Chapter 12
Calpains in Health and Disease

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12.1 Introduction

Since the first report in 1964 (Guroff 1964) of a cytosolic Ca^{2+}-dependent neutral proteolytic activity, now known as calpain-1 (also called μ-calpain), other members of the calpain family of proteinases have been discovered (Croall and DeMartino 1991; Molinari and Carafoli 1997; Goll et al. 2003; Bertipaglia and Carafoli 2007; Croall and Ersfeld 2007; Sorimachi et al. 2010, 2011a, b). The calpains are defined as having amino acid (aa) sequences significantly similar to that of the protease domain of human calpain-1. Calpains also contain a variety of protein structural motifs including C2, calpain-type β-sandwich (CBSW; once called C2-like), Zn-finger and penta-EF-hand (PEF) domains. This important class of intracellular cysteine proteases is found in almost all eukaryotes and some bacteria. To date, 15 calpain genes have been identified in humans (see Fig. 12.1). However, mammalian calpain-1 and calpain-2 (also called m-calpain, calpain-II or mCANP), sometimes referred to as the "ubiquitous," "conventional," or "classical" calpains, are the best-characterized members of the calpain superfamily (Clan CA, family C02, EC 3.4.22.17). The conventional calpains have a very specific proteinaceous inhibitor *in vivo* called calpastatin. The ubiquitous calpain-1 and -2 and their natural inhibitor calpastatin are the three members of the calpain system that have been

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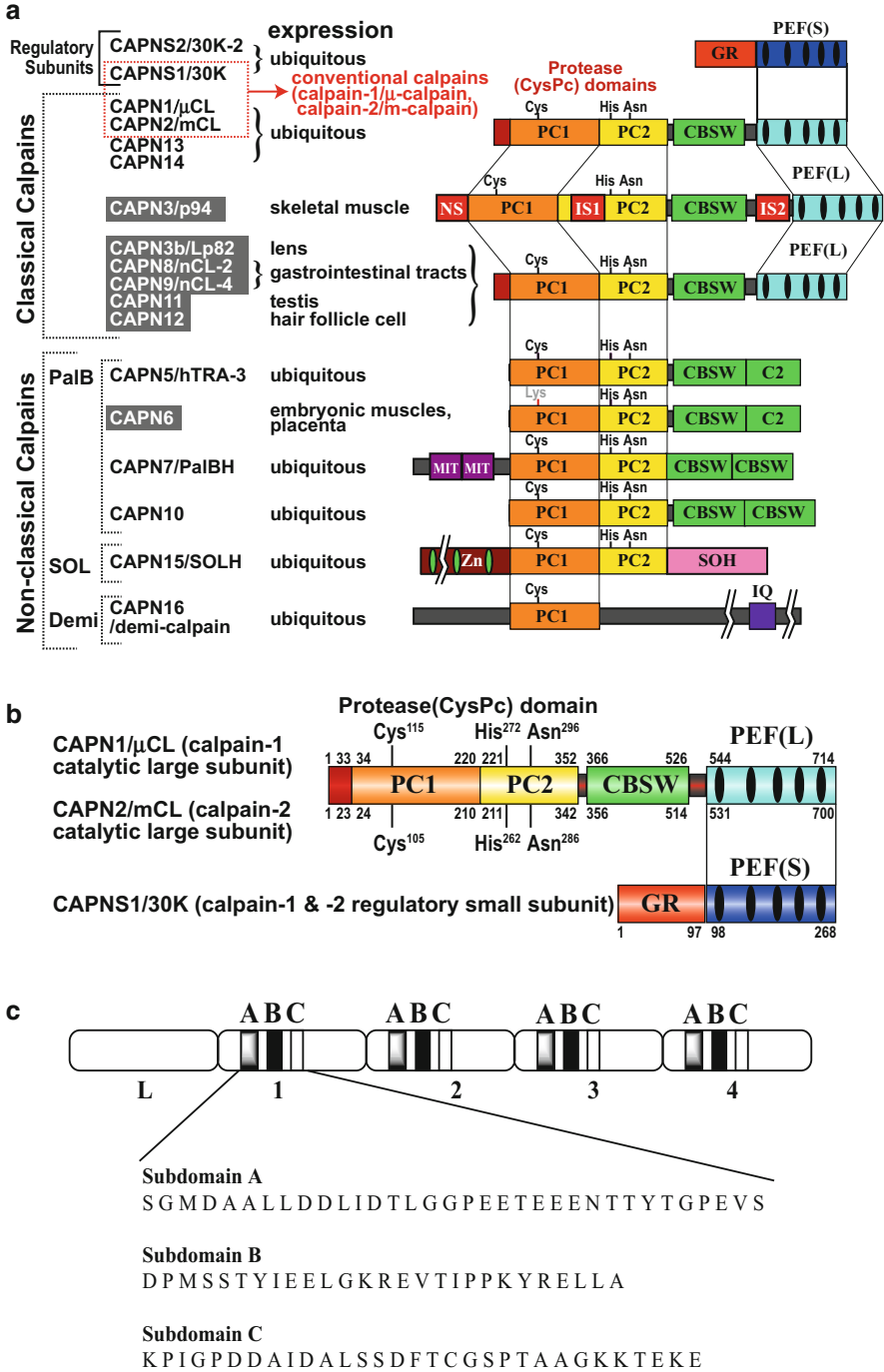
Fig. 12.1 Schematic structures of human calpains and the related molecules. (**a**) Schematic structures of 15 human calpains. Names for calpains are by the general nomenclature of gene product (*e.g.*, *CAPN1* → CAPN1) used in this review, and their previous names, if any, are shown

studied most extensively at the structure-functional level. Under normal physiological conditions, calpains exist at very low activity in cells and are proposed to participate in important cellular activities, including signal transduction, cell motility and apoptosis. However, excessive calpain activation following ischemic and traumatic cell injury or aberrant expression of components of the calpain system results in cell death or impaired cellular function. This review summarizes current knowledge of the biological significance of regulated calpain activity and the pathological consequences of uncontrolled or excessive calpain activation, with special emphasis on the pathophysiological roles of the calpains in ischemic and traumatic brain injury and some tissue-specific diseases.

12.2   Conventional Calpains

12.2.1   Calpain-1/μ-Calpain and Calpain-2/m-Calpain

12.2.1.1   Discovery and Nomenclature

Ca^{2+}-activated neutral proteinase (CANP) activity was first discovered in the soluble fraction of rat brain in 1964 (Guroff 1964). Similar observations were subsequently made in rabbit, human and chicken skeletal muscle as well as in rat liver (Meyer et al. 1964; Huston and Krebs 1968; Busch et al. 1972; Reddy et al. 1975; Kar and Pearson 1976; Takai et al. 1977; Ishuira et al. 1978). Further characterization of the biochemical properties of the Ca^{2+}-dependent proteolytic activity led to the isolation of distinct molecules of the calpain system. The cDNA for calpain was finally cloned by Ohno et al. (1984). Hundreds of calpain-related genes, including tissue-specific and non-classical forms, have been cloned and sequenced since the original cloning of the ubiquitously expressed calpain, but the gene products of a majority of them remain to be characterized. The conventional calpains are represented by two isoforms of calpains, calpain-1 and calpain-2, that are activated at neutral pH by micro- and milli-molar concentrations of Ca^{2+} *in vitro*, respectively, and are ubiquitously expressed in all eukaryotic cells examined so far (Croall and DeMartino 1991; Goll et al. 2003; Croall and Ersfeld 2007;

Fig. 12.1 (continued) after the general names. *Black* and *reversed letters* indicate ubiquitous and tissue/organ-specific calpains, respectively. Symbols: PC1 and PC2, protease core domains 1 and 2 in the calpain protease (CysPc) domain; CBSW calpain-type β-sandwich domain; PEF(L/S), penta-EF-hand domains in the large(L)/small(S) subunit; GR, glycine-rich hydrophobic domain; MIT, microtubule interacting and transport motif; C2, C2 domain; Zn, Zn-finger motif; SOH, SOL-homology domain; IQ, a motif interactive with calmodulin; NS/IS1/IS2, CAPN3/p94-characteristic sequences. (**b**) Domain architecture of human calpain-1 and calpain-2. Calpain-1 and -2 are heterodimers composed of a common regulatory small subunit (CAPNS1/30K) and each catalytic large subunit (CAPN1/μCL and CAPN2/mCL, respectively). See (**a**) for abbreviations of domain names. (**c**) Domain architecture of calpastatin

Sorimachi et al. 2010). They are also called μ-calpain and m-calpain, calpain-I and calpain II, or μCANP and mCANP, respectively. An endogenous specific inhibitor of calpain-1 and -2, called calpastatin, was later discovered in the late 1970s (Dayton et al. 1976; Suzuki and Murachi 1977; Nishiura et al. 1978). To date, 15 human calpain genes have been discovered and numbered *CAPN1–3* and *5–16*, as shown in Fig. 12.1a. μCL and mCL correspond to the gene products of *CAPN1* and *CAPN2*, and the systematic names, calpain-1 and calpain-2, have gained popularity. However, since "calpain" originally was a term for an enzyme consisting of two distinct subunits, this nomenclature is confusing and misleading. Therefore, here, the formal gene product nomenclature, *i.e.*, CAPN1 and CAPN2, is used. Accordingly, calpain-1 (= μ-calpain) is a heterodimer of CAPNS1 and CAPN1. In this review, to include the older name, CAPN1/μCL and CAPN2/mCL, is used for clarity.

12.2.1.2   Structural Features

Both calpain-1 and -2 are heterodimers, composed of a common 28 kDa regulatory subunit (CAPNS1/30K) and two distinct 80 kDa catalytic subunits (CAPN1/μCL and CAPN2/mCL, respectively). The catalytic and regulatory subunits of the conventional calpains are divided into four and two region/domains, respectively (see Fig. 12.1b). The protease (CysPc) domain is the catalytic site containing the cysteine, histidine, asparagine triad (Saido et al. 1994). In the absence of Ca^{2+}, CysPc domain is divided into protease core domains PC1 and PC2, which are folded into one domain upon Ca^{2+} binding (Hanna et al. 2008; Hosfield et al. 1999; Strobl et al. 2000). Domains PC1, PC2, CBSW, PEF(L) and PEF(S) bind at least one Ca^{2+} with varying affinities (Moldoveanu et al. 2002, 2003; Tompa et al. 2001). An extensive knowledge of the structures of the inactive Ca^{2+}-free and the active (Ca^{2+} and calpastatin bound) forms of calpain-2 is available, and has provided useful insights into the mechanism for the Ca^{2+}-dependent activation of the enzyme but the mechanism by which the protease is regulated *in vivo* is still not clear, in spite of many suggestions (Hanna et al. 2008).

The classical calpains normally exist as inactive proteases because their catalytic triad residues are not properly aligned in the Ca^{2+}-free form of the protein. It has been deduced from the crystal structures that upon binding of Ca^{2+} to specific penta-EF-hands in PEF domains and other non-EF-hand sites within PC1, PC2, and CBSW domains, multiple conformational changes occur within the protein to align the catalytic machinery of the active site (Hosfield et al. 1999, 2004; Strobl et al. 2000; Moldoveanu et al. 2002). Furthermore, site-directed mutagenesis studies have allowed the identification of multiple intramolecular sites in the NH_2-terminal peptide (residues 1–20) and the domain CBSW-PEF(L) linker peptide ("transducer") that regulate the response of calpain to Ca^{2+} binding to CysPc, CBSW, PEF(L) and PEF(S) domains (Moldoveanu et al. 2002; Hosfield et al. 2004; Bozoky et al. 2005). The exact sequence of the steps involved in the relay of the Ca^{2+} message is however unknown. On activation, calpain autolyzes by truncating the

N-terminal portion of the large subunit and most of Gly-rich (GR) domain of the small subunit. The activated form appears to have greater calcium sensitivity (Hathaway et al. 1982; Imajoh et al. 1986; Suzuki et al. 1981).

An endogenous proteinaceous inhibitor of calpain-1 and -2, called calpastatin, was discovered in the late 1970s (Dayton et al. 1976; Suzuki and Murachi 1977; Nishiura et al. 1978; Takahashi-Nakamura et al. 1981). Among the other calpain homologues, CAPN8/nCL-2 and CAPN9/nCL-4, but not CAPN3/p94, are also inhibited by calpastatin. The primary structure of calpastatin consists of a non-inhibitory L-domain and four repeating (1–4) inhibitory domains, each having an independent inhibitory activity against calpain (Emori et al. 1988) (see Fig. 12.1c). The repeating inhibitory domains have highly conserved internal subdomains A, B, and C, with subdomain B representing the calpain inhibitory moiety. A 27-mer synthetic peptide corresponding to subdomain 1B of human calpastatin has been found to exhibit potent and specific inhibition toward calpain-1 and -2 *in vitro* (Maki et al. 1989; Betts et al. 2003; Betts and Anagli 2004; Fiorino et al. 2007) and *in vivo* in a rat model of ischemic stroke (Anagli et al. 2009).

### 12.2.1.3   Biological Significance

Because the conventional calpains are ubiquitously expressed in vertebrate cells, their function is considered to be fundamental and essential. The physiological role of calpain-1 and -2 is not fully understood, but it has been linked to Ca^{2+}-regulated signaling pathways. Their involvement in the regulation of physiological processes such as apoptosis (Benetti et al. 2001; Danial and Korsmeyer 2004; Gomez-Vicente et al. 2005; Norberg et al. 2008; Sanges et al. 2006), signal transduction (Kishimoto et al. 1983; Noguchi et al. 1997; Pontremoli and Melloni 1988), membrane repair (Mellgren and Huang 2007; Mellgren et al. 2007), modulation of cell motility (Satish et al. 2005; Wells et al. 2005) and synaptic function and memory formation (see review by Liu et al. 2008a) has been described. Calpains are somewhat selective with regard to substrate selection; only 5 % of cellular proteins are degraded or fragmented by calpain-1 and -2 (Wang et al. 1989). A number of substrates have been reported for calpains, but few of them have been linked to physiological functions. These include, focal adhesion kinase (FAK) and ezrin, which are involved in fibroblast cell attachment and mobility (Potter et al. 1998; Huang et al. 2004), and phosphotyrosine phosphatase 1 B (PTP1B), which is involved in platelet aggregation (Kuchay et al. 2007). Possible substrates in the central nervous system (CNS) that might be critical to synaptic function and memory formation include αII- and βII-spectrin, the postsynaptic density-95 (PSD95) scaffolding protein, calcium- and calmodulin-dependent protein kinase II (CaMKII), and the *N*-methyl-D-aspartate (NMDA)-type and other glutamate receptors (Bi et al. 1998; Wang 2000; Liu et al. 2008b).

12.2.1.4 Pathologic Role of Calpain

Evidence accumulated over the past 15 years incriminates calpain hyper-activation in neural cell death following severe cellular challenge or damage in cerebral ischemia (Hong et al. 1994; Roberts-Lewis et al. 1994; Markgraf et al. 1998; Yokota et al. 1999; Liebetrau et al. 1999; Kambe et al. 2005; Kawamura et al. 2005; Anagli et al. 2009), traumatic brain injury (TBI) (Taft et al. 1992; Nath et al. 1996; Postmantur et al. 1996; Buki et al. 1999, 2003; Newcomb et al. 1999; Kampfl et al. 1997; Pike et al. 1998; Pike et al. 2000; Ringger et al. 2004), spinal cord injury (SCI) (Banik et al. 1997; Ray et al. 1999, 2001a, b, 2011; Schumacher et al. 2000; Ray et al. 2003; Sribnick et al. 2007; Yu et al. 2008; Zhang et al. 2003), sub-arachnoid hemorrhage (SAH) (Germano et al. 2002; Zhang et al. 2000; Wang and Yuen 1994; Lee et al. 1997; Yamaura et al. 1993) cerebral vasospasm (Minami et al. 1992), multiple sclerosis (Shields and Banik 1999; Schaecher et al. 2001, 2002; Benjamins et al. 2003; Nakanishi 2003; Shields et al. 1999), Parkinson's Disease (Crocker et al. 2003; Ray et al. 2000; Chera et al. 2002; Kim et al. 2003; Samantaray et al. 2008, 2011), Alzheimer's Disease (Veeranna et al. 2004; Chen and Fernandez 2005; Higuchi et al. 2005; Fifre et al. 2006; Rao et al. 2008; Liu et al. 2011), Huntington's Disease (Gafni and Ellerby 2002; Gafni et al. 2004; Bizat et al. 2003, 2005), and amyotrophic lateral sclerosis (ALS) (Das et al. 2008; Tradewell and Durham 2010).

Investigation of the neuronal pathobiology following cerebral ischemia (Hong et al. 1994; Chen et al. 1998; Kristian et al. 1998) and TBI (Povlishock et al. 1983, 1992; Povlishock 1992; Maxwell et al. 1997; Meaney et al. 2001) indicates that the majority of injured neurons are not damaged at the time of impact but show progressive degenerative changes over the ensuing hours or days. The initial injurious event triggers a destructive cascade of biochemical events that results in cell death within the core of the injured tissue and which spreads out into surrounding tissue over time. As the initial step, ischemic (Szatkowski and Attwell 1994; Rothman and Olney 1986) or traumatic (Hayes et al. 1992; McIntosh et al. 1998) insult induces massive release of glutamate from damaged synapses which leads to activation of glutamate receptor-associated and voltage-dependent calcium channels. Such activation induces influx of calcium ions into the neuron (Fineman et al. 1993; Pettus et al. 1994; Pettus and Povlishock 1996) and release of calcium ions from intracellular stores (Berridge 1993) (see Fig. 12.2). Loss of intracellular calcium homeostasis contributes to cell death by (1) activating various enzymes including proteases, kinases, phosphatases, and phospholipases (Siesjo et al. 1989; Wieloch et al. 1991; Morioka et al. 1992; Verity 1992; Nishida et al. 1994; Wang and Yuen 1994; Dash et al. 1995), (2) induction of free radical release (Kontos 1989; Bading et al. 1993; Hall 1998), and (3) mediating detrimental changes in gene expression (Bading et al. 1993; Rink et al. 1995). Integral to the mechanism of calcium-mediated neuronal degeneration in both ischemic (Yamashima 2000; Rami 2003) and traumatic (Kampfl et al. 1997; Newcomb et al. 1997; Posmantur et al. 1997; Saatman et al. 1996a; Wang 2000) brain injury

Fig. 12.2 Conventional calpains in disease. (**a**) Schematic of the destructive Ca^{2+}-mediated cascade that results in progressive cell damage following traumatic or ischemic brain injury. Initial injury induces massive glutamate release from damaged synapses leading to hyper-activation of NMDA and AMP/kainite receptors (NMDA-R and AMPA/KA-R) leading to influx of calcium and sodium ions. Ca^{2+} ions can also enter the cell through sodium-activated voltage-sensitive calcium channels (VSCC), via leakage through damaged cell membranes, and they can be released from internal stores. The resulting increase in intracellular Ca^{2+} levels activates a number of Ca^{2+}-sensitive proteases, including calpain. Once activated, calpain proteolyses critical cellular proteins, including the cytoskeletal protein αII-spectrin, resulting in compromise of the cell membrane and cell death. (**b**) Calpain as a therapeutic target for ameliorating brain injury. Calpain inhibitors, such as SNJ-1945 or B27-HYD, can block calpain hyper-activation following injury, thereby shutting down the destructive cascade and preserving cell integrity. Thus, calpain inhibitors are neuroprotective
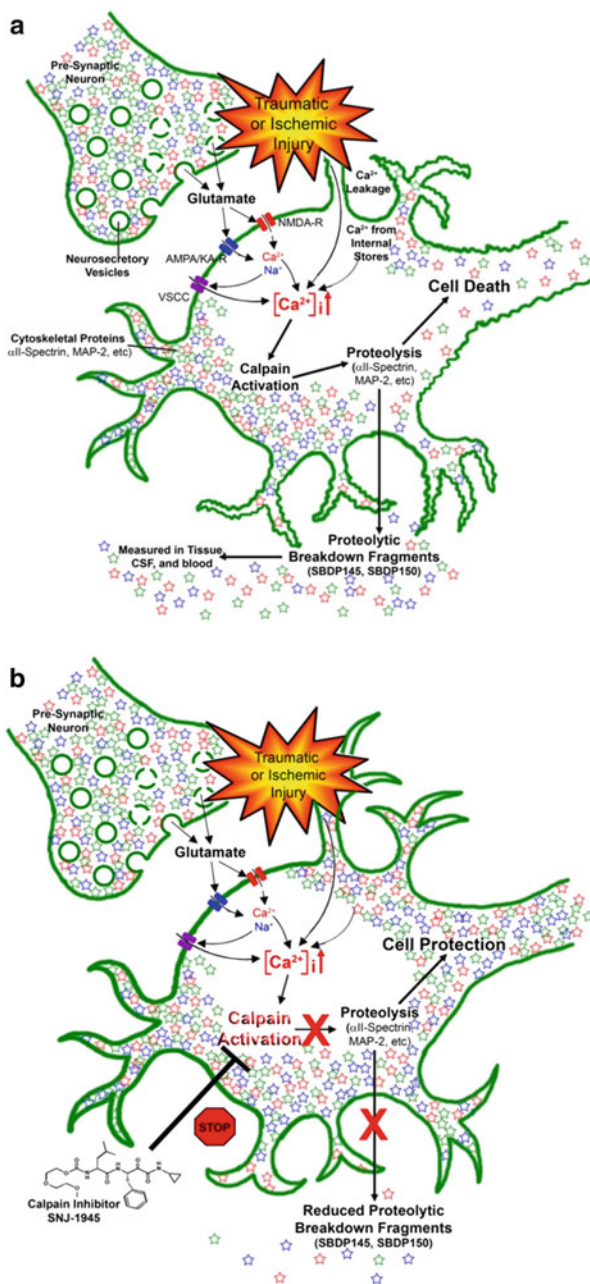
is the pathological activation of calpain-1 (CAPN1/μCL + CAPNS1/30K) and calpain-2 (CAPN2/mCL + CAPNS1/30K), which results in proteolytic destruction of many cellular proteins including receptor proteins, calmodulin binding proteins,

signal transduction enzymes, transcription factors, and cytoskeletal proteins (Wang and Yuen 1997, 1999). Such uncontrolled proteolysis can result in disruption of axonal transport and structural collapse culminating in oncotic and/or apoptotic cell death (Buki et al. 2000; Medana and Esiri 2003) (see Fig. 12.2).

#### 12.2.1.5   Conventional Calpain-Generated Biomarkers of CNS Injury

During brain injury, neural proteins or their breakdown products generated by calpains (calpain-1 and -2) are released into the extracellular environment and eventually reach the cerebrospinal fluid (CSF) in relatively high concentration (Wang et al. 2005). In due time the proteins reach the blood stream either via the compromised blood brain barrier (BBB) or *via* filtration of the CSF (see Fig. 12.3). Clearance and half life of the biomarkers contribute to the final concentration that can be measured in the blood. The CSF volume of an adult human (CSF 125–150 mL) is about 30- to 40-fold less than the blood volume (4.5–5 L) which explains why the brain biomarker concentration is significantly higher in the CSF samples versus blood samples and makes the former valuable for drug development. Enabled by recent technological advances in proteomics, novel brain injury biomarkers that have elevated levels in biofluids such as cerebrospinal fluid or blood after traumatic brain injury have been discovered (Vitzthum et al. 2005; Kobeissy et al. 2006; Liu et al. 2006; Mondello et al. 2011). The most well-studied calpain target is the cytoskeletal structural protein αII-spectrin which is cleaved by calpain into signature breakdown products of 150 kDa (SBDP150) and 145 kDa (SBDP145) (Saido et al. 1993; Nath et al. 1996; Wang et al. 1998; Pike et al. 1998; Pike et al. 2000; Zhao et al. 1999; Newcomb-Fernandez et al. 2001). Calpain-generated neural protein breakdown products (BDPs) such as SBDP150, SBDP145, c-Tau, PARP, as well as MAP2, GFAP and CRMP-2 BDPs can be used to monitor acute necrotic/oncotic neural cell death and diagnose brain injury severity and progression (Siman and Noszek 1988; Siman et al. 1989, 2004; Pike et al. 2001, 2003; Ringger et al. 2004; Liu et al. 2006, 2008a, b, 2011; Anagli et al. 2009; Zhang et al. 2009). In addition, sensitive and selective sandwich ELISAs for such biomarkers have allowed quantification of the extent of brain injury in animal studies and clinical studies of brain injury alike (Liu et al. 2010; McGinn et al. 2009; Mondello et al. 2010; Siman et al. 2009).

12.2.1.6   Calpain-Target-Based Theranostics for CNS Injury

Although brain (ischemic and traumatic) and spinal cord injury is a significant medical crisis, there are no FDA-approved therapies that have been demonstrated to improve functional outcomes. This is the case despite a large number of clinical trials with promising animal model efficacy data. Major pharmaceutical companies and biotech companies have been trying for years to tackle acute brain injury without success. New therapeutic development traditionally has an extremely

Fig. 12.3 Generation and detection of brain injury biomarkers
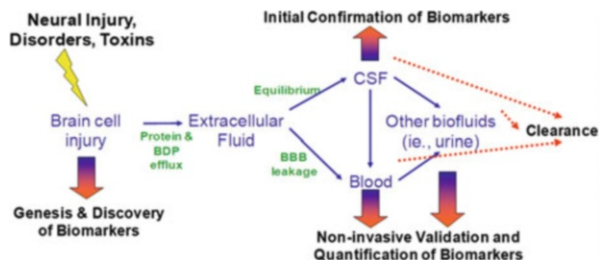
high triage rate because more than 90 % of drugs that advance to Phase I clinical trials fail. Some argue such extreme loss can be overcome by guiding all new therapeutic development and clinical trials with a disease-relevant diagnostic test. Discovery of translational biomarkers (from animal studies to clinical trials) might help to finally deliver the long sought after clinical trial success.

Since the discovery of calpain in the mid-1960s (Guroff 1964; Huston and Krebs 1968), scientists have sought methods to inhibit its activity. Early attempts used calcium chelators, but as knowledge of calpain substrate specificity grew, and inhibitors from natural products screening programs became available, more selective inhibitors were designed and synthesized (Aoyagi and Umezawa 1975; Parkes et al. 1985; Crawford 1987; Crawford et al. 1988; Anagli et al. 1991; Angliker et al. 1992; Wikstrom et al. 1992; Fukiage et al. 1997; Schroder et al. 1993; Wang and Yuen 1997; Shirasaki et al. 2005; Pietsch et al. 2010; Donkor 2011). Although synthetic calpain inhibitors have proved efficacious in *in vivo* models of TBI, SCI and cerebral ischemia they have limited therapeutic value because (1) they inhibit other cysteine and serine proteases, (2) they have poor membrane permeability, and (3) they are readily degraded *in vivo* (Wang and Yuen 1997; Yuen and Wang 1998). The endogenous calpain inhibitor, calpastatin, is the most potent and selective inhibitor identified to date (Murachi 1989; Maki et al. 1991). Other proteins, including the heavy chains of L- and H-kininogens and α_2-macroglobulin, also inhibit calpain (Crawford 1987; Salvesen et al. 1986). However, these high molecular weight inhibitors have limited therapeutic utility due to their poor cell permeability and lack of specificity. Calpain inhibition has the potential to shut down the destructive calcium-mediated cascade, thereby reducing proteolysis of essential cell proteins, resulting in cell preservation and protection (see Fig. 12.4). Previous therapeutic strategies for ameliorating the post-injury calcium-mediated destructive cascade have focused on antagonizing glutamate release, glutamate receptors, or voltage-sensitive calcium channels (Hossman 1988; Siesjo and Bengtsson 1989; Bullock and Fujisawa 1992; McIntosh 1994). These strategies have produced limited success because (1) the glutamate response occurs very rapidly after injury leaving only a narrow window for treatment efficacy, and (2) it is difficult to distinguish which of the various receptor and ion channel subclasses are primarily involved in perpetuating the cytotoxic cascade (Hossman 1988; Saatman et al. 1996b). Treatment aimed at a downstream neuropathological event could provide a longer window of opportunity for effective intervention, and therefore be
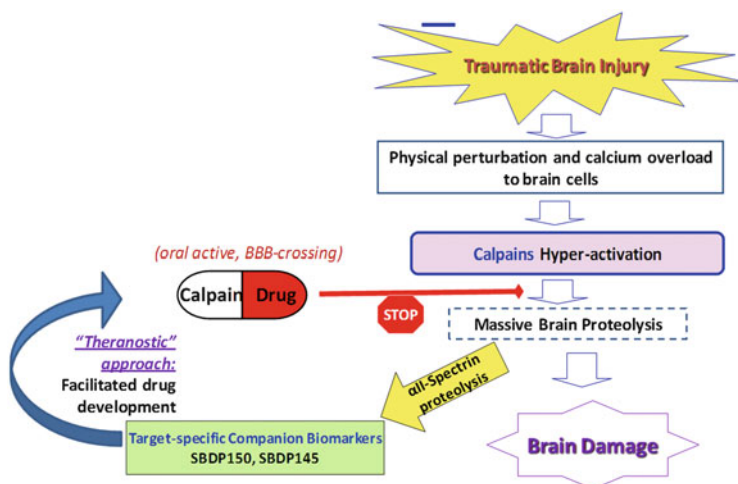
Fig. 12.4 "Theranostic Approach" to treat traumatic brain injury with a calpain inhibitor drug

Table 12.1 Unique characteristics of calpain as a therapeutic target for CNS injury

Unique characteristics of calpain as a therapeutic target for CNS injury	Translation into therapeutic advantage
Calpain has very low basal activity and is only hyperactivated by calcium overload under pathological conditions	Calpain inhibitor should have minimal adverse effects
Calpain activation is downstream from glutamate receptors	Calpain inhibition should provide an extended therapeutic window
Calpain is dually involved in both necrotic and apoptotic cell death	Calpain inhibition can suppress both acutely occurring necrosis and delayed apoptosis
Proteases as a class are well-established therapeutic targets (e.g. angiotensin converting enzyme, HIV proteases)	Calpain inhibitors have high likelihood of being formulated into molecules that can be used as human drugs

valuable for a greater number of patients. Calpain antagonists may have a further advantage over glutamate and calcium channel receptor antagonists in that calpain exists predominantly in its inactive proenzyme form under normal physiological conditions, and only becomes significantly activated under pathological conditions. Therefore, it would be reasonable to assume that calpain inhibition would not lead to any untoward adverse events. On the other hand, glutamate and calcium channel receptors play a critical neurotransmitter role in and outside of the CNS, and their inhibition could be expected to have profound side effects. Indeed, although glutamate receptors have proved to provide neuroprotection in animal models of cerebral ischemia, they have also been shown to have significant psychotomimetic effects (Olney et al. 1991). Therefore, calpain has unique characteristics as a therapeutic target for CNS injury (Liu et al. 2008a, b) (see Table 12.1). Taken together, these data suggest that brain injury is in need of a paradigm shift with

regards to therapeutic development. Calpain-target-based therapeutics and companion diagnostic biomarkers might be the missing component that helps guide successful clinical trials for brain injury.

"Theranostics" represents the convergence between Therapeutics and diagnostics. "Theranostics is the term used to describe the proposed process of diagnostic therapy for individual patients—to test them for possible reaction to taking a new medication and to tailor a treatment for them based on the test results" (Warner 2004). Theranostics encompasses the possible utilization of a wide range of procedures including: predictive medicine, personalized medicine, integrated medicine, pharmaco-diagnostics and Dx/Rx partnering. It has been viewed as the parallel use of new therapy and diagnostic tests for a human disease or disorder so as to facilitate drug development and clinical trials and to achieve optimal clinical outcomes in a population of patients. Importantly, in recognizing the emerging role of theranostic approach, FDA has recently drafted a "*Drug-Diagnostic Co-Development Concept Paper*" with the goal of setting guidelines for prospective co-development of a drug or biological therapy (drugs) and a device test in a scientifically robust and efficient way. Lastly, another advantage of the theranostic approach is its built-in ability to achieve a post-marketing personalized medicine paradigm (i.e., drug treatment could be tailored according to a patient's diagnostic biomarker profile over time. Most importantly, as proposed in this review, a novel theranostic approach that combines calpain-generated acute brain injury-tracking biomarkers with potent and selective calpain inhibitor drug candidates could fast-track and improve the chances of successful drug development for CNS injury.

12.3   Unconventional Calpains

12.3.1   Skeletal-Muscle-Specific Calpain

12.3.1.1   Discovery and Nomenclature

In 1989, in the course of the cDNA cloning of human calpain-1 and -2 catalytic large subunits (CAPN1/μCL and CAPN2/mCL, respectively), a cDNA encoding a novel molecule that was similar to but distinct from both was discovered (Sorimachi et al. 1989). In contrast to the ubiquitous expression of CAPN1/μCL and CAPN2/mCL, the identified mRNA was predominantly expressed in skeletal muscle, *i.e.*, skeletal muscle-specific calpain was the first tissue-specific calpain to be discovered. The novel calpain catalytic subunit was about 50 % identical in aa sequence to those of CAPN1/μCL and CAPN2/mCL. It had the same basic domain structure as the known calpain catalytic subunits, consisting of four region/domains: the N-terminal specific region (NS), CysPc, CBSW, and PEF domains (see Fig. 12.1a) (Ono et al. 1999; Sorimachi et al. 2010, 2011a, b; Sorimachi and Suzuki 2001).

Initially, this molecule was called p94 for its putative molecular mass (Sorimachi et al. 1989), because there was no information about its tertiary structure. Since the overall aa sequence of p94 was highly similar to those of CAPN1/μ CL and CAPN2/mCL, it was thought likely that an as-yet-unknown regulatory small subunit, similar or identical to CAPNS1/30K, would associate with p94 to make the active enzyme, which would be called a novel calpain with a new name such as n-calpain. Four years later, another novel calpain homolog was identified, clarifying the need for a method to systematize the names of the calpain-related molecules (Sorimachi et al. 1993a). For this purpose, it was proposed that novel calpains, including as-yet-undiscovered ones, would be numbered as n-calpain-1, n-calpain-2, *etc.*, where 'n' stands for novel, and p94 would be the catalytic large subunit of n-calpain-1 (for short, nCL-1). In 1990, the human genes for CAPN1/μ CL, CAPN2/mCL, p94/nCL-1, and CAPNS1/30K were identified and named *CAPN1*, *CAPN2*, *CAPN3*, and *CAPN4*, respectively (Ohno et al. 1990). In 1995, a defect of *CAPN3* was shown responsible for limb-girdle muscular dystrophy (LGMD) type 2A (LGMD2A, also called calpainopathy), and p94/nCL-1 was termed calpain 3 after the gene name *CAPN3* (Richard et al. 1995). As discussed in the conventional calpain nomenclature, the skeletal-muscle-specific calpain should now be called CAPN3. In this review, CAPN3/p94 is used.

12.3.1.2   Structural Features

The basic domain structure of CAPN3/p94 is identical to those of CAPN1/μCL and CAPN2/mCL, as shown in Fig. 12.1a, b. However, CAPN3/p94 contains three unique regions, NS, IS1, and IS2 (insertion sequence 1 and 2, respectively), which are neither found in the other calpains nor related to other peptide sequences in the databases.

The NS is a Pro-rich structure of unknown function that is cut off upon the autolysis of CAPN3/p94 (Hayashi et al. 2008; Ono et al. 2004). CysPc domain, as in other calpain homologs, is the most highly conserved. Site-directed mutagenesis identified that Cys129, His334, Asn358 are essential for CAPN3/p94's activity. IS1 is inserted into the PC2 domain.

IS2 locates between the CBSW and PEF domains and contains a nuclear-localization signal-like sequence, PXKKKKXKP. Deletion of either IS1 or IS2 suppresses autolysis, and thus stabilizes CAPN3/p94 expression. In addition, the IS2 region is necessary and sufficient for CAPN3/p94's binding to the N2A region of connectin/titin (Sorimachi et al. 1995).

The CBSW and PEF domains of CAPN3/p94 are highly homologous to those of the conventional calpains, strongly suggesting that these domains bind Ca^{2+} regardless of the Ca^{2+}-independence of CAPN3/p94's protease activity in the presence of enough Na^+ (see below). Many of the pathogenic mutations found in LGMD2A/ calpainopathy patients reside in these domains as well as the CysPc domain (Richard et al. 1999).
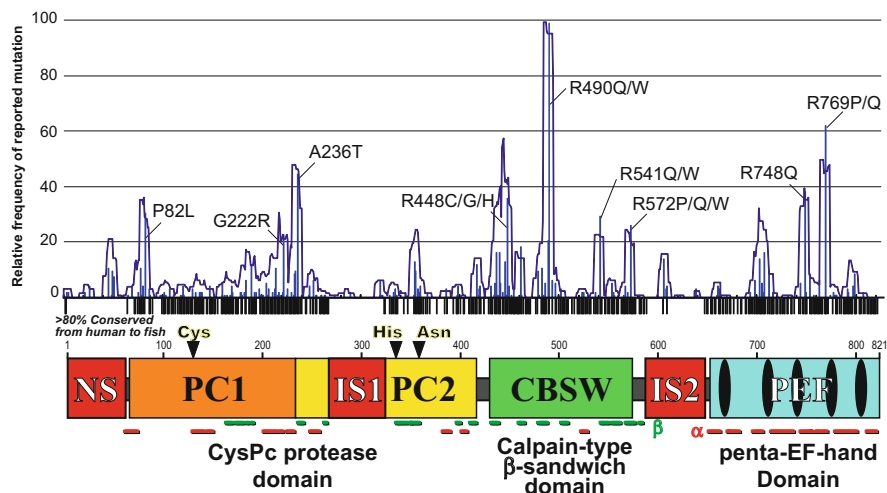
Fig. 12.5 Pathogenic *CAPN3* missense mutations and their frequencies in LGMD2A/calpainopathy patients. Frequencies are relative to the maximum value. *Black vertical lines* indicate aar that are conserved more than 80 % of vertebrates. α (*red*) and β (*green*) indicate α-helix and β-strand secondary structures of the corresponding calpain-2 3D structure. See Fig. 12.1 legend for other symbols

A comparison of the CAPN3/p94s of different vertebrates showed that more than half aa residues (aar) are more than 80 % conserved between species (Sorimachi et al. 2011a). These conserved aar include more than 70 % of the missense loci found in LGMD2A/calpainopathy pathogenic mutations (see below and Fig. 12.5). Conservation within the CAPN3/p94-characterizing regions (NS, IS1, and IS2) is low; however, it should be noted that some missense mutations, such as the R49C/H, P319L, and S606L mutations within the NS, IS1, and IS2 regions, respectively, occur relatively frequently (Sorimachi et al. 2011a) (Fig. 12.5). This observation suggests that strict sequence conservation within the CAPN/p94-characterizing regions is not necessarily required, but a few important aar, usually near the borders of these regions, must be conserved to maintain proper function.

12.3.1.3 Proteolytic Activity

The mRNA for CAPN3/p94 is expressed predominantly in skeletal muscle, about ten times more abundant than the those for CAPN1/μCL and CAPN2/mCL. For a long time, however, neither the CAPN3/p94 activity nor the protein itself could be detected in skeletal muscle extract by the isolation procedures used successfully for calpain-1 and -2. Then, expression experiments using mutated CAPN3/p94 began to reveal its unique properties (Baghdiguian et al. 1999; Benayoun et al. 2008;

Fukiage et al. 2002; Hayashi et al. 2008; Kramerova et al. 2006, 2008, 2009; Ono et al. 1998, 2004, 2010; Richard et al. 1995; Sorimachi et al. 1993b, 1995; Taveau et al. 2003; Ueda et al. 2001). These studies showed that the CAPN3/p94 protein undergoes extremely rapid autolysis (its half-life *in vitro* is less than 10 min), indicating that CAPN3/p94 has strong protease activity, which, on the other hand, makes studying it at the protein level very difficult. Moreover, this autodegradation is Na^+-dependent in the absence of Ca^{2+}, establishing CAPN3/p94 as the first example of an intracellular Na^+-dependent enzyme. Specific inhibitors for calpain-1 and -2, including calpastatin, E-64, and leupeptin, have little effect on CAPN3/p94's autolytic activity. The *in vitro* substrates of CAPN3/p94 include CAPN3/p94 itself, myotonin protein kinase, fodrin, heat-shock protein (HSP) 60, calpastatin, muscle-specific ankyrin-repeat proteins (MARPs), and connectin/titin, the gigantic filamentous muscle protein.

Although the precise *in vivo* mechanism of CAPN3/p94's regulation remains unclear, it is very likely that the activity is suppressed by the interaction of the enzyme with connectin/titin (Hayashi et al. 2008). The physiological relevance of the Na^+-dependency of CAPN3/p94 is still unclear, but its substrate specificity differs depending on whether it is activated by Ca^{2+} or Na^+ (Ono et al. 2010). One of the splicing variants of *CAPN3* encodes CAPN3:ex1–5|7–14|17–24 (called p94Δ), which lacks both IS1 and IS2, and has markedly reduced autolytic activity. Therefore, recombinant p94Δ could be purified relatively stable, and was used for enzyme characterization studies. The results showed that p94Δ has characteristics very similar to those of conventional calpains, except that it proteolyzes, and is not inhibited by, calpastatin (Ono et al. 2004).

The lens-specific splicing variant, CAPN3:ex1B|2–5|7–14|17–24 (called Lp82), also shows characteristics very similar to those of conventional calpains (Fukiage et al. 2002). Lp82 shows Ca^{2+}-dependent protease activity against βA3 and αB crystallins, and autolyzes at the N-terminus and between the CBSW and PEF domains. Lp82's activity is inhibited by E-64 and iodoacetamide (Ueda et al. 2001). Like p94Δ, Lp82 is not inhibited by calpastatin, and proteolyzes it. Some other splicing variants with structures similar to Lp82 are expressed in embryonic skeletal muscles (Fougerousse et al. 2000). These variants show a number of unique features compared with CAPN3/p94, although their physiological functions are not clear (Herasse et al. 1999; Ojima et al. 2007).

12.3.1.4 Biological Significance

In 1995, human *CAPN3* was identified as a gene responsible for LGMD2A/calpainopathy (Richard et al. 1995). The mutations, including missense point mutations, nonsense mutations, frame-shift mutations, and splice-site mutations, are widely distributed in *CAPN3*. The absence of any 'hot-spot' in *CAPN3* makes the diagnosis of LGMD2A/calpainopathy difficult (Sorimachi et al. 2011a). So far, more than 450 unique mutations have been reported in *CAPN3*. More than half of the *CAPN3* pathogenic mutations are point mutations, and the majority of these are

missense mutations, which are distributed throughout the protein at more than 175 independent loci among the 821 aar (Sorimachi et al. 2011a) (Fig. 12.5). Then, knock-out ($Capn3^{-/-}$) mice were shown to emulate a human LGMD2A/calpainopathy-like phenotype (although less severe), indicating that defects in *CAPN3/Capn3* and LGMD2A/calpainopathy have a cause-effect relationship (Kramerova et al. 2004; Richard et al. 2000). This is the first and only example so far of a clear cause-effect relationship between human disease and calpain gene mutations.

The following evidence showed that LGMD2A/calpainopathy is primarily caused by compromised CAPN3/p94 protease activity, rather than by damaged structural properties. First, it was shown that 10 pathogenic missense mutants of CAPN3/p94 all showed a deficient ability to proteolyze fodrin (Ono et al. 1998). Second, transgenic mice expressing a structurally intact but inactive CAPN3/p94: C129S protein showed an accumulation of the inactive protein and myopathic phenotypes similar to LGMD2A/calpainopathy (Tagawa et al. 2000). Finally, CAPN3/p94 knock-in ($Capn3^{CS/CS}$) mice, which express CAPN3/p94:C129S in place of wild-type CAPN3, showed a muscular dystrophy phenotype (Ojima et al. 2010). Intriguingly, however, the $Capn3^{CS/CS}$ mice showed a less severe phenotype than $Capn3^{-/-}$ mice did, indicating that the proteolytically inactive CAPN3/p94 retains some function. One of possible scenarios is that CAPN3/p94 is a novel structural components of the SR, considering its association with ryanodine receptors (Ojima et al. 2010, 2011). On the other hand, transgenic mice expressing a CAPN3/p94 splicing variant lacking exon 6 (CAPN3:ex1–5|7–24) showed muscle phenotypes that were more severe than those of the knock-out mice (Spencer et al. 2002). These observations indicate that autolytic unstability of CAPN3/p94 is related to a certain aspect of its regulatory mechanism.

Studies using $Capn3^{CS/CS}$ mice have provided some insights into the molecular mechanisms underlying the pathogenesis of LGMD2A/calpainopathy. First, CAPN3/p94 shows a stretch-dependent distribution. The amount of CAPN3/p94 at the M-line relative to that in the N2A region of myofibrils decreases as the sarcomere lengthens. This change in localization is delayed when CAPN3/p94 is inactive, as in $Capn3^{CS/CS}$ mouse muscle, and is likely to be one of the molecular mechanisms by which LGMD2A/calpainopathy develops. Second, $Capn3^{CS/CS}$ mice show an impaired ability to adapt to physical stress, accompanied by a compromised exercise-induced upregulation of MARP2 and HSPs. These findings suggest that the stretch-induced dynamic redistribution of p94, which is dependent on its protease activity, functions in the surveillance of myofibrillar conditions, and that this system is essential for protecting muscle tissue from degeneration, particularly under physical stress conditions (Ojima et al. 2011).

12.3.2   Gastrointestinal-Tract-Specific Calpains

12.3.2.1   Discovery and Nomenclature

In 1993, in the course of cDNA screening for tissue-specific calpains, a cDNA was obtained from rat stomach that encoded a novel molecule similar to but distinct from existing calpain catalytic large subunits, CAPN1/μCL, CAPN2/mCL, and CAPN3/p94 (Sorimachi et al. 1993a). The novel catalytic subunit had the same basic domain structure as these (Sorimachi et al. 2010, 2011a) (see below, Fig. 12.1a). As mentioned above, next to the first tissue-specific calpain, CAPN3/p94, which was called nCL-1 at that time, this stomach-specific calpain was designated nCL-2, and is now called CAPN8 (Sorimachi et al. 1993a). In 1997, Dear and his colleagues discovered the third novel calpain, CAPN5/hTRA-3, and called it nCL-3 in their database submission. However, nCL-3 was not used in the published report, and they named it Capn5 (Dear et al. 1997). Almost simultaneously, the fourth one was found, which was named nCL-4; it is now called CAPN9 (Lee et al. 1998).

Comparative examinations revealed that CAPN8/nCL-2 and CAPN9/nCL-4 show predominant expression in the surface mucus-secreting cells (pit cells) of the stomach, and that smaller amounts are expressed by the goblet cells in the intestines (Hata et al. 2006; Lee et al. 1998). Thus, these calpains are categorized as gastrointestinal-tract-specific calpains (Hata et al. 2010). Their physiological functions and mode of action were unclear until 2010, when they were shown to form a complex with each other in the stomach and to function in gastric mucosal protection against stress-induced ulcer (Hata et al. 2010). For CAPN8/nCL-2 and CAPN9/nCL-4 to be proteolytically active, they must form a complex; this complex is called G-calpain (G stands for gastric). After calpain-1 and -2, G-calpain was the third mammalian calpain enzyme complex shown to function *in vivo*, and the first to be composed of two different calpain catalytic subunits.

12.3.2.2   Structural Features

The basic domain structures of both CAPN8/nCL-2 and CAPN9/nCL-4 are identical to those of CAPN1/μCL and CAPN2/mCL, as shown in Fig. 12.1a. In cultured cells, CAPN9/nCL-4 is active only in the presence of CAPNS1/30K, and, therefore, is probably similar to, if not exactly the same as, those of the conventional calpains (CAPN1/μCL or CAPN2/mCL + CAPNS1/30K). CAPN8/nCL-2, however, is active without CAPNS1/30K *in vitro*, and shows homo-oligomerization *via* the CBSW domain (see below). Some C2-domain-like (β-sandwich) structures are known to form homo-oligomers (Jones et al. 1989; Lu et al. 2010), and the CAPN8/nCL-2 homo-oligomer may have a structure similar to one of these.

In contrast, these calpains show rather different characteristics *in vivo*. Endogenous CAPN8/nCL-2 and CAPN9/nCL-4 in mouse stomach form a hybrid complex,
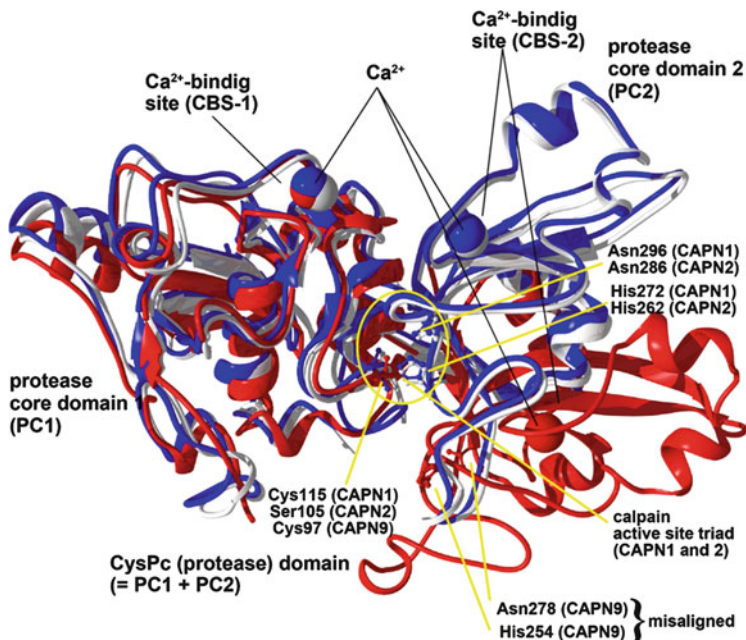
Fig. 12.6 Superimposed schematic 3D structures of CysPc domains of the active CAPN1/μCL, CAPN2/mCL, and CAPN9/nCL-4. Schematic 3D ribbon structures of the active (Ca²⁺-bound) forms of CysPc domains of CAPN1/μCL (*blue*), CAPN2/mCL (*white*), and CAPN9/nCL-4 (*red*); their PC1 domains are superimposed by Deli server (PDB data: 2ARY, 1MDW, and 1ZIV) (Davis et al. 2007; Moldoveanu et al. 2003). The active sites are *circled* in *yellow*. PC1 subdomains of the three CysPc domains fit very well (Root-Mean-Square Deviation (RMSD) = 1~4 Å). CAPN1/μCL and CAPN2/mCL show overall fitting with very low RMSD (1.0 Å) including Ca²⁺, but with one significant difference at Trp116 (CAPN1/μCL) and Trp106 (CAPN2/mCL). On the other hand, the PC2 domains of CAPN1/μCL and CAPN9/nCL-4 are markedly displaced. Note that, in the CAPN9/nCL-4 structure, His254 and Asn278 are too far away from Cys97 to form an active triad

G-calpain, and neither CAPN8/nCL-2 nor CAPN9/nCL-4 forms a stable complex with CAPNS1/30K (Hata et al. 2010). Gel filtration analysis of the endogenous mouse stomach G-calpain showed a molecular mass of about 180,000, indicating that G-calpain may contain other small subunit(s) of up to 20,000 (Hata et al. 2010).

The 3D structural analysis of the active (Ca²⁺-bound) protease domains of CAPN2/mCL and CAPN9/nCL-4 as well as CAPN1/μCL revealed locally distinct structures. Compared with the structure of CAPN1/μCL, that of CAPN2/mCL has a reversible intrinsic silencing mechanism by Trp106 (Moldoveanu et al. 2002, 2003) (Fig. 12.6). In contrast, the structure of CAPN9/nCL-4 shows that its activity can be auto-inhibited by misalignment of the catalytic triad, mediated by large intra-domain movements (Davis et al. 2007) (Fig. 12.6). These findings indicate that, in addition to the common activation mechanism, each calpain family member evolved molecule-specific one, even though they share high levels of sequence conservation.

12.3.2.3   Activity and Substrates

Of the human calpains, CAPN8/nCL-2 shows the highest similarity (~60 % identity in aa sequence) to CAPN2/mCL. However, unlike calpain-2 (CAPN2/mCL + CAPNS1/30K), recombinant CAPN8/nCL-2 expressed in *Escherichia coli* exhibits Ca^{2+}-dependent activity without CAPNS1/30K, and forms a homodimer~oligomer *via* its CBSW domain *in vitro* (Hata et al. 2007). Recombinant mouse CAPN8/nCL-2 shows half-maximal activity at approximately 0.3 mM, which is similar to calpain-2 (Hata et al. 2007). Its autolytic activity in the presence of Ca^{2+} is very rapid, with more than 80 % of the protein being cleaved within 30 s (Hata et al. 2007). Several autolytic sites have been identified in the N-terminal anchor helix region, and in the CBSW and PEF domains (Hata et al. 2007). Although most of these features are unique to CAPN8/nCL-2, their physiological relevance remains unclear. On the other hand, recombinant human CAPN9/nCL-4 requires CAPNS1/30K for its activity *in vitro*, and the activity of this complex is Ca^{2+}-dependent, with half-maximal activity at approximately 0.1 mM Ca^{2+} under the optimal condition (Lee et al. 1999). The activity of both CAPN9/nCL-4 and CAPN8/nCL-2 is inhibited by calpastatin and other cysteine protease inhibitors, similar to the conventional calpains (Hata et al. 2007; Lee et al. 1999).

The β-subunit (β-COP) of the coatomer complex, which is involved in retrograde membrane trafficking from the Golgi to the endoplasmic reticulum, is expressed in the stomach pit cells, and was shown to be proteolyzed by CAPN8/nCL-2 in vitro (Hata et al. 2006). Furthermore, β-COP and CAPN8/nCL-2 co-expressed in COS7 cells co-localize to the Golgi, and Ca^{2+}-ionophore stimulation causes limited proteolysis of β-COP near the linker region after Ser528 (as in NP_057535). This cleavage probably results in the dissociation of β-COP from the Golgi, strongly suggesting that CAPN8/nCL-2 is involved in the membrane trafficking of mucus cells via interactions with coat protein.

12.3.2.4   Biological Significance

Xenopus laevis has a CAPN8/nCL-2 orthologue named xCL-2, which causes severe developmental defects when disrupted (Cao et al. 2001). CAPN9/nCL-4 was reported to be down-regulated in gastric cancer (Yoshikawa et al. 2000), and is involved in the formation of a lumen by breast epithelial cells induced by carcinoembryonic antigen-related cell adhesion molecule 1 (Chen et al. 2010).

In mouse stomach *in vivo*, the disruption of either mouse *Capn8* or *Capn9* causes a down-regulation of the other gene product, similar to the effect of $Capns1^{-/-}$ on the levels of CAPN1/μCL and CAPN2/mCL. This suggests a co-dependency in terms of the stability and functionality of both gene products. This point is supported by the finding that the residual CAPN9/nCL-4 in $Capn8^{-/-}$ mice does not display autolytic activity, and *vice versa* (Hata et al. 2010). However, $Capn8^{-/-}$ and $Capn9^{-/-}$ mice appear healthy under normal conditions. Nevertheless, they are

significantly more susceptible to ethanol-induced gastric ulcers (Hata et al. 2010). CAPN8/nCL-2 knock-in (*Capn8$^{CS/CS}$*) mice expressing a protease-inactive CAPN8/nCL-2:C105S mutant also show stress-induced gastropathy, indicating that CAPN8/nCL-2 and CAPN9/nCL-4 (in the form of G-calpain) mediate key components of gastric mucosal defense (Hata et al. 2010).

Gastric mucosal defense is a complex process involving mucus secretion and the migration of the pit cells that differentiate from stem-cell progenitors. Considering that β-COP is a substrate for G-calpain, it might be involved in the regulation of mucus secretion. On the other hand, the expression of CAPN8/nCL-2 in cranial neural crest cells, where it is involved in cell motility, is regulated by ADAM13, a transmembrane metalloprotease containing a disintegrin domain (Cousin et al. 2011), suggesting that G-calpain is involved in pit cell migration.

In another study on the physiological functions of G-calpain, a single nucleotide polymorphism (SNP) database search showed that human *CAPN8* and *CAPN9* contain several SNPs that result in aa substitutions (Hata et al. 2010). *In vitro* expression experiments showed that the G-calpain variants resulting from these SNPs have compromised proteolytic activity, suggesting that individuals expressing certain *CAPN8* and *CAPN9* variants may suffer from gastrointestinal dysfunction.

12.3.3 Other Calpains

12.3.3.1 PalB Homologs

PalB was first identified in *Emericella* (*Aspergillus*) *nidulans* as a product of the gene responsible for the fungus's adaptation to alkaline conditions (Denison et al. 1995). Subsequently, its orthologs were identified in *S. cerevisiae* (Rim13/Cpl1) and humans (CAPN7/PalBH) (Futai et al. 1999; Sorimachi et al. 2011a). *E. nidulans*, like many other microorganisms, grows over a wide pH range. The *palB* gene locus was identified as one of the *pal* genes that show defective alkaline adaptation when disrupted. PalB is involved in the proteolytic activation of the PacC transcription factor, a key regulator of pH-dependent gene expression. Here, we refer to this pH adaptation system as the Pal-PacC pathway.

The Pal-PacC pathway contains *palA*, *palB*, *palC*, *palF*, *palH*, *palI*, and *pacC*. PalF is a distant homolog of mammalian arrestin and binds to the large cytosolic domain of PalH. The phosphorylation and ubiquitination of PalF under alkaline conditions depend on PalH and PalI, suggesting that pH-sensing is mediated *via* the interaction between PalF and PalH (similar to that observed for the arrestin receptor, which regulates various processes in mammals, such as photo-sensing). PacC usually adopts an inactive conformation during intramolecular interactions, and is activated by proteolytic removal of its C-terminus. PalB is primarily responsible for the processing of PacC in response to increased pH, which is followed by further proteolysis by the proteasome to yield the active form.

The Pal-PacC pathway is well conserved in yeasts. The yeast calpain, Rim13/Cpl1, was identified as a product of the *RIM* genes. Like PacC and PalB, Rim101 is proteolytically regulated by Rim13/Cpl1 (Futai et al. 1999), although in this case the proteasome is not involved. Under normal conditions, the environment for *Saccharomycetes* is rather acidic and this adaptive system (the Rim pathway) functions in near-neutral pH conditions. One example of a biological event in which the Rim pathway plays a role is the infection and invasion of mammalian skin by pathogenic and saprophytic yeasts such as *Candida albicans* and *Yarrowia lipolytica* at neutral pH. Disrupting the Rim pathway compromises the pathogenicity of these organisms (Mitchell et al. 2007).

The Pal-PacC and Rim pathways relate to membrane trafficking and involve the ESCRT (endosomal sorting complex required for transport) and Vps (vacuolar protein sorting) proteins. The Pal-PacC and Rim pathways are the first examples of genetic studies that are being used to thoroughly elucidate the molecular components and mechanisms underlying calpain-mediated systems (Hayashi et al. 2005; Rodriguez-Galan et al. 2009). These pathways also exemplify the fact that calpains function as modulator proteases, affecting the function of their substrates through limited proteolysis. Theoretical extension of the knowledge of these pathways is anticipated to provide important keys to understanding other calpain systems, especially those that involve CAPN7/PalBH (Osako et al. 2010).

12.3.3.2 The TRA-3 Homologs

TRA-3/CLP-5 was first identified as the product of one of the genes involved in the sex determination cascade of *C. elegans*. The Ca^{2+}-dependent protease activity of TRA-3/CLP-5 is necessary for the processing of TRA-2A, which is required for female development in XX hermaphrodites. On another front, both TRA-3/CLP-5 and CLP-1 are components of a neuronal necrotic death cascade, which acts upstream of the aspartic proteases, ASP-3 and ASP-4, orthologs for mammalian cathepsins D and E (Syntichaki et al. 2002). In addition, an SNP in *tra-3* is reported to be involved in nematode body-size determination (Kammenga et al. 2007).

Mammals have two TRA-3 orthologs, CAPN5/hTRA-3 and CAPN6 (Sorimachi et al. 2011a). CAPN5/hTRA-3 has Ca^{2+}-dependent autolytic activity and is sensitive to several calpain inhibitors (Waghray et al. 2004). *CAPN5* is expressed at varying levels in almost all tissues. Analysis of $Capn5^{-/-}$ mice shows that CAPN5/hTRA-3 is expressed by a subset of T cells, but is not required for development. SNPs within CAPN5/hTRA-3 are associated with polycystic ovary syndrome, diastolic blood pressure, and cholesterol levels (Saez et al. 2007), and expression of both *Capn5* and *Capn2* is upregulated in caerulein-induced acute pancreatitis in mice (Nakada et al. 2010).

CAPN6 proteins expressed in eutherians (placental mammals) and schistosomes have a naturally occurring aa substitution at the most important residue in the active site triad (Cys->Lys in humans); strongly suggesting that these CAPN6 proteins have no proteolytic activity. Interestingly, CAPN6 proteins expressed in

marsupialia and birds retain all the active-site residues. Moreover, frogs and fish express three TRA-3 homologs, all of which retain the active-site residues (Sorimachi et al. 2011a). Mammalian CAPN6 is predominantly expressed in embryonic muscles, placenta, and in several cultured cell lines. CAPN6 is involved in regulating microtubule dynamics (Tonami et al. 2007) and motility (Tonami et al. 2011) *in cellulo*, although the *in vivo* physiological functions of CAPN6 are still unclear (Tonami et al. 2013).

12.3.3.3 The CAPN10 Homologs

A large-scale genetic association study identified an SNP within intron 3 of *CAPN10* that is linked to susceptibility to non-insulin-dependent diabetes mellitus (NIDDM, or type 2 diabetes) (Horikawa et al. 2000), although there is no clear molecular explanation for the reason. *Capn10* is also a candidate gene responsible for the NIDDM phenotype in the Otsuka Long-Evans Tokushima Fatty (OLETF) rat. Quantitative trait locus (QTL) analyses using $Capn10^{-/-}$ mice and two other strains with low and high obesity phenotypes (LG/J and SM/J) show that *Capn10* is a component of the obesity QTL, *Adip1*. This indicates that CAPN10 is involved in obesity in mice (Cheverud et al. 2010).

Studies using Capn10−/− or CAPN10-overexpressing mice suggest that CAPN10 is involved in type 2 ryanodine receptor-mediated apoptosis (Johnson et al. 2004). Although phenotype of Capn10−/− mice was not described, they are not embryonic lethal. CAPN10 is ubiquitously distributed and its cellular localization is dynamic. When localized in the mitochondria, it mediates mitochondrial dysfunction by cleaving Complex I subunits and promotes mitochondrial permeability transition (Arrington et al. 2006). CAPN10 is also involved in GLUT4 vesicle translocation during insulin-stimulated glucose uptake in adipocytes (Paul et al. 2003).

12.3.3.4 The SOL Homologs

The first genetic calpain study identified the *Drosophila* gene, *small optic lobes* (*sol*), in 1991 (Delaney et al. 1991). A mutation in *sol* results in the absence of certain classes of columnar neurons from the optic lobes, the differentiation and/or survival of which is dependent on the protease activity of SOL. SOL comprises an N-terminal six Zn-finger motifs and C-terminal CysPc domain (Delaney et al. 1991; Sorimachi et al. 2011a). Unfortunately, there are no other reports analyzing the molecular mechanisms involved in this process. Mammals express a single SOL ortholog, CAPN15/SOLH, but its physiological role remains unclear. In parallel with the PalB subfamily, the SOL subfamily (including their mammalian homologs) is a group of evolutionarily interesting molecules that warrant further study.

12.3.3.5 Phytocalpains

A plant calpain, phytocalpain, was first identified from the sugarcane expressed sequence tag database (Correa et al. 2001). Subsequently, many other phytocalpains were identified from dicotyledons, monocotyledons, and gymnospermae. A genetic study identified phytocalpain as the *defective kernel 1* (*dek1*) gene product, DEK1, which is required for aleurone cell development in the maize endosperm (Lid et al. 2002).

DEK1 contains a potential signal peptide sequence followed by possible three 7-trans-membrane (TM) regions, and CysPc and CBSW domains (Lid et al. 2002; Sorimachi et al. 2011a). Although the CysPc domain of DEK1 is divergent from mammalian calpains, the C-terminal CBSW domain is significantly similar to that of classical calpains such as CAPN1/μCL, suggesting that DEK1 consists of evolutionarily-different modules. Recombinant maize DEK1 CysPc-CBSW fragment show Ca^{2+}-activated caseinolytic activity, depending on the predicted active site Cys residue. DEK1 is the only calpain homolog in the *Arabidopsis* genome, and it is important for regulating growth in this plant. Although the full-length DEK1 protein localizes to membranes, intramolecular autolytic cleavage releases the CysPc-CBSW domains into the cytoplasm. These domains are sufficient to fully complement *dek1* mutants (Tian et al. 2007).

12.3.3.6 Other Calpain Members

Leishmania and *Trypanosoma* each expresses around 20 calpain homologs, which likely contribute to cell morphogenesis, drug resistance, and stress-response mechanisms (Olego-Fernandez et al. 2009; Sangenito et al. 2009). Some trypanosome calpains have N-terminal domains with weak similarity to calpastatin. As described above, some calpain homologs show substitutions in one or more of the well-conserved active-site triad residues. This non-proteolytic family of calpain homologs includes eutherian and schistosome CAPN6, several of the schistosome and nematode calpains, insect CALPC, and all the *Trypanosoma* homologs (Croall and Ersfeld 2007; Sorimachi et al. 2011a). The evolutionary background for the occurrence of these calpain species is quite interesting, and elucidating their physiological functions will expand our knowledge regarding the functions of the calpain superfamily, *e.g.*, possible non-proteolytic functions (Ojima et al. 2011).

12.4 Concluding Remarks

Here we reviewed pathological and biological situations where involvement of calpains has gained great interest from multiple angles. For example, conventional calpains (calpain-1 and -2) are involved in neurodegeneration, as well as producing

several well documented proteolytic biomarkers that can help track brain injury progress, severity and as a theranostic tool for drug development. In addition, other calpains such as tissue-specific calpains (CAPN3 in skeletal-muscle, and CAPN8 and CAPN9 in gastrointestinal tract) and calpain homologs originally identified in non-mammals represent various physiological, cellular and biochemical pathways.

Although many studies suggest that activation of calpain, especially the conventional ones, is involved in pathophysiological processes as an exacerbating factor, it is absolutely false to recognize calpain as an existence for making diseases worse. Genetic manipulations causing constitutive and conditional disruption of *Capn2* in mice result in embryonic lethality and cardiac hypertrophy, respectively. In other words, calpain could be a two-edged sword in that its activity is indispensable for certain aspects of life, yet, its regulation gets somehow fragile within the cells undergoing pathogenic chaos.

Therefore, in addition to investing our efforts in developing novel theranostic approaches aimed at the conventional calpains as useful promising targets for biomarkers and inhibitory drugs, further application fields should be simultaneously pursued with the view that some pathological contexts may be related to insufficient conventional calpain activity. In these cases, appropriate calpain activators, rather than inhibitors, will be candidate drug targets to be developed. In fact, genetic inactivation of tissue-specific calpains causes disease states, indicating that activators/stabilizers for these calpains, if developed, may be a drug for these tissue-specific diseases such as muscular dystrophy and gastropathy.

To date, only the conventional calpains' over-activation has been associated with diseases. It is, however, equally possible that an inappropriate activation of other calpains including tissue-specific ones is laterally or centrally linked to disease aggravation. In any case, calpain protease research is a rich area for both basic research as well as implicational research in biomedical science. The open question is how to extract and profile its activity with a resolution that is consistently competent for both normal and disease conditions.

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