Structure, Activation, and Biology of Calpain

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Variation in the calpain 10 gene has recently been shown to be associated with type 2 diabetes by positional cloning. Since then, studies on calpain 10 have been started in correlation with diabetes and insulin-mediated signaling. In this review, the activation mechanism of calpain by calcium ions, which is essential to understand its physiological functions, is discussed on the basis of recent X-ray structural analyses. Further, special features of the structure of calpain 10 that differ from those of typical μ- or m-calpain used in most studies are summarized together with discussion of the physiological function of calpain with respect to type 2 diabetes. *Diabetes* 53 (Suppl. 1):S12–S18, 2004

Calpain is a cytoplasmic cysteine protease requiring calcium ions for activity. Although its physiological function is still not fully understood, it is implicated in a variety of calcium-regulated cellular processes such as signal transduction, cell proliferation, cell cycle progression, differentiation, apoptosis, membrane fusion, and platelet activation (1–5). Deregulation of its activity has been implicated in various pathological conditions such as neuronal degeneration, Alzheimer's disease, metastasis, and cataract.

Molecular biological studies have shown that calpains constitute a superfamily, which exists ubiquitously in organisms ranging from humans to microorganisms. Calpain has attracted much attention because of the recent discovery of correlations between calpain gene mutations and human diseases, together with elucidation of its three-dimensional structure (6,7) and calcium-induced activation mechanisms (8,9). Positional cloning of genes responsible for diseases has revealed association of the calpain 3 gene (*CAPN3*) with limb-girdle muscular dystrophy type 2A (LGMD2A) (10) and the calpain 10 gene (*CAPN10*) with type 2 diabetes (11). The link between calpain 3 and LGMD2A has been analyzed at the molecular level (12), but information on molecular and physiological mechanisms explaining the association of the calpain 10 gene with type 2 diabetes remains unclear and limited (13,14). To understand how mutations of the calpain 10 gene or abnormal calpain activity affects susceptibility to type 2 diabetes, knowledge of the activation mechanism of calpain at the molecular level and its physiological function are essential. In this review, the current status of mammalian calpain studies is summarized with reference to calpain 10.

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LGMD2A, limb-girdle muscular dystrophy type 2A.

PROPERTIES AND STRUCTURE OF CALPAINS

Calpain is not specific for certain amino acid residues or sequences but recognizes bonds between domains. As a consequence, calpain hydrolyzes substrate proteins in a limited manner, and large fragments retaining intact domains are produced by hydrolysis. Calpain is regarded as a bio-modulator, because properties of the substrate proteins are often modulated upon hydrolysis by calpain (1–5). For example, treatment of protein kinase C with calpain produces an intact kinase domain that is active by itself without addition of effectors such as diacylglycerol and calcium (1–5).

Two calpain activities are detected when tissue extracts are separated by Diethylaminoethyl column chromatography. These correspond to two conventional calpain species in mammals: μ-calpain and m-calpain. They differ in calcium sensitivity in vitro and are activated at levels of micromoles per liter and millimoles per liter of Ca^{2+}, respectively. They are heterodimers composed of a large ~80-kDa catalytic subunit (80K encoded by *CAPN1* and *CAPN2* for μ- and m-calpains, respectively) and a common ~30-kDa regulatory subunit (30K encoded by *CAPN4*). As shown in Fig. 1, 80K and 30K contain four (I–IV) and two (V and VI) domains, respectively. The protease domain II, like other cysteine proteases such as cathepsins B and L, is composed of two subdomains (IIa and IIb) with its substrate binding cleft in-between (Fig. 2). The active site Cys-105 on IIa is >10 angstrom apart from other catalytic triad residues (His-262 and Asn-286) on IIb, indicating that IIa and IIb are slightly too separated and open to constitute a functional catalytic triad (6,7). This inactive conformation of domain II is stabilized by interaction of domain I with domain VI on one side and interaction of IIb with domain III, especially an acidic loop, on the other side (7–9). Domain III has no apparent sequence homology to other proteins, but its higher order structure resembles C2 domains found in phospholipase C, protein kinase C, synaptotagmin, etc., and it binds Ca^{2+} and phospholipids (15–17). Both domains IV and VI contain five sets of EF-hand Ca^{2+}-binding motifs similar to those found in calmodulin. The extreme COOH-terminal fifth EF-hand motif in IV and VI cannot bind Ca^{2+} but interacts with each other to form a heterodimer comprising 80K and 30K (Fig. 2). The NH_2-terminal domain V of 30K is rich in glycine and

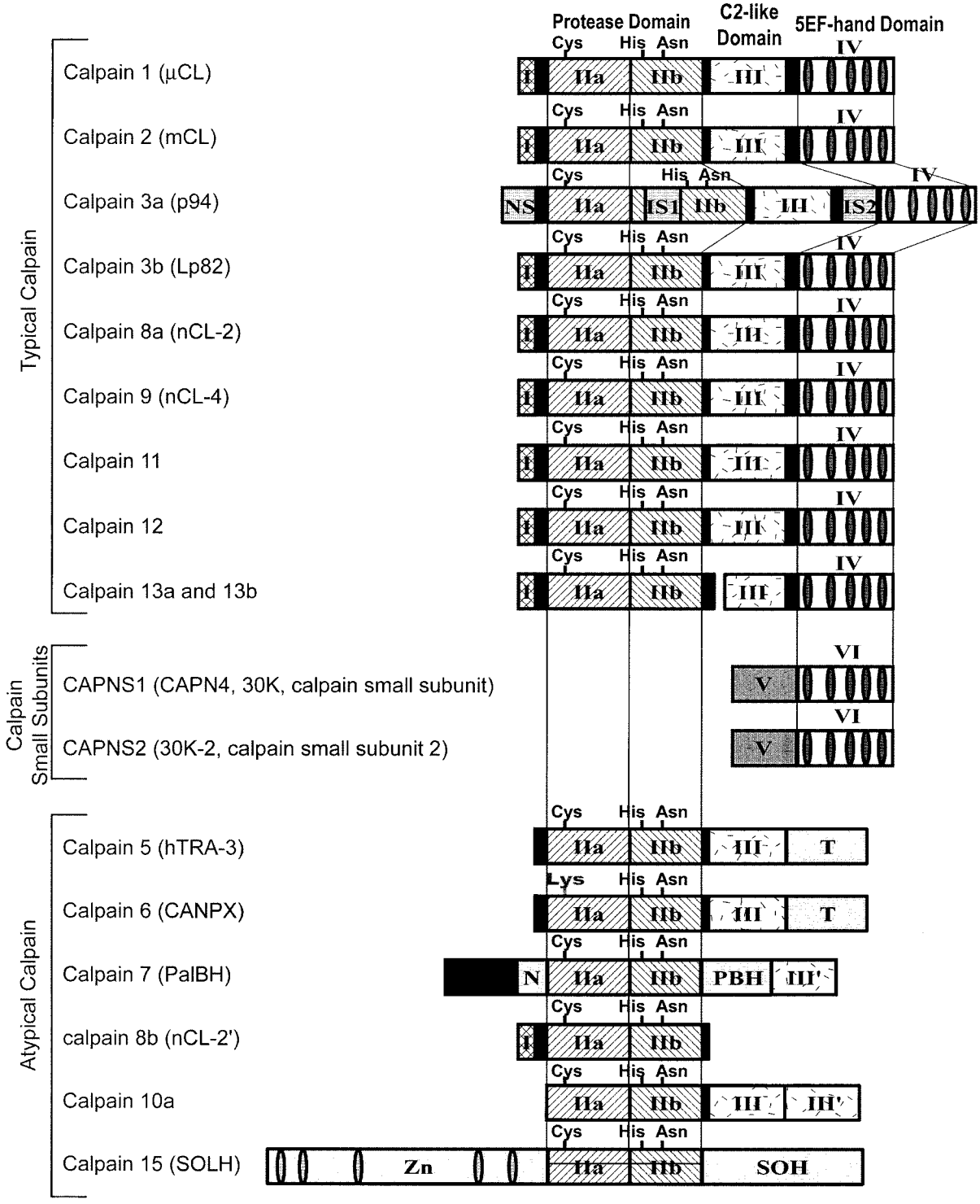

FIG. 1. Domain structures of human calpain family members. Typical calpains (80K) are composed of four domains (I–IV), whereas in the case of atypical calpains, certain domains of typical calpains have been deleted or replaced. The small subunit of calpain (30K) is composed of two domains (V and VI). The extreme COOH-terminal 5th EF-hand structure of domain IV of calpains 1 and 2 interacts with the 5th EF-hand structure of domain VI and forms a heterodimeric structure comprising 80K and 30K for μ- and m-calpains.

cannot be seen in the crystal structure because of its mobile conformation.

The 80K of μ- and m-calpains isolated by dissociation in urea and that obtained by expression of the cDNA possess a full protease activity if properly folded, indicating that 30K is not essential for protease activity (18,19). And, 30K plays a role as a chaperon and is essential for 80K to have correct conformation. μ and m-calpains dissociate into subunits in the presence of Ca^{2+}, and dissociated 80K probably functions as an active species in vivo (8,18,19). This dissociation theory of calpain, although a full consensus has not yet been reached, is apparently consistent with the existence of various 80K homologues that appear to function without 30K (Fig. 1). Dissociated 30K might have

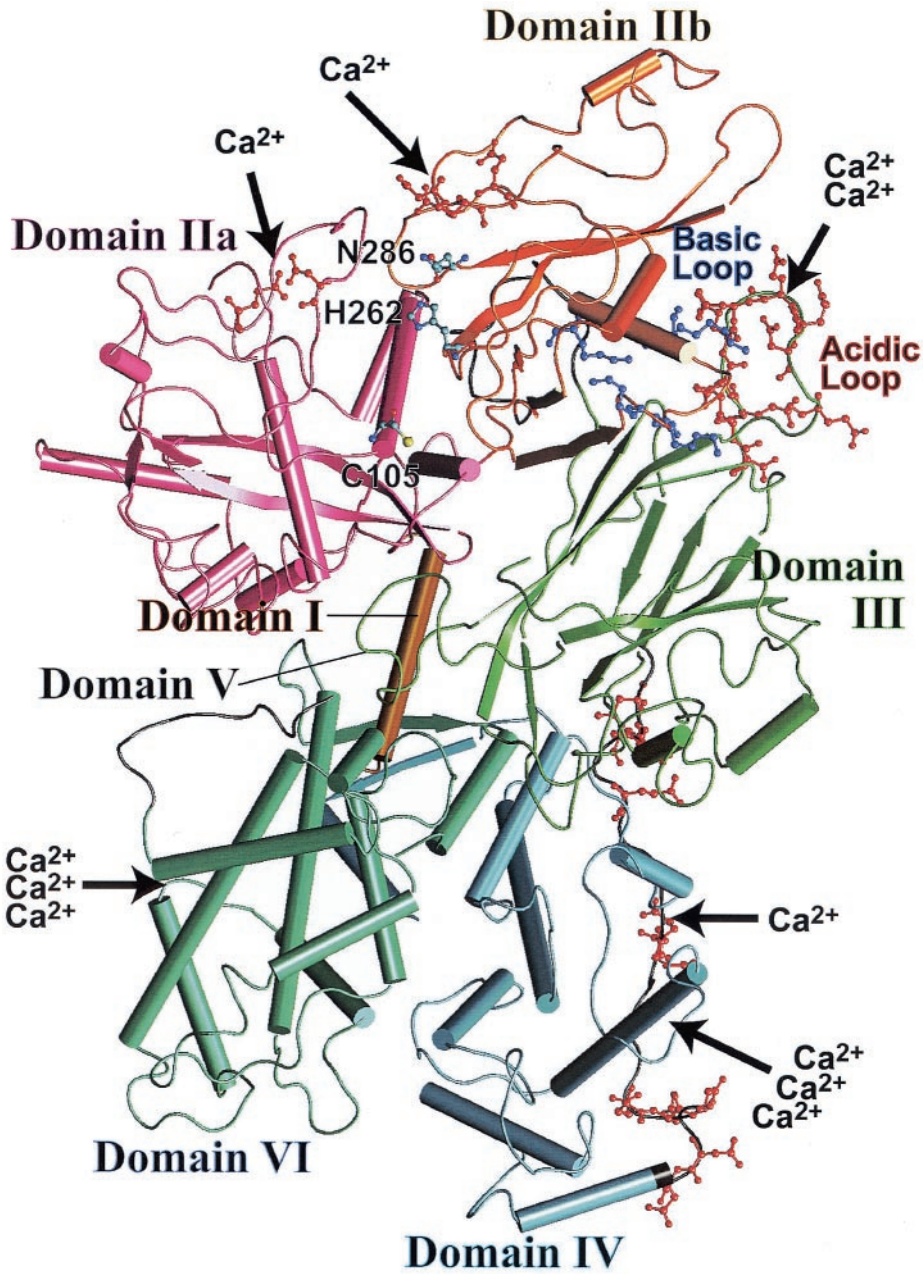

FIG. 2. Three-dimensional structure of human m-calpain without Ca^{2+}.

a function different from proteolysis after forming a homodimer (19). The word "calpain" was originally used for conventional μ- and m-calpains, heterodimers of 80K and 30K, but because 80K itself is fully active, the word "calpain" is now used for 80K as well.

Both μ- and m-calpains and 80K homologues are inhibited by reagents that react with the active site cysteine residues, such as E64, leupeptin, and N-Ac-Leu-Leu-norleucinal (2). These inhibitors also react with other cysteine proteases and are not specific for calpain. The nonpeptidic inhibitor PD150606 inhibits calpain by interacting with the calmodulin domain. This inhibitor is relatively specific for calpain, and the inhibition mechanism is different from other inhibitors (20).

An endogenous calpain inhibitor, calpastatin, exists in the cytosol. It contains four equivalent inhibitory domains of ~140 residues, having three conserved regions (A, B, and C) that are important for inhibition. A and C interact with IV and VI, respectively, in a Ca^{2+}-dependent manner, and B shows inhibitory activity by itself, probably by binding at or near the active site (21). Presence of the two calmodulin-like domains IV and VI are necessary for effective inhibition by calpastatin. Thus, calpastatin inhibits only dimeric calpain (namely μ-calpain, m-calpain, and calpain 9) with 30K. Calpain 80K homologues including calpains 1 and 2 are not inhibited and thus escape from the regulatory actions of calpastatin.

FAMILY MEMBERS OF CALPAIN

At present, 14 human genes have been identified as members of the calpain 80K family, together with two

TABLE 1
Human calpain genes

	Gene	Other names	Tissue distribution	Chr.	Amino acid residues	Protease activity	Association with 30K
Calpain protein							
Calpain 1	*CAPN1*	μ-calpain large subunit	Ubiquitous	11q12–13.1	714	+	+
Calpain 2	*CAPN2*	m-calpain large subunit	Ubiquitous	1q32-41	700	+	+
Calpain 3	*CAPN3*	p94, nCL-1	Skeletal muscle	15q15	821	+	−
Calpain 5	*CAPN5*	hTRA-3, nCL-3	Ubiquitous	11q14	640	+	−
Calpain 6	*CAPN6*	CANPX	Placenta, embryonic muscles	Xq23	641	−	−
Calpain 7	*CAPN7*	PalBH	Ubiquitous	3p24	813	+	−
Calpain 8*	*CAPN8*	nCL-2	Stomach	1q32–41	703	+	−
Calpain 9	*CAPN9*	nCL-4	Digestive tract	1q42.1–43	690	+	+
Calpain 10*	*CAPN10*	—	Ubiquitous	2q37.3	672	ND^+	ND
Calpain 11	*CAPN11*	—	Testis	6p12	702	ND^+	ND
Calpain 12*	*CAPN12*	—	Hair follicle	19q13	720	ND^+	ND
Calpain 13	*CAPN13*	—	Ubiquitous	2p21–22	423	ND^+	ND
Calpain 14	*CAPN14*	—	ND	2p21–22	?	ND^+	ND
Calpain 15	*SOLH*	SOLH	Ubiquitous	16p13.3	1086	ND^+	ND
CAPNS1	*CAPNS1*	Calpain small subunit, 30K, CAPN4	Ubiquitous	19q13	268	−	+
CAPNS2	*CAPNS2*	Calpain small subunit 2	Ubiquitous	16q13	248	−	+

Some alternative splicing products are known. For other details, including GeneBank accession numbers, see References 4 and 5. ND, not determined. *Longest translational product among alternatively spliced gene products.

genes for 30K, and their gene products are summarized in Fig. 1 and Table 1. Calpains 1, 2, 3, 8, 9, 11, 12, and 13 are composed of four domains originally found in μ- and m-calpains and thus are called typical calpains. Calpains 1, 2, and 9 associate with 30K, but calpains 3, 8, 11, and 12 do not apparently interact with 30K, although they have a similar calmodulin-like domain corresponding to domain IV, which is important for association with 30K. Calpains 5, 6, 7, 8b, 10a, and 15 small optic lobe homology (*SOLH*) are atypical calpains, where certain domains have been deleted or replaced (Fig. 1 and Table 1). Atypical calpains lack domain IV and cannot form a dimer with 30K.

Not all calpain family members have been fully analyzed at the protein/enzyme level, and protease activity has not yet been identified for calpains 7, 10, and 15. The active site Cys of calpain 6 has been replaced with lysine, and hence it plays a role not as a protease but as a protein with some other function. Calpains can be classified on the mode of expression. Calpains are usually expressed ubiquitously in cytosol, but some are expressed in a tissue-specific or preferred manner. Calpains 1, 2, 5, 7, 10, 13, and 15 are regarded as ubiquitous calpains, whereas calpains 3, 6, 8, 9, 11, and 12 are tissue-specific calpains expressed mainly in certain tissues (Table 1). For example, calpain 3 is skeletal muscle specific and calpain 8 is stomach specific (4,5).

Alternative splicing of *CAPN8* (nCL-2) generates a shorter form (nCL-2′, calpain 8b), where most of domain III and the whole domain IV are missing. Calpain 8b and corresponding fragments of calpains 1 and 2, which apparently do not contain known calcium-binding motifs, showed activity only in the presence of Ca^{2+}, indicating that other calcium-binding sites exist in domain II as proved by recent studies (8,22).

ACTIVATION MECHANISM OF CALPAIN

Studies on calpain thus far performed using mainly μ- and m-calpains have elucidated following the activation mechanism at the macroscopic level. Calpain exists in the cytosol as an inactive enzyme and translocates to membranes in response to increases in the cellular Ca^{2+} level. At the membrane, calpain is activated in the presence of Ca^{2+} and phospholipids. Autocatalytic hydrolysis of domain I takes place during activation, and dissociation of 30K from 80K occurs as a result. Activated calpain or 80K hydrolyzes substrate proteins at membranes or in cytosol after release from membranes (23).

In the absence of Ca^{2+}, two protease subdomains IIa and IIb are separated by structural constraints imposed by domain interaction. Ca^{2+}-induced structural changes that release the constraints are prerequisite for activation to form a functional catalytic site (6–8,24). Recent X-ray structural analyses have revealed a Ca^{2+}-induced activation mechanism at the molecular level (8). There are at least three different Ca^{2+}-binding sites in m-calpain, two calmodulin-like domains IV and VI, an acidic loop region in C2-like domain III, and a protease domain II. A proposed mechanism for activity regulation of m-calpain by Ca^{2+} comprises two stages (Fig. 3; 8,9,24). The first stage is the release of constraints imposed by domain interactions. Binding of Ca^{2+} to domains IV, VI, and III releases domain I from VI and domain II from III and leads to dissociation of 30K from 80K. The second stage is the rearrangement of the active site cleft caused by binding of two Ca^{2+} atoms to the protease domain (one each to the IIa and IIb subdomains). Activation by the second stage occurs only after release of the constraints freed by the first stage (8).

The two-stage activation mechanism is a general mechanism applicable to calpains without 30K or atypical calpains, because the protease domains are always composed of IIa and IIb, and the calcium-coordination residues for the two Ca^{2+}-binding sites in IIa and IIb are conserved among calpains (4,8). In the cases of calpains without 30K or without domain III or IV, the first stage of activation might be bypassed and they are activated di-

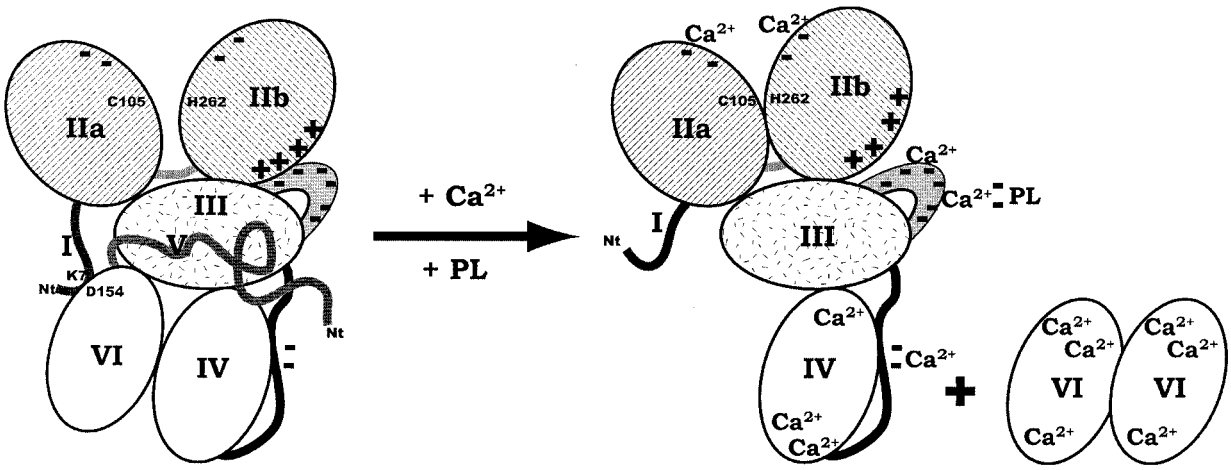

FIG. 3. Activation mechanism of calpain by Ca^{2+}. Binding of Ca^{2+} and phospholipids (PL) to m-calpain induces conformational changes, which brings IIa and IIb closer together to form a functional catalytic site and causes dissociation of 30K from 80K, resulting in 30K homodimer formation. There are at least three different calcium-binding sites in m-calpain, two calmodulin-like EF-hand structures in domains IV and VI, an acidic loop in domain III, and two non–EF-hand calcium-binding sites in IIa and IIb. C105 and H262 are catalytic residues. K7 and D154 form a salt bridge in the absence of calcium ions. Ca^{2+}, calcium atom bound to calpain; Nt, NH_2-terminal residue; + and −, basic and acidic amino acid residues important for binding of calcium ions.

rectly by the second stage. Autolysis of domain I facilitates dissociation of 30K from 80K (24), and binding of Ca^{2+} at domain III is responsible for translocation of calpain to membranes, as observed with protein kinase C and phospholipase C (16).

Activity of calpain is tightly regulated both temporally and spatially mainly by Ca^{2+}, because deregulation of calpain activity causes excessive degradation or accumulation of coexisting cellular proteins resulting in serious cellular damage and pathological conditions. Phosphorylation of calpain might be another important mechanism for activity regulation. Phosphorylation of calpain at Ser-369 in domain III by protein kinase A restricts domain movement and freezes m-calpain in an inactive state (25). Further studies along this line would evaluate the physiological importance of phosphorylation in the regulation of calpain activity.

PHYSIOLOGICAL FUNCTION AND IMPLICATIONS OF CALPAIN IN HUMAN DISEASES

The physiological function of calpain is still not fully understood, although many potential substrate proteins have been identified in vivo and in vitro. To understand the physiological function of calpain, disruption of calpain genes has been performed. Homozygous disruption of the murine *CAPN4* (30K) eliminated both μ- and m-calpain activities, but survival and proliferation of cultured embryonic stem cells were not affected (26,27). *CAPN4*$^{+/-}$ mice possessed calpain activity and were phenotypically normal. *CAPN4*$^{-/-}$ embryos, however, died at embryonic day 11.5 with apparent defects in cardiovascular system and erythropoiesis. Hence, 30K is indispensable for correct folding of 80K (8,18). Essential function of 30K for normal embryonic development exerts its effect probably by causing complete loss of μ- and m-calpain activities, indicating an indispensable role of calpain that cannot be replaced by other proteolytic systems. μ-Calpain knockout mice (*CAPN1*) were apparently normal, although they showed some defects in platelet aggregation (28). Calpain activity is essential for normal embryonic development, but *CAPN1* null mice show normal development, indicating that m-calpain compensates for μ-calpain in *CAPN1*$^{-/-}$ mice. μ-Calpain null platelets showed impaired tyrosine phosphorylation of proteins, including the integrin β3 subunit, suggesting that μ-calpain plays a role in signal transduction important for normal platelet functions (28). Similar experiments with *CAPN2* have not yet been performed.

Positional cloning of a gene responsible for LGMD2A, an autosomal recessive disorder, identified the gene for calpain 3/p94 (*CAPN3*) (10). Analyses of the point mutations found among LGMD2A patients have shown that a functional rather than structural defect of calpain 3 is responsible for the disease (12). Calpain 3, a skeletal muscle–specific calpain, binds to titin/connectin at the N2 line, M-line, and probably at the Z-line. It also protects titin and other skeletal muscle–associated proteins from degradation and modulates signal transduction around skeletal muscle important for normal muscle cell development (29,30).

Various calpain substrate proteins are associated with carcinogenesis, including products of oncogenes and tumor suppressor genes (transcription factors such as ras, c-fos, c-jun, p53, pp60src, and merlin). In gastric cancer, digestive tract–specific nCL-4 (*CAPN9*) is downregulated (31). Calpain 9 might be a new type of tumor suppressor that degrades oncogene products important for carcinogenesis in digestive tracts. Calpain also plays important roles in the p53 apoptotic response. Calpain inhibits p53-dependent apoptosis by hydrolyzing and hence lowering its in vivo level. Gas2, a death substrate cleaved by caspase 3, inhibits hydrolysis of p53 by calpain and consequently enhances the cellular apoptotic response. Gas2 appears to act like calpastatin and regulates a specific cellular response. Calpastatin is a unique in vivo regulator of calpain, but other cellular proteins interacting with calpain like Gas2 might regulate calpain activity (32).

Calpain degrades cyclin-dependent kinase inhibitor p27 during the mitotic clonal expansion phase of preadipocyte differentiation (33,34). Degradation of p27 releases the cyclin D-CDK4 complex from inhibition and phosphorylates retinablastoma gene product (Rb). As a result, tran-

scription factors necessary for differentiation are released from sequestration by Rb and become active. Although the results indicate important roles of calpain in cell cycle regulation and differentiation, how calpain is activated at an early specific stage of preadipocyte differentiation and what calpain species is activated are still not clear. Further, calpain-mediated turnover of p27 might not be critical, because p27 deficiency appears to be permissive in p27-deficient mice (33,34).

Calpain participates in various calcium-regulated cellular functions, and many substrate proteins have been identified in vivo and in vitro, but its precise physiological functions are still not clear. To analyze physiological functions of calpain, inhibitors specific for calpain, especially those specific for each calpain homologue, are essential. Various calpain inhibitors have been developed, but none of them are truly specific for calpain (2,20,35,36). Because the activation mechanism and three-dimensional structure of calpain are known, development of mechanism-based calpain-specific inhibitors is an urgent critical issue.

CALPAIN 10 AND TYPE 2 DIABETES

Calpain 10 is an atypical calpain in that domain IV of typical calpain has been replaced with a domain III–like structure. Thus, its protease domain is followed by a tandem repeat of two domains similar to domain III (Fig. 1). The extreme COOH-terminal III–like domain (III′) was originally called domain T, but is now regarded as a III-like domain (4). Because domains III and T are structurally similar and evolutionarily related, calpains 5, 6, and 10 can be regarded as close members, at least with respect to domain structure. Although the sequences around the active site residues (Cys-105, His-262, and Asn-286 for m-calpain and Cys-73, His-238, and Asn-263 for calpain 10) are highly conserved among family members of calpain, calpain 10 differs from calpains 5 and 6 and other calpains in the amino acid sequence. First, residues in domain II important for Ca^{2+}-binding and common among typical calpains are not conserved. Second, acidic and basic loops in domain III important for Ca^{2+} and phospholipid binding are not found in the two domain III–like structures of calpain 10 (4,8). As a consequence, calpain 10 probably has no Ca^{2+}-binding sites in domains II and III identified for typical calpains. The activity regulation of calpain 10 by Ca^{2+} would be different from that observed with typical calpains. It should be noted that calpain 5 (*hTRA-3*) retains residues important for Ca^{2+}-binding in domains II and III, and its Ca^{2+}-dependent protease activity has been identified (4,37), although the protease activity of calpain 10 has not yet been detected. There are several possibilities for the unsuccessful efforts to detect protease activity, such as *1*) inadequate assay conditions, including substrates and metal ions; *2*) calpain 10 in an inactive state, including improper folding; and *3*) the presence of an inhibitor or inactivation by extraction.

The message of calpain 10 is expressed ubiquitously in all adult and fetal human tissues examined (11), and similar ubiquitous expression has been confirmed in rat and mouse (38,39). The message level varies with tissues but is highest in the heart, followed by the brain, liver, kidney, and pancreas in humans (11). At least eight splice variants of calpain 10 (calpains 10a through 10h) have been identified, including three variants that lack intact domain II and thus have no protease activity (11). Some calpain 10 is detectable in the soluble fraction by Western blotting using an antibody raised against a synthetic peptide, but it is preferentially found in the water-insoluble fraction in rat tissues (38). In skeletal muscle fibers from young mice, calpain 10 is detected predominantly at the sarcolemma. Interestingly, Ca^{2+} affects both the expression level and subcellular distribution of calpain 10 in rat cultured lens epithelial cells. These results might suggest that calpain 10 is responsive to Ca^{2+}, although its Ca^{2+}-binding properties would be different from typical calpains as discussed above.

Genetic variants in the *CAPN10* gene are associated with elevated free fatty acids and insulin resistance (40). In vitro studies have shown that free fatty acids activate protein kinase C, which results in hyperphosphorylation of insulin receptors, leading to reduction in the kinase activity of insulin receptors and thus enhancing insulin resistance (41). Therefore, downregulation of protein kinase C activity appears to be an important factor to maintain proper phosphorylation levels of insulin receptors (42). Polymorphism within intron 3 of *CAPN10* (UCSNP-43) affects mRNA expression, and those with the G/G genotype have lower calpain mRNA levels (13). Because protein kinase C is a well-known in vivo substrate of calpain, a lower calpain level leads to upregulation of protein kinase C activity, reduces insulin signaling, and results in insulin resistance.

Short-term exposure of mouse pancreatic islets to calpain inhibitors enhanced glucose-induced insulin secretion but reduced insulin-stimulated glucose uptake into adipocytes and skeletal muscle, and reduced glycogen synthesis rates in muscle (43). This partly supports the above consideration, but the exact site of calpain action in the insulin-signaling pathway is not clear. But because calpain inhibition showed similar reduced effects on a hypoxia-associated increase in glucose uptake, which is not mediated by the insulin receptor system, the calpain-regulated step might not be directly involved in this pathway (43). The fact that another calpain inhibitor PD147631, which interacts with calpain at domain IV, had similar effects, suggests that the observed effect of calpain is not due to inhibition of calpain 10.

The protease activity of calpain 10 has not yet been analyzed. Its calcium sensitivity appears to be significantly different from typical calpains on the basis of the structure. Calpain plays roles in membrane fusion (44) and hydrolyzes various proteins that participate in cellular signaling, such as kinases, receptors, and transcription factors. Further, calpain is also important for differentiation of preadipocytes into adipocytes. These facts raise the possibility that calpain modulates both insulin secretion and action. In skeletal muscle, liver, and pancreas, several calpain species are expressed, including typical and atypical calpains. Studies on the action of calpain using calpain inhibitors suggest that calpain species other than calpain 10 also participate in insulin secretion and action and susceptibility to type 2 diabetes. Because many steps could be modulated by calpain, further precise experiments are essential to clarify the molecular and physiological mechanism explaining the association of calpain with type 2 diabetes.

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