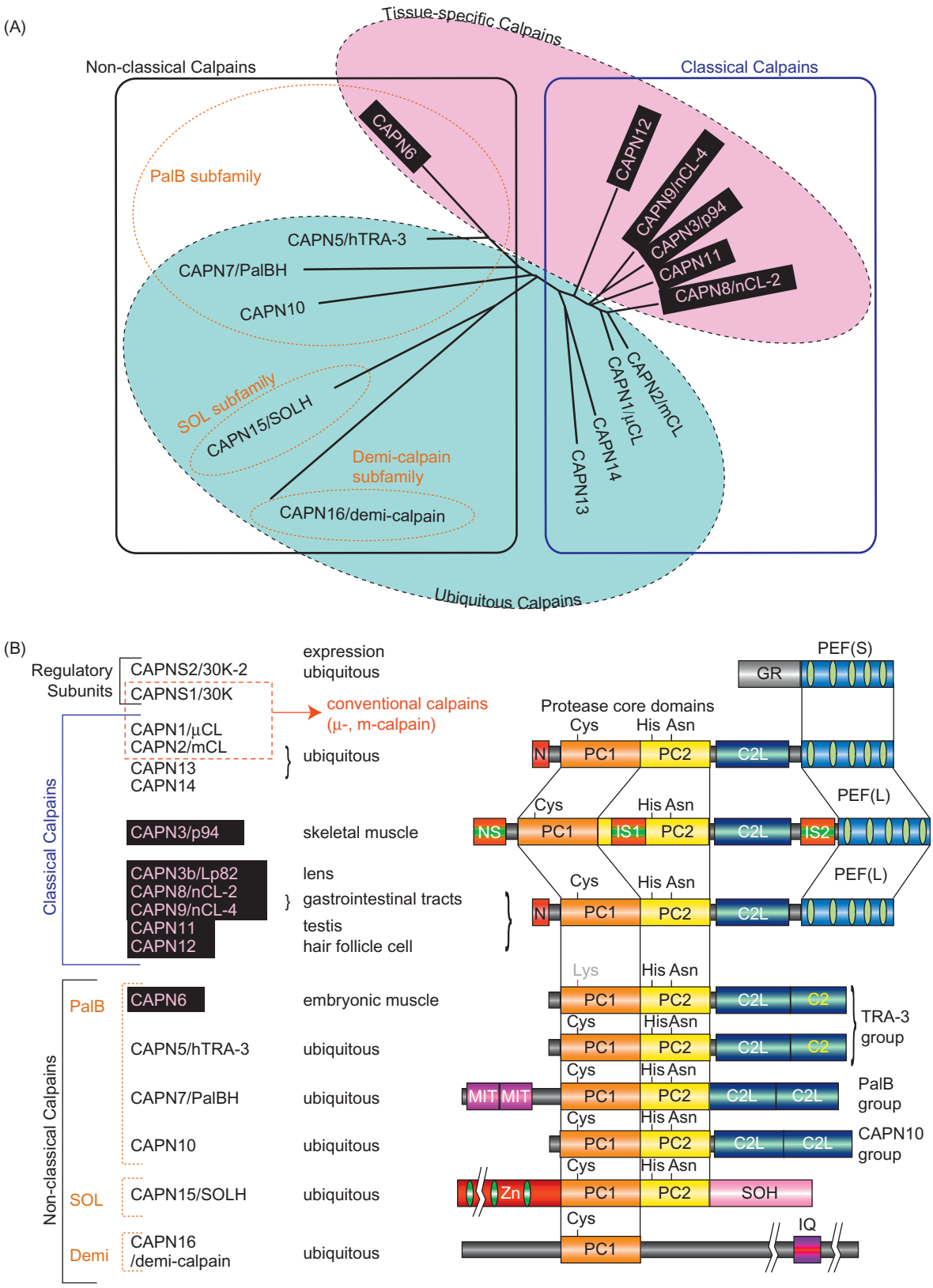
[a] + indicates that the molecule has well-conserved aar consisting of the active site triad, and – means that it has other aar in that position.
[b] + or – indicates that the molecule has, or does not have, a corresponding domain.
** Not yet reported.*

often used and are commercially available. These include Suc-LLY-AMC [18], Suc-LLVY-AMC [18], FAM-KEVY↓GMMK-Dabcyl [19], (Dabcyl-TPLK↓SPPPSPR-EDANS [20], and its cell-permeable version, Dabcyl-TPLK↓SPPPSPRE(EDANS)-RRRRRRR-NH_2 [21], and Boc-LM-AMC, which is also cell permeable [22]. Note that short oligopeptidyl substrates are cleaved not solely by calpain, but also by other proteases, to some extent; *e.g.* Suc-LLVY-AMC is also cleaved by chymotrypsin and the proteasome [23]; Suc-LY-AMC is a good substrate for *Schistosoma mansoni* cathepsin L-like protease [24]; and Boc-LM-AMC is cleaved by fiber cell globulizing aminopeptidase (FCGAP) [25].

The rules governing calpains' substrate specificity remain unclear. Calpains seem to recognize the overall three-dimensional structure of their substrates, more than the primary structure. Even so, some sequence preferences have been reported, such as Leu, Thr/Arg, and Pro residues in positions P2, P1, and P3′, respectively (see 'Calpain for Modulatory Proteolysis Database (CaMP DB)') [20,26]. Figure 454.2 shows the preferred residues of calpain substrates at each position relative to the cleavage site [27]. Protein kinases, phosphatases, phospholipases, cytoskeletal proteins, membrane proteins, cytokines, transcription factors, lens proteins, calmodulin-binding proteins and others have been suggested as *in vivo* substrates for calpain, but clear evidence has not yet been obtained. Calpains proteolyze these proteins in a limited manner to produce large fragments partially retaining their intact structures, rather than digesting them to small peptides, suggesting that it may modulate the functions of the substrate proteins by cutting their inter-domain regions [17,28,29]. Although there is no clear evidence of a difference in substrate specificity between μ- and m-calpains, some statistically significant differences can be found between them concerning sequence lengths of substrate recognizing regions [27].

Calpains have a very specific *in vivo* protein inhibitor, named calpastatin, which contains four repeats of the inhibitory unit, each of which can inhibit calpain independently. Both μ- and m-calpains have similar susceptibilities to calpastatin. Peptides (20∼40 mers) corresponding to the calpastatin-reactive site are also used as specific inhibitors for calpains. A number of low-molecular-weight inhibitors for μ- and m-calpains have been reported, most of which are commercially available. The classic ones include leupeptin [30] and E-64 [31,32], and its membrane permeable analog, E-64-d, also called EST or loxistatin) [33]. These are efficient inhibitors for calpains, but also for many cysteine proteases, such as cysteine cathepsins and papain.

There is also a series of molecules named 'Calpain Inhibitors': Calpain Inhibitor I (Ac-LLN-CHO, also called MG-101); II, Ac-LLM-CHO; III, Z-VF-CHO, MDL-


(A)
Tissue-specific Calpains
Non-classical Calpains
Classical Calpains
PalB subfamily
CAPN6
CAPN12
CAPN9/nCL-4
CAPN3/p94
CAPN11
CAPN8/nCL-2
CAPN5/hTRA-3
CAPN7/PalBH
CAPN10
SOL subfamily
CAPN15/SOLH
Demi-calpain subfamily
CAPN16/demi-calpain
CAPN2/mCL
CAPN1/μCL
CAPN14
CAPN13
Ubiquitous Calpains
(B)
Regulatory Subunits
CAPNS2/30K-2
CAPNS1/30K
expression ubiquitous
conventional calpains (μ-, m-calpain)
CAPN1/μCL
CAPN2/mCL
CAPN13
CAPN14
ubiquitous
CAPN3/p94
skeletal muscle
CAPN3b/Lp82
CAPN8/nCL-2
CAPN9/nCL-4
CAPN11
CAPN12
lens
gastrointestinal tracts
testis
hair follicle cell
Classical Calpains
Non-classical Calpains
PalB
SOL
Demi
CAPN6
embryonic muscle
CAPN5/hTRA-3
ubiquitous
CAPN7/PalBH
ubiquitous
CAPN10
ubiquitous
CAPN15/SOLH
ubiquitous
CAPN16 /demi-calpain
ubiquitous
PEF(S)
GR
Protease core domains
Cys
His Asn
N
PC1
PC2
C2L
PEF(L)
NS
IS1
IS2
Lys
C2
TRA-3 group
MIT
PalB group
CAPN10 group
Zn
SOH
IQ

28170); IV, Z-LLY-CH_2F or Z-LLL-CHO (MG-132); V, morpholinoureidyl-Val-homophenylalanyl-CH_2F; VI, 4-fluorophenylsulfonyl-VL-CHO; VII, LLF-CH_2Cl; X, Z-L-Abu-$CONHC_2H_5$; XI, Z-Leu-Abu-$CONH(CH_2)_3$-morpholine; XII, Z-Leu-L-norvaline-CONH-CH_2-2-pyridyl. Calpeptin (Z-LN-CHO) is also often used. However, all of these inhibitors are active-site-directed and react with other cysteine proteases and/or proteasomes more or less. On the other hand, PD150606 (3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid) is unique in that it is directed to the calpain PEF domain. However, this compound is not specific for calpain either [34], and it has weaker inhibition activity than calpeptin [35]. Therefore, to show if calpains are involved in a phenomenon of interest, it is important to use several different inhibitors. In addition, several endogenous activator proteins of calpains have also been reported [36–38].

The Ca^{2+} concentration required for the activation of μ-calpain (pKa) is lowered by the addition of phosphoinositides such as PIP, PIP2, and PIP3 [39]. In the presence of phosphoinositide, the Ca^{2+} concentration required for the activity is reduced to 100 nM-1 μM. During the activation of μ-calpain, the autolysis of a few N-terminal residues occurs. This autolysis precedes the appearance of proteolytic activity under normal conditions *in vitro* [40], causes subunit dissociation, and changes the substrate specificity [41]. However, the physiological significance of this autolysis remains unclear.

Structural Chemistry

As mentioned above, mammalian μ-calpain is a heterodimer of about 110 kDa. At least in mammals, the μ-calpain larger catalytic subunit is similar to but distinct from that of m-calpain, whereas these calpains use the identical smaller regulatory subunit. The different Ca^{2+} dependencies of the μ- and m-calpains must therefore be due to structural difference in the catalytic subunits. The structures of the μ- and m-calpain catalytic and regulatory subunits are divided into four and two domains/regions, respectively, as shown in Figure 454.1B. The domain boundaries were first defined from the proteins' primary structure, but were later revised slightly on the basis of three-dimensional structural studies of m-calpain with and without Ca^{2+}.

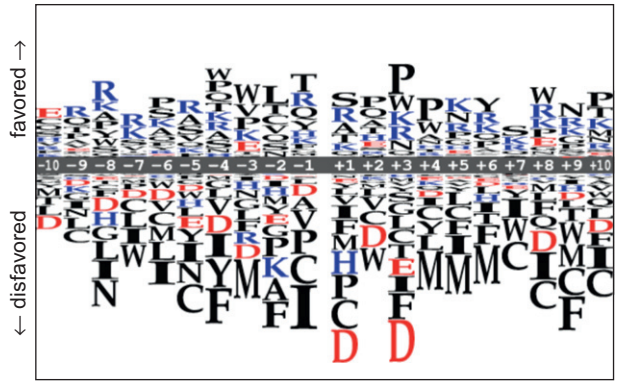


FIGURE 454.2 Substrate sequence specificity of calpains. Data are from the CaMP DB (Calpain for Modulatory Proteolysis Database). Colors indicate charge (blue: +, red: −) of aar; numbers indicate the positions of the aar from the cleavage site, at zero (minus: P10, P9, . . ., P1; plus: P1′, P2′, . . .P10′). Although no clear specificity is observed, Lys and Arg are generally favored, and Trp at P3, Leu at P2, and Pro at P3′ are the most favored.

The N-terminus of the catalytic subunit is autolyzed upon calpain's activation by Ca^{2+}, and is therefore thought to be important for regulating the activity of μ- and m-calpain (see also Chapter 455). The N-terminal regions of μ- and m-calpains are small α-helices of only 28 and 18 aar, whereas those of other classical calpains, such as CAPN3/p94, drosophila CALPA and CALPB, and some *Schistosome* calpains, are much larger. The N-terminal anchor helix is thought to be involved in subunit association/dissociation in some cases (see Chapter 455) [41].

The cysteine protease (CysPc) domain next to the N-terminus has a local weak similarity to those of papain and other members of peptidase family C1. That is, the Cys, His, and Asn residues that compose the active site triad can be identified by similarity to papain and cysteine cathepsins, and have been confirmed by site-directed mutagenesis [42,43]. The CysPc domain is the most conserved among calpain family members, suggesting that it has indispensable functions. Three-dimensional structural studies revealed that the protease domain in the absence of Ca^{2+} is divided into two **p**rotease **c**ore domains, PC1

FIGURE 454.1 Phylogenetic tree and schematic structures of human calpains. (A) Phylogenetic tree of human calpains and their classification. The tree was drawn using the neighbor-joining/bootstrap method after aligning all the sequences using MAFFT v6.240 (strategy: E-INS-i). Human calpains are classified into two groups according to their domain structures (see below): classical (in the blue rectangle) and non-classical (in the black rectangle). Non-classical calpains further consist of three subfamilies, one of which shows further divergence, *i.e.* the PalB subfamily is composed of the strict PalB, TRA-3, and CAPN10 groups. In addition, human calpains are also divided into two groups: tissue-specific (pink) and ubiquitous (light blue) calpains, according to their expression patterns; (B) Schematic structures of human calpains. Black and highlighted letters indicate ubiquitous and tissue/organ-specific calpains, respectively (see also Table 453.1). Symbols: N, N-terminal region/domain (also called domain I); PC1/PC2, protease core domains 1/2 (also called domain I/II or subdomain IIa/IIb); C2L, Ca^{2+}-binding C2-domain-like domain (also called domain III); PEF(L/S), penta EF-hand domains in the large(L)/small(S) subunit (also called domain IV/VI); Gr, glycine-clustering hydrophobic domain (also called domain V); MIT, microtubule interacting and transport motif; C2, C2-domain (also called domain T); Zn, Zn-finger motif; SOH, SOL-homology domain; IQ, a motif interactive with calmodulin; NS/IS1/IS2, CAPN3/p94-characteristic sequences.

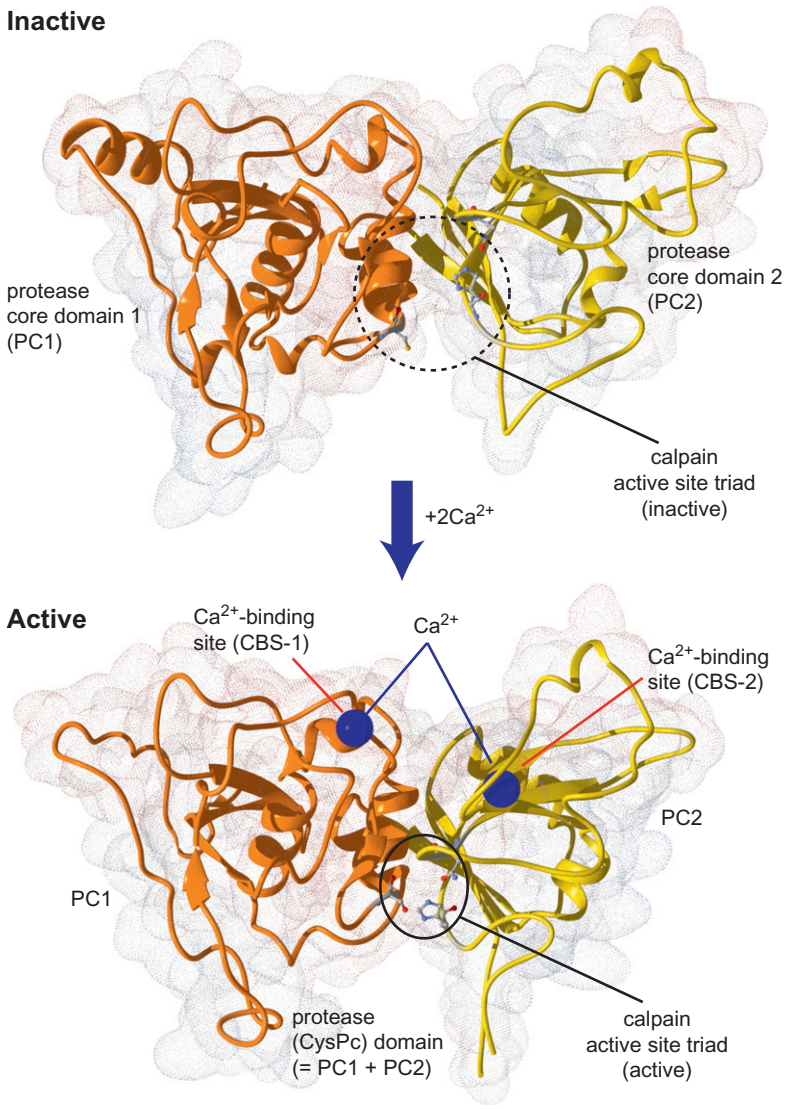


FIGURE 454.3 Schematic three-dimensional structures of inactive and active calpain CysPc domains. Schematic three-dimensional ribbon structures superimposed on the surface-type structures of the inactive (Ca^{2+}-free) and active (Ca^{2+}-bound) forms of human and rat m-calpain using PDB data, 1KFX and 3DF0, respectively [45,47]. The active protease (CysPc) domain is formed by fusion of the PC1 and PC2 core domains after each of these domains binds a single Ca^{2+}. The active site is circled in black. Blue balls represent Ca^{2+}.

and PC2 (also called domain-I and -II [44] or subdomains IIa and IIb [45], respectively) that are fused into one domain upon Ca^{2+} binding [46–49] (Figure 454.3). The active (Ca^{2+}-bound) CysPc domains of CAPN1/μCL and CAPN2/mCL have very similar structures (Root Mean Square Deviation (RMSD) = 1.0 Å) with a few exceptions (Figure 454.4; see also Chapter 455).

Surprisingly, the protease domain alone of μ- and m-calpains shows Ca^{2+}-dependent protease activity [50]. This finding is supported by a three-dimensional structural study of the protease domain in the presence of Ca^{2+}, which showed that one Ca^{2+} molecule binds to each protease core domain (Figures 454.3 and 454.4) [46–49]. These Ca^{2+}-binding sites have a novel and unique structure. Only recently was a Ca^{2+}-binding site in another molecule shown to have a similar Ca^{2+}-binding geometry to that in the CAPN1/μCL PC1 domain: the three-dimensional structure of the 'Ca^{2+}-bowl' of the high-conductance voltage- and Ca^{2+}-activated K^+ (BK or SLO1) channel; however, there is no similarity in the primary sequences of these domains [51]. When the structural information for calpains was provided only by their primary structure, their Ca^{2+}-dependency was attributed to the C-terminal penta EF-hand (PEF) domain, because only this domain consisted of known Ca^{2+}-binding motifs. However, because all the domains (PC1 and PC2 domains, C2-domain-like domain [see below], and PEF domains of both subunits) have since been found to bind Ca^{2+}, calpain now appears to be regulated in multiple and concerted ways by plural Ca^{2+} molecules.

The active-site cleft of the calpain protease domain is deeper and narrower than that of papain. Due to this constraint, calpain substrates must be in a fully extended conformation with an outstretched backbone; this was verified by three-dimensional structures of the Ca^{2+}-bound active μ-calpain protease domain co-crystallized

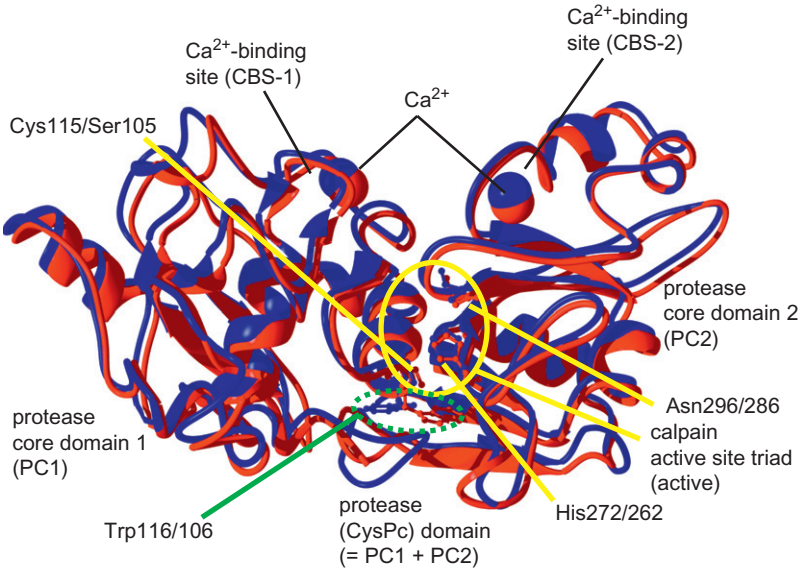


FIGURE 454.4 Schematic three-dimensional structures of the active CAPN1/μCL and CAPN2/mCL CysPc domains. Schematic three-dimensional ribbon structures of the active (Ca^{2+}-bound) forms of CAPN1/μCL (blue) and CAPN2/mCL (red) CysPc domains; their PC1 domains are superimposed (PDB data: 2ARY and 1MDW [49,93]. The active sites are circled in yellow. The two structures have very low RMSD (1.0 Å) including Ca^{2+}, but with one significant difference at Trp116 (CAPN1/μCL) and Trp106 (CAPN2/mCL) (for details see Chapter 453). The pairs of numbers indicate the residues for CAPN1/μCL and CAPN2/mCL at corresponding positions.

with leupeptin or E64 [52]. This finding explains calpain's preference for proteolyzing inter-domain unstructured regions.

The three-dimensional structure of the third domain/region, originally called domain III, consists of eight antiparallel β-strands (β-sandwich structure), a three-dimensional structure very similar to that of the C2-domains found in several Ca^{2+}-regulated proteins such as PKCs and synaptotagmins, and thus is now called the C2-domain-like (C2L) domain (see Figure 459.3). Although the primary structure of this domain is highly conserved among various calpain homologs, it has no similarity to any other known protein sequences in proteins, including those of C2-domains. Although crystallography showed no Ca^{2+} bound in this domain, biochemical analysis showed that this domain binds Ca^{2+} [53] and may play an important role in the Ca^{2+}-dependent membrane translocation of calpains.

The C-terminal PEF(L) domain of the catalytic subunits (also called domain IV) is very similar to that of the regulatory subunit, PEF(S) (also called domain VI), and each contains five EF-hand motifs. *In vitro* experiments together with three-dimensional structural studies showed that not all of these EF-hands bind Ca^{2+} [46–49,54–56]. The fifth EF-hand motif of both subunits is instead involved in heterodimer formation [57]. The entire three-dimensional structure has only been elucidated for m-calpain so far; however, μ-calpain probably has a very similar overall three-dimensional structure, as judged from the highly conserved sequences between μ- and m-calpains (see also Chapter 455).

The N-terminal domain of the regulatory subunit is a hydrophobic, Gly-rich region, therefore called the GR region (also called domain V). Most of this domain is cleaved upon calpain's activation by Ca^{2+}, indicating that it is not directly involved in calpain's protease activity.

Chicken also has μ- and m-calpains, whose catalytic subunits have protein sequences about 80% identical to their orthologs in mammals [14]. In addition to these two forms, the chicken has the intermediate-type μ/m-calpain. As in the case of the μ- and m-calpains, μ/m-calpain consists of a larger catalytic and smaller regulatory subunit. Its structure and Ca^{2+} requirements are intermediate between those of the μ- and m-calpains. The catalytic subunit of μ/m-calpain was once thought not to exist in mammals [14], but was later found to correspond to mammalian CAPN11 [16]. However, mammalian CAPN11 is expressed testis-specifically and at low levels, whereas chicken CAPN11 is ubiquitously and abundantly expressed. In contrast, the levels of chicken CAPN1 and -2 are quite low compared to their counterparts in mammals. The physiological significance of these differences remains unclear. Quail (*Coturnix coturnix*) and amphibia (*Xenopus laevis*) also have CAPN11 orthologs.

Lower vertebrates and invertebrates such as fish, insects, and schistosomes also possess homologs of these conventional calpain catalytic subunits, *e.g.* CAPN1, CAPN2, and CAPN11; however, their calpains cannot be designated as orthologs of specific calpains, because they are almost equally similar to all of the mammalian classical calpains. Therefore, plural calpain species appear to be required for these animals rather than orthologs of calpains with specific roles, suggesting they may have redundant roles. For example, mammals have 7∼9 classical calpains, fish such as *Danio rerio* have about double that, *Drosophila* and *Anopheles gambiae* have three each, and *Schistosoma mansoni* has four. Other animals, such

as nematodes and non-animal organisms such as fungi, yeasts, and plants, do not have classical calpains (see Chapter 459 for details). The classical calpains were previously called typical calpains. They, however, are not 'typical' among all living organisms, so 'typical' calpain is misleading and should not be used.

Preparation

Although μ-calpain is ubiquitously expressed in mammalian tissues, it is often prepared from relatively large tissues like skeletal muscle and spleen, in which μ-calpain is relatively abundant. Since mammalian erythrocytes do not contain m-calpain, they are sometimes used for the purification of μ-calpain [58]. Usually, successive steps of DEAE-cellulose anion-exchange column chromatography, gel filtration, phenyl-Sepharose chromatography, and Mono-Q fast protein liquid chromatography yield more than 1 mg of μ-calpain from 1 kg of rabbit skeletal muscle at a specific activity of 300−500 units/mg, representing a more than 10 000-fold purification. Some new purification methods for specific calpains using affinity column chromatography have been reported [59,60]. Recombinant μ-calpain large and small subunits have been expressed in the baculovirus/insect cell system and purified on a scale of tens of milligrams with a specific activity comparable to natural μ-calpain [61].

Biological Aspects

μ-Calpain is ubiquitously distributed in mammalian cells, strongly suggesting that it has a fundamental and essential function. Genetic studies in mice revealed that conventional calpain is essential for mammalian life, which greatly inspired researchers seeking to understand this enigmatic enzyme. In 2000, Arthur *et al.* [62] and Zimmerman *et al.* [63] independently demonstrated that disrupting the mouse gene (*Capns1*) for the conventional calpain regulatory subunit CAPNS1/30K causes embryonic lethality before E11.5. The disruption of CAPNS1/30K causes the down-regulation of both the CAPN1/μCL and CAPN2/mCL proteins, indicating that CAPNS1/30K is required for the stable presence of both calpain catalytic subunits *in vivo* and that it probably functions as an intramolecular chaperone. In contrast, *in vitro*, CAPN2/mCL alone (without CAPNS1/30K) shows full proteolytic activity after being denatured and then renatured by a long incubation with PEG or GroE [64]. In cells, unfolded calpain large subunits are probably degraded by other proteases before they take on active conformations.

Surprisingly, disrupting the mouse gene for CAPN1/μCL or CAPN2/mCL, *i.e.* *Capn1* or *Capn2*, respectively, leads to different results: *Capn2*$^{-/-}$ mice are lethal in embryo whereas *Capn1*$^{-/-}$ mice appear normal and are fertile [65,66]. This suggests that μ- and m-calpain have different functions and/or expression levels, at least at specific developmental stage(s). The growth and adhesion of *Capns1*$^{-/-}$ embryonic stem (ES) cells are not noticeably different from those of wild-type ES cells [62,63]. Cells from *Capns1*$^{-/-}$ mice have served as useful tools for unequivocally demonstrating calpain's roles in specific cellular events: Dourdin *et al.* [67] showed that calpain is required for cell migration, Mellgren *et al.* [68] showed that calpain is required for the rapid, Ca^{2+}-dependent repair of wounded plasma membrane, and Demarchi *et al.* [69] showed that calpain is required for macroautophagy.

Calpastatin is the only known endogenous inhibitor protein for the conventional calpains. It is highly effective and specific, and does not inhibit any enzyme other than calpains so far examined. Calpastatin has an inhibitor unit that is repeated four times, and each unit inhibits one calpain molecule, although the units' inhibitory efficiencies vary [70−72]. Calpastatin inhibits μ- and m-calpains with similar efficiencies. Among other calpain homologs, CAPN8/nCL-2 and CAPN9/nCL-4, but not CAPN3/p94, are inhibited by calpastatin *in vitro* [73−75].

Disruption of *Cast*, the mouse gene for calpastatin, does not produce a significant phenotype under normal, unstressed conditions [76]. This suggests that conventional calpains are not normally activated dynamically, and that calpastatin is dispensable as a safety system for calpain regulation. On the other hand, the intra-hippocampal injection of kainic acid (KA), which causes apoptotic neuronal cell death by excitotoxicity, results in significantly more DNA fragmentation in *Cast*$^{-/-}$ mice than in wild-type mice [76]. Moreover, this KA effect is reduced in transgenic (Tg) mice overexpressing calpastatin in neuronal cells, whereas Tg mice overexpressing the baculoviral caspase inhibitor p35 show no change [77]. These results indicate that KA-induced apoptotic neuronal cell death is mediated by the conventional calpains, and that caspases are not involved in this process.

Spencer and colleagues [78,79] developed Tg mice that overexpress calpastatin in the muscles. These mice appear healthy, without observable body or muscle mass changes or gross physiological, morphological, or behavioral defects. These mice were crossed with *mdx* mice (a mouse model for human Duchenne-type muscular dystrophy, DMD), which have a nonsense mutation in the dystrophin gene, *Dmd*, and show mild muscular dystrophic phenotypes. In the resulting calpastatin-overexpressing *mdx* mice, the dystrophic phenotype was significantly ameliorated [78]. Even in wild-type mice, calpastatin overexpression slows muscle atrophy during muscle unloading [79].

Studies using Tg mice have also impacted food science, *e.g.* regarding the postmortem tenderization of

muscles. Both calpastatin-overexpressing [80] and *Capn1*$^{-/-}$ mice [81] show reduced postmortem proteolysis of muscle proteins. Thus, the calpain system has drawn attention as a possible target for meat quality control.

In addition, various pathological states that may involve conventional calpains and/or calpastatin have been reported, including cataract formation, Alzheimer's disease, ischemia, inflammation, and cardiovascular disorders [40,82]. The primary cause of human DMD is a defect in dystrophin, which leads to membrane permeability and an influx of Ca^{2+} into skeletal muscle cells; this activates calpain, resulting in the degradation of muscle structural proteins. However, this mechanism is different from that of limb-girdle muscular dystrophy type 2A, which is caused by a loss of function of the skeletal muscle-specific calpain, CAPN3/p94 [83,84] (see Chapter 456).

Distinguishing Features

For Calpain portals, see The Calpain Family of Proteases (http://ag.arizona.edu/calpains/), Calpain for Modulatory Proteolysis (CaMP DB) (http://www.calpain.org/), and The Calpain Research Portal (http://calpain.net/).

Related Peptidases

See also Chapters 455–459 for accounts of m-calpain (CAPN2/mCL and CAPNS1/30K), muscle calpain (CAPN3/p94), gastrointestinal calpain (CAPN8/nCL-2 and CAPN9/nCL-4) CAPN10/Calpain-10, and other calpains, respectively.

Further Reading

For recent reviews on conventional calpain functions and various biological phenomena, see Sorimachi *et al.* [12,17], Donkor [85], Portbury *et al.* [86], Sorimachi *et al.* [87], Murphy [88], Liu *et al.* [89], Das *et al.* [90], Azuma & Shearer [91], and Dargelos *et al.* [92].

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