How calpain is activated by calcium

Ahmad Khorchid and Mitsuhiko Ikura

The discovery of two unexpected Ca^{2+}-binding sites in the structure of a minimal catalytic domain of μ-calpain reveals a new mechanism underlying the Ca^{2+}-dependent activation of calpains.

Calpains are a family of cytosolic cysteine proteinases whose enzymatic activities depend on Ca^{2+}. Members of the calpain family are believed to function in various biological processes, including integrin-mediated cell migration, cytoskeletal remodeling, cell differentiation and apoptosis[1]. At the pathological level, over-activation of calpain as well as mutations abrogating calpain activity have been implicated in muscular dystrophy, cardiac and cerebral ischemia, platelet aggregation, neurodegenerative diseases, rheumatoid arthritis, cataract formation and Alzheimer's disease[2]. Despite numerous biochemical and structural studies[3], there has been no definitive description of the molecular mechanism by which Ca^{2+} activates calpain enzymatic activity.

In a recent issue of *Cell*, Davies and colleagues[4] report the crystal structure of an enzymatically active fragment of μ-calpain that contains two new Ca^{2+}-binding sites within the catalytic core. This crystallographic study, together with biochemical data, indicates that Ca^{2+} binding to these sites is cooperative and required for full enzymatic activity. These findings finally resolve the long-standing mystery of calpain's Ca^{2+}-dependent activity.

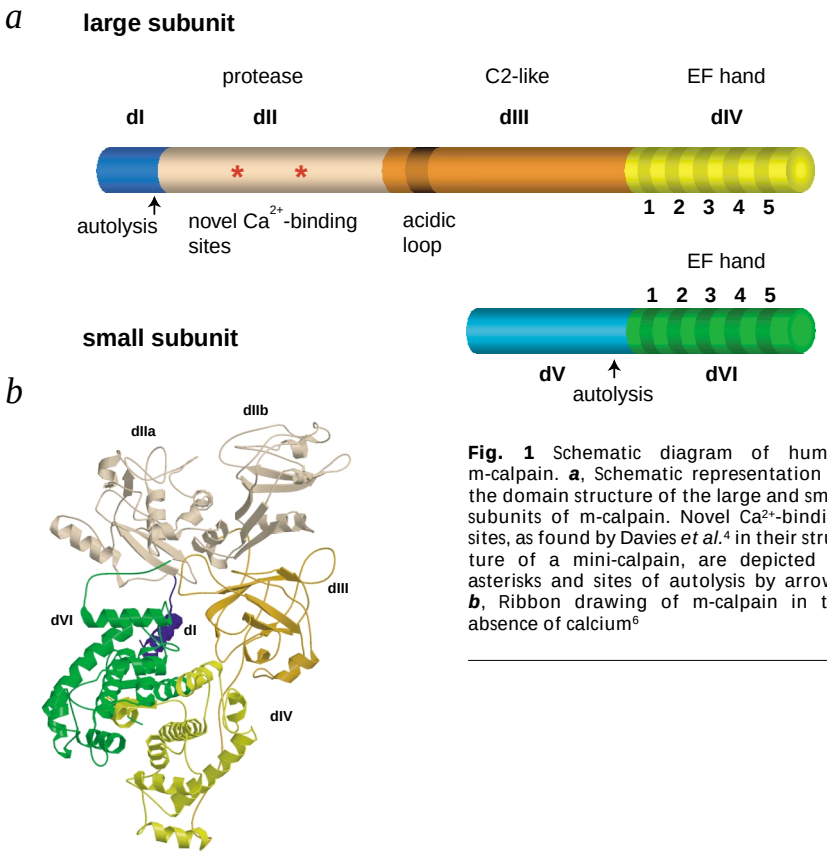

Fig. 1 Schematic diagram of human m-calpain. ***a***, Schematic representation of the domain structure of the large and small subunits of m-calpain. Novel Ca^{2+}-binding sites, as found by Davies *et al.*[4] in their structure of a mini-calpain, are depicted by asterisks and sites of autolysis by arrows. ***b***, Ribbon drawing of m-calpain in the absence of calcium[6]

Domain architecture of 'classic' calpains

The calpain family includes numerous members found in organisms ranging from mammals to *Drosophila melanogaster* and *Caenorhabditis elegans*, as well as homologs in both yeast and bacteria[3,5]. Currently, at least 12 different calpains have been identified in mammals with ubiquitous (such as m- and μ-calpains) and tissue-specific (such as p94 calpain 3 in skeletal muscle) expression patterns. The m- and μ-calpains are the best characterized members in the family. Both proteins are heterodimers composed of a large 78–80 kDa catalytic subunit and a common small 29 kDa regulatory subunit. The large subunit comprises four domains (dI–dIV), while the small subunit has two domains (dV and dVI) (Fig. 1*a*).

A previous crystal structure of the full-length human m-calpain in the Ca^{2+}-free state[6] revealed the overall architecture of this protein (Fig. 1*b*). Domain dI comprises a single α-helix anchored in a cavity of dVI, thereby stabilizing the circular domain arrangement of the protein. Domain dII contains the catalytic site, and can be further divided into two subdomains, dIIa and dIIb. These subdomains have been shown to contain a putative catalytic triad similar to the one found in cysteine proteinases such as papain. Domain dIII consists of eight strands with a topology similar to the C2 domain found in various proteins including protein kinase C and phospholipase C, which is known to interact with Ca^{2+} and phospholipids[7]. Indeed, an acidic loop within dIII has been suggested to play a role in the Ca^{2+}-promoted activation of calpain[6,8]. Domains dIV and dVI are well-characterized Ca^{2+}-binding domains, each containing five EF-hand motifs. These domains are responsible for heterodimerization of the large and small subunits through a unique interaction between their fifth EF-hand motifs. Domain dV of the small subunit contains a cluster of Gly residues in the N-terminal region, which is largely unresolved in the crystal structure. While the structure of the apo m-calpain explained why the protein is inactive without Ca^{2+}, it could not explain the mechanism of activation upon Ca^{2+} binding. While the structure of Ca^{2+}-bound calpain would provide insights into this process, efforts in this regard have been hampered by the dissociation of the small subunit and the aggregation of the large subunit under crystallization conditions.

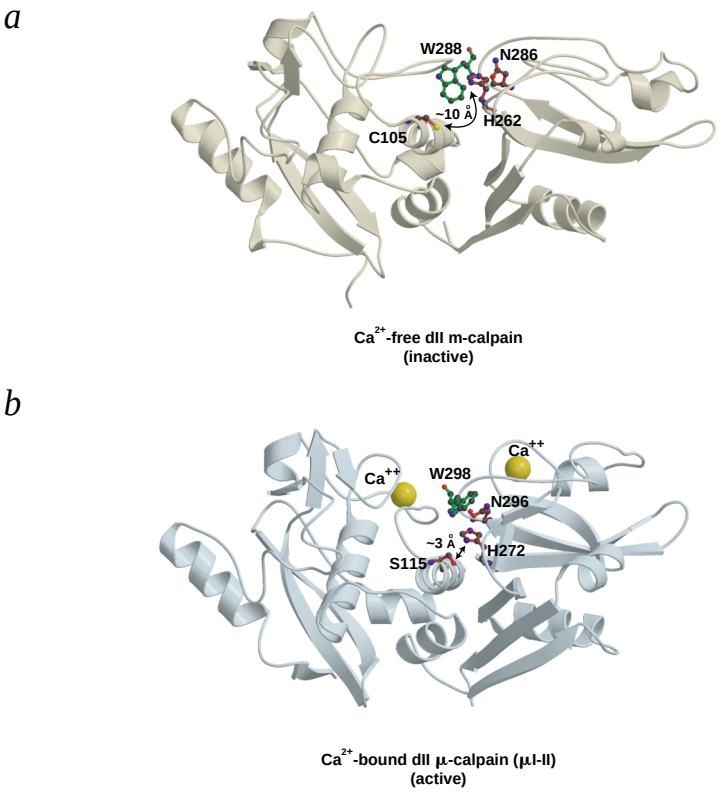

Fig. 2 Ribbon drawings of the protease domain (dII) of calpain in ***a***, the absence[6] and ***b***, the presence[4] of calcium. Catalytic triad residues are colored in red, and the active site Trp in green.

Mini-calpain

The recent success by Davies and colleagues[4] depended upon the construction of a recombinant mini-calpain that comprises the catalytic domain IIa-IIb (referred to as μI-II). This mini-calpain is catalytically active only upon Ca^{2+}-loading, and tryptophan fluorescence analysis demonstrated that Ca^{2+}-binding is positively cooperative involving multiple sites.

The crystal structure of the mini-calpain revealed two novel Ca^{2+}-binding sites. The first site, located in dIIa, involves residues Asp 106, Val 99 and Gly 101 on the loop preceding α-helix 3 (that contains the catalytic Cys 115) and residue Glu 185, which is positioned on the loop leading to the N-terminus of the core α-helix 5. The second site is in dIIb, and Ca^{2+}-binding is coordinated by four acidic residues and a methionine: Glu 302 and Asp 309 in the loop containing Trp 298, another residue in the active site, and residues Asp 331, Glu 333 and Met 329 in the loop between α-helices 8 and 9.

Based on the inactive Ca^{2+}-free m-calpain structure, Bode and colleagues[3,6] had previously predicted the involvement of Asp 321 in Ca^{2+}-binding to m-calpain. Indeed, the corresponding residue in μ-calpain, Asp 331, is part of the second Ca^{2+}-binding site in the structure of μI-II. Most of the Ca^{2+}-coordinating residues found in the μI-II structure, however, were not predicted earlier. Bode and colleagues[3,6] had also suggested that the 'acidic loop' in dIII (Fig. 1*a*) binds Ca^{2+} and that binding of Ca^{2+} to this site may result in a conformational change of dIIa and dIIb *via* an 'electrostatic switch' mechanism.

Mechanism of Ca^{2+}-activation

Not surprisingly, the active site of Ca^{2+}-bound μ-calpain markedly resembles the active site seen in other cysteine proteases such as papain and cathepsins. The location and spacing between the critical active site residues — Cys 115, His 272, Asn 296, Gln 109 and Trp 298 — overlap well with the corresponding residues in papain (a root mean square (r.m.s.) deviation of 0.66 Å for all side chain atoms). Most remarkably, the Oγ atom of the active site Ser 105 is in very close proximity (3.7 Å) to the immidazole Nδ of His 262, a central histidine in the catalytic triad of μ-calpain (Fig. 2*b*). By contrast, the distance between these residues in Ca^{2+}-free calpain is 10.5 Å (Fig. 2*a*).

What are the backbone conformational changes that trigger these side chain rearrangements required for the activation of μI-II, which are also likely for native μ-calpain? Interestingly, the differences in backbone conformation between Ca^{2+}-bound and Ca^{2+}-free calpain are relatively small (r.m.s. deviation of 1.6 Å for dIIa and 3.6 Å for dIIb). A major change occurs within the two loop regions involving Gln 96–Cys 108 in dIIa and Ile 254–Val 269 in dIIb. In the presence of Ca^{2+}, residues in the loop region Ile 254–Val 269 come together to form a β-sheet that provides supporting van der Waals contacts to the active site Trp 298. The net effect of this rearrangement is that the Trp 298 side chain, which acts as a wedge between domains IIa and IIb in the apo state, moves away from the active site cleft allowing for the proper formation of the catalytic triad (Fig. 2).

In addition to domain II, the full-length calpains contain two other domains that bind Ca^{2+} — that is, the EF-hands in domains IV and VI. How does Ca^{2+}-binding to all three domains orchestrate the activation of calpains? Davies and colleagues[4] suggest a two-step mechanism for activation. The first step is the release of constraints imposed by the circular arrangement of the domains. This would involve subtle conformational changes in dIV and dVI upon Ca^{2+} binding, leading to the abrogation of the interaction between the N-terminal α-helix of dI and the second EF-hand motif of dVI[9] and perhaps promoting conformational changes in dIII[8] and dissociation of the small subunit from the large subunit. The second step in the activation is the realignment of the active site cleft caused by the cooperative binding of Ca^{2+} to dIIa and dIIb. Such a two-step mechanism adds an extra layer of control over calpain function. This makes sense physiologically considering the ubiquitous expression of calpains and their role as biomodulators of a wide variety of cytoskeletal, membrane and cytosolic proteins[1,10].

Remaining questions

The residues involved in the newly characterized Ca^{2+}-binding sites in dII are highly conserved in calpains from *Drosophila* to humans. However, members in the calpain family have very different Ca^{2+} sensitivities. For example, μ-calpain is active in the Ca^{2+} concentration range of 5–50 μM, where as m-calpain is active in the 200–1,000 μM range; the activity of the tissue-specific human calpain 3 is Ca^{2+}-independent. A possible explanation for

these differences is the high sequence diversity in the acidic loop of dIII among the calpains. μ-calpain has three more acidic residues in the dIII loop relative to m-calpain; human calpain 3 has no acidic residue in this loop. As the central domain, dIII may play a major role in the Ca^{2+}-mediated activation of calpain[6,8]. It is conceivable that changes in dIII affect the catalytic and structural integrity of the catalytic dII, as well as that of the other Ca^{2+}-binding motifs in calpain. Further studies are needed to understand the variability of calpain's Ca^{2+}-sensitivity.

Another major unresolved question concerns the intracellular Ca^{2+} level, which is generally 1 μM at most, even in stimulated cells, and never reaches the high concentration range at which the calpains become active. Thus, it may be that other biological molecules such as protein inhibitors (calpastatin[11] and Gas-2[12]) as well as phospholipids[13] are required to modulate the Ca^{2+}-sensitivity of calpains. Recently, Tompa *et al.*[14] reported that the isolated C2-like dIII of classic calpains binds Ca^{2+} and that its affinity to Ca^{2+} is enhanced by the presence of di- and triphosphoinositide-containing liposomes. Furthermore, Ca^{2+}-binding to calpain promotes several processes: (i) translocation of the enzyme to the plasma membrane[15] (ii) autolysis in both the large and small calpain subunits[16,17] (Fig. 1*a*), and (iii) dissociation of the two subunits into truncated fragments[1]. Together these processes contribute to the biological activity of calpain in the cell, adding a complexity to the mechanism underlying the Ca^{2+}-dependent activation of calpains. The next challenge for calpain structural biology would be the determination of a membrane-bound, fully Ca^{2+}-activated enzyme.

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Single-handed recognition of a sorting traffic motif by the GGA proteins

Tom Kirchhausen

Selective transport of cargo between membrane-bound organelles is vital for the well-being of cells. The crystal structure of a short peptide signal from the cytoplasmic tail of the mannose-6-phosphate receptor bound to the VHS domain of GGA proteins gives hints to how sorting works.

In most cells, it takes an hour or less to recycle lipids and many transmembrane proteins between various cellular membranes. Vesicles and tubulo-vesicular carriers shuttle these components, but remarkably, the compositions of the various membrane compartments remain distinct (Fig. 1). This feat depends in part on the controlled formation of the vesicles and tubulo-vesicular structures that bud from the donor membrane, their movement inside the cell, and their targeting and fusion with the acceptor membrane. Much like the zip code addressing system for letters and packages, sorting at the beginning of the process ensures correct selection of cargo molecules for transport and delivery. Sorting of transmembrane proteins involves recognition of short peptide signals in their cytoplasmic tails by special cytosolic proteins, which function as adaptors to link the cargo and the coat machinery responsible for membrane deformation and budding[1].

Cellular processes that depend on accurate membrane traffic range from endocytosis of hormones, nutrients and viruses to protease secretion, antigen presentation and membrane recycling during neurotransmission. The bi-directional traffic along the secretory and endocytic pathways intersect at the interface between the trans Golgi network (TGN, a tubulo-vesicular network abutting the distal side of the characteristic Golgi membrane stacks) and the endosomal/lysosomal compartment (a tubulo-vesicular network that is more dispersed throughout the cells). One class of cargo proteins in this bi-directional traffic comprises the mannose-6-phosphate receptors (MPRs), transmembrane proteins that are essential for normal lysosomal function in mammalian cells. For example, these receptors recognize the mannose-6-phosphate groups on lysosomal hydrolases and are involved in transporting these enzymes from the TGN to endosomes. After making such a delivery, the receptors cycle back to the TGN for another round of traffic.

How is this trafficking pattern maintained? Enter the GGAs (Golgi-localized γ-ear-containing ARF binding proteins)[2–5]. Unknown until barely two years ago, GGAs are ubiquitous cytosolic proteins of 613–721 amino acids that cycle between the cytosol and the TGN and link clathrin to membrane-bound ARF–GTP. The three mammalian GGAs — GGA1, GGA2 and GGA3 — are responsible for the accurate trafficking of MPRs and