Subcellular localization and \textit{in vivo} subunit interactions of ubiquitous \(\mu\)-calpain*

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**Running title:** Interaction and localization of calpain subunits
SUMMARY

Ubiquitously expressed calpains are Ca\(^{2+}\)-dependent, intracellular cysteine proteases comprising a large catalytic subunit (domains DI to DIV) and a non-covalently bound small regulatory subunit (DV and DVI). It is unclear whether Ca\(^{2+}\)-induced calpain activation is followed by subunit dissociation or not. Here, we have applied advanced fluorescence microscopy techniques to study calpain subunit interactions in living cells using recombinant calpain subunits or domains fused to fluorescent reporter proteins ECFP and EYFP. All over-expressed variants of the catalytic subunit (DI-IV, DI-III, DI-IIb) were active and Ca\(^{2+}\)-dependent. The intact large subunit, but not its truncated variants, associates with the small subunit under resting and ionomycin-activated conditions. All variants were localized in cytoplasm and nuclei, except DI-IIb, which accumulates in the nucleus and in nucleoli as shown by microscopy and cell fractionation. Localization studies with mutated and chimeric variants indicate that nuclear targeting of the DI-IIb variant is conferred by the two N-terminal helices of DI. Only those variants that contain DIII migrated to membranes upon addition of ionomycin, suggesting that DIII is essential for membrane targeting. We propose that intracellular localization and in particular membrane targeting of activated calpain, but not dissociation of its intact subunits, contribute to regulate its proteolytic activity in vivo.
INTRODUCTION

Calpains are intracellular cysteine endopeptidases (clan CA) requiring Ca\(^{2+}\) ions for activity. The family members can be classified as typical calpains, which are further divided into ubiquitous and tissue-specific calpains, and atypical calpains (reviewed in (1)). Ubiquitous calpains are heterodimers (Mr \(\sim 110\) kDa) made up of a catalytic (Mr \(\sim 80\) kDa; 80K) and a common regulatory (Mr \(\sim 30\) kDa; 30K) subunit (2, 3). Two calpain isoforms are known, which share about 62% identical residues of their catalytic subunits, but differ in the Ca\(^{2+}\) concentrations required for activation \textit{in vitro}: While \(\mu\)-calpain is activated by 5-50 \(\mu\)M Ca\(^{2+}\), m-calpain requires from 0.2 to 1 mM Ca\(^{2+}\) concentrations for activation (4). Calpain activity is regulated \textit{in vivo} by phosphorylation (5) and by the endogenous intracellular inhibitor, calpastatin (6). The latter are widely distributed heat-stable proteins acting specifically on calpains \textit{in vitro} in the presence of Ca\(^{2+}\) (7). In addition, calpain inhibition by kininogen domain II (8) and growth-arrest specific factor 2 (Gas-2)\(^{1}\) (9) has been reported.

On exposure to Ca\(^{2+}\), ubiquitous calpains undergo \textit{in vitro} limited autolysis of both subunits, which reduces Ca\(^{2+}\) requirement for activity (10). Following initial autolysis at the N-terminus of both subunits, further degradation of the large subunit eventually leads to loss of enzymatic activity (11). Controversial hypotheses of the activation mechanism of calpain \textit{in vivo} and the role of Ca\(^{2+}\) in this process have been presented (1, 11, 12). Important open questions in this regard are whether or not calpain activation results in dissociation of the large and small subunits, and the probable role of autolysis.

Recent crystal structures of full-length, Ca\(^{2+}\)-free rat and human m-calpain (13, 14) confirmed that the catalytic subunit is organized in four domains, termed DI to DIV; the 30K subunit consists of domains DV and DVI (Fig. 1A,F). Compared to the structurally homologous papain, subdomains DIIa and DIIb are misplaced; with catalytic residues Cys\(^{105}\) and His\(^{262}\) located about 10 Å apart. Moreover, the substrate binding cleft is disrupted and

\textbf{Footnote 1}
open, which is incompatible with productive binding of peptide substrates. These features explain the inactivity of calpains in the absence of Ca\(^{2+}\). The crystal structure of Ca\(^{2+}\)-bound DII domain discloses two Ca\(^{2+}\) ions that bridge subdomains IIa and IIb\(^2\) to form the catalytically competent active site, highlighting an important effect of calcium for calpain activity (15). There are, however, additional Ca\(^{2+}\) binding sites with important roles for activity.

Domains DIV and DVI contain five EF-hand motifs highly similar to those of the major intracellular Ca\(^{2+}\)-binding protein, calmodulin. Accordingly, the first three N-terminal EF-hands bind Ca\(^{2+}\) (16). Calcium-mediated calpain activation was therefore proposed to result from Ca\(^{2+}\) binding to these domains, mainly to the third EF hand in DIV. Ion binding is expected to promote release of the N-terminal α-helix that anchors the catalytic and regulatory subunit, with concomitant displacement of DIIa toward DIIb (17). An alternative hypothesis points up the role of the synaptotagmin C2-like domain DIII, which harbors several acidic residues within a prominent solvent-exposed loop. This ‘acidic loop’ is spatially adjacent to DIIb, and engages in electrostatic interactions with the latter (14). It was suggested, therefore, that Ca\(^{2+}\) binding to this loop could release subdomain IIb to move toward IIa, thus allowing formation of a functional catalytic center (the ‘electrostatic switch’ hypothesis). Furthermore, it was proposed that the Ca\(^{2+}\) coordination spheres might be only incompletely formed by protein oxygen atoms, and that additional ligands, provided by negatively charged head groups of acidic phospholipids, complete these coordination spheres. Indeed, acidic phospholipids such as phosphorylated phosphatidyl inositols reduce the Ca\(^{2+}\) concentration required for autolysis in vitro (10, 18, 19), and mutation of the acidic residue Glu\(^{504}\) of m-calpain significantly affects Ca\(^{2+}\) sensitivity of the enzyme (13). Finally, DIII alone binds Ca\(^{2+}\) with an affinity comparable to that of DIV, which is increased 2- to 10-fold upon addition of liposomes (20). Conversely, Ca\(^{2+}\) significantly promotes phospholipid

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Footnote 2

Footnote 4
binding in a similar manner as observed with the C2 synaptotagmin domain. These findings suggest a connection between calpain activation and membrane translocation (see e.g. Ref. 21).

In the light of these findings, we have decided to study subcellular localization and subunit interactions of endogenous calpains and over-expressed human µ-calpain subunits and several engineered variants thereof, aiming to provide insight into the calpain activation mechanism in vivo. Firstly, we have followed the subcellular distribution of endogenous ubiquitous human calpains and calpastatin under resting and Ca²⁺-activated (ionomycin-induced) conditions. Next, we have over-expressed the active 80K subunit of human µ-calpain and its truncated variants DI-III, DI-IIb and DIII, N- or C-terminally fused to ECFP, in COS 7 and in LCLC 103H cells. Co-expression with 30K chimeras that incorporate EYFP allowed us to analyze the interactions between the 80K and 30K subunits in vivo using fluorescence energy transfer techniques (FRET). This technique is particularly suited to demonstrate intimate (below 80 nm) contact between reaction partners. Recent progress in fluorescence microscopy has made it possible to apply this technique also to interaction analysis in living cells (22). At the same time, over-expression of the 80K-EYFP chimera in cells stably over-expressing a membrane marker, mem-ECFP, combined with co-localization analysis (23) supported the membrane association of this subunit. We provide evidence against dissociation of calpain subunits upon activation in vivo. Both endogenous and over-expressed calpain variants were localized in the cytoplasm and/or nucleus of cells, respectively, except DI-IIb, which accumulated in the nucleus and even in nucleoli. Localization studies with five additional truncated or mutant variants of the DI-IIb construct identify the N-terminal helices of DI as a putative nuclear-targeting motif.
EXPERIMENTAL PROCEDURES

Materials - The plasmid vectors pECFP-C1, pECFP-N1, pEYFP-C1 and pEYFP-N1 were purchased from Clontech; restriction endonucleases and DNA modifying enzymes were from Roche Biochemicals and New England Biolabs. Antibodies anti-peptide 80K and anti-peptide 76K were produced by Biogenes on request. Commercially available antibodies used were anti-DII (804-051-R100, Alexis), anti-80K (MAB3104, Chemicon), anti-30K (MAB3083, Chemicon), anti-calpastatin (MA3-945, ABR), anti-living colors (8367-1, Clontech), and anti-calpain (laboratory stock). The fluorogenic substrate Suc-LLVY-amc was purchased from Bachem. The calpain inhibitor, AC27P, and ionomycin were from Sigma. All other reagents were of the highest purity commercially available.

Plasmids construction and preparation - All variants of human µ-calpain large subunit for expression in mammalian cells were constructed using as scaffold a self-prepared semi-synthetic gene, pUC18_80K DNA, or a variant thereof with the active site residue Cys\textsuperscript{115} mutated to alanine (pUC18\_\{C^{115}A\}\_80K).\textsuperscript{3} Variants DI-IV (S\textsuperscript{2}-A\textsuperscript{711}), DI-III (S\textsuperscript{2}-D\textsuperscript{523}), and DIII (I\textsuperscript{363}-Q\textsuperscript{527}) were obtained via PCR, using primers listed in Table I. The DNA for variant DI-IIb (S\textsuperscript{2}-T\textsuperscript{390}) was obtained by digestion of pECFP_80K DNA with the restriction enzymes EcoRI and SacII. The small subunit DNA was amplified via PCR using as template a plasmid coding for the His-tagged 30K subunit\textsuperscript{3} (Table I). The DNA fragments were subcloned into the appropriate vectors, pECFP-N1 (80K–ECFP, [C\textsuperscript{115}A]80K–ECFP), pECFP-C1 (ECFP–80K, ECFP–[C\textsuperscript{115}A]80K, ECFP–DI-III, ECFP–DI-IIb, ECFP–DIII), pEYFP-N1 (30K–EYFP, 80K–EYFP, DI-III–EYFP), and pEYFP-C1 (EYFP–30K), after digestion with the indicated restriction enzymes (Table I). Competent TG1 cells were transformed with the ligation mixtures and the resulting isolated plasmids with the correct sequences were used for further transfections. The variants ECFP–[K\textsuperscript{20}P]DI-IIb, ECFP–[R\textsuperscript{48}P]DI-IIb and ECFP–[R\textsuperscript{376}A,R\textsuperscript{377}A]DI-IIb were prepared via PCR using as template pECFP_DI-IIb DNA.

Footnote 3

Table I
according to the QuickChange™ site-directed mutagenesis kit instructions (Stratagene, USA), using the primers listed in Table II. Variants that either lack the two N-terminal helices (ECFP–Δ(S²-L⁵⁵)DI-Illb) or consist of these two helices fused to ECFP (ECFP–(S²-L⁵⁵)), were obtained by double-digesting pECFP_Dl-Illb DNA with EcoRI/KpnI or KpnI/BamHI, respectively, followed by ligation into pECFP-C1 digested with the same pairs of enzymes. The identity of cloned DNAs was verified by sequencing both DNA strands.

**Cell culture and transfection** - For routine cell culture RPMI 1640 (LCLC 103H cells, DSMZ ACC 384) or DMEM (COS 7, ATCC CRL 1651) media (Gibco BRL) were supplemented with 10% fetal calf sera (Sigma), 0.6% L-glutamine (Gibco BRL). Transfection was carried out using the FuGENE™6 Reagent (Roche Molecular Biochemicals) according to the general protocol suggested by the manufacturer. COS 7 and LCLC 103H cells transiently over-expressing the corresponding construct were used in this study. For membrane targeting studies a LCLC 103H clone constitutively expressing the membrane marker mem-ECFP (Clontech) was transfected with 80K–EYFP and DI-III–EYFP DNAs. For localization experiments of ECFP–DI-Illb and variants thereof, a LCLC 103H clone expressing constitutively histone H2A coupled to EYFP was used. Histone H2A–EYFP and mem-ECFP expressing cell clones of LCLC 103H were obtained as described earlier (24).

**Isolation and analysis of cytoplasmic proteins** - Cytoplasmic extracts from approx. 10⁶ cells were prepared according to Ref. 25. Briefly, pools of cells transfected with the corresponding constructs were either kept untreated or incubated with the ionophore for 30 min before cell lysis and fractionation. Protein content was assayed by the bicinchoninic acid method (26). Samples were electrophoretically resolved on SDS-Tris-Glycine (12.5%) gels, transferred to nitrocellulose membranes (Schleicher & Schuell), and probed against the

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indicated antibodies; the resulting complexes were detected with anti-mouse or anti-rabbit HRP-linked IgG from New England Biolabs using ECL (Amersham).

**Immunoprecipitation** - Cell lysates were pre-incubated with the antibody, anti-80K, bound to Protein G-Agarose (Roche Diagnostics), and eluted according to Ref. 12. The immunoprecipitated proteins were separated in SDS-Tris-Glycine (12.5%) gels, transferred to nitrocellulose membranes, and detected with anti-calpain, a polyclonal antibody that recognizes both large and small calpain subunits.

**Epifluorescence microscopy** - Cells were cultured on 4.2 cm diameter coverslips (Langenbrinck) or in Lab-Tec™ II cover glass chambers (Nunc) and used for microscopical studies 24 to 72 h after transfection with the indicated constructs. Prior to observation, the coverslips were mounted in POC-holders (PeCon) that were kept at 34-36 °C and 5% CO₂ during observation. The microscope (Axiovert S100 TV, Zeiss) was equipped with objectives Fluor 40/1.3 oil Ph2, Apochromat 40/1.2 W korr, or Neofluar 63/1.25 oil Ph3, filterwheels and shutters (Ludl); Orca 4742-95 CCD camera (Hamamatsu), and controlled by OpenLab software (Improvision). Filter systems for ECFP, EYFP and FRET were from Chroma Technologies (Ex: 435/10 and 515/10; single band: DM₉ 455, Em₉ 480/40 and DMᵧ 530, Emᵧ 560/40; doubleband: DM₉ 475, Em₉ 470/30, DMᵧ 556, Emᵧ 555/40 and FRET: DM 460, Em 535/30). All observations were carried out in the corresponding growth medium. Images were captured prior and after addition of ionomycin to 2 µM end concentration during 30 min in 5 min intervals. To obtain optical section series a piezo motor (Physical Instruments) was used to drive the C-Apochromat 40x objective in appropriate steps. Images were processed by deconvolution and false LUTs were applied to them for the final presentation. Co-localization was analyzed by the respective Openlab™ software module, which operates on a pixel-by-pixel comparison of pairs of images (23).

**Immunocytofluorescence detection of endogenous calpain and calpastatin** - Cells were grown on glass coverslips and stained according to Becton Dickinson Transduction
Laboratories protocol using the indicated antibodies. For fluorescence microscopy, coverslips were mounted on microscope slides with PermaFluor (Immunotech) and inspected in the microscope (LSM 410, Zeiss) equipped with the objective Fluor 63/1.4 oil Ph2. Images were acquired using LSM410 software version 3.95 (Zeiss). For cytoskeletal immunostaining (F-actin) cells were pre-incubated with ALEXA633-phallolidin (Molecular Probes). Second antibodies used were ALEXA488 anti-mouse and anti-rabbit (Molecular Probes), detected at excitation 488 nm, beamsplitter FT 488/543, emission 515-525 nm. F-actin was visualized with the same beamsplitter at excitation 633 nm, emission >665 nm. Image parameters: 512 x 512 pixels, pinhole 20.

Confocal laser scanning microscopy - Cells were prepared as for epifluorescence microscopy. The microscope (Leica TCS SP) was equipped with objectives, Fluor 40/1.3 oil Ph2 and Neofluar 63/1.25 oil Ph3, and the following filter sets: ExC 453, EmC 495/60; ExY 514, EmY 585/70. Images were taken prior and then after addition of 2 µM ionomycin during 30 min in 5 min intervals. Imaging parameters: 512 x 512 pixels, pinhole 1. Other relevant parameters are given with the respective images. All images were processed with a 3x3 median filter.

Calpain activity in living cells - was determined as previously described (27), using the fluorogenic substrate Suc-LLVY-amc. The specificity of calpain cleavage was evaluated by pre-incubating culture cells with 50 µM AC27P (28) for 1 h before treatment with 2 µM ionomycin.

Measurement of free Ca²⁺ concentrations - Free Ca²⁺ concentrations in COS 7 cells were determined prior and after addition of 2 µM of ionomycin, as previously described (27).
RESULTS

Subcellular localization of endogenous calpain and calpastatin by immunocytofluorescence - The subcellular localization of endogenous calpain and calpastatin was characterized by immunocytofluorescence microscopy using the monoclonal antibodies anti-DII (specific for \( \mu \)-calpain large subunit residues G\(^{245}\)-F\(^{265}\)), anti-30K and anti-calpastatin, respectively. Prior to Ca\(^{2+}\)-mobilization, both calpain subunits and calpastatin are homogenously distributed in the cytoplasm, with a slight preponderance around the nuclear region (Figs 2A-C). Besides, calpastatin accumulated in the nuclei (Fig. 2C). After ionomycin addition, fluorescence labeling due to calpain detection increased at cell membranes (Figs 2A-B), while calpastatin did not relocalize to membranes under calcium-stimulating conditions (Fig. 2C).

Over-expression of ECFP- and EYFP-fused human \( \mu \)-calpain variants in COS 7 and LCLC 103H cells - For expression in mammalian cells, DNA fragments coding for the human \( \mu \)-calpain 80K subunit (residues Ser\(^{2}\)-Ala\(^{711}\)) or its truncated variants S\(^{2}\)-D\(^{523}\) (DI-III), S\(^{2}\)-I\(^{390}\) (DI-IIb), and I\(^{363}\)-Q\(^{527}\) (DIII), as well as the active site mutant [C\(^{115}\)A]80K were ligated into vector pECFP-C1 to generate chimeric proteins N-terminally extended with ECFP. DNAs coding for the wild type and active site-mutated large subunit were ligated into pECFP-N1 to produce variants C-terminally tagged with ECFP, respectively. 80K and DI-III were also fused to the N-terminus of EYFP. Similarly, the 30K DNA was ligated into pEYFP-C1 or pEYFP-N1 to allow expression of chimeric constructs with EYFP attached to the N- or C-terminus of the small subunit, respectively (see Fig. 1F for a schematic representation of the variants).

Firstly, we determined whether fusion of enhanced fluorescence protein at either terminus of the 80K calpain subunit influences the protein expression level. LCLC 103H cells were transfected with plasmids encoding for either the individual large subunit...
(pECFP_80K, p80K_ECFP) or small subunit chimeras (p30K_EYFP, pEYFP_30K), or co-transfected with all possible combinations of chimeric 80K- and 30K-constructs. Cells were lysed 72 h after transfection, and cytoplasmic extracts were analyzed by Western blot using anti-DII antibody (Fig. 3A). All variants were successfully over-expressed, but the expression levels of variants in which ECFP is fused to the N-terminus of the 80K subunit were systematically lower than those of the C-terminal fusions (Fig. 3A). The latter species were therefore selected for subsequent cellular localization studies. Of note, the 80K subunit of endogenous calpain was also detected in mock-transfected cells (Fig. 3A).

**Intracellular calcium concentrations in COS 7 and LCLC 103H cells** - Addition of a calcium ionophore to cells leads to an increase in the intracellular calcium concentration with concomitant generation of calpain activity (27, 29). Fura-2 measurements of intracellular calcium concentrations were carried out in living cells before and after addition of 2 µM ionomycin. In both cell lines, the addition of ionomycin caused a fast transient increase ("burst") in the intracellular Ca\(^{2+}\) concentration before equilibrium was reached (Fig. 3B). Interestingly, we observed remarkable differences regarding (i) the peak values of intracellular Ca\(^{2+}\) concentration (between 800 nM and 1.6 µM in LCLC 103H cells (27), but only between 180 nM and 240 nM in COS 7 cells), and (ii) the intracellular Ca\(^{2+}\) concentration at equilibrium (180 nM in LCLC 103H cells (27), 50 nM in COS 7 cells; Fig. 3B).

**Calpain activity in living cells** - The fluorogenic peptide substrate Suc-LLVY-amc (30) was used to investigate calpain activity *in vivo* after calcium stimulation. Cells were treated with 160 µM Suc-LLVY-amc and 2 µM ionomycin, and fluorescence at 460 nm was measured in 5 min intervals for up to 1 h. We have previously shown that addition of the calcium ionophore results in an approximately two-fold increase in calpain activity, which was specifically inhibited by AC27P (27). We have now confirmed that calpain activity was stimulated by addition of ionomycin in both COS 7 (Fig. 3C) and LCLC 103H cells (Fig. 3D),

**FIG. 3**
also in agreement with previous reports in other cell lines (31-33). Furthermore, cells over expressing μ-calpain chimeras that include the catalytic domain showed similar levels of Ca\(^{2+}\)-dependent activity as variants of full-length 80K. Calpain activities of these fusion proteins were ~four-fold (in COS 7 cells) or ~three-fold higher (in LCLC 103H cells) than those of wild type and mock-transfected cells. Most of the ionophore-induced hydrolytic activity was inhibited by preincubation of cells with the specific calpain inhibitor, 50 µM AC27P (Fig. 3C-D), thus confirming that the observed activity results almost exclusively from calpain activation. No significant differences in activity were detected between cells co-expressing large and small subunit variants and those transfected with the large subunit chimeras alone. Interestingly, cells that exclusively over-expressed the small subunit variants showed significantly higher hydrolytic activities than wild type and mock-transfected cells.

*Subcellular localization of human μ-calpain variants and calpain subunit interactions*

- Epifluorescence microscopy was used to study (i) the localization of over-expressed μ-calpain subunits and truncated variants of the large subunit, and (ii) the interaction between large and small calpain subunits *in vivo*. Both under resting and activated conditions (Fig. 4A), the 80K and 30K subunits co-localized in the cytosol, as indicated by the fluorescence (colored yellow-brown) observed upon excitation with the FRET filter (Fig. 4A). Upon addition of the ionophore the two subunits seem to migrate together, mainly to the plasma membrane (Fig. 4A, white arrows). In cells co-expressing non-fused ECFP and EYFP we observed uniform patterns of cytoplasmic localization but no quenching of the ECFP fluorescence either under resting or activated conditions (data not shown), thus ruling out that the observed FRET effects were due to unspecific interactions. These results were corroborated by co-immunoprecipitation of the 30K and 80K subunits under resting and Ca\(^{2+}\)-activated conditions (Fig. 4B). No FRET was observed in cells transiently co-expressing truncated variants of μ-calpain large subunit fused to ECFP and 30K-EYFP (data not shown), in agreement with the severely reduced interaction interfaces after deletion of either C-
terminal domains (chimeras of DI-III, DI-IIb) or both N- and C-terminal domains (DIII; compare Fig. 1A-D).

Confocal laser scanning microscopy was further used to study the intracellular localization and trafficking of the μ-calpain subunits and of truncated variants thereof. Chimeras 80K–ECFP and 30K–EYFP homogenously co-localized in the cytoplasm under resting conditions, without obvious accumulation in subcellular organelles. Further, we could confirm their migration to membranes upon addition of ionomycin (Fig. 5A-C). Again, comparison of both fluorescence patterns provides additional evidence that the two subunits co-localize throughout (Fig. 5C). The migration of the small subunit variant seems to occur in vesicle-like structures of about 1 µm diameter (Fig. 5B). Whether the large subunit is included within these structures is unknown, but co-localization of both subunits throughout would point to its inclusion in these vesicles (see Discussion).

Over-expressed ECFP–DI-III was distributed homogenously in the cytoplasm and within the nucleus (Fig. 5D). In this figure, images corresponding to the same cell are shown at two different levels (whole cell, upper panels or nucleus, lower panels) to highlight the ionomycin-dependent migration of ECFP–DI-III to both the plasma and nuclear membranes. In contrast, over-expressed ECFP–DI-IIb was mainly observed in the cell nucleus including the nucleoli, and this localization pattern did not change after ionomycin addition (Fig. 5E).

To confirm the plasma membrane association of calpain or its subunits, 80K and DI-III DNAs were fused to EYFP and supertransfected into LCLC 103H-clone cells constitutively expressing a membrane marker, mem-ECFP. Fluorescence image series were taken in the respective channels, processed by deconvolution algorithms and analyzed comparatively pixel-by-pixel with a co-localization program. The result of this process is given by a representative example of the 80K-EYFP construct (Fig. 6); it clearly demonstrates the plasma membrane association of the 80K subunit. Experiments conducted with the DI-III construct had similar outcomes (data not shown).
Subcellular localization of calpain variants by Western blot analysis - To further confirm the results obtained with microscopic techniques, the relative distribution of calpain chimeras in transfected LCLC 103H cells was assessed via Western blot analysis. Equal amounts of cells ($10^6$) either untreated or incubated with 2 µM ionomycin were fractionated by centrifugation. The purity of isolated fractions was verified by measuring a ~15-fold higher activity of lactate dehydrogenase (LDH) in the cytoplasmic than in the nuclear fractions (data not shown), indicating a successful separation (34). Moreover, only the nuclear but not the cytoplasmic fraction was stained by propidium iodide (data not shown).

The separated fractions were analyzed by Western blot using either anti-DII or a polyclonal antibody that recognizes the fused fluorescent proteins, anti-living colors. The human µ-calpain large subunit was detected in the cytoplasmic, membrane and nuclear fractions of cells over-expressing 80K–ECFP before and after ionomycin addition (Fig. 7A). After ionomycin treatment, however, the relative amount of calpain large subunit variant in the membrane fractions increased substantially. In the cytoplasmic fraction of resting cells over-expressing ECFP, a faint band corresponding to endogenous calpain was detected, which disappeared in activated cells due to autolysis (data not shown). Unexpectedly, the intensity of the 80-kDa-band -corresponding to endogenous calpain- was increased in cells over-expressing the chimeric 80K-ECFP form, compared to wild type cells. This feature may reflect the existence of an uncharacterized mechanism(s) of up-regulation of calpain expression by its isolated subunits, also suggested by the results of activity measurements (Fig. 3C, D).

The truncated variant, ECFP–DI-III, was primarily detected in the nucleus of the cells, but it was also detectable in the cytoplasmic fraction (Fig. 7B). After ionomycin addition, this large subunit variant was enriched in the membrane fractions as well. In striking contrast,
ECFP–DI-IIb was localized mainly in the nuclear and nucleolar fractions, and did not re-localize after ionophore treatment (Fig. 7C).

Domain DIII contributes to membrane targeting of calpain – We had determined that chimeric variants of μ-calpain that contain domain DIII were targeted to membranes upon ionomycin-mediated activation. Considering also the proposed membrane-targeting role of DIII (14), we investigated the intracellular localization of over-expressed ECFP–DIII, as well as possible interactions between the ECFP–DIII / 30K–EYFP pair in COS 7 and LCLC 103H cells. The chimeric protein ECFP–DIII was primarily localized in the cytoplasmic fraction, but it was also detectable in the membrane fraction in resting cells (Fig. 7D). After addition of ionomycin, ECFP–DIII was enriched in the membrane fraction (Fig. 7D).

N-terminal helices in domain DI are a putative nuclear targeting motif - Ubiquitous calpains lack stretches of positively charged residues like those of the SV40 large tumor antigen (PKKKRKV\textsuperscript{132}, (35)), or the protooncogene c-myc (PAAKRVKLD, (36)), which have been repeatedly implicated as nuclear localization signals (NLS, recently reviewed in (37)). The nuclear and nucleolar localization of the truncated ECFP–DI-IIb variant was therefore unanticipated and provoked an investigation regarding structural elements that could be responsible for this finding. With this aim, we produced five additional chimeras derived from this variant, and over-expressed them in LCLC 103H cells to perform subcellular localization studies similar to those described above. The selected chimeras either lack the two helices within the N-terminal DI and the linker to DIIa (ECFP–Δ(S\textsuperscript{2}-L\textsuperscript{55})DI-IIb), or have positively charged residues within this domain replaced by a helix-disrupting proline within the first (ECFP–[K\textsuperscript{20}P]DI-IIb) or the second helix (ECFP–[R\textsuperscript{48}P]DI-IIb). An additional charge-mutated variant had two consecutive basic residues in domain DIIb, Arg\textsuperscript{376} and Arg\textsuperscript{377}, replaced by alanines (ECFP–[R\textsuperscript{376}A,R\textsuperscript{377}A]DI-IIb). Finally, a chimera consisting of the N-terminal helices fused to the N-terminus of ECFP was constructed (ECFP–(S\textsuperscript{2}-L\textsuperscript{55})).
To clearly document their subcellular localization we transfected these constructs into a LCLC 103H cell clone that constitutively expresses histone H2A fused to EYFP (Fig. 8), enabling us to study the possible influence of the mutated calpain DI-IIb constructs on nuclear morphology and chromatin structure. Already within 24 h post transfection the chromatin segregated, with chromatin clusters appearing at the inner nuclear envelope. The morphology of nuclei changed dramatically from slightly indented to multilobed "kidney" forms (Fig. 8C). Interestingly, with the only exception of the ECFP–[R376A,R377A]DI-IIb chimera, all the investigated variants accumulate in the cell nucleus (Fig. 8). Deletion of both helices, however, diminished the nuclear localization of DI-IIb. The over-expressed chimeric proteins do not appear within organelles (ER, vesicles). These findings suggest an important role of the N-terminal helices of DI in the nuclear localization of ubiquitous calpains.
DISCUSSION

Differential subcellular distribution of ubiquitous calpains and calpastatin - It is becoming increasingly clear that ubiquitous calpains play important physiological roles in vivo (see for instance Ref. 38), and are implicated in pathological processes such as ischemia/reperfusion injury (39, 40). The mechanisms of calpain assembly, activation and regulation as well as of cellular trafficking are therefore of considerable interest, but they are only scarcely understood. Here we have investigated the activation mechanism of ubiquitous calpains in vivo, in particular the subcellular localization of calpain subunits and their interaction under resting and Ca^{2+}-activated conditions.

Addition of 2 µM ionomycin increased the intracellular Ca^{2+} level (Fig. 3B and Ref. (27)), and this moderate increase in Ca^{2+} concentration activates endogenous calpains in the two cell lines studied (Fig. 3C,D). We had previously shown that calpain activation triggers the intrinsic apoptotic pathway, with first signals of caspase activity detected 3 h after ionomycin treatment (27). The ionomycin concentration employed in the current investigation, however, did not cause cytotoxic effects on wild type cells up to 30 min (data not shown), the time period during which the microscopic localization experiments were carried out. Thus, all observations discussed below relate to cells before the onset of apoptosis.

We have detected a differential distribution of calpains and calpastatin in LCLC 103H cells: While calpains are distributed homogenously in the cytoplasm, their endogenous inhibitor, calpastatin, was localized not only in the cytoplasm but also in the cell nucleus. Of particular note, we detected ionomycin-induced redistribution of calpain toward membranes, while the nuclear localization of calpastatin was unaffected by the increase in intracellular Ca^{2+} concentration. Similar results have also been documented in proliferating culture cells using immunofluorescent light microscopy (41). Although calpains are usually viewed as
cytoplasmic enzymes (Fig. 2A–B and Ref. (42)), they have also been detected in the nucleus of C-33A cervical carcinoma cells (41, 43). In contrast, calpastatin is mainly localized in the nucleus (Fig. 2C, see also (41)) or in nuclear invaginations (44). Active transport of fluorescein-labeled \( \mu \)-calpain to the nucleus has been reported, while labeled m-calpain and calpastatin were poorly transported at best (45). Our results corroborate the variable distribution of calpain and calpastatin in different human cells (41), but in particular stress the physical separation between calpains and their endogenous protein inhibitor.

*Calpain subunits do not dissociate upon activation* - The hypothesis that calpain subunits dissociate in the presence of \( \text{Ca}^{2+} \) has previously been tested *in vitro* using techniques that cannot avoid interference from \( \text{Ca}^{2+} \)-induced aggregation phenomena, and/or that are probably obscured by large subunit autolysis (1, 46). To overcome these limitations, and to study for the first time the interaction between calpain subunits *in vivo*, we developed a strategy based on FRET visualization between fluorescence-tagged calpain subunits.

First we verified that all ECFP fusion proteins that contain the catalytic subunit were enzymatically active, as shown by cleavage of the cell-permeable small peptide substrate Suc-LLVY-amc (Fig. 3C,D). These activities were \( \text{Ca}^{2+} \)-dependent, independent of the position of the fluorescence label, and were reduced to the level of the endogenous background activity in control cells preincubated with AC27P. In our hands, the minimum active \( \mu \)-calpain fragment comprises residues Ser\(^2\)-Thr\(^{390}\) (variant DI-IIb), in agreement with recent results (15, 47). Thus, domains DIII and DIV are dispensable for calpain activity on small peptide substrates, although they could play roles in enzyme localization and protein substrate recognition, as suggested (14). Surprisingly, not only cells over-expressing the 80K catalytic subunit alone, but also those that exclusively over-express the 30K regulatory subunit showed significantly higher \( \text{Ca}^{2+} \)-induced activities than wild type and mock-transfected cells. This observation points to a previously unanticipated cross talk between the two calpain subunits,
which results in up-regulation of the 80K by the 30K subunit and vice versa. In this regard, we have observed that down-regulation of the m- or \( \mu \)-calpain catalytic subunit results in a concomitant decrease in the levels of the regulatory subunit\(^4\). The association and stabilization of the exogenous 30K subunit with endogenous m-/\( \mu \)-calpain 80K subunits and vice versa could contribute to the observed effect.

Because of their higher over-expression levels, variants 80K–ECFP, ECFP–DI-III, ECFP–DI-IIb, ECFP–DIII and 30K–EYFP were selected for subunit interactions studies. We only observed the FRET effect in cells co-expressing 80K–ECFP and 30K–EYFP, both under resting and \( \text{Ca}^{2+} \)-activated conditions. This observation, together with the results of immunoprecipitation experiments conducted with endogenous calpains, indicates that under the given experimental conditions the subunits of \( \mu \)-calpain associate not only under resting conditions, but also after activation. Domain IV seems to be indispensable in subunit interaction since we could not detect energy transfer between the small subunit and variants of the large subunit lacking DIV.

These results are in agreement with several previous findings in vitro: (i) Both subunits of m-/\( \mu \)-calpain were co-precipitated by monoclonal antibodies directed against each single subunit in the presence of \( \text{Ca}^{2+} \)-concentrations supporting catalytic activity (12). (ii) The 80K subunit alone lacks proteinase activity in vitro, even in the presence of \( \text{Ca}^{2+} \), but \( \text{Ca}^{2+} \)-dependent calpain activity is observed following association of the two subunits (48). (iii) Subunit exchange does not occur in mixtures of wild type and \( [\text{C}^{105}\text{S}]80\text{K} \) rat m-calpain in the presence of \( \text{Ca}^{2+} \) (11). (iv) His-tagged rat \( [\text{C}^{105}\text{S}]80\text{K} \) m-calpain does not dissociate in the presence of \( \text{Ca}^{2+} \) (49). These authors also demonstrated that (v) natural bovine m-calpain, after irreversible inhibition with z-LLY-CHN\(_2\), bound to immobilized casein and was eluted as a heterodimer. Finally, (vi) the crystal structures of m-calpain disclose large (~3,000 Å\(^2\)) interaction interfaces between large and small subunits.
In spite of this overwhelming body of evidence in support of the indivisibility of the calpain heterodimer, some authors have argued that both calpain subunits are only loosely associated, and therefore prone to dissociation. For instance, it was suggested that the small subunit is exclusively needed to assist folding of the 80K subunit. Along these lines, it has been reported that dissociation of the large subunit from the small subunit on exposure to Ca\textsuperscript{2+} was required for the expression of activity. Moreover, the 80K subunit shows a calcium sensitivity identical to that of the activated form of calpain but not of the original control calpain (50). In a recent report, Pal and co-workers (46) discuss the results of crystallization trials conducted with Ca\textsuperscript{2+}-activated m-calpain. Crystals were grown that contained only the 30K subunit, while the large subunit was largely found as an amorphous precipitate. However, these results were obtained with samples maintained for large periods of time at the non-physiologically high protein and calcium concentrations needed for crystallization.

In contrast, the current studies were performed under cytosolic calcium and protein concentrations and are at odds with the possibility of subunit dissociation in vivo. Of course, we cannot completely exclude reversible dissociation in a time scale much lower than the observation time and/or as a minor process, but dissociation of calpain subunits seems to be unlikely according to our current results, and functional and structural evidence cited above.

*Calpain autolysis products are not detected in vivo* - Calpain autolysis in vitro has been repeatedly reported (see e.g. (51, 52)). These results seeded the speculation that calpain autolysis could contribute to the differential subcellular targeting of the enzyme, for instance via dissociation of truncated subunits. Antibodies raised against the N-terminal peptide of the 80K subunit, S\textsuperscript{2}-Q\textsuperscript{31}, (anti-peptide 80K) recognize the intact (dissociated) large subunit in Western blots of purified calpain and in cytoplasmic extracts (Ref. (51) and data not shown). In a similar manner, antibodies raised against peptide L\textsuperscript{28}-N\textsuperscript{33} (anti-peptide 76K) recognize 76-kDa and 40-kDa autolysis fragments in vitro. However, these anti-peptide antibodies failed to detect the catalytic subunit or its 76/40-kDa N-terminal autolysis products in living
cells, both under resting and under activated conditions. Either these antibodies are not sensitive enough for immunocytofluorescence studies, or these fragments are not produced in vivo. Alternatively, the N-terminal region of the 80K subunit may not be accessible to these antibodies within the heterodimeric calpain molecule in vivo, or may adopt a conformation(s) that differs from the one(s) recognized by these antibodies. Regardless, additional experimental evidence presented here exclude unrestricted degradation of calpain in our current setting. We cannot rule out, however, that autolysis at the N-terminus of both subunits and other sites could occur in other cell lines and/or in different experimental settings, with concomitant dissociation of calpain subunits (53). This might in turn affect the differential intracellular distribution of the protease, allowing calpainolysis to take place, e.g. in the nuclei (54-57).

**Nuclear localization of variant DI-IIb** - ECFP–DI-IIb was almost exclusively localized in the nucleus and nucleoli (Fig. 5E). It seems unlikely that simple diffusion through nuclear pores is responsible for this localization, because the variants ECFP, EYFP, ECFP–DIII and 30K–EYFP, which are of similar size or smaller, are distributed homogenously in the cytoplasm and nucleus.

The three-dimensional structure of m-calpain reveals a positively charged region at the N-terminus of the large subunit comprising two α-helices (see Fig. 1A-E), one of which is largely solvent-exposed in m-calpain, and by analogy in all analyzed µ-calpain variants containing the homologous N-terminal peptide stretch (see Fig. 1F). Recently, positively charged helical structures have been found responsible for nuclear targeting of several unrelated proteins (58-60). In the light of these findings, and considering the lack of a consensus sequence for nuclear localization in ubiquitous calpains, we speculated that the α-helices at the N-terminus of the large subunit were important for nuclear targeting. Indeed, deletion of both helices from the DI-IIb chimera results in a form that is localized mainly in
the cytoplasm, while the helices alone were able to direct ECFP to the cell nucleus. Our data and reports of calpain involvement in signaling pathways (38, 61) suggest that the nucleocytoplasmic distribution of calpains may be altered in response to cell activation, differentiation, stress or other stimuli.

Domain III is critical for membrane targeting of \( \mu CP \) – The over-expressed catalytic subunit and its truncated variants, DI-III and DIII, migrated to cell membranes under activating conditions, in agreement with previous reports (62, 63). In contrast, ECFP–DI-IIb did not migrate to membranes, indicating a critical role of the synaptotagmin C2-like domain DIII in the membrane targeting mechanism of calpains. This finding is in line with the \( \text{Ca}^{2+} \)-dependent phospholipid binding ability of the isolated domain \textit{in vitro} (20). Further, we detected relocalization of the over-expressed light chain chimera 30K–EYFP upon ionomycin addition. Remarkably, redistribution of this form was associated with the formation of organized structures, possibly vesicles (Fig. 5B). This suggests that 30K small subunit could oligomerize in the absence of the large subunit, as observed by others \textit{in vitro} (46).

In summary, we provide insight into the mechanisms of calpain assembly, activation and regulation \textit{in vivo}. Our results suggest that calpain activity in the cells is regulated not only by calpastatin, but also by differential intracellular localization and in particular membrane targeting of activated calpain. Dissociation of its intact subunits, in contrast, appears to play at most a secondary role in its regulation. Further, we have begun to unravel the roles of non-catalytic domains for calpain activity \textit{in vivo}. Most interestingly, we confirm and extend results implicating the synaptotagmin C2-like domain III as well as the light chain domains in membrane targeting. In addition, we identify a putative novel role for the N-terminal domain I, regulation of nuclear localization.
Acknowledgements - We are grateful to Mathias Hafner for facilitating intracellular activity and calcium measurements in his laboratory, Axel Ullrich and Axel Choidas for valuable suggestions. We also thank Dusica Gabrijelic-Geiger for providing anti-calpain antibody. Claudia Huber, Barbara Meisel, and Anna Heckel-Pompey are acknowledged for their excellent technical assistance. S.G.P. was supported by a research fellowship from the Deutschen Akademischen Austauschdienst.
REFERENCES


FOOTNOTES

1The abbreviations used are: AC27P, acetyl calpastatin 27-peptide; ECFP, enhanced cyan fluorescent protein; ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; EYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; HRP, horse radish peroxidase; LUT, color look up table; SDS, sodium dodecyl sulfate; Suc-LLVY-ame, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin.

2 We follow the domain nomenclature proposed by Strobl et al. (14), illustrated in Fig. 1A. In this nomenclature, the papain-like catalytic domain DII is further subdivided into two subdomains, DIIa and DIIb.


FIGURE LEGENDS

FIG 1. **Calpain variants used in subcellular localization and subunit interaction studies.**

A-D, Ribbon drawings of m-calpain three-dimensional structure and variants studied in this work (B, 80K; C, DI-III and D, DI-Iib). Figures were prepared with SETOR using the Protein Data Bank entry for human m-calpain (1DKV). Color labels of the domains: I, green; IIa, yellow; IIb, red; III, blue; IV, gold-brown; V, orange-brown and VI, orange. E, GRASP electrostatic surface potential of DI-Iib. Positively and negatively charged surfaces are blue and red colored, respectively. F, Summary of variants of human µ-calpain over-expressed in COS 7 and LCLC 103H cells. Asterisks indicate the position of mutated amino acid residues.

FIG 2. **Differential subcellular localization of endogenous ubiquitous calpains and calpastatin by immunofluorescence.** Cells were permeated, fixed and stained as explained in "Experimental Procedures". Confocal laser scanning microscopy images were taken before or after treatment with 2 µM ionomycin as indicated. Cells were pre-incubated with anti-DII (A), anti-30K (B) or anti-calpastatin monoclonal antibodies (C), as well as with phalloidin (A-C) to detect F-actin. Notice the nuclear localization of calpastatin in panel C. Arrows indicate membrane association of the large and small subunit. Bars represent 12.5 µm (A upper panel), 25 µm (A lower panel, C) and 40 µm (B).

FIG 3. **Over-expressed chimeras of human µ-calpain are Ca^{2+}-activatable.** A, Western blots of cytoplasmic fraction of cells expressing the indicated variants probed with anti-DII antibody. The molecular weights of 80K–ECFP and ECFP–80K (107 kDa) or the large subunit of endogenous human µ-calpain (80 kDa) are indicated with arrows. B, increase of intracellular calcium concentration upon ionomycin addition to COS 7 cells. Cells were loaded with Fura-2 for 30 min and then treated with 2 µM ionomycin as explained in
"Experimental Procedures". The average calcium concentration in five different cells is represented (black squares). C and D, calpain activity in COS 7 (C) and LCLC 103H cells (D) transiently over-expressing fluorescence-tagged variants of human μ-calpain subunits. Relative increase of Suc-LLVY-amc hydrolyzing activity compared to wild type cells (activity = 1, dotted lines) is shown by black (for cells that were not treated with AC27P) or white bars (cells pre-incubated with 50 μM AC27P), respectively. Standard deviations of three measurements are indicated (bars).

FIG 4. **Calpain subunits associate under resting and ionomycin-activated conditions.** A, fluorescence analysis of COS 7 cells over-expressing large and small calpain subunits. Fluorescence channels detected FRET (left panels) and YFP (right panels), phase contrast images are shown in the middle panels. Images of the same cell were taken under resting and activated conditions as indicated. White arrows mark the observed fluorescence accumulation at the plasma membrane. Bars represent 5 μm. B, endogenous calpain subunits do not dissociate under calcium-activated conditions. Cytoplasmic proteins extracted from wild type LCLC 103H cells were immunoprecipitated with anti-80K antibody and detected by Western blots using anti-calpain antibody. Ion, cells were treated with 2 μM ionomycin for the indicated time; AC27P, cells were pre-incubated with 50 μM AC27P for 1 h and then treated with 2 μM ionomycin for the indicated time as explained in “Experimental Procedures”.

FIG 5. **Intact and truncated variants of the μ-calpain subunits show different subcellular localization.** Confocal images of COS 7 cells over-expressing 80K–ECFP (A), 30K–EYFP (B), 80K–ECFP and 30K–EYFP (C, leakage from ECFP in EYFP has been corrected), ECFP–DI-III (D, upper and lower images show the whole cell or the cell nucleus, respectively), and
ECFP–DI-IIb (E, both images show the cell nucleus). The fluorescence channel detected CFP, except in figures B and C (lower images), where YFP have been detected. Images were taken under resting (left column) and activated (right column) conditions. Series of images with a distance of 50 µm were taken for each channel using single band emission filters. Notice the increased fluorescence at the plasma membrane of ionomycin-treated cells (white arrows in A-D), indicating ionomycin-dependent migration to the membrane. Bars represent 2.5 µm (A), 5 µm (B left, C, D upper panel, E) and 1 µm (B right, D lower panel).

FIG 6. Co-localization of 80K-EYFP and mem-ECFP in LCLC 103H cells. Cells of a clone constitutively expressing mem-ECFP were super-transfected with 80K-EYFP DNA. Fluorescence microscopical analysis was performed two days post transfection in ionomycin-activated cells. Series of images were taken in 500 nm distances for both channels, alternatively, subsequently processed by deconvolution algorithms and a LUT was applied to the central image choosing green and red colors for ECFP and EYFP, respectively. The results are given in the upper panels (A and B). These images were analyzed pixel by pixel for co-localization. The result of this analysis is shown in the scatter plot (C). According to theory co-localization events are located in the line x = y. An area around this line was selected (surrounded by the dotted line). The pixels of this area are overlaid in yellow color on the merged reconstruction of the original images (colocalization image, panel D). Bars represent 5 µm. Co-localization of 80K-EYFP and mem-ECFP is very prominent in the plasma membrane.

FIG 7. The synaptotagmin C2-like domain of human µ-calpain contributes to membrane targeting. Western blot analysis of subcellular fractions of COS 7 cells over-expressing fluorescence-tagged calpain variants 80K–ECFP (A), ECFP–DI-III (B), ECFP–DI-IIb (C),
and ECFP–DIII (D). Molecular masses of the variants (107, 87, 75 and 46 kDa, respectively) are indicated on the right margins. Blotted proteins were detected with antibodies anti-DII (A) or anti-living colors (B-D). Subcellular fractions: C, cytoplasmic; M, membrane; N, nuclear; Nu, nucleolar; upper panels, untreated cells; lower panels (Ionomycin-activated), cells treated with 2 µM ionomycin; ECFP, lysates from cells over-expressing ECFP; wt, wild type COS 7 cells.

**FIG 8. N-terminal domain DI of human μ-calpain as nuclear targeting motif.** Subcellular localization by fluorescence microscopy of ECFP–DI-IIb variants, ECFP–Δ(S2-L55)DI-IIb, ECFP–(S2-L55), ECFP–[K20P]DI-IIb, ECFP–[R48P]DI-IIb and ECFP–[R376A,R377A]DI-IIb, over-expressed in a LCLC 103H clone expressing constitutively histone H2A coupled to EYFP. A, expression pattern of the respective fragment in the CFP-channel (green); C, expression pattern of histone H2A in the YFP channel (red); B, merged expression patterns (A+C). Series of images with a distance of 200 nm were taken for each channel using single band emission filters, processed by volume deconvolution and false colorized (ECFP = green; EYFP = red) before merging. For the final presentation the images were contrast-enhanced by a logarithmic function to strengthen the weak intensities in the cytoplasm. Bars represent 25 µm (Δ(S2-L55)DI-IIb, [R376A,R377A]DI-IIb), 20 µm ([S2-L55], [K20P]DI-IIb) and 13 µm ([R48P]DI-IIb).
### Table I. Primers used for the construction of large and small subunit variants of human μ-calpain fused to ECFP and EYFP.

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Sequence</th>
<th>Variant</th>
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<tbody>
<tr>
<td><strong>Forward primers</strong></td>
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<tr>
<td><em>HindIII_DIII</em></td>
<td>5'-CCC AAG CTT CGA TGA TCC GCA AAT GGA ACA CCA -3'</td>
<td>ECFP–DIII</td>
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<td><em>BglII_30K</em></td>
<td>5'-GGA AGA TCT ATG TTC CTG GTT AAC TCG TTC-3'</td>
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<td><strong>Reverse primers</strong></td>
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<tr>
<td><em>BamHI_80K</em></td>
<td>5'-CGC GGA TCC TGC AAA CAT GGT CAG CTG-3'</td>
<td>80K–ECFP, 80K–EYFP [(C115A]80K–ECFP</td>
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<td><em>BamHI_DIII</em></td>
<td>5'-CGC GGA TCC TCA TTA CTG GTC ATC CAG CTC CAC AGT-3'</td>
<td>ECFP–DI-III, DI-III–EYFP</td>
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<td><em>SmaI_30KH</em></td>
<td>5'-CCC CGG GAG TGA TGA TGA TGA TGG GAA TAC ATA GTC AGC TGC AG -3'</td>
<td>30K–EYFP</td>
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*The name of the primer includes the DNA restriction enzyme used for cloning (in *italics*). The corresponding cleavage sites are also in *italics*. 
**Table II.** Primers used for the construction of ECFP–DI-IIb variants

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<td>5’-GCT CAG GTT CAG <strong>CGG</strong> CAG CTG GCT CGA GAA-3’</td>
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<td>K20.Reverse</td>
<td>5’-TTC TCG AGC ACG CTG <strong>CGG</strong> CTG AAC CTG AGC-3’</td>
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<td>R376/377_Forward</td>
<td>5’-TCC CGT ACC ATC <strong>CGG</strong> GCG TGG AAC ACC ACC-3’</td>
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<td>5’-GGT GGT GTT CCA <strong>CGG</strong> CGC GAT GGT ACG GGA-3’</td>
<td>R&lt;sup&gt;376&lt;/sup&gt;A, R&lt;sup&gt;377&lt;/sup&gt;A</td>
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*Mutations are underlined.*
FIG. 1

Variants of the large subunit:

- 80K-ECFP, ECFP-80K, 80K-EYFP
- [C115A]80K-ECFP, ECFP-[C115A]80K
- ECFP-DIII, DI-III-EYFP
- ECFP-DI-Ib
- ECFP-DIII
- ECFP-DIII
- ECFP-[K20P]DI-Ib
- ECFP-[R48P]DI-Ib
- ECFP-[R376A,R377A]DI-Ib

Variants of the small subunit:

- 30K-EYFP, EYFP-30K
FIG. 2

A

anti-DII  phalloidin

Resting

B

anti-30K  phalloidin

Ionomycin-activated

C

anti-calpastatin  phalloidin
FIG. 3

A

B

C

D
FIG. 4

A

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<td></td>
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<tr>
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B

μ-calpain  Lysates immunoprecipitated with anti-80K

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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</tbody>
</table>

80 kDa  anti-calpain  30 kDa
FIG. 5

A
80K-ECFP

B
30K-EYFP

C
80K-ECFP
30K-EYFP

D
ECFP-DI-III

E
ECFP-DI-IIb
FIG. 6

mem-ECFP-construct 80K-EYFP-construct

A B C D

scatter plot colocalization
FIG. 7

A

80K-ECFP detected with anti-DII

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<tr>
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<tr>
<td>Ionomycin-activated</td>
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B

ECFP-DI-III detected with anti-living colors

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<tr>
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<td>Ionomycin-activated</td>
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C

ECFP-DI-IIb detected with anti-living colors

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D

ECFP-DIII detected with anti-living colors

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FIG. 8

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<th>A</th>
<th>Δ(S²-L⁵⁵)DI-IIb</th>
<th>(S²-L⁵⁵)</th>
<th>[K²⁰⁸]DI-IIb</th>
<th>[R⁴⁸⁸]DI-IIb</th>
<th>[R³⁷⁶A,R³⁷⁷A]DI-IIb</th>
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<tr>
<td>B</td>
<td>ECFP-construct</td>
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<td>H₂A-EYFP</td>
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Subcellular localization and in vivo subunit interactions of ubiquitous $\mu$-calpain
Shirley Gil-Parrado, Oliver Popp, Tobias A. Knoch, Stefan Zahler, Felix Bestvater, Marcel Felgenträger, Andreas Holloschi, Amaury Fernández-Montalván, Ennes A. Auerswald, Hans Fritz, Pablo Fuentes-Prior, Werner Machleidt and Eberhard Spiess

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