A novel human small subunit of calpains

Éva SCHÁD, Attila FARKAS, Gáspár JÉKELY, Peter TOMPA and Peter FRIEDRICH¹

Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, H-1518 Budapest, P.O. Box 7, Hungary

Typical calpains are heterodimeric cysteine proteases which have distinct large catalytic subunits (80 kDa) but share a common small regulatory subunit (30 kDa; css1). Here we report the identification, cloning and characterization of a novel human small subunit (css2) encoded by an intronless gene, *capns2*, located on chromosome 16. This new protein displays 73 % sequence identity within the Ca²⁺-binding region but lacks two oligo-Gly stretches characteristic of the N-terminal domain of the conventional small subunit. css2 appears to be the functional equivalent of the conventional small subunit *in vitro* in that it helps the large subunit fold into the active conformation of similar Ca²⁺ sensitivity when the two proteins are co-expressed in

Escherichia coli. The purification of various chimaeric rat 80 kDa–human css2 constructs, on the other hand, shows that css2 binds the large subunit much more weakly than css1. Further, it does not undergo the autolytic conversion typical of the classical small subunit. The expression of this protein *in vivo*, as assessed from its appearance in expressed sequence tag clones, is rather limited, making it an example of a tissue-specific, rather than ubiquitous, small subunit.

Key words: 30 kDa subunit, calpain evolution, calpain small subunit 2, regulatory subunit, tissue-specific calpain.

INTRODUCTION

Calpain, the Ca²⁺-dependent cytoplasmic cysteine protease, is involved in the control of diverse physiological and pathological processes [1–3]. According to the long-recognized basic design, the best-studied conventional calpains, μ -, m- and μ /m-calpain, are heterodimeric proteins with a large catalytic subunit (80 kDa) and a small regulatory subunit (30 kDa or css1). It is generally held that these enzymes have distinct large subunits but share a common small subunit. The other tissue-specific and atypical forms of calpain all have different catalytic subunits with apparently no associated small subunit. Currently, about a dozen human genes encoding proteins that share significant homology with the catalytic subunit are known, but only a single small subunit has so far been described. As a matter of fact, the small subunit is also homologous with other proteins such as sorcin [4], grancalcin [5], ALG2 [6] and peflin [7], which together constitute the penta-EF-hand (PEF) family of proteins. Although certain features of these proteins, such as Ca²⁺-dependent translocation to the cell membrane [8] and homo- [9] and heterodimerization [10], resemble the behaviour of the calpain small subunit, none has so far been assumed to function in regulating calpain action.

The function of this apparently single small subunit is unclear. It is denoted as regulatory due to the general notion that it regulates the action of the catalytic large subunit. One suggestion is that its association with the large subunit keeps the latter in a dormant state and that Ca²⁺-induced dissociation of the heterodimeric molecule is essential for the catalytic activity to be expressed [11,12]. Other data challenge this scheme of dissociation [13,14]. Although inhibitory to activity, the small subunit is indispensable for the proper folding of the large subunit into the

catalytically competent conformation. Both denaturation–renaturation [15] and expression [16] studies have shown that css1, very much like a chaperone, assists the 80 kDa subunit in finding its native fold. Still other studies claim that autolytic processing of the small subunit [17] and/or its membrane/lipid binding [18,19] contribute to activation and Ca²⁺ sensitivity of the enzyme. Recent X-ray crystallographic studies of rat [20] and human [21] m-calpain revealed that anchoring of the N-terminal 19-mer of 80 kDa subunit to css1 prevents the proper assembly of the active site. The Ca²⁺-induced release of this segment from the small subunit enables the alignment of active-site residues and activation of the enzyme. In earlier crystallographic studies the Ca²⁺-free and Ca²⁺-bound forms of the small subunit homodimer were described [22,23]. A small but significant conformational change has been reported; this apparently contributes to activation of calpain.

Whatever its exact mechanistic role, the small subunit is indispensable for calpain activity and function *in vivo*. In recent knockout experiments the gene for murine calpain small subunit (*capns1*) was disrupted [24,25]. This intervention abolished calpain activity and was lethal early in fetal development. This observation underlines the fact that calpain, and the small subunit itself, is essential for life.

In this paper we report the identification, cloning, expression and characterization of a novel human protein that shares significant sequence similarity with the *capns1* gene product. This protein (denoted human new calpain small subunit, hcsc2) associates transiently with rat m-calpain large subunit, assists its folding and appears to be an authentic calpain small subunit by this criterion. Note that human calpain genes are numbered *capn1* to *capn15*, of which *capn4* encodes the conventional small subunit. It is recently recommended that some alternative name,

Abbreviations used: css1, conventional calpain small regulatory subunit; css2, new calpain small subunit; hcsc2, human css2; EST, expressed sequence tag; PEF, penta-EF-hand; Ni-NTA, Ni²⁺-nitrilotriacetate.

¹ To whom correspondence should be addressed (e-mail friedric@enzim.hu).

The nucleotide sequence data for the novel small subunit of human calpain has been deposited in the GenBank® Nucleotide Sequence Database under the accession number AY052551.

perhaps *capns1*, be given to this gene. In compliance, the gene encoding the new small subunit is denoted *capns2* here; the protein products are called *css1* and *css2*. For an update, please visit the calpain website at <http://ag.arizona.edu/calpains>.

MATERIALS AND METHODS

Materials

cDNA clones (Image IDs 669499, 1088399, 327492 and 2405872) were purchased from Genome Systems. Casein (catalogue no. 102242) was from Merck. [³H]Formaldehyde was a DuPont NEN product. Restriction enzymes and the SDS protein marker were from New England Biolabs. Ni²⁺-nitrilotriacetate (Ni-NTA) metal affinity resin was from Qiagen. The anti-His-tag monoclonal antibody was from Novagen, the ECL[®] kit was from Amersham Bioscience. For PCR the proofreading Pfx Platinum polymerase was used, purchased from Gibco. All other chemicals were from Sigma. Buffers and solutions were prepared with ion-exchanged distilled water.

Construction of expression vectors

The expression vectors of rat m-calpain [80/21; composed of an intact (80 kDa) large subunit and a truncated (21 kDa) small subunit] used for these studies were kindly provided by Professor John S. Elce (Department of Biochemistry, Queen's University, Kingston, Ontario, Canada). The large subunit was subcloned from pET-24-m-80K-CHis6 (coding for the large subunit of rat m-calpain with a C-terminal His tag) to a pET-22b vector. The new human small subunit was also cloned to a pET-22b vector with PCR from the original clone 327492. *css2* alone and *css2* fused with the C-terminal His tag were reamplified with PCR containing the ribosome-binding site 5'-upstream of *css2*. These products were cloned into the vector downstream of the six-His-tagged rat 80 kDa subunit, resulting in bicistronic constructs under the control of a common promoter region. The constructs are termed rat80/hcss2 and rat80/hcss2CHis6, respectively. The construct rat80/hcss2NHis10, encoding hcss2 with an N-terminally fused His tag, was made the same way, but using the pET-16 vector. The initial clones were sequenced from both directions; the constructs were verified by sequencing on an ABI 373 DNA Sequencer (Applied Biosystems).

Expression and purification of various rat 80 kDa/hcss2 constructs

The constructs were transformed into the *Escherichia coli* strain BL21(DE3) and the cells were grown at 37 °C and 250 rev./min in 500 ml of NZYM medium [1 % NZ amine (Sigma catalogue no. C0626)/0.5 % bacto-yeast extract (Sigma catalogue no. Y0875)/85 mM NaCl/8 mM MgSO₄·7H₂O] containing 100 µg/ml ampicillin and 50 µg/ml kanamycin (the latter for 80/21 only) until a *D*₆₀₀ value of 0.7–1.0 was reached. Expression was induced by 0.4 mM isopropyl β-D-thiogalactoside and growth was continued for 3 h at 30 °C and 250 rev./min. The cells were harvested by centrifugation (3600 *g* for 20 min; IEC Centra MP4R centrifuge) and resuspended in lysis buffer (50 mM phosphate, 300 mM NaCl, 10 mM imidazole, 5 mM benzamidine, 0.5 mM PMSF and 20 mM β-mercaptoethanol). The cells were disrupted by sonication for six bursts of 15 s at 16 µm (MSE sonicator) at 0 °C, leaving 1 min between bursts to allow for cooling. The sonicate was centrifuged at 100 000 *g* for 60 min and the pellet was discarded.

The supernatant was applied to a 3 ml Ni-NTA column equilibrated with Ni-NTA buffer (50 mM phosphate, 300 mM

NaCl, 20 mM imidazole, 1 mM benzamidine, 0.1 mM PMSF and 20 mM β-mercaptoethanol). The column was washed with 10 vol. of Ni-NTA buffer and calpain was eluted with 250 mM imidazole in the same buffer.

Activity assay and autolysis

Calpain activity was assayed in calpain buffer (10 mM Hepes, pH 7.5, 1 mM EDTA, 20 mM β-mercaptoethanol, 0.1 mM PMSF and 1 mM benzamidine) at various free Ca²⁺ concentrations as indicated, by measuring the liberation of trichloroacetic acid-soluble peptides from [³H]casein, as described in [26]. Autolysis of the small subunit was tested at room temperature in calpain buffer with 1 mM free Ca²⁺. The reaction was started by the addition of CaCl₂ and was run for the time indicated; then SDS sample buffer containing 10 mM EDTA was added and the sample was boiled for 5 min. The samples were subjected to SDS/PAGE and stained with Coomassie Brilliant Blue.

Other procedures

Pig kidney m-calpain was prepared as described previously [26]. SDS/PAGE was carried out as described by Laemmli [27]. Western-blot analysis was carried out using a monoclonal antibody recognizing His tags on the expressed proteins, following the instructions of the supplier. As a secondary antibody, horseradish peroxidase-linked mouse IgG was used, visualized by the ECL[®] Western-blotting detection kit.

RESULTS

Identification of the new small subunit

The human expressed sequence tag (EST) database was screened with the sequence of human *css1*. A number of clones with partial sequence similarity were found and four of them were sequenced. Clone W20468 (Image ID 327492) was found to contain an open reading frame that codes for a full-length protein with a remarkable sequence similarity to the conventional small subunit. The respective gene is intronless and is located on chromosome 16 (determined via the human genome link on the NCBI server, <http://www.ncbi.nlm.nih.gov>). We designate the protein encoded by this gene as hcss2; it contains 248 amino acids with a predicted molecular mass of 27 659 Da. This new homologue has a high sequence similarity to human *css1* (Figure 1): the overall similarity is 85 %, with 63 % identity. A BLAST search in GenBank[®] revealed a mouse homologue of the same length with 97 % overall similarity and 92 % identity to hcss2 (GenBank[®] accession no. AK009171). We designate this uncharacterized protein mouse *css2*.

Similarity between human (and mouse) *css1* and *css2* is confined mostly to the C-terminal calmodulin-like domain, where identity is 73 %; the N-terminal Gly-rich domains differ significantly, as two oligo-Gly stretches are missing from *css2*. A comparison of the calcium-binding EF-hand motifs shows that most of the residues (13 out of 17) involved in calcium coordination [23] are conserved; for residues not conserved, only conservative substitutions are tolerated. Residues responsible for interaction with the large subunit are also mostly conserved (35 identity out of 44); certain non-conservative substitutions, such as D111P, D219N, Q253L and Q259K, however, might have functional consequences (see below).

Multiple alignment of human and mouse *css1* and *css2* (results not shown) indicates that the orthologues are sequentially much closer to each other than the paralogues, which points to an early evolutionary separation of the two subunits. To establish their

Figure 4 Ca^{2+} sensitivity of the active enzyme species

The rat80/21 ($\square$), rat80/hcss2 ($\circ$) and rat80/hcss2NHis10 (Δ) constructs were expressed and purified as described for Figure 3. Their activity was measured at various free Ca^{2+} concentrations by the casein assay as described in the Materials and methods section. Half-maximal activity of the various enzyme forms invariably occurred at around $150 \mu\text{M}$ Ca^{2+} .

Characterization of various calpain constructs

The interaction of calpain subunits has been rather conserved in evolution; various chimaeric constructs show that subunits of different mammalian species are freely interchangeable [29]. Our study of the behaviour of hcss2 is based on this observation and the sturdiness of rat m-calpain expression in *E. coli*. In accordance with this, the following constructs were expressed in *E. coli*: rat80/21, rat80, rat80/hcss2, rat80/hcss2CHis6 and rat80/hcss2NHis10. The proteins were purified by Ni-NTA metal affinity chromatography, and then separated by SDS/PAGE (12.5% gel; Figure 3A) and their enzyme activity was determined (Figure 3C). In the case of rat80/21 (Figure 3, lanes 1), both subunits were seen on the gel and activity was taken as 100%. Rat80 alone (Figure 3, lanes 2) did not yield an active enzyme. With rat80/hcss2 (Figure 3, lanes 3), the new subunit could not be seen on the gel, probably because it did not bind to the large subunit strongly enough to co-purify with six-His-tagged rat80

on the metal affinity resin. Nevertheless, it restored the majority (about 70%) of enzyme activity, which is one function of a competent small subunit. It appears that css2 is able to act as a chaperone for the 80 kDa subunit, but that it does not bind strongly to the folded protein. To check whether css2 was expressed at all, we put a six-His tag on the C-terminus of css2 (Figure 3, lanes 4). This time the protein could be detected, but there was no enzyme activity. We surmised, based on the three-dimensional structure, that a His tag at the C-terminus of css2 might affect interaction with the 80 kDa subunit. Therefore, we placed a 10-His tag on the N-terminus of css2 (Figure 3, lane 5). Indeed, this construct produced enzyme activity and css2 could be seen on SDS/PAGE. The identity of band at 26 and 31 kDa as hcss2 was verified by Western blotting (Figure 3B). In conclusion, our experiments demonstrate that the novel calpain small subunit is able to help folding of the large 80 kDa calpain subunit during expression in *E. coli*, without later binding strongly to the mature, folded protein.

Ca^{2+} sensitivity and autolysis

To further compare the function of css1 and css2, the Ca^{2+} sensitivity of the three active enzyme species (rat80/21, rat80/hcss2 and rat80/hcss2NHis10) was compared. Their Ca^{2+} sensitivities were nearly identical, as half-maximal activity was seen in all three cases at about $150 \mu\text{M}$ free Ca^{2+} (Figure 4).

Activation of m-calpain is accompanied by autolysis, i.e. specific cleavage of its small subunit into an 18 kDa truncated form [26]. This was demonstrated with pig m-calpain that has an intact 30 kDa small subunit (Figure 5), and could also be seen clearly with rat80/21 (results not shown). css2, however, displayed distinct behaviour in this regard: when rat80/hcss2NHis10 was incubated with Ca^{2+} , no autolytic product was seen at 18 kDa (Figure 5).

DISCUSSION

It is generally held that the two ubiquitous calpains, μ - and m-calpain, are heterodimers that share a common small subunit [1,2]. This small subunit, often described as regulatory, is thought to control activity and action of calpain(s) in some way that is not yet understood fully. The small subunit is encoded by the *capns1* gene, which has 11 exons and is located on chromosome 19. The novel small subunit cloned and characterized in this study is encoded by an intronless gene located on chromosome 16. Co-expression of css2 with the 80 kDa subunit in *E. coli* shows that this protein acts as a chaperone for the large subunit, i.e. it helps the formation of a catalytically competent conformation of the latter. Such a function is thought to be the major attribute of css1 as well [15,16]; in this respect, css2 is a functional small subunit that can substitute for css1. Other considerations also support the view that css2 is an authentic small subunit. The sequence alignments clearly place this protein closest to css1 in the PEF family: within the calmodulin-like domain, its sequence identity with css1 (73%) is far greater than with 80 kDa (58%) or other PEF proteins (< 40%; see also [1]). If similarity within functionally important regions, i.e. Ca^{2+} -co-ordinating residues within the five EF-hand regions [23] and residues involved directly in subunit–324 subunit interaction [20,21], is considered, conservation is remarkable. In accordance with the expectations based on this match, the Ca^{2+} sensitivity of 80/21 and 80/hcss2 are very close to each other. Thus the new small subunit appears to be the functional equivalent of css1 at the biochemical level.

Other observations, however, indicate clearly that they are not equivalent. Firstly, css2 binds the 80 kDa subunit more weakly than css1, so that it does not co-purify with the large subunit via

Figure 5 Autolysis of pig m-calpain and rat80/hcss2NHis10

rat80/hcss2NHis10 (lanes 1–4) and pig m-calpain (lanes 5–9) were incubated in the presence of 1 mM Ca^{2+} at room temperature. Aliquots of the reaction mixture were withdrawn at the times indicated (where ' is min and '' is s), boiled for 5 min with SDS sample buffer containing 10 mM EDTA, and separated by SDS/PAGE (12.5% gel). The gel was stained with Coomassie Brilliant Blue. The autolysis product of pig m-calpain small subunit appears at 18 kDa.

the His tag attached to the latter. Consequently, this subunit might have an effect on calpain stability and action *in vivo* that differs from that of the conventional small subunit. This difference may result from certain non-conservative substitutions found within the residues which interact directly with the large subunit. The difference may also stem from the N-terminal region, where two hydrophobic oligo-Gly stretches present in *css1* are missing from *css2*. Of particular functional interest is a putative membrane-binding site of the small subunit, G₁₇TAMRILGG, between residues 39 and 62, which has been identified in liposome-binding studies [18,19]. Although this region probably does not contribute to interaction with the large subunit, it might be involved in controlling calpain action by lipid activation/membrane translocation, a recurring theme in calpain action [12,30]. As this region is not present in *css2*, the enzyme with this small subunit might behave differently in physiological situations. A further point worthy of note is the difference in autolysis of the two subunits. The conventional small subunit is thought to undergo specific, functionally important autolytic cleavage upon activation of m-calpain, resulting in a stable 18 kDa fragment (see [26]); the novel subunit shows no sign of such a specific cleavage. Although these differences are not necessarily important *in vivo*, they nevertheless stress that the new subunit is not a simple functional replica of the conventional small subunit. One may speculate that, as *css2* is not trapped by the folded 80 kDa subunit, its chaperone function is to catalytically assist several 80 kDa polypeptides.

Some physiological considerations also underline the difference of the two small subunits. Recent knockout experiments have shown that disruption of the ubiquitously expressed *capns1* gene abolishes μ - and m-calpain activity and leads to embryonic lethality [24,25] despite the presence of *css2*, which is expressed, as its sequence can be found in EST clones. The inability of *css2* to substitute for *css1* indicates that the two small subunits are not redundant, probably because the expression of *css2* is limited to certain tissues/developmental stages and/or due to the biochemical differences described in this article. The gene structure provides some clues for the fundamental difference in the expression pattern of the two small subunits. *capns2* is an intronless, i.e. processed, gene located on chromosome 16. This gene must therefore have arisen by reverse transcription of the message of the original small subunit. As such genes necessarily lose control of their original promoter, they are usually no longer expressed or display a basically altered expression pattern; often they become pseudo-genes. The *capns2* gene probably escaped this fate by acquiring a promoter region that enables it to be expressed, albeit more limited in space and time than *capns1*.

In conclusion, our work is the first indication that the superfamily of calpain genes contains more than one member encoding a functional small subunit. At the biochemical level, the product of this gene seems a good substitute for the conventional small subunit. Nevertheless, several considerations suggest that the effect of this protein *in vivo* is rather limited. As large-subunit genes are divided classically into ubiquitous and tissue-specific categories, we propose that this novel small-subunit gene should be considered as tissue-specific.

This work was supported by grants T 22069, T 32360 and T 34255 from the Hungarian Scientific Research Fund (OTKA), FKFP 100/2000 from the Ministry of Education and AKP 98-45 3,3 from the Hungarian Academy of Sciences. P.T. acknowledges the support of a Bolyai János Scholarship. We thank Professor John S. Elce (Department of Biochemistry, Queen's University, Kingston, Ontario, Canada) for kindly providing the expression vectors encoding rat m-calpain (80/21) and D. Reverter (Max-Planck Institut für Biochemie, Martinsried, Germany) for his help in identifying residues involved in subunit interaction. We are grateful to Klára Majzik for the highly skilled technical assistance.

REFERENCES

- Sorimachi, H., Ishiura, S. and Suzuki, K. (1997) Structure and physiological function of calpains. *Biochem. J.* **328**, 721–732
- Ono, Y., Sorimachi, H. and Suzuki, K. (1998) Structure and physiology of calpain, an enigmatic protease. *Biochem. Biophys. Res. Commun.* **245**, 289–294
- Friedrich, P., Tompa, P., Jékely, G., Farkas, A. and Schád, É. (2001) Calpains in cellular signalling. In *Protein Modules in Cellular Signalling*, vol. A/318 (Heilmeyer, L. and Friedrich, P., eds.), pp. 363–373, IOS Press, Amsterdam
- Meyers, M. B., Spengler, B. A., Chang, T. D., Melera, P. W. and Biedler, J. L. (1985) Gene amplification-associated cytogenetic aberrations and protein changes in vincristine-resistant Chinese hamster, mouse, and human cells. *J. Cell Biol.* **100**, 588–597
- Lollike, K., Johnsen, A. H., Durussel, I., Borregaard, N. and Cox, J. A. (2001) Biochemical characterization of the penta-EF-hand protein grancalcin and identification of L-plastin as a binding partner. *J. Biol. Chem.* **276**, 17762–17769
- Vito, P., Lacana, E. and D'Adamio, L. (1996) Interfering with apoptosis: Ca(2+)-binding protein ALG-2 and Alzheimer's disease gene ALG-3. *Science* **271**, 521–525
- Kitaura, Y., Watanabe, M., Satoh, H., Kawai, T., Hitomi, K. and Maki, M. (1999) Peflin, a novel member of the five-EF-hand-protein family, is similar to the apoptosis-linked gene 2 (ALG-2) protein but possesses nonapeptide repeats in the N-terminal hydrophobic region. *Biochem. Biophys. Res. Commun.* **263**, 68–75
- Meyers, M. B., Zamparelli, C., Verzili, D., Dicker, A. P., Blanck, T. J. and Chiancone, E. (1995) Calcium-dependent translocation of sorcin to membranes: functional relevance in contractile tissue. *FEBS Lett.* **357**, 230–234
- Teahan, C. G., Totty, N. F. and Segal, A. W. (1992) Isolation and characterization of grancalcin, a novel 28 kDa EF-hand calcium-binding protein from human neutrophils. *Biochem. J.* **286**, 549–554
- Kitaura, Y., Matsumoto, S., Satoh, H., Hitomi, K. and Maki, M. (2001) Peflin and ALG-2, members of the penta-EF-hand protein family, form a heterodimer that dissociates in a Ca(2+)-dependent manner. *J. Biol. Chem.* **276**, 14053–14058
- Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S. and Suzuki, K. (1995) Calpain dissociates into subunits in the presence of calcium ions. *Biochem. Biophys. Res. Commun.* **208**, 376–383
- Suzuki, K. and Sorimachi, H. (1998) A novel aspect of calpain activation. *FEBS Lett.* **433**, 1–4
- Zhang, W. and Mellgren, R. L. (1996) Calpain subunits remain associated during catalysis. *Biochem. Biophys. Res. Commun.* **227**, 890–896
- Dutt, P., Arthur, J. S., Croall, D. E. and Elce, J. S. (1998) m-Calpain subunits remain associated in the presence of calcium. *FEBS Lett.* **436**, 367–371
- Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S. and Suzuki, K. (1995) A catalytic subunit of calpain possesses full proteolytic activity. *FEBS Lett.* **358**, 101–103
- Graham-Siegenthaler, K., Gauthier, S., Davies, P. L. and Elce, J. S. (1994) Active recombinant rat calpain II. Bacterially produced large and small subunits associate both *in vivo* and *in vitro*. *J. Biol. Chem.* **269**, 30457–30460
- Saïdo, T. C., Nagao, S., Shiramine, M., Tsukaguchi, M., Yoshizawa, T., Sorimachi, H., Ito, H., Tsuchiya, T., Kawashima, S. and Suzuki, K. (1994) Distinct kinetics of subunit autolysis in mammalian m-calpain activation. *FEBS Lett.* **346**, 263–267
- Crawford, C., Brown, N. R. and Willis, A. C. (1990) Investigation of the structural basis of the interaction of calpain II with phospholipid and with carbohydrate. *Biochem. J.* **265**, 575–579
- Arthur, J. S. and Crawford, C. (1996) Investigation of the interaction of m-calpain with phospholipids: calpain-phospholipid interactions. *Biochim. Biophys. Acta* **1293**, 201–206
- Hosfield, C. M., Elce, J. S., Davies, P. L. and Jia, Z. (1999) Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. *EMBO J.* **18**, 6880–6889
- Strobl, S., Fernandez-Catalan, C., Braun, M., Huber, R., Masumoto, H., Nakagawa, K., Irie, A., Sorimachi, H., Bourenkow, G., Bartunik, H. et al. (2000) The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 588–592
- Lin, G. D., Chattopadhyay, D., Maki, M., Wang, K. K., Carson, M., Jin, L., Yuen, P. W., Takano, E., Hatanaka, M., DeLucas, L. J. and Narayana, S. V. (1997) Crystal structure of calcium bound domain VI of calpain at 1.9 Å resolution and its role in enzyme assembly, regulation, and inhibitor binding. *Nat. Struct. Biol.* **4**, 539–547
- Blanchard, H., Grochulski, P., Li, Y., Arthur, J. S., Davies, P. L., Elce, J. S. and Cygler, M. (1997) Structure of a calpain Ca(2+)-binding domain reveals a novel EF-hand and Ca(2+)-induced conformational changes. *Nat. Struct. Biol.* **4**, 532–538
- Arthur, J. S., Elce, J. S., Hegadorn, C., Williams, K. and Greer, P. A. (2000) Disruption of the murine calpain small subunit gene, *Capn4*: calpain is essential for embryonic development but not for cell growth and division. *Mol. Cell Biol.* **20**, 4474–4481
- Zimmerman, U. J., Boring, L., Pak, J. H., Mukerjee, N. and Wang, K. K. (2000) The calpain small subunit gene is essential: its inactivation results in embryonic lethality. *IUBMB Life* **50**, 63–68

- 26 Tompa, P., Baki, A., Schád, E. and Friedrich, P. (1996) The calpain cascade. Mu-calpain activates m-calpain. *J. Biol. Chem.* **271**, 33161–33164
- 27 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 28 Jékely, G. and Friedrich, P. (1999) The evolution of the calpain family as reflected in paralogous chromosome regions. *J. Mol. Evol.* **49**, 272–281
- 29 Elce, J. S. (1999) Expression of calpain in bacteria and in insect cells. In *Calpain: Pharmacology and Toxicology of Calcium-Dependent Protease* (Wang, K. K. W. and Yuen, P.-W., eds.), pp. 51–62, Taylor and Francis, Philadelphia
- 30 Goll, D. E., Thompson, V. F., Taylor, R. G. and Zalewska, T. (1992) Is calpain activity regulated by membranes and autolysis or by calcium and calpastatin? *Bioessays* **14**, 549–556

Received 5 October 2001/26 November 2001; accepted 21 December 2001