REVIEW ARTICLE

Structure and physiological function of calpains

Hiroyuki SORIMACHI[1], Shoichi ISHIURA and Koichi SUZUKI

Laboratory of Molecular Structure and Function, Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

For a long time now, two ubiquitously expressed mammalian calpain isoenzymes have been used to explore the structure and function of calpain. Although these two calpains, μ- and m-calpains, still attract intensive interest because of their unique characteristics, various distinct homologues to the protease domain of μ- and m-calpains have been identified in a variety of organisms. Some of these 'novel' calpain homologues are involved in important biological functions. For example, p94 (also called calpain 3), a mammalian calpain homologue predominantly expressed in skeletal muscle, is genetically proved to be responsible for limb-girdle muscular dystrophy type 2A. Tra-3, a calpain homologue in nematodes, is involved in the sex determination cascade during early development. PalB, a key gene product involved in the alkaline adaptation of *Aspergillus nidulans*, is the first example of a calpain homologue present in fungi. These findings indicate various important functional roles for intracellular proteases belonging to the calpain superfamily.

INTRODUCTION

Recently, the biological importance of various proteinases in cellular function has been emphasized. The processing rather than digesting activity of these enzymes makes it possible to modulate directly the activities and/or functions of other proteins both precisely and irreversibly. Calpain (EC 3.4.22.17; Clan CA, family C02) is one such modulating intracellular proteinase. One of the features of calpain that attracts investigation is its structure: a cysteine-proteinase domain is combined with a calmodulin-like Ca^{2+}-binding domain, as described below in detail (see Figure 1; for other reviews of calpain, see [1–5]). In fact, the activities of the representative 'conventional' mammalian calpains, μ- and m-calpains (also called calpains I and II respectively), are regulated by Ca^{2+} concentration. Thus calpain is considered to participate in various intracellular signalling pathways mediated by Ca^{2+}. The precise functions of calpain *in vivo*, however, have not yet been clearly identified. The ubiquitous and constitutive expression of mammalian μ- and m-calpains strongly suggests that they are involved in basic and essential cellular functions. This may be one of the reasons why we cannot specify precisely the physiological functions of calpain.

The existence of a Ca^{2+}-dependent neutral proteinase in rat brain was first reported in 1964 [6,7] and identified as a proteinase identical with 'calpain' in 1968 [8]. Calpain was re-identified as a Z-line hydrolysing enzyme and a protein kinase C-activating factor by Goll's and Nishizuka's groups respectively [9,10]. Calpain was first purified to homogeneity in 1978 [11] and, in 1984, the cDNA for the large subunit of a chicken calpain, which is now recognized as a type intermediate between the μ- and m-types (called the μ/m-type) [12], was cloned for the first time [13]. This was followed by the determination of the whole structure of the large subunit. Since then, various types of calpain subunits and their homologues have been identified and their primary structures determined by cDNA cloning [12–41]. It was later agreed that the enzyme would be called 'calpain' following the recommendations made at the International Conference on Intracellular Protein Catabolism in 1991 [42,43].

Some calpain homologues in mammals have been found to be predominantly expressed in a limited number of organs, in contrast with the ubiquitous expression of the 'conventional' μ- and m-calpains. These 'tissue-specific' calpains, such as skeletal-muscle-specific 'p94' (also called 'nCL-1' or 'Calpain 3') and stomach-specific 'nCL-2' and 'nCL-2′', are probably closely related to the specific functions of the organs in which they are predominantly expressed.

Various 'atypical' homologues of calpain are now also being reported in lower organisms such as insects, nematodes, fungi and yeast. These contain a cysteine-protease domain of about 30 kDa that shows more similarity to the mammalian conventional calpain large subunit than to other cysteine proteinases such as papain or cathepsins. However, most of these homologues are atypical in that their other domains do not necessarily resemble those of conventional calpain large subunits. Instead, they possess unique domains possibly responsible for any specific functions they may possess.

Thus a superfamily of homologues of the calpain large subunit has now been identified, as shown in Figure 1. Members of the calpain superfamily characteristically contain homologous protease domains. In other words, these molecules form an evolutionary group distinct from other cysteine proteinases such as papain, cathepsins, caspases, etc. [44], suggesting evolutional conservation of the function(s) of this proteinase superfamily. Moreover, as shown in Figure 2, calpain small subunits (30K) also form a superfamily distinct from other Ca^{2+}-binding proteins such as calmodulin [45,46]. In the present Review Article we

Abbreviations used: ICE, IL-1β-converting enzyme; μCL, μ-calpain large subunit; mCL, m-calpain large subunit; nCL-2, novel calpain-2 large subunit; 30K, calpain small subunit; LGMD, limb-girdle muscular dystrophy; Z-, benzyloxycarbonyl-; -MCA, -4-methylcoumaryl-7-amide; PEG, poly(ethylene glycol); IL-6, interleukin-6; DMD, Duchenne-type muscular dystrophy; CMD, congenital muscular dystrophy; EST, expressed-sequence-tag; PBH, PalB homologous domain; SoH, *sol* homologous region; for brevity the systematic names of various calpain inhibitors are given only in the text.

[1] To whom correspondence should be addressed.

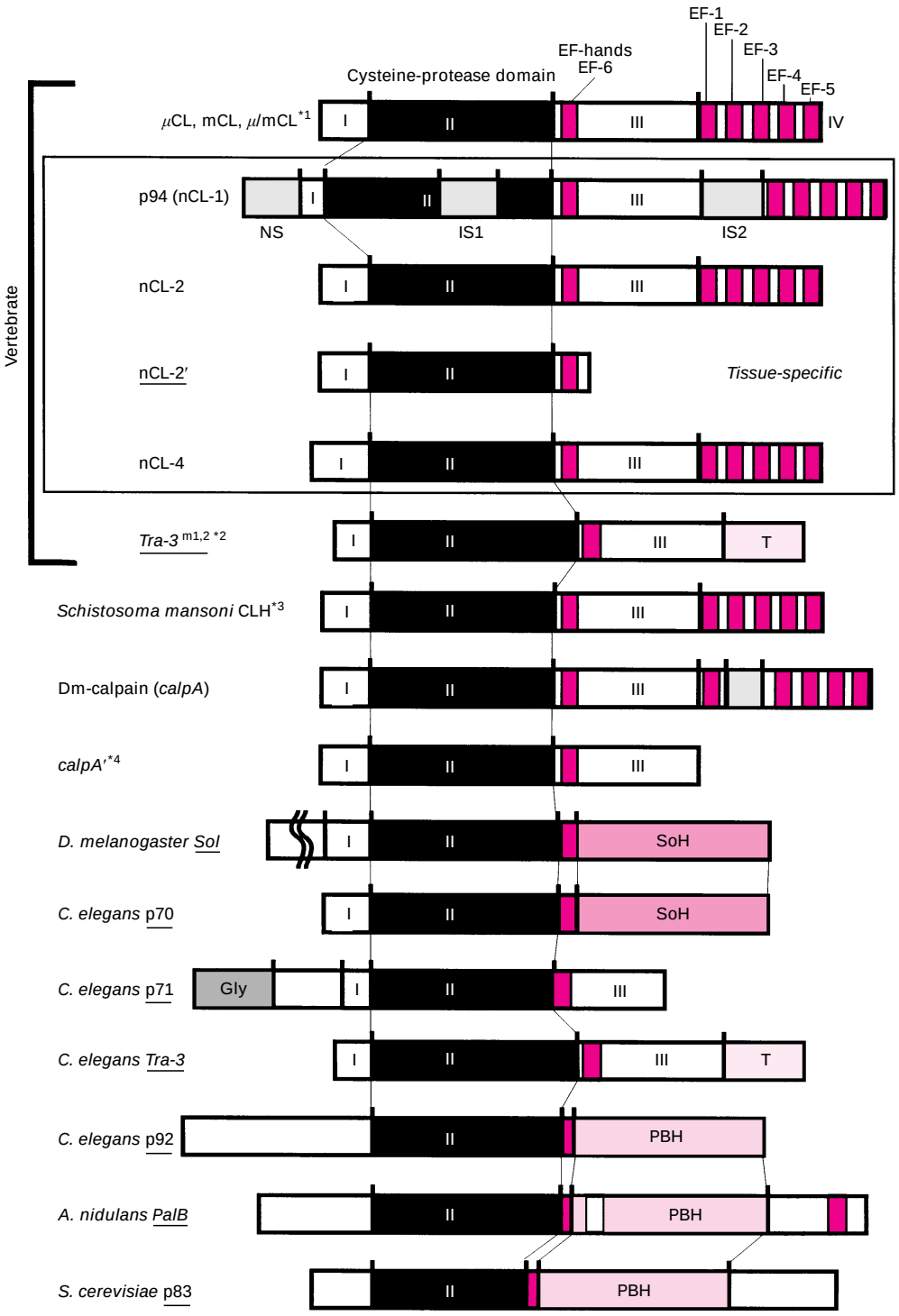
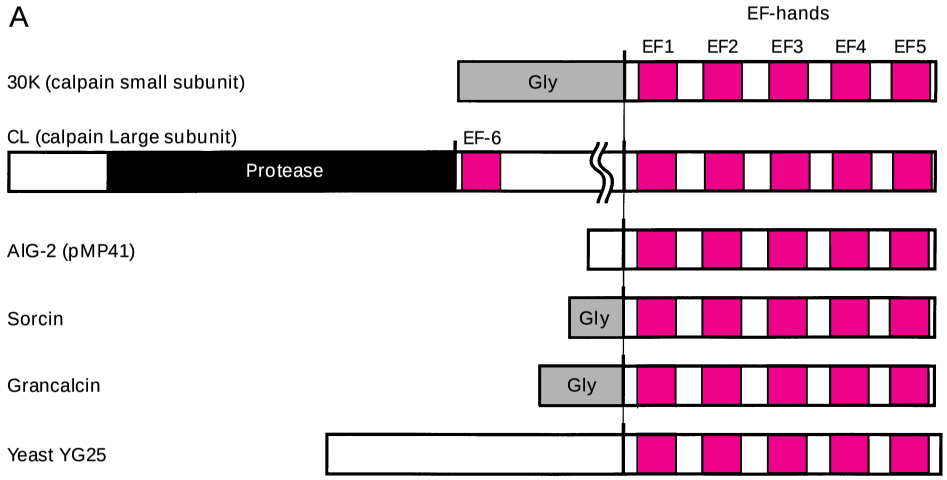


Figure 1 Schematic structures of the large subunit of the calpain superfamily

II is the cysteine-protease domain. I, III, IV and T are domains I, III, IV, and T respectively. Notes: *1μ/mCL was identified in chicken only; *2corresponding to two mammalian Tra-3 homologues (accession nos. Y10552 and AJ000388; see the text for details); *3calpain-large-subunit homologue; *4corresponding to the deduced amino acid sequence of the shorter transcript of CalpA. EF-hands are shown in red, SoH regions are shown in dark pink and the PBH regions in mid-pink. Domain II regions are shown in black, Gly regions in dark grey and NS, T, IS1 and IS2 regions in light grey.



C

	30K	ALG-2	Sorcin	Grancaicin	YG25
p94 (Domain IV)	58.3	30.7	34.0	34.0	25.6
30K		31.3	36.5	34.6	19.4
ALG-2			35.4	33.0	27.5
Sorcin				58.9	25.4
Grancaicin					25.7

B

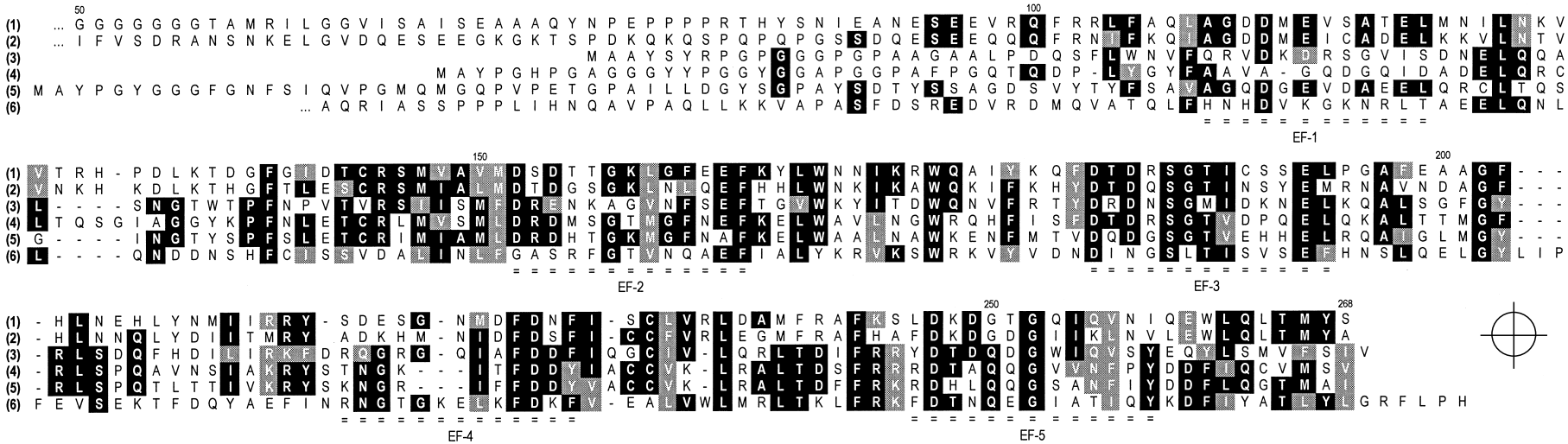


Figure 2 Structures of the calpain-small-subunit superfamily

(**A**) Schematic structure. The protease region is shown in black, the EF-hands in red and the Gly regions in grey. (**B**) Amino-acid-sequence alignment. The numbers in parentheses at the extreme left are identified as follows: (1) human 30K; (2) human p94 (domain IV); (3) mouse ALG-2 (pMP41); (4) human sorcin; (5) human grancalcin; (6) *S. cerevisiae* YG25. Identical amino acids among more than three sequences are shown as white letters on black. Conservative substitutions are shown as white letters on grey. (**C**) Sequence identity (%) of 30K homologues. Domain IV of human p94 was used as representative of calpain large subunits.

discuss the structures of each member of these superfamilies in detail and speculate on their physiological functions.

STRUCTURAL STUDIES OF CONVENTIONAL CALPAINS

As the names indicate, μ- and m-calpains are activated by micro- and milli-molar *in vitro* Ca^{2+} concentrations respectively. These calpains consist of two subunits: a distinct larger (about 80 kDa) subunit (called 'μCL' or 'mCL' for the μ- or m-calpain large subunit respectively) and a common smaller (about 30 kDa) subunit (called '30K' on the basis of the molecular mass). As shown in Figure 1 (top line), large subunits can be divided into four domains. The second and fourth domains are the cysteine protease (domain II) and Ca^{2+}-binding (domain IV) domains respectively. Thus the proteinase activity of calpain is ascribed to the large subunit. The functions of the first and third domains (domains I and III) are not clear at present. 30K is composed of two domains, an N-terminal glycine-clustering hydrophobic region (domain V) and a C-terminal Ca^{2+}-binding domain [domain IV′ (or VI)] similar to domain IV of the large subunit (Figure 2A) and is thought to regulate calpain activity as described below. The subunits are associated through their Ca^{2+}-binding domains (domains IV and IV′), at least in the absence of Ca^{2+} [47–49].

A three-dimensional analysis of the structure of conventional calpain is not yet available because of its high molecular mass (about 110 kDa for both large and small subunits). Before it is possible to elucidate the structure–function relationship or determine the activation mechanism and develop specific synthetic inhibitors, it is essential to establish the three-dimensional structure of calpain. A number of groups have reported the expression of active recombinant calpain [23,50]. Quite recently, the crystal structure of the Ca^{2+}-binding domain of 30K has been reported, as described in the next section [51–53].

The expression of a large molecule such as the calpain large subunit in its native form in *Escherichia coli* is generally very difficult, and many past attempts, including ours, ended in failure. However, Elce's group recently succeeded in obtaining an active recombinant rat m-calpain expressed in *E. coli* strain BL21(DE3) [23]. The subunits of the recombinant m-calpain were expressed separately or in the same cells, purified through several column steps, and reconstituted by dialysis. The reconstituted recombinant m-calpain showed almost the same relative activity and Ca^{2+}-requirement as native m-calpain. According to the method used by these investigators, pure active calpain can be obtained in 3–4 days in amounts of 5–10 mg per 4 litre culture of *E. coli*. By applying this expression system to analyses of the structure–function relationship using site-directed mutagenesis, they showed that the presumed active-site residues, Cys^{105}, His^{262} and Asn^{286}, are responsible for the protease activity of calpain [54]. The $Ala^{9} \rightarrow Phe$ mutant showed resistance to autolysis, but no change in its Ca^{2+}-dependency, suggesting that autolysis itself is not essential for the increased Ca^{2+}-sensitivity often observed on autolysis [55]. On the other hand, when Asp^{104} adjacent to the active-site cysteine residue is changed to serine, the Ca^{2+}-requirement for half-maximal activity increases greatly, implying that the Ca^{2+}-dependency is not necessarily determined by domain IV alone [56].

On the other hand, Siman's group used a baculovirus/Sf-9-cell system to express and obtain large amounts of human μ-calpain [50]. The profiles of the expressed protein are almost identical with those of native μ-calpain. Furthermore, Carafoli's group expressed human μ-calpain in both *E. coli* and baculovirus/Sf-9-cell systems and compared various parameters of each protein. In order to purify the expressed recombinant proteins, they used their original affinity-chromatographic methods [57,58]. Interestingly, the features of μ-calpains expressed in *E. coli* and Sf-9 cells differed slightly.

STRUCTURE OF THE Ca^{2+}-BINDING DOMAIN OF CALPAIN

The C-terminal domains [domains IV and IV′ (or VI)] of both the small and large subunits of calpain are Ca^{2+}-binding domains whose ancestral gene, because of the sequence similarities, is presumed to be calmodulin [13]. As shown in Figures 1 and 2(A), domains IV and IV′ each contain four EF-hand structures (EF2–EF5). In addition to these structures, two more EF-hand structures are reported to be present [36,59], one (EF6) at the boundary of domains II and III, and the other (EF1) located in the region N-terminal to EF2. EF6 was first reported by Andresen and colleagues [59] and was shown to bind Ca^{2+} in a gel-overlay experiment. EF1 is predicted to be present in *Drosophila* Dm-calpain (or CalpA) [36].

Recently the precise three-dimensional structure of 30K domain IV′ (or VI) expressed in *E. coli* was elucidated and indicates that 30K binds four Ca^{2+} molecules at positions EF1, EF2, EF3 and EF4 [52,53]. EF1 and EF2 comprise an 'open' pair almost identical with that seen in calmodulin, whereas EF3 and EF4 show 'closed' conformations similar to the two C-terminal EF-hand structures in troponin C. Furthermore, domain IV′ constitutes a homodimer through its hydrophobic association with EF5, which does not bind Ca^{2+}, and is in a 'closed' conformation. These results suggest that EF5 in both 30K and the calpain large subunit may be important for maintaining the natural heterodimeric subunit conformation, consistent with the observation that a mutant lacking the C-terminal 24 residues of EF5 can no longer interact with the large subunit [49].

PROTEINS HOMOLOGOUS WITH THE Ca^{2+}-BINDING DOMAIN OF CALPAIN

Although domain IV is structurally similar to calmodulin in that it contains four EF-hand structures, the primary sequences of both are rather diverged (for an exhaustive review, see [60]). Moreover, there are proteins that are significantly more similar to calpain domain IV than calmodulin, including sorcin [61], ALG-2 [62] (identical with previously cloned pMP41 [63]) and grancalcin [64,65]. Thus another superfamily, a family of calpain small subunit homologues, is now identified (Figure 2). Sorcin is involved in the multi-drug resistance of cultured cell lines, and was recently reported to associate with the cardiac ryanodine receptor [66]. Grancalcin possibly plays a role in granule–membrane fusion and degranulation. Sorcin and grancalcin function as homodimers [64,67]. The N-terminal regions of both proteins are rich in glycine residues and translocate to cell membranes in a Ca^{2+}-dependent manner [65,68]. All these are also features of 30K, suggesting some unknown function conserved among 30K, sorcin and grancalcin. ALG-2 was identified by a method called 'death trap' and is thought to be involved in apoptosis (genetically programmed cell death) [62]. pMP41, which is identical with ALG-2, was originally described as possibly induced by tumour promoters such as PMA and shown to be induced by nerve-growth-factor-treated PC12 cells [63,69]. The relationship between ALG-2 and apoptosis is interesting in that calpain is potentially involved in apoptosis as described in the next section.

In 1996 the complete nucleotide sequence of the *Saccharomyces cerevisiae* genome was determined. The sequence was found to include one 30K homologue along with a calpain (*pal*B) homologue described later. The hypothetical coding protein

(YGR058w, YG25 [70]) consists of a unique N-terminal region and a potential C-terminal Ca^{2+}-binding domain. As shown in Figure 2(B), the C-terminal domain shows considerable similarity to other 30K homologues, suggesting some conserved functions of the 30K superfamily through the long evolutionary process.

INHIBITORS FOR CALPAIN

Conventional calpains in mammals co-exist in cells with the very specific endogenous inhibitor protein calpastatin, strongly suggesting the pivotal role of this inhibitor in the regulation of calpain activity [71–75]. Calpastatin was first cloned in 1987 [27] and then found not to have exact secondary structures [76]. Maki's group, the successors of the late Professor T. Murachi, one of the great pioneers of calpain research, examined the interaction between calpastatin and calpain subunits in a real-time biomolecular interaction analysis using a BIAcore instrument [77]. They showed that three subdomains of the reactive site of calpastatin bind to domain IV of the large subunit, the active site, and domain IV′ (VI) of the small subunit respectively, a finding consistent with their previous observations [47,48,78–80]. Interestingly, the muscle-specific homologue of calpain, p94, described below, is not inhibited by calpastatin and, moreover, proteolyses it in the COS-cell expression system [81]. One possible explanation for this is as follows. Calpastatin binds to the Ca^{2+}-binding domains of both the large and small subunits, but p94 is not associated with the conventional calpain small subunit as described below and, thus, calpastatin cannot bind firmly to p94, resulting in the digestion of rather than inhibition by calpastatin.

As for synthetic calpain inhibitors, their history goes back to 1980 when Sugita and colleagues used derivatives of E-64 {*N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]agmatin; also known as *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane} [82] to prevent muscle degradation in patients with muscular dystrophy [83]. E-64 was first isolated as a papain inhibitor from the culture medium of *Aspergillus japonicus*. Typical derivatives, E-64c {*N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]isoamylamine} and E-64d {*N*-[*N*-(L-3-*trans*-ethoxycarbonyloxyoxirane-2-carbonyl)-L-leucyl]isoamylamine}, a membrane-permeable derivative of E-64c, have together with E-64 been widely used for various purposes [84–86]. Although E-64, E-64c, E-64d, and leupeptin (*N*-acetyl-Leu-Leu-argininal) [87] efficiently inhibit both μ- and m-calpains, they are not very specific as they also inhibit other cysteine proteinases. Leupeptin inhibits proteasome at the same, or even lower, concentrations. It is noteworthy that E-64 and leupeptin do not suppress the autolysis of p94 at all, as described later [88]. Calpain inhibitors I (*N*-acetyl-Leu-Leu-norleucinal) and II (*N*-acetyl-Leu-Leu-methioninal) are frequently used and commercially available [89], but they also inhibit proteasome and other cysteine proteinases [90,91]. In this respect the results concerning the differential inhibition of calpain and proteasome by di- and tri-leucyl aldehydes as shown by Tsubuki and colleagues are noteworthy [91]. They synthesized benzyloxycarbonyl-Leu-Leu-leucinal (ZLLLal) and benzyloxycarbonyl-Leu-leucinal (ZLLal) and showed that both ZLLLal and ZLLal strongly inhibit calpain ($K_i = \approx 1\ \mu$M), but that only ZLLLal inhibits proteasome [$K_i = \approx 1\mu$M and 0.1 μM for the ZLLL-4-methyl-coumaryl-7-amide (ZLLL-MCA) and succinyl-LLVY-MCA-degrading activity of proteasome respectively], whereas the K_i for ZLLal is above 100 μM for both activities. These synthetic inhibitors are potentially useful for identifying the functions of calpain and proteasome in cell biology.

Recently, Powers and co-workers [92] developed oxoamide inhibitor molecules, AK295 [benzyloxycarbonyl-leucylamino-butyric acid-CONH$(CH_2)_3$-morpholine], AK275 (benzyloxycarbonyl-leucylaminobutyric acid-CONH-CH_2CH_3) and CX275 (the active isomer of the diastereomeric mixture of AK275), which are more effective and more calpain-specific than the above inhibitors. The K_i value of AK295 is about 30 nM for μ- and m-calpain and about 1000 times higher for cathepsin B. The same researchers also screened derivatives of peptidyl α-oxo compounds to improve the specificity and K_i value, and found that benzyloxycarbonyl-leucylaminobutyric acid-CONH-CH_2-CHOH-C_6H_5 ($K_i = 15$ nM for m-calpain) and benzyloxycarbonyl-leucylnorvalyl-CONH-CH_2-2-pyridyl ($K_i = 19$ nM for μ-calpain) are the best inhibitors among over 100 molecules tested [93,94].

On the other hand, Wang and colleagues [95] developed a novel inhibitor with a distinct inhibitory mechanism compared with the other active-site-directed inhibitors described above. Their inhibitor, PD150606, showed a K_i value for μ- and m-calpains of about 0.3 μM, but greater than 100 μM for cathepsin B and papain, indicating its high specificity for calpains relative to other proteinases. It binds to the Ca^{2+}-binding domain of calpain, thus inhibiting calpastatin binding. Therefore PD150606, when used in combination with other active-site-directed inhibitors such as AK295, produces a very specific inhibition of calpain, a characteristic that is essential for *in vivo* studies of the physiological functions of calpain.

BRAIN FUNCTION AND CALPAIN

Calpain is believed to be strongly related to certain brain functions: the involvement of calpain in ischaemia was first suggested in 1989 [96,97]. Since then, there have been many reports that examined the relationship between calpain and ischaemia ([98–103]; for reviews, see [104–106]). For example, Saido and colleagues [98] observed drastic spatial changes in the proteolysis of fodrin in the hippocampus after 1–24 h of transient ischaemia using a proteolysed α-fodrin-150-kDa-fragment-specific antibody. Fodrin is a major cytoskeletal protein and one of the best substrates for calpain. The method developed in their laboratory is widely applicable, and has been used not only in studies of ischaemia, but also in various other proteinase studies such as β-amyloid processing [107], integrin proteolysis [108] and calpain autolysis [109,110]. Calpain inhibitors can suppress these post-ischaemic changes as described below, suggesting a therapeutic potential for calpain inhibitors in brain ischaemia [111,112]. These observations suggest that calpain functions as a mediator of the pathological process, not necessarily as the ultimate destroyer of cells.

Calpain may also be involved in long-term potentiation and Alzheimer's disease [113–116]. Nixon's group [117] reported that m-calpain is overexpressed in the brain of patients with Alzheimer's disease. Although indirect evidence was provided, more direct data confirming the involvement of calpain in these phenomena are needed. An interesting problem that needs explanation is why ubiquitously expressed μ- and/or m-calpains are specifically involved in brain-specific functions. Alternatively, brain-specific calpain species may exist that function in co-operation with the ubiquitous species.

The inhibitors described above effectively prevent fodrinolysis upon ischaemia, and could be applied as therapeutic agents in the future. For example, Saatman et al. [118,119] showed that AK295 attenuates motor and cognitive deficits following experimental brain injury in rats. Thus calpain inhibitors are one of the most competitive molecules for development in the near future. The road to success is, of course, the specificity of the inhibitor.

p53 AND CALPAIN

The p53 protein plays an essential role in tumour suppression, and mutational defects in p53 function are the most frequently detected genetic event in human cancers [120]. Most normal cells express very low levels of p53 due to the relatively short half-life of the protein. There is evidence that p53 can be degraded through ubiquitin-dependent proteolysis [121]. p53 interacts with the E6 protein encoded by the human papilloma virus, and E6, in association with the cell protein E6-AP, can function as a ubiquitin ligase that targets p53 for degradation [122]. Recently, and almost simultaneously, four separate research groups independently reported that p53 is sensitively proteolysed, at least *in vitro*, and may be regulated by calpain [123–126]. Kubbutat and Vousden [123] showed that calpain cleaves p53 *in vitro*, and that calpain inhibitors I (16 μM in the medium) and II (100–200 μM) enhance endogenous p53 levels in MCF-7 (breast carcinoma) and RKO (colon cancer) cells. They also showed that the E6/ubiquitin-dependent degradation of p53 is not affected by calpain inhibitor I (up to 200 μM). Pariat and colleagues [124] also showed that oligomerization of p53 through the C-terminal domain does not affect the cleavage kinetics, but that heat-denatured p53, which retains its sensitivity to trypsin, is resistant to calpain proteolysis. Their results indicate that an intact p53 quaternary structure is not a prerequisite for cleavage, but that the tertiary structure is essential for recognition by calpain. Furthermore, they observed that the over-expression of calpastatin in H358a and SAOS cells leads to the accumulation of p53 in a dose-dependent manner, and that the addition of the Ca^{2+} ionophore A21187 reduces p53 levels, an effect that can be prevented by the further addition of E-64d, calpain inhibitor I or calpain inhibitor II. Ciechanover's group [125] also showed, in *in vitro* experiments, that calpain can digest p53 as well as N-myc, c-Fos or c-Jun, and that the digestion can be stopped by E-64, EGTA, MG115 (carbobenzoxyl-Leu-Leu-norvalinal) or calpain inhibitor I or II. Mellgren's group [126] used a calpain-selective cell-permeable inhibitor benzyloxycarbonyl-Leu-Leu-Tyr diazomethane on serum-stimulated WI-38 human fibroblasts and observed similar results.

In addition, the first two groups examined the susceptibility of naturally occurring p53 mutants to calpain [123,124]. These mutants can be classified into three groups based on their susceptibility to calpain. The first group includes $Ser^{15} \rightarrow Ala$, $Ser^{15} \rightarrow Asp$, $Met^{246} \rightarrow Val$, $Arg^{256} \rightarrow Ala$, $Val^{272} \rightarrow Met$ and $Arg^{273} \rightarrow Cys$, all showing sensitivities similar to that of wild-type p53. The second group, including $Ala^{138} \rightarrow Val$, $Arg^{175} \rightarrow His$, $Met^{237} \rightarrow Ile$ and $Arg^{273} \rightarrow Pro$, show decreased sensitivity, whereas the third group, including $Arg^{248} \rightarrow Trp$, $Arg^{273} \rightarrow Cys$ and $Arg^{273} \rightarrow His$, are more sensitive to calpain. These results strongly suggest the importance of the local tertiary structure of p53 in its proteolysis by calpain.

As discussed by Gonen et al. [125], the results described in the previous four reports are essentially from *in vitro* experiments. To prove the involvement of calpain in cellular functions, it is important to obtain more rigorous evidence that it is actually involved in the *in vivo* regulation of p53. However, unlike proteasome, the ability of calpain to process substrates into only a few fragments is suitable for modulating such ubiquitous and functional proteins as p53. One critical question is whether calpain truly functions in the nucleus *in vivo*. In this regard, Kubbutat and Vousden [123] observed calpain activity in the nuclear fraction, and Mellgren's group [126,127] reported that μ-calpain, but not m-calpain, is transported into nuclei in an ATP-dependent fashion. Thus it is possible that calpain translocates into the nucleus under certain cell conditions, resulting in interaction with, and regulation of, the p53 molecule. More recently, the oncoprotein Mdm2 has been reported to promote the rapid degradation of p53 [128,129]. However, this pathway is under the regulation of proteasome, not calpain.

POSSIBLE CALPAIN INVOLVEMENT IN APOPTOSIS

Apoptosis is the common phenotype of programmed or physiological cell death, a process in which IL-1β-converting enzyme (ICE) and ICE-like proteinases (now called 'caspases') play important roles (for reviews, see [130–133]). The possible involvement of calpain in apoptosis was first suggested in 1993 [134,135] and has been reported for several cells including thymocytes, hippocampal neurons and hepatocytes [136–140]. Two of the hallmarks of apoptosis are the proteolytic cleavage of fodrin and DNA fragmentation. As previously reported, fodrin is a very good *in vitro* substrate for calpain and is thought to be one of the physiological substrates. Thus it is suspected that calpain is involved in apoptosis via fodrinolysis [141,142], but fodrinolysis coupling with apoptosis does not seem to be related to calpain [143–145]. In either case, observations concerning apoptosis differ depending on the cell line studied, and it is not yet established whether the fundamental mechanism of apoptosis is identical for all cells. Within a given cell, however, it seems that various distinct triggers can produce a very similar final apoptotic phenotype. In thymocytes, for example, Squier and Cohen [136] recently observed that PD150606 (at about 10 μM) as well as E-64d (300 μM), MDL 28170 (carbenzoxyvalylphenylalaninal) and calpain inhibitor I (20 μM) prevent dexamethasone-induced apoptosis in thymocytes, but that valinomycin- and heat-shock-induced apoptosis are not inhibited. Moreover, DNA fragmentation induced by the addition of Ca^{2+} (5 mM in medium) is not inhibited by calpain inhibitor I (20 μM). On the basis of these results they argued that calpain acts as a factor in the initiation of apoptosis of thymocytes, rather than in a final common pathway or mechanism [136]. Thus the involvement of calpain seems to be independent of the caspase family, and the interaction between p53 and calpain described above is not unrelated to calpain-mediated apoptosis, since p53 also triggers apoptosis [146–148]. In addition, a number of calpain homologues in *Caenorhabditis elegans* (described below) may be related to the well-studied apoptotic mechanisms in nematodes involving the *ced-3* gene product [149,150]. It should be noted, however, that the specificity of the inhibitors used for experiments is crucial in interpreting these results, and only the specific inhibition of calpain can lead to a correct interpretation of the experimental results. The effects of proteasome and lysozomal proteases must be eliminated in order to obtain a true understanding of the mechanism of calpain-mediated apoptosis.

FUNCTIONS OF THE CALPAIN LARGE SUBUNIT MONOMER AND 30K

As described above, both μ- and m-calpains consist of one μCL (or mCL) and one 30K, forming a heterodimer. It has been postulated that 30K is a regulator of calpain activity acting as an inhibitor or pseudo-substrate. Direct evidence for the function of 30K, however, has not yet been reported. Previous observations indicate that the large subunit alone is very unstable and has only trace activity (about 5 % compared with the native subunit). In contrast, the complex of the large subunit and 30K shows full activity upon reconstitution after denaturation and separation of the subunits [151,152]. Recently, we found that poly(ethylene glycol) (PEG) and/or chaperone proteins can stabilize the large subunit in the absence of 30K, and that the isolated large subunit expresses full activity comparable with that of the native calpain

complex [153]. Meyer and colleagues [50] also reported the existence of an active monomeric recombinant human μ-calpain large subunit using the baculovirus/Sf-9-cell system. Furthermore, the Ca^{2+}-sensitivity of the non-autolysed large subunit renatured in the presence of PEG is equal to that of autolysed native calpain, that is, more sensitive than non-autolysed native calpain. This observation, in combination with another recent finding that 30K dissociates from the large subunit in the presence of Ca^{2+} [154], suggests that the Ca^{2+}-sensitivity observed in our study is in fact the Ca^{2+} concentration at which the calpain subunits dissociate. In other words, 30K may regulate the Ca^{2+}-sensitivity of calpain by association and dissociation. In contrast, Zhang and Mellgren [155] recently showed that the calpain subunits remain associated during catalysis. As is often pointed out, the *in vitro* Ca^{2+} requirement for calpain activation and dissociation is unrealizable inside actual cells. Thus we need to clarify important factors lying between *in vitro* and *in vivo* conditions, i.e., some kind of activator and/or effector molecule(s) as reported by Pontremoli's group [156,157]; only the *in vivo* situation is meaningful to investigate and discuss. Only then will the physiological importance of the observed dissociation, if any, be elucidated.

Recently, Tompa and colleagues [158] proposed a 'calpain cascade' in which μ-calpain activated by Ca^{2+} lowers the Ca^{2+}-sensitivity of m-calpain. This may be able to explain the physiological importance of the very low Ca^{2+}-sensitivity of m-calpain, a point that has been argued for a long time. Although this possibility has been excluded previously on the basis of results obtained *in vitro* [159], re-evaluation of the results might be necessary. More importantly, evidence is required that μ-, m- or other calpains are activated under *in vivo* conditions in order to confirm that this heterolysis actually occurs *in vivo*. One potential solution lies in the autolysed form-specific antibody first described by Saido and colleagues [110]. It should be remembered that autolysis does not necessarily mean activation, although it does facilitate the dissociation of calpain into subunits [154].

p94, A SKELETAL-MUSCLE-SPECIFIC CALPAIN HOMOLOGUE

p94 has many unique properties that are probably related to its physiological function in skeletal muscles. The mRNA shows skeletal-muscle-specific expression, and the amount of mRNA is about 10 times that of conventional calpain subunits [16]. However, the protein undergoes extremely rapid autolysis (half-life is less than 10 min *in vitro*), resulting in its disappearance from skeletal muscle under normal conditions. Previous studies have shown that specific inhibitors of μ- and m-calpains, such as calpastatin, E-64 and leupeptin, have no effect on p94 activity [88]. When over-expressed in COS cells, p94 autolysis results in the tentative accumulation of a C-terminal 55 kDa fragment of p94, which also eventually disappears. In addition, regardless of the fact that p94 contains conserved EF-hand structures in its C-terminus (domain IV), its proteinase activity does not require Ca^{2+}, and the addition of Ca^{2+} or EGTA does not produce a detectable alteration in its activity. Moreover, as described above, p94 has a domain IV sequence similar to that of μCL and mCL, but does not bind to 30K [160]. p94 possesses a nuclear-localization-signal-like basic sequence (Arg-Pro-Xaa-Lys-Lys-Lys-x-Lys-Pro) in one of its unique regions, IS2 (see Figure 1) and is localized in the nucleus as well as cytosol when expressed in cultured cells [88]. The *in vivo* situation, however, is not yet clear. In skeletal muscle, p94 binds to a gigantic muscle protein, connectin/titin, specifically through IS2 [81,160]. Finally, the gene for p94 is responsible for limb-girdle muscular dystrophy type 2A (LGMD2A) [17].

Because of the extreme autolytic activity described above, studies of p94 at the protein level are very difficult. The proteinase activity of p94 should be regulated *in vivo*, but the mechanism remains unclear. Connectin/titin, a huge filamentous muscle protein, is a candidate for the suppression of p94 proteolytic activity [81]. As described below, it is essential to clarify the mechanism by which p94 activity is regulated and the *in vivo* substrates for p94. The potential substrates so far identified include p94 itself, myotonin protein kinase, fodrin and the 65 kDa protein of COS cells; p94 does not cleave μ- or m-calpain subunits, connectin/titin or most endogenous proteins in COS cells [81,88].

The basic domain structure of p94 is identical with that of the large subunits of μ- and m-calpains, as shown in Figure 1. However, p94 contains three unique regions, NS (N-terminal specific region), IS1 and IS2, that show no similarity to any other peptide sequences in the databases. The NS region is rich in proline, but its function has not yet been determined. This region overlaps domain I, which is thought to play important roles in the whole structure of μ- and m-calpains.

The protease domain (domain II) is the most conserved domain among calpain-large-subunit homologues, and high conservation around the active-site (Cys^{129}, His^{334} and Asn^{358}) is also observed. Mutagenesis studies [88] indicate that Cys^{129} and His^{334} are essential for proteinase activity, consistent with the observation for rat mCL described above [54]. This domain is intercalated by the IS1 region, which is encoded by an extra exon 6 [17,161,162] compared with the conventional calpain large subunit [18]. Our recent results show that deletion of the IS1 region stops the rapid autolysis, suggesting the involvement of the IS1 region in the regulation of p94 activity, which we postulated previously [163,164]. Deletion of the IS2 region in the C-terminal part of the domain also stabilizes p94 expression. Furthermore, the IS2 region is necessary and sufficient for binding to the N2-line portion of connectin/titin [81]. Thus the p94-specific regions, IS1 and IS2, are thought to be closely related to the autolysis and function of p94.

Recently, Tsujinaka and colleagues [165] made an interesting suggestion regarding the physiological functions of p94. Transgenic mice that over-express interleukin-6 (IL-6), a potential muscle-proteolysis-inducing factor, suffer from muscular atrophy. In these mice, the levels of the mRNAs for ubiquitins and cathepsins B and L are increased, whereas that of p94 is decreased. Moreover, treatment with anti-(mouse IL-6 receptor) antibody (2 mg/body intravenously at 5 weeks of age, followed by 0.1 mg/body subcutaneously twice a week from 6 to 14 weeks of age), which completely blocks atrophy, also eliminates the changes in mRNA levels. These results suggest the involvement of the IL-6/IL-6 receptor in the transcriptional regulation of p94, and might provide a clue as to the molecular mechanism of muscular dystrophy as described in the next section.

LGMD2A AND p94

In 1995, Beckmann's group [17] used the positional-cloning method to show that the gene for p94 is responsible for LGMD2A, and identified various mutations of the p94 gene in LGMD2A patients. The positions of mutation are distributed widely throughout the p94 gene, including missense point mutations, nonsense mutations, frameshift mutations and splice-site mutations [17,166–168]. No 'hot point' in the p94 gene was found, making diagnosis very difficult. Generally speaking,

frameshift and nonsense mutations correspond to more severe symptoms than missense mutations. Some of the frameshift mutant genes encode only a few residues of the N-terminus, resulting in a complete lack of domains II, III and IV. This almost corresponds to a null genotype, consistent with the idea that the function of p94 is essential to the muscle system.

All other muscular dystrophies, including Duchenne-type muscular dystrophy (DMD), congenital muscular dystrophy (CMD) and other LGMDs, are caused by deficiencies in structural proteins around the muscle-cell membrane (sarcolemma), such as dystrophin, merosin and sarcoglycans respectively [169]. On the other hand, in LGMD2A, a defect in a cytosolic enzyme, p94, results in symptoms similar to those in other muscular dystrophies. In LGMD2A, dystrophin and sarcoglycans are normally expressed, whereas very low levels of these proteins are present in DMD and all the sarcoglycans are down-regulated in the four different types of LGMD (2C, 2D, 2E, and 2F) [167,169]. In contrast, and on the basis of observations by Spencer and colleagues [170], p94 protein, which is very difficult, if not impossible, to identify by Western-blot analysis, seems to exist in the muscles of patients with LGMD2B, BMD and DMD. The muscles of these patients contain roughly the same amounts of p94, μCL and mCL. These workers used an anti-peptide antiserum against a synthetic 20-mer peptide corresponding to the common proteolytic domain II of the chicken μ/m-calpain large subunit (EQLIRIRNPWGQVEWTGAWS), which shares 16, 15 and 14 out of 20 residues with the corresponding sequences of μCL, mCL, and p94. Thus, although it is expected that this antiserum reacts equally with μCi, mCL and p94, the specificity and titre of the antiserum need to be verified with purified calpain proteins.

It seems that the mechanisms of LGMD2A and other muscular dystrophies are totally distinct. We have speculated that some signal-transduction pathways from the sarcolemma to the cytosol and/or nucleus involving p94, connectin/titin and the conventional calpains etc. must play an important role in LGMD2A [81]. The LGMD2A/p94 study is one of the best examples in which a top-down approach of pathological evaluation and a bottom-up approach of molecular biochemical study have met successfully. However, the tunnel connecting both sides is at present very narrow and dark. In order to admit light into the tunnel, both the pathological and biochemical approaches must be co-operatively and efficiently performed. Such studies should uncover the mechanisms not only of LGMD2A but also of other muscular dystrophies.

OTHER TISSUE-SPECIFIC CALPAIN HOMOLOGUES: nCL-2, -2′ AND -3

nCL-2 is another calpain-large-subunit homologue and its structure closely resembles those of μCL and mCL [22]. Unlike p94, nCL-2 contains no significant specific sequences. However, an alternatively spliced molecule, nCL-2′, which co-exists in the stomach, lacks the Ca^{2+}-binding domain and most of domain III, suggesting that the function of nCL-2′ is independent of Ca^{2+}, although the EF6 structure remains in nCL-2′. nCL-2 and nCL-2′ are predominantly expressed in the stomach, although weak expression is observed in other organs. Thus, nCL-2 and nCL-2′ may play a specific role in the stomach, just as p94 has a specific role in skeletal muscles. More recently, however, another tissue-specific calpain, nCL-4, was identified in our laboratory (H. S. Lee, H. Sorimachi, S.-Y. Jeong, S. Ishiura and K. Suzuki, unpublished work). The expression of nCL-4 predominates in the digestive tubules, i.e., in both the stomach and intestines, but not in the uterus. Furthermore, other mammalian calpain homologues are being reported on the databases as described below. We speculate that in addition to the conventional calpains, most organs have their own specific calpain(s), such as brain-specific, immune-system-specific, germline-specific, etc. The very rapidly developing expressed-sequence-tag (EST) projects will certainly reveal an outline of these tissue-specific calpain systems. In fact, some ESTs that probably encode novel calpain homologues are being identified. Thus the reported mammalian calpain homologues may be only the visible tip of an enormous iceberg.

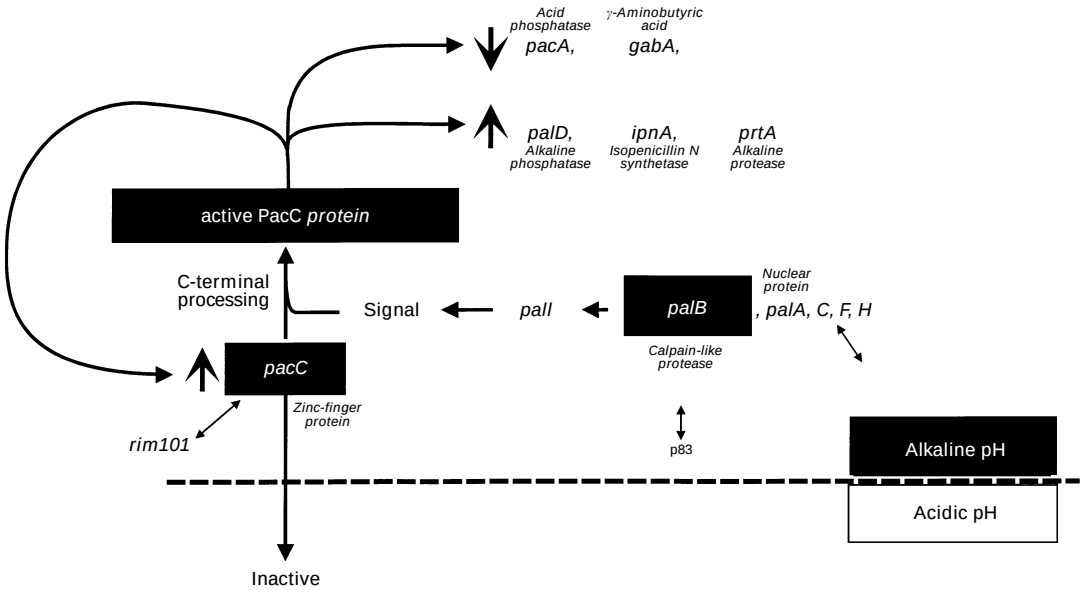
CALPAIN HOMOLOGUES IN FUNGI AND YEAST: THE *palB* PRODUCT (PalB) AND ITS HOMOLOGUES

At the end of 1995, Denison and colleagues [39] reported an interesting gene product in *Aspergillus nidulans*. The product of the *palB* gene has a cysteine-protease domain showing significant similarity to that of mammalian conventional calpains. However, PalB does not have domains similar to those of the conventional calpains other than a protease domain. Although some sequences similar to the EF-hand motif are present, it is not clear that PalB can actually bind Ca^{2+}. Thus, strictly speaking, PalB is a proteinase homologous with the calpain protease domain. We are calling all molecules 'calpain homologues' in the present Review Article, including proteinases like PalB.

Regardless of the nomenclature, PalB is noteworthy, since the genetic approach has already revealed that PalB is involved in the adaptation process of *A. nidulans* to alkaline conditions (see Scheme 1). This adaptation gene cascade consists of genes for a transcription factor, PacC [171], and a possible nuclear protein, PalA [172], as well as several other genes whose products have not yet been identified. As shown in Scheme 1, a key transcription factor, the *pacC* product, up-regulates the transcription of various genes for enzymes that function under alkaline conditions and down-regulates those that function under acidic conditions [171]. In order to express activity, PacC must be proteolysed from 73 to 29 kDa. PalB might be involved in this process, which occurs in more than one step and probably involves more than one proteinase, suggesting a crucial role for PalB in the signal-transduction pathway. Although no PalB substrates have been identified yet, these will be easily found by the genetic method in the near future.

One PalB homologue, i.e., the 'calpain homologue', was found in the sequence of the *S. cerevisiae* genome [173], although the sequence is rather diverged from the protease domain of the conventional calpain large subunit. This hypothetical gene product (YMR154c), which we call 'p83' on the basis of its molecular mass, has a cysteine-protease domain showing significant similarity to that of conventional calpain, but no Ca^{2+}-binding motif. However, p83 contains one domain homologous with PalB that can be found in p73, one of the calpain homologues of *C. elegans* (see Figure 1). Thus this domain may play a certain role, such as substrate recognition, that is conserved among these evolutionally distant organisms, fungi, nematodes and yeast. We propose calling this domain 'PBH', for **P**alB **h**omologous **d**omain.

Interestingly, the yeast genes called *rim101* [174] and *bro1* [175] encode proteins similar to PacC and PalA in *A. nidulans*, respectively. Moreover, YlRim101, a Rim101 homologue in the yeast *Yarrowia lipolytica*, was recently reported to be involved in the alkaline adaptation process of the yeast [176]. Thus a signal-transduction pathway similar to the *pal*-gene cascade in fungi must also exist in *S. cerevisiae*. We have cloned the p83 gene and disrupted it in order to characterize the function of p83 in relation to PalB function. We are hopeful that this will help to elucidate the cascade in *S. cerevisiae*.



Scheme 1 Alkaline adaptation cascade of *Aspergillus nidulans*

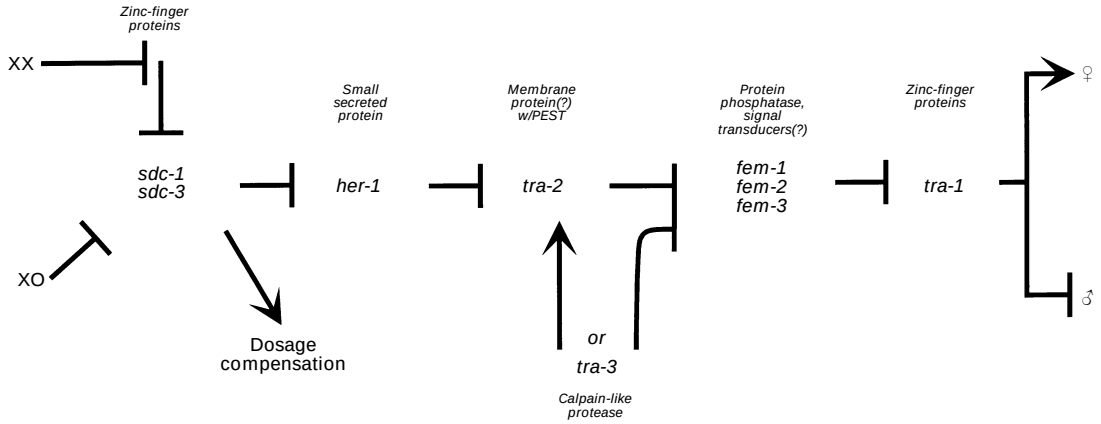
See the text for details.

SEX DETERMINATION IN NEMATODES INVOLVES THE CALPAIN HOMOLOGUE Tra-3

The *C. elegans* genome project has so far revealed three different genes presumably encoding calpain homologues. According to their molecular sizes, we tentatively call these gene products p70, p71 and p92 [177]. In addition to these, the EST project has also revealed the existence of at least one more calpain homologue, although a short cDNA sequence is the only information available at present [178]. Although the biochemical properties of these proteins remain unclear, their deduced amino acid sequences imply a unique function. As shown in Figure 1, the N-terminus of p71 contains a glycine cluster, which is a characteristic structure of 30K. Although p71 does not possess EF-hand structures, the results suggest that p71 is an ancestral integrated molecule of mammalian conventional calpain which is composed of two distinct subunits.

As described above, p92 has a PBH domain. Similarly, p70 has a region homologous with that of *Drosophila* calpain, sol (indicated as SoH in Figure 1). p70 and p92 also do not have EF-hand structures, suggesting that these three calpain homologues may play roles rather different from those played by mammalian conventional calpains.

Recently, one of the sex-determination cascade genes of nematodes, *tra-3*, has been shown to encode a calpain homologue with a sequence similar to that of domains I, II and III of the conventional calpain large subunit [179]. Thus the *tra-3* product does not contain a calmodulin-like Ca^{2+}-binding domain (domain IV), but rather possesses another domain, called domain T, at the C-terminus. Unfortunately, since the exons encoding domain



Scheme 2 Sex determination cascade of *Caenorhabditis elegans*

See the text for details.

T are outside the minimum rescuing region, this domain is not required for the critical function of Tra-3. On the other hand, the EF6 region as described above is well conserved in Tra-3, suggesting that Ca^{2+} signalling might be involved in the sex-determination cascade.

There are two sexes of *C. elegans*, the hermaphrodite, which has two X chromosomes, and the male, which has one X chromosome. Determination of sex is normally governed by the ratio of the X chromosome to the number of sets of autosomes (the X/A ratio) [180]. As shown in Scheme 2, various genes involved in the sex-determination cascade of nematodes have been identified. They include a possible transcription factor containing zinc-finger motifs (Tra-1), a protein phosphatase (Fem-2), a possible membrane receptor (Tra-2), and others. Tra-3 is thought either to activate *tra-2* function or to inactivate *Fem* function in a *tra-2*-dependent manner. Since Tra-2A contains a PEST sequence [181] in its C-terminal domain and Fem-1 contains ankyrin-like repeats [182], these are the most likely candidates for Tra-3 substrates. Tra-3 seems to be a very specialized molecule, since its genetic function is entirely dispensable in males and required only for correct hermaphrodite sex determination.

Two of the recent human calpain homologue database entries (accession nos. Y10552 and AJ000388, whose mouse counterparts are Y10656 and Y12582 respectively) show significant amino-acid-sequence similarity to nematode Tra-3 and can be aligned with Tra-3 from the N-terminus to the C-terminus without significant insertions or deletions (total amino acid sequence identities are 37.6 and 35.2 % respectively). Interestingly, both AJ000388 and Y12582 are localized at X-chromosomes of human and mouse respectively. If these molecules are mammalian counterparts of nematode Tra-3, it is possible that these calpain homologues are involved in a similar sex-determination cascade in mammals.

OTHER CALPAIN HOMOLOGUES: SCHISTOSOME, *DROSOPHILA* AND NEMATODE CALPAIN HOMOLOGUES

The *Drosophila sol*-gene product contains zinc-finger motifs in the N-terminal portion and shows embryo-specific expression [37]. Furthermore, *sol* is clearly related to a biological defect, i.e., lack of the *sol* gene leads to atrophy of the optic lobes, a defect in the adult nervous system as a result of neurodegeneration. The *sol* gene has two alternative splicing products, the shorter of which lacks the calpain-like protease domain, suggesting some regulatory mechanism produced by switching the protease domain on and off.

In addition to Sol, a conventional-type homologue of the mammalian calpain large subunit is also present in *Drosophila*. This calpain homologue, called Dm-calpain [35] or CalpA [36], is similar to the large subunit of conventional calpain from the N-terminus to the C-terminus, but has one significant insertion sequence of about 80, rather hydrophobic, amino acid residues. Moreover, Dm-calpain (CalpA) has an alternative splicing product that lacks domain IV, as in the case of nCL-2 and nCL-2′ [36].

CONCLUDING REMARKS AND PERSPECTIVES

The first phase of calpain study began with its discovery and characterization. Then research entered the second phase when several investigators vigorously studied the role of calpain in muscular dystrophies. The next phase was marked by molecular-biological studies, which soon expanded to laboratories around the world. During this phase, the molecular cloning of various calpain molecules started and progressed vigorously. When the 'cloning wars' settled, calpain study went into a quiescent state. Now, a new phase of calpain research is starting, especially focused on investigating its role in brain function, apoptosis, structural organization, muscular dystrophy and signal transduction, as described in the present Review Article. Furthermore, the three-dimensional structure of calpain is being elucidated by several groups. Gene targeting should be in progress in a number of laboratories. On the other hand, a genetic approach in nematodes, *Drosophila*, fungi and yeast will shed light on the functions of the calpain superfamily in various signal-transduction pathways. Together, these studies will clarify the physiological significance of the intracellular proteinases encompassing the calpain superfamily.

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