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μ-Calpain binds to lipid bilayers via the exposed hydrophobic surface of its Ca^{2+}-activated conformation

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Abstract

μ- and m-calpain are cysteine proteases requiring micro- and millimolar Ca^{2+} concentrations for their activation *in vitro*. Among other mechanisms, interaction of calpains with membrane phospholipids has been proposed to facilitate their activation by nanomolar [Ca^{2+}] in living cells. Here the interaction of non-autolysing, C115A active-site mutated heterodimeric human μ-calpain with phospholipid bilayers was studied *in vitro* using protein-to-lipid fluorescence resonance energy transfer and surface plasmon resonance. Binding to liposomes was Ca^{2+}-dependent, but not selective for specific phospholipid head groups. $[Ca^{2+}]_{0.5}$ for association with lipid bilayers was not lower than that required for the exposure of hydrophobic surface (detected by TNS fluorescence) or for enzyme activity in the absence of lipids. Deletion of domain V reduced the lipid affinity of the isolated small subunit (600-fold) and of the heterodimer (10- to 15-fold), thus confirming the proposed role of domain V for membrane binding. Unexpectedly, mutations in the acidic loop of the 'C2-like' domain III, a putative Ca^{2+} and phospholipid-binding site, did not affect lipid affinity. Taken together, these results support the hypothesis that *in vitro* membrane binding of μ-calpain is due to the exposed hydrophobic surface of the active conformation and does not reduce the Ca^{2+} requirement for activation.

Keywords: acidic loop; C2 domain; Ca^{2+} requirement; domain V; membrane binding; surface plasmon resonance.

Introduction

Calpains (EC 3.4.22.52) are intracellular cysteine proteinases exhibiting a unique dependence on Ca^{2+} for enzyme activity. Members of the calpain superfamily have been found from lower organisms, plants and invertebrates through to mammals. As known so far, their functions are related to cytoskeleton remodelling, signal transduction, cell cycle regulation and cell death pathways (see Goll et al., 2003, for a review). The best characterised mammalian calpains are the ubiquitous μ- and m-isoforms, heterodimeric enzymes activated *in vitro* by Ca^{2+} concentrations of 3–50 μM and 0.4–0.8 mM, respectively. They are inhibited by nanomolar concentrations of their endogenous inhibitor, calpastatin (see Wendt et al., 2004, for a review).

μ-Calpain (μCP) and m-calpain (mCP) comprise homologous, 80-kDa, large (L) 'catalytic' subunits and a common, 28-kDa, small (S) 'regulatory' subunit. Activation of the calpains by Ca^{2+}, resulting in the formation of a functional active site from subdomains IIa and IIb of the L-subunit, has been investigated at the molecular level in recent years (Moldoveanu et al., 2002). However, after four decades of research, the mechanisms by which calpain activity is spatially and temporally controlled at the low Ca^{2+} concentrations prevailing in resting or stimulated cells (50–300 nM) remain enigmatic (Goll et al., 2003). Although unautolysed calpain is evidently active on protein substrates, autoproteolytic processing yields an enzyme with trimmed 76-kDa L- and 18-kDa S-subunits and a reduced Ca^{2+} requirement for enzymatic activity. However, the Ca^{2+} concentration required for activity by the autolysed enzyme *in vitro* (0.5–2 μM for μCP and 50–150 μM for mCP) is still far above the cellular concentrations (Goll et al., 2003). The initial Ca^{2+} concentrations for autolysis *in vitro* are comparable to those needed for proteolytic activity of the unautolysed enzyme. Additional mechanisms have been proposed to account for the reduction in activation threshold by Ca^{2+} *in vivo*: association with membrane phospholipids (Pontremoli et al., 1985), activator molecules (Melloni et al., 1998), phosphorylation by protein kinases (Glading et al., 2004), and calpain activation cascades (Tompa et al., 1996; Ono et al., 2004). The existing reports on how these factors contribute to calpain activation are often controversial and their modes of action remain largely unexplored (Goll et al., 2003).

The putative role of membranes in calpain activation is based on the *in vitro* observation that membrane phospholipids reduce the Ca^{2+} requirement for autolysis (Coolican and Hathaway, 1984; Saido et al., 1992). Therefore, the commonly accepted 'membrane activation hypothesis' (Suzuki et al., 1987; Zalewska et al., 2004) emerged, postulating that calpain is activated at the cellular membranes, where the interaction with membranes somehow increases its Ca^{2+} sensitivity. Under resting conditions, only small amounts of erythrocyte calpain have been detected in the detergent-soluble fraction (Hatanaka et al., 1984) and at cellular membranes *in situ* (Samis and

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Elce, 1989). Upon treatment with Ca^{2+} ionophores, added amounts of μ-calpain have been found associated with the membrane fraction of lysed cells or located at the cell periphery *in situ* (Saido et al., 1993; Gil-Parrado et al., 2003). Recent work revealed an increased association of calpains with the endoplasmic reticulum and the Golgi apparatus after stimulation of cells with laminin. In these studies m-calpain co-localised with phosphatidylinositol-4,5-bisphosphate (PIP_2) and with membrane lipid rafts (Hood et al., 2004). However, Zalewska et al. (2004) reported that inside-out erythrocyte vesicles did not lower the Ca^{2+} requirement for autolysis of μ- and m-calpain. To be activated by interaction with membranes, the calpains should be able to bind to the membranes at significantly lower Ca^{2+} concentrations than are required for enzymatic activity. To date, *in vitro* data on the Ca^{2+} requirement for the binding of isolated calpains to membranes and on their affinity for membranes are lacking.

Owing to their structural features, domain V of the S-subunit and the 'C2-like' domain III of the L-subunit are favourite candidates for phospholipid binding sites in calpains. Domain V is an unusual, hydrophobic, Gly-rich sequence that did not diffract in X-ray crystallography (Strobl et al., 2000). Studies with calpains lacking domain V, as well as with synthetic peptides mimicking parts of this domain, suggested a role of domain V in the interaction of calpain with membranes (Imajoh et al., 1986; Crawford et al., 1990; Arthur and Crawford, 1996; Brandenburg et al., 2002; Dennison et al., 2005). Domain III weakly resembles C2 domains, which are Ca^{2+}- and phospholipid-binding modules found in a number of signalling and membrane trafficking proteins (Rizo and Sudhof, 1998; Cho, 2001). In the 'canonical' C2 domains, Ca^{2+} is complexed by negatively charged side chains of the β-strand interconnecting loops. Although these motifs in domain III of calpains differ from the classical C2 domains in sequence and topology, a cluster of acidic residues, the so-called 'acidic loop', may have the potential to bind up to three Ca^{2+} ions (Strobl et al., 2000). Mutations within this 'acidic loop' have been shown to strongly affect Ca^{2+}-dependent activation of the calpains (Alexa et al., 2004; Fernandez-Montalvan et al., 2004). Recent work has demonstrated that recombinant domain III of rat μ-calpain and *Drosophila* calpain B can bind Ca^{2+} in a phospholipid-dependent manner and that this Ca^{2+} binding is abolished by deletion of the acidic loop of domain III (Tompa et al., 2001; Alexa et al., 2004). In cell culture experiments with overexpressed EYFP fusion proteins, Gil-Parrado et al. (2003) provided evidence that domain III is required for translocation of the large subunit of μCP from the cytosol to the cell periphery.

Here we address some basic issues related to membrane binding of μ-calpain in a simplified *in vitro* model. Active-site mutants of μ-calpain were used that do not hydrolyse substrates or autolyse (and subsequently inactivate) on Ca^{2+} exposure, but undergo essentially the same conformational changes as the non-mutated enzyme (Fernandez-Montalvan et al., 2004). The interaction of these recombinant proteins with free or immobilised phospholipid vesicles was analysed by fluorescence resonance energy transfer (FRET) and surface plasmon resonance (SPR) (Cho et al., 2001). We determined the Ca^{2+} requirement and the affinity of binding to free and immobilised liposomes of various compositions. Moreover, mutated proteins were used to analyse the contributions of domain V and of the acidic loop of domain III to phospholipid binding, with the objective of delineating a molecular mechanism for the interaction of calpains with membrane lipids. We provide experimental evidence supporting the novel hypothesis that *in vitro* Ca^{2+}-dependent binding of μ-calpain to lipid bilayers is mostly due to the increased hydrophobicity of the activated molecules and does not itself lower the Ca^{2+} demand for activation.

Results

Heterologous expression and purification of functional μ-calpain variants

In a recent communication we described the generation and use of baculoviral vectors for the expression in *Sf*21 insect cells of full-length heterodimeric active and C115A active-site mutated μ-calpain and seven variants thereof containing further point mutations within the acidic loop of domain III (Fernandez-Montalvan et al., 2004). The C115A variants are catalytically inactive and therefore not prone to autolysis, but fully functional in terms of Ca^{2+}-dependent conformational changes and binding of calpastatin. For the present study, C115A μ-calpain and its mutants D402A, E403A, D405A, D406A, D408A, D412A, and E414A (Figure 1A) were expressed and purified using the same methodology. The recombinant proteins were obtained in high purity (Figure 1B).

In addition, we expressed a heterodimeric C115A μ-calpain in which the first 85 N-terminal residues of the small subunit were deleted (Figure 1A). This variant, L+(ΔV)S, lacks domain V almost completely and its truncated small subunit resembles the fragment resulting from autoproteolytic cleavage of the natural enzyme (Gabrijelcic-Geiger et al., 2001; Li et al., 2004). In contrast to rat m-calpain expressed in *E. coli* (Elce et al., 1997), replacement of the small subunit by its truncated form did not improve the expression yields of the μ-calpain heterodimer in insect cells. Ca^{2+}-dependent conformational changes detected by 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) fluorescence indicate that (C115A)L+(ΔV)S is functional (Figure 1C).

For the first time we succeeded in expressing the single full-length small subunit (28 kDa) of μ-calpain in sufficient quantities for functional characterisation. Western blot analysis of the lysates indicated the expression of a soluble protein of the expected molecular weight in the insect cells. Microgram quantities of the isolated small subunit were obtained after affinity purification on a calpastatin BC-peptide (subdomains B and C of calpastatin domain 1) column. SDS-PAGE of the purified protein (Figure 1B) showed only minor contaminating bands. Detection of Ca^{2+}-dependent binding to immobilised calpastatin domain 1 and to the calpastatin BC-peptide in SPR experiments confirmed that the isolated recombinant protein was properly folded and functional (Figure 1D). These features, however, deteriorated very rapidly during storage. Therefore, experiments with this protein

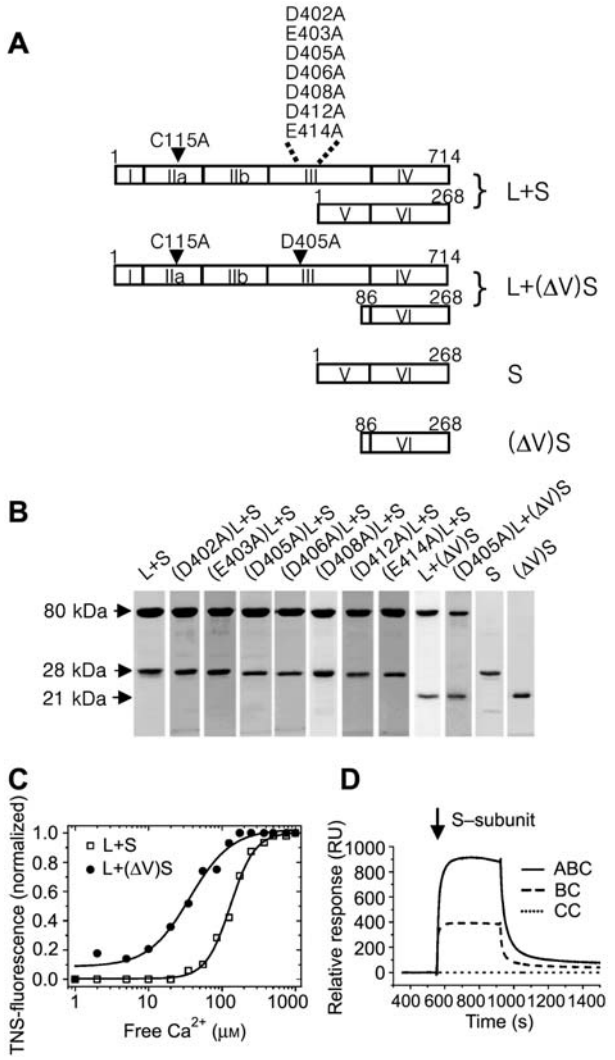

Figure 1 Design and functional integrity of the recombinant calpain constructs.
(A) Schematic representation of the calpain constructs used in this work: L+S, heterodimeric μ-calpain composed of C115A active site-mutated large (L) and full-length small (S) subunit; L+(ΔV)S, heterodimeric μ-calpain comprising the C115A large (L) and a truncated small subunit lacking residues 1–85 of domain V; S, single full-length small subunit; (ΔV)S, single truncated small subunit lacking residues 1–85. In addition, a series of heterodimers containing acidic-loop single mutations of (C115A)L were prepared as indicated above the bars. L+S, L+(ΔV)S and S were expressed in *Sf*21 insect cells using the baculovirus system; (ΔV)S was expressed in *E. coli*. (B) SDS-PAGE analysis of the calpain variants shown in panel A, purified either on a BC-calpastatin or a Ni-NTA affinity column (see materials and methods). The upper bands represent the large (80 kDa) and the lower the small (28 kDa or 21 kDa) subunits. (C) Ca^{2+} titration of L+S and L+(ΔV)S monitored by TNS fluorescence (see materials and methods). (D) Sensorgram showing the interaction of the S subunit with immobilised domain 1 (ABC) and a fragment containing subdomains B+C (BC) of calpastatin at 0.5 mM free Ca^{2+}. Immobilised chicken cystatin (CC), which does not interact with calpain, was used as a control.

were performed shortly after elution from the affinity column.

Bacterial expression of the single truncated small subunit, (ΔV)S, of μ-calpain (Figure 1A) and its isolation by Ni-NTA affinity chromatography (Figure 1B) were performed as previously described (Minami et al., 1987; Diaz et al., 2001). The functional integrity of the protein isolated was confirmed by measuring Ca^{2+}-dependent conformational changes using TNS fluorescence spectroscopy (data not shown) and binding to immobilised calpastatin in SPR experiments as previously reported (Diaz et al., 2001). Unlike the full-length small subunit, the 21-kDa protein conserved its functional properties during long-term storage.

Membrane binding of μ-calpain is Ca^{2+}-dependent, but lacks headgroup selectivity

Interaction of the recombinant calpain proteins with free liposomes was studied by protein-to-lipid FRET measurements with *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (dansyl DHPE)-labelled small unilamellar vesicles (Cho et al., 2001). To mimic more closely the lipid bilayer of biological membranes, the vesicles were captured on a Pioneer L1 sensor chip, where they fuse to form a lipid bilayer (Erb et al., 2000). Binding of the proteins to the immobilised lipid bilayers was analysed by SPR. With both methods we observed Ca^{2+}-dependent binding of heterodimeric μ-calpain to the model membranes (Figure 2A, B). As can be observed from the SPR sensorgrams in Figure 2B, binding was quickly reversed when Ca^{2+} was washed out using a Ca^{2+}-free running buffer. In the presence of a saturating Ca^{2+} concentration (1 mM), concentration-dependent binding of μCP was observed (Figure 2C). By fitting the data to a 1:1 Langmuir binding model, association (k_a) and dissociation (k_d) rate constants were determined separately from the association and dissociation phases of the sensorgrams. The equilibrium dissociation constants ($K_d=k_d/k_a$) calculated from these rate constants were in the nanomolar range (Table 1).

The SPR experiments were repeated with captured liposomes of various composition, representing different ratios of zwitterionic and anionic head groups, as well as mimicking typical plasma and nuclear envelope membranes (Stahelin et al., 2003) (Table 1). Furthermore, in some liposomes, PI, PIP and PIP_2 (see materials and methods for abbreviations) were included. As shown in Table 1, the lipid composition of the vesicles had no significant influence on the binding affinity (K_d). This lack of headgroup selectivity was unexpected on the basis of (i) previous papers reporting that PIP_2, and to a lesser extent PI, were more effective than other phospholipids in lowering the Ca^{2+} concentration required for proteolytic activity of μ-calpain (Saido et al., 1991, 1992); and (ii) published results on Ca^{2+}-dependent membrane binding of C2 domains indicating a clear preference for either anionic (e.g., PS) or zwitterionic (e.g., PC) lipids (Stahelin et al., 2003).

Membrane binding has a similar Ca^{2+} requirement as TNS binding and enzymatic activity in the absence of lipids

The free calcium concentration for half-maximal membrane binding, $[Ca^{2+}]_{0.5}$, was determined in Ca^{2+} titration experiments monitored by protein-to-lipid FRET meas-

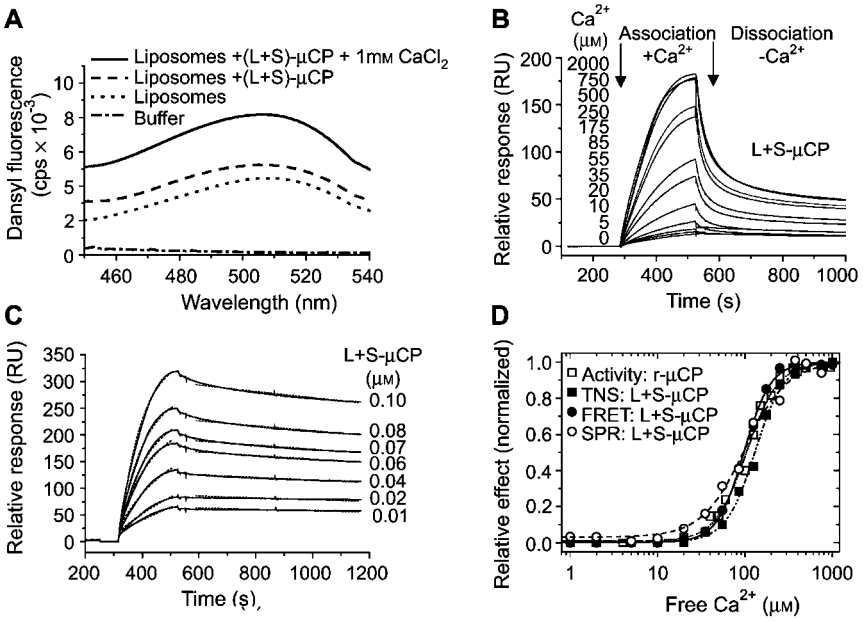

Figure 2 Ca^{2+}-dependent interaction of μ-calpain with liposomes.
(A) Spectra recorded at 280 nm excitation showing fluorescence resonance energy transfer (FRET) from L+S-μCP to dansyl-DHPE labelled PC/PG (70:30) liposomes in the absence and in the presence of 1 mM $CaCl_2$. (B) Representative Biacore sensorgrams recorded at increasing Ca^{2+} concentrations (as indicated) after injection of 50 nM L+S-μCP to a Pioneer L1 sensor chip coated with plasma membrane-mimicking vesicles (see Table 1). (C) Representative sensorgrams recorded at 1 mM $CaCl_2$ after injection of increasing concentrations of L+S-μCP (as indicated) to an identical chip as in panel (B). For composition of the running buffer and conditions for data collection, see the materials and methods section. Solid lines, experimental data; dashed lines, data globally fitted to a 1:1 interaction model, as described in materials and methods. (D) Semilogarithmic Ca^{2+} titration curves for peptidolytic activity (active recombinant μCP), TNS fluorescence (L+S-μCP), protein-to-liposome FRET (L+S-μCP) and SPR-monitored binding to immobilised liposomes (L+S-μCP). The different effects were normalised and fitted with the Hill equation as described in materials and methods (lines). Data for activity and TNS fluorescence are from Fernandez-Montalvan et al. (2004).

urement and/or SPR. The mean $[Ca^{2+}]_{0.5}$ for the association of heterodimeric full-length C115A μ-calpain with membranes composed of PC/PG was 97.1±1.5 μM when determined by FRET assay (PC/GG=70:30) and 135±20 μM by SPR measurements (PC/PG=60:40). Like the K_d values, $[Ca^{2+}]_{0.5}$ values were not significantly different for membranes of different lipid composition (Table 1). Hill coefficients (Weiss, 1997) in the region of 2 indicated positive cooperativity (see the discussion section).

Unexpectedly, the $[Ca^{2+}]_{0.5}$ values for membrane binding were very similar to the corresponding values that we had previously determined for Ca^{2+}-induced conformational changes of C115A μ-calpain, probed by TNS fluorescence, and for enzymatic activity of active μ-calpain

Table 1 Ca^{2+} requirement and kinetic constants for membrane binding of human (C115A)L+S μ-calpain determined from SPR analysis[a].

Lipid vesicles[b]	$[Ca^{2+}]_{0.5}$ (μM)	Hill coefficient[c]	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_d (nM)
PC/PE (60:40)	125±19	1.7±0.4	(1.4±0.2)×10⁵	(3.1±0.3)×10⁻⁴	2.2±0.4
PC/PS (60:40)	117±17	1.8±0.4	(1.2±0.1)×10⁵	(2.8±0.2)×10⁻⁴	2.3±0.3
PC/PG (60:40)	135±20	1.6±0.3	(1.4±0.2)×10⁵	(4.1±0.4)×10⁻⁴	2.9±0.5
PC (100)	106±16	1.9±0.5	(1.6±0.1)×10⁵	(4.0±0.5)×10⁻⁴	2.5±0.3
PC/PE/PS (60:20:20)	111±5.7	2.3±0.2	(1.7±0.2)×10⁵	(3.2±0.3)×10⁻⁴	1.9±0.3
PC/PE/PS/PI (60:20:18:2)	115±6.3	2.3±0.3	(1.5±0.2)×10⁵	(3.0±0.6)×10⁻⁴	2.0±0.5
PC/PE/PS/PIP (60:20:18:2)	109±5.7	2.2±0.2	(1.6±0.3)×10⁵	(3.9±0.7)×10⁻⁴	2.4±0.6
PC/PE/PS/PIP_2 (60:20:18:2)	108±5.3	2.2±0.2	(1.6±0.2)×10⁵	(4.2±0.8)×10⁻⁴	2.6±0.4
Plasma membrane mimic[d]	99±2.5	2.2±0.3	(1.4±0.2)×10⁵	(2.2±0.3)×10⁻⁴	1.6±0.3
Nuclear envelope mimic[e]	111±8.8	1.8±0.1	(1.6±0.2)×10⁵	(2.7±0.3)×10⁻⁴	1.7±0.3

[a]Values represent the mean±SD from two to three different estimations, each comprising six to ten different concentrations.
[b]See the materials and methods section for explanation of the abbreviations.
[c]Hill coefficient for Ca^{2+}-dependent binding.
[d]PC/PE/PS/PI/cholesterol (12:35:22:9:22).
[e]PC/PE/PS/PI/cholesterol (61:21:4:7:7).

in the absence of lipids (Fernandez-Montalvan et al., 2004). Almost coincident Ca^{2+} titration curves were obtained for membrane binding and these effects (Figure 2D), suggesting that *in vitro* activation by calcium ions and membrane binding are closely linked processes. Attempts to measure calpain binding to liposomes at physiological cellular Ca^{2+} concentrations (below 1 μM) were not successful because of methodological limitations imposed by undetectably low signals in FRET and non-compensatable unspecific binding in SPR experiments of high sensitivity.

Acidic loop mutations in domain III lower the Ca^{2+} requirement for membrane binding, but do not affect membrane affinity

From the reported ability of the acidic loop of isolated domain III to bind Ca^{2+} in a lipid-dependent manner (Alexa et al., 2004), a significant role of this region in the Ca^{2+}-dependent interaction of the calpain heterodimer with membrane lipids could be anticipated. It has been reported that mutations within the binding loops of C2 domains affect binding to lipid vesicles and subcellular targeting in living cells (Stahelin et al., 2003). In previous work we performed an Ala-scan of the acidic residues in the acidic loop of C115A μ-calpain (Fernandez-Montalvan et al., 2004). Here we determined the Ca^{2+} requirement for membrane binding of these mutants by protein-to-lipid FRET (Figure 3, Table 2). Unexpectedly, abrogation of single potential Ca^{2+} coordination sites within the acidic loop did not increase the Ca^{2+} requirement, but even resulted in two- to three-fold lower $[Ca^{2+}]_{0.5}$ values for membrane binding (Table 2). Accordingly, a 2.5-fold lower $[Ca^{2+}]_{0.5}$ for membrane binding of D405A C115A-μCP was found in SPR experiments. The reduced $[Ca^{2+}]_{0.5}$ values for vesicle binding of the mutants correlate with reduced $[Ca^{2+}]_{0.5}$ values for conformational changes probed by TNS binding and for enzymatic activity, which we observed in the absence of lipids (Table 2). We explain the increased calcium sensitivity of these mutants by weakened electrostatic interactions of the acidic loop, facilitating the conformational changes required for activation (Fernandez-Montalvan et al., 2004). In SPR experiments, the D405A mutant (D405A)L+S had similar phospholipid affinity as the 'wild-type' L+S and also lacked headgroup selectivity (Table 3).

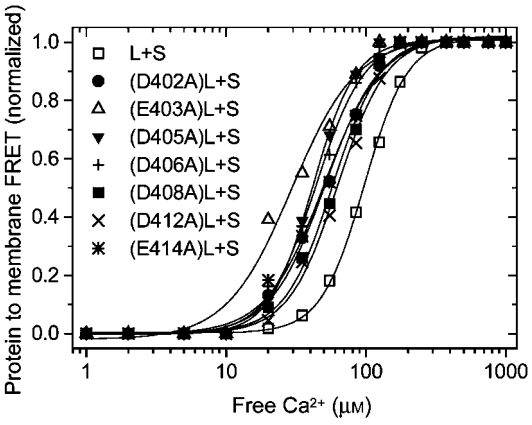

Figure 3 Effect of acidic loop mutations on Ca^{2+}-dependent interaction with liposomes.
Ca^{2+} titration of FRET from L+S-μCP and seven acidic loop mutants (as indicated) to dansyl-DHPE labelled PC/PG (70:30) vesicles. The FRET signals were normalised for comparison and fitted with the Hill equation (lines). Results are listed in Table 2.

Table 2 Ca^{2+} requirement for half-maximal membrane binding of domain III acidic loop mutants of (C115A)L+S μ-CP determined by protein-to-lipid FRET.

	$[Ca^{2+}]_{0.5}$ (μM)		
	FRET[a]	TNS[b]	Activity[b]
L+S	97.1±1.5	130.6±3.8	104.4±4.9
(D402A)L+S	50.9±1.5	82.8±2.7	44.9±3.4
(E403A)L+S	29.4±2.5	66.7±2.5	34.9±2.0
(D405A)L+S	41.5±0.6	31.7±3.0	15.2±1.1
(D406A)L+S	43.8±1.2	57.7±2.1	24.0±1.5
(D408A)L+S	58.1±2.1	73.5±5.6	34.1±0.9
(D412A)L+S	58.1±2.6	93.0±4.1	46.7±1.1
(E414A)L+S	50.1±2.1	86.5±3.1	48.1±2.0

[a]$[Ca^{2+}]_{0.5}$ from the data shown in Figure 3 obtained with POPC/POPG (70:30) vesicles supplemented with 5% dansyl-DHPE.
[b]$[Ca^{2+}]_{0.5}$ values for TNS binding of (C115A)L+S μCP and peptidolytic activity of L+S μ-CP were taken from Fernandez-Montalvan et al. (2004).

Deletion of domain V lowers the membrane affinity of the heterodimer

Deletion of domain V, leading to the L+(ΔV)S heterodimer, resulted in 10- to 15-fold reduced membrane binding affinity measured in SPR experiments (Figure 4A, Table 3). No significant effects on headgroup selectivity were observed. Previous reports (Imajoh et al., 1986; Crawford et al., 1990; Arthur and Crawford, 1996) suggesting a role for domain V in the interaction of the calpains with membranes were confirmed and extended by our quantitative data. Interestingly, even for the (D405A)L+(ΔV)S variant, in which the contribution of domain V is eliminated, the additional acidic loop D405A mutation had no significant effect on lipid binding affinity (Table 3).

Membrane affinity of the single small subunit strongly depends on the presence of domain V

To further investigate the role of domain V in membrane binding, we analysed the interaction with captured liposomes of the single full-length small subunit, S, and of a truncated mutant thereof lacking residues 1–85 of domain V, (ΔV)S. Surprisingly, the Ca^{2+}-dependent association of the full-length small subunit alone with immobilised phospholipid bilayers in SPR experiments (Figure 4B) was only four- to eight-fold lower than the membrane affinity of the L+S heterodimer (Table 3). However, removal of domain V from the single small subunit, leading to the (ΔV)S variant, resulted in a 600-fold reduced Ca^{2+}-dependent affinity for immobilised bilayers when compared with the full-length small subunit (Figure 4C, Table 3).

Table 3 Membrane binding parameters for calpain variants determined from SPR analysis[a].

	Lipid vesicles[b]	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_d (nM)	Fold increase in K_d[c]
(D405A)L+S	PC/PE	$(1.1\pm0.1)\times10^5$	$(3.0\pm0.6)\times10^{-4}$	2.7±0.6	1.2
	PC/PS	$(1.1\pm0.1)\times10^5$	$(2.7\pm0.7)\times10^{-4}$	2.5±0.7	1.1
	PC/PG	$(1.0\pm0.1)\times10^5$	$(3.3\pm0.7)\times10^{-4}$	3.3±0.8	1.1
	PC	$(1.1\pm0.2)\times10^5$	$(4.2\pm0.6)\times10^{-4}$	3.8±0.9	1.5
L+(ΔV)S	PC/PE	$(3.1\pm0.9)\times10^3$	$(1.1\pm0.2)\times10^{-4}$	35.5±12.2	16
	PC/PS	$(3.5\pm0.5)\times10^3$	$(1.1\pm0.1)\times10^{-4}$	31.4±5.3	14
	PC/PG	$(4.0\pm1.0)\times10^3$	$(1.4\pm0.3)\times10^{-4}$	35.0±11.5	12
(D405A)L+(ΔV)S	PC/PE	$(3.2\pm0.3)\times10^3$	$(1.1\pm0.1)\times10^{-4}$	34.4±4.5	16
	PC/PS	$(3.7\pm0.1)\times10^3$	$(1.1\pm0.2)\times10^{-4}$	29.7±5.5	13
S	PC/PE	$(6.5\pm0.2)\times10^3$	$(1.1\pm0.4)\times10^{-4}$	16.9±6.2	7.7
	PC/PS	$(8.4\pm0.5)\times10^3$	$(1.5\pm0.5)\times10^{-4}$	17.9±6.1	7.8
	PC/PG	$(1.3\pm0.4)\times10^4$	$(1.6\pm0.3)\times10^{-4}$	12.3±4.4	4.2
	PC	$(1.4\pm0.4)\times10^4$	$(1.6\pm0.4)\times10^{-4}$	11.4±4.3	4.6
(ΔV)S	PC/PE	$(7.0\pm0.1)\times10^2$	$(6.1\pm1.0)\times10^{-3}$	8700±1430	4000
	PC/PS	$(5.5\pm1.5)\times10^2$	$(6.2\pm1.1)\times10^{-3}$	11300±3700	4900
	PC/PG	$(4.5\pm0.4)\times10^2$	$(3.2\pm0.4)\times10^{-3}$	7100±1090	2500

[a]Values represent the mean±SD from two to three different estimations, each comprising six to ten different concentrations.
[b]See Table 1 for lipid composition and the materials and methods section for explanation of the abbreviations.
[c]Relative to the binding of (C115A)L+S μ-calpain shown in Table 1.

Discussion

In vitro membrane binding is due to increased hydrophobicity of the activated calpain

Using two distinct biophysical methods, we determined congruent $[Ca^{2+}]_{0.5}$ values for Ca^{2+}-dependent binding of μ-calpain to lipid bilayers *in vitro*. Our data reveal the surprising fact that the association of C115A μCP with phospholipid vesicles requires a similar non-physiologically high Ca^{2+} concentration as unautolysed μCP for enzymatic activity in the absence of lipids (see Figure 2D). These *in vitro* findings question a major premise of the popular 'membrane activation hypothesis'. According to this hypothesis, significant membrane binding should be observable at much lower Ca^{2+} concentrations than required for activation/autolysis in the absence of lipids (see the introduction section). However, because of methodological limitations (see the results section) we cannot directly exclude a small amount of binding at Ca^{2+} concentrations below 1 μM that might be relevant to the interaction of calpain with membranes *in vivo*.

When our *in vitro* results are applied to living cells, only a very small proportion of calpain molecules (less than 1%) would be active and able to bind to the lipids of cellular membranes. This scenario can principally explain the rather low quantities of calpain found to be associated with cellular membranes under 'resting conditions' and the added membrane-associated localisation of calpain after activation by moderately increased Ca^{2+} levels due to physiological stimuli or Ca^{2+} ionophores (see introduction). As active calpain is required for autolysis, selective binding of the active conformation to membranes would also explain the observed prevalence of autolysed calpains at the plasma membrane (Anagli et al., 1993).

We noted a close correlation between the Ca^{2+} titration curves for membrane binding and those for TNS fluorescence (see Figure 2D and Table 2). The increase in TNS fluorescence is a well-established indicator for the exposure of hydrophobic regions on proteins (Brand and Gohlke, 1972) and has been observed previously with calpain (Hong et al., 1990). Thus, we argue that the exposure of a hydrophobic surface after calpain transition to its active conformation (Pal et al., 2001; Dainese et al., 2002; Li et al., 2004) should be responsible for the Ca^{2+}-dependent membrane binding in our experiments. This type of membrane binding would occur simultaneously with or shortly after activation. A close connection between activation and membrane binding is corroborated by the Ca^{2+} requirement for vesicle binding of a set of domain III acidic-loop point mutants, which were previously shown to have up to seven-fold increased Ca^{2+} sensitivity for activation due to the disruption of electrostatic interactions of the acidic loop (Fernandez-Montalvan et al., 2004). The $[Ca^{2+}]_{0.5}$ values for phospholipid binding of the corresponding active-site mutants are similar to those measured previously for enzyme activity in the absence of lipids (see Figure 2D and Table 2). Notably, the Hill coefficients for the Ca^{2+} dependence of both processes were also not significantly different, e.g., 2.8 for lipid binding of (C115A)L+S μ-CP (determined by FRET) and 2.4 for enzyme activity of L+S μ-CP (Fernandez-Montalvan et al., 2004). This is illustrated by almost identical shapes of the Ca^{2+} titration curves (Figure 2D). As expected from previous work (Dutt et al., 2000; Moldoveanu et al., 2004), activation by Ca^{2+} is positively cooperative, but this cooperativity is likely not enhanced by the presence of lipids.

Our data are in line with earlier results questioning the role of membrane phospholipids in calpain activation (Cong et al., 1989) and with the recently reported inability of inside-out erythrocyte membranes to decrease the Ca^{2+} requirement for calpain autolysis (Zalewska et al., 2004). In fact, we observed no consistent effects on the Ca^{2+} requirement for hydrolysis of the substrate Suc-LY-AMC by human erythrocyte calpain when we added the same phospholipid vesicles as used in the membrane binding studies (data not shown). We explain the effects

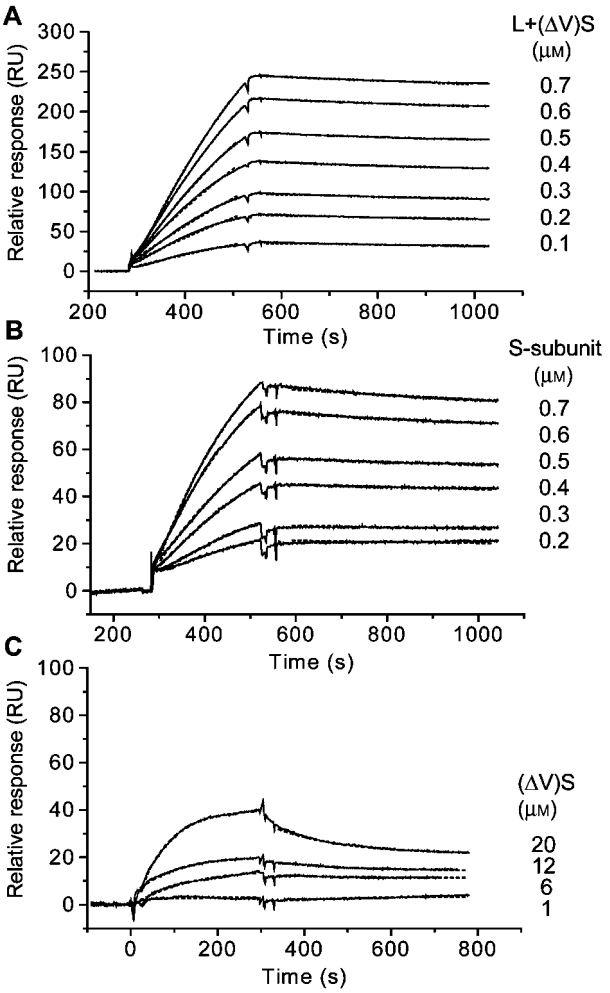

Figure 4 Effect of domain V deletion on membrane binding. Representative sensorgrams recorded at 1 mM Ca^{2+} for the interaction of L+(ΔV)S-μCP heterodimer (A), single S subunit (B) and single (ΔV)S subunit (C) with PC/PS (60:40) liposomes coated on a Pioneer L1 sensor chip. See Figure 1A for the design of the analytes. Solid lines, experimental data; dashed lines, data globally fitted to a 1:1 interaction model, as described in the materials and methods section.

of phospholipid vesicles on the Ca^{2+} requirement for calpain autolysis and activity that have been reported in numerous studies (see Goll et al., 2003, and Zalewska et al., 2004, for references) by hydrocarbon chain-mediated stabilisation of the hydrophobic Ca^{2+}-activated calpain structure. This would increase the effective concentration of active molecules able to activate more molecules by (intermolecular) autoproteolysis and would reduce the formation of inactive large protein aggregates (Li et al., 2004). Interestingly, we have observed a stabilising effect of non-ionic detergents such as Triton X-100 and Brij-35 on the Ca^{2+}-dependent peptidolytic activity of erythrocyte μ-calpain (data not shown).

Interaction of calpain with membrane lipids is non-selective

For the first time we obtained quantitative data on the binding affinity of a calpain to lipid bilayers captured on a L1 sensor chip (Tables 1 and 3). The dissociation constants (K_d) were within the nanomolar range and are therefore similar to those for other Ca^{2+}-dependent membrane binding proteins such as annexins (Tait et al., 1989) and typical C2 domain-containing proteins, such as PKC (Bazzi and Nelsestuen, 1987), synaptotagmin (Niinobe et al., 1994) and phospholipase A2 (Kim et al., 1997). This observation underlines the role of phospholipids as potential binding partners of activated calpain at the cellular membranes, a function once attributed exclusively to membrane-associated proteins (Inomata et al., 1990).

Interestingly, μ-calpain showed no preference for negatively charged phospholipid headgroups (as described for most of the annexins and many C2 domain proteins, which preferentially bind phosphatidyl serine), nor for neutral/zwitterionic groups (as in the case of cPLA) (Cho, 2001; Gerke and Moss, 2002; Murray and Honig, 2002). Unlike C2 domain-containing proteins, such as the tumour supressor PTEN (Das et al., 2003), the binding affinities of μ-calpain to nuclear envelope and plasma membrane-mimicking bilayers (Stahelin et al., 2003) were similar, and the affinity was not changed by inclusion of 2% PIP_2 in the liposomes. The lack of headgroup selectivity supports a membrane-binding mechanism involving hydrophobic interactions with the non-polar moieties of phospholipid vesicles. Indeed, recent work using velocity gradient centrifugation suggested that the large and small subunits of μ- and m-calpain may be embedded within the organelle membranes, similar to integral membrane proteins (Hood et al., 2004).

Lipid-binding sites of μ-calpain: roles of domain V and the 'C2-like' domain III

We have analysed for the first time the individual contributions of the two putative lipid-binding sites, domain V and domain III, in the context of a 'stable', non-autolysing active-site mutated heterodimeric μ-calpain.

A role for the hydrophobic Gly-rich domain V in membrane binding seems likely owing to the 10- to 15-fold reduction in the affinity for phospholipid vesicles we observed with heterodimeric μCP lacking domain V. This is strongly supported by our novel binding data for the single small subunit. Ca^{2+}-dependent binding of the full-length small subunit to phospholipids had only a four- to eight-fold lower affinity than the Ca^{2+}-dependent binding of the heterodimer to phospholipids (Table 3). These *in vitro* results are consistent with our previous observation of the Ca^{2+}-dependent translocation of an overexpressed small subunit to cellular membranes (Gil-Parrado et al., 2003) and the recently reported association of the small subunit with the endoplasmic reticulum and the Golgi apparatus (Hood et al., 2004). The ~600-fold reduced phospholipid affinity of the N-terminally truncated single small subunit, (ΔV)S, clearly underlines the essential contribution of domain V to lipid binding of the small subunit. It should be noted that our (ΔV)S mutant lacks the ^{56}GTAMRILGGVI^{66} sequence that has been implicated in lipid binding of calpain (Arthur and Crawford, 1996; Brandenburg et al., 2002).

The small calpain subunit is a member of the penta-EF-hand (PEF) protein family and, within this family, is most similar to sorcin and grancalcin (Maki et al., 2002). Typical PEF proteins are composed of a Ca^{2+}-binding EF-

hand domain corresponding to domains VI and IV of calpain, and a Pro/Gly-rich N-terminal domain of variable length, corresponding to domain V of calpain. Domain V of calpain is significantly different from the N-terminal extensions of all other PEF proteins because of its outstanding length and peculiar sequence. A common feature of non-calpain PEF proteins is their Ca^{2+}-induced translocation from the cytosol to membranes, where they interact with different target proteins. Depending on the protein targets, Ca^{2+}-dependent interactions can occur either through the Ca^{2+}-binding domains or, induced by Ca^{2+} binding to these domains, through the hydrophobic N-terminal extension (see Maki et al., 2002, for a review). To date, little is known about the interaction of non-calpain PEF proteins with phospholipids, but recently a Ca^{2+}-dependent increase in hydrophobicity was reported for a PEF protein from maize lacking an N-terminal extension (Barry et al., 2006). This is consistent with our results showing that the truncated small subunit still binds to liposomes in a Ca^{2+}-dependent manner, although with greatly reduced affinity.

In SPR experiments, $[Ca^{2+}]_{0.5}$ for membrane binding of the L+(ΔV)S heterodimer was approximately two-fold lower when compared to the L+S heterodimer containing the full-length small subunit. This correlates with the three-fold reduced $[Ca^{2+}]_{0.5}$ for TNS fluorescence (Figure 1C) and eight-fold lower $[Ca^{2+}]_{0.5}$ for enzyme activity of non-active-site mutated L+(ΔV)S μ-calpain in the absence of lipid vesicles (data not shown), suggesting a role for domain V in the activation process.

The only 10- to 15-fold reduction in membrane affinity by truncation of domain V within the heterodimer points to an almost equally important contribution of the large subunit to lipid binding. The regions of the large subunit involved in membrane binding are not known. Surprisingly, the domain III acidic-loop D405A mutants with and without domain V bind to immobilised vesicles with similar affinity as the corresponding non-mutated enzymes (Table 3). As expected from structural and functional data, the D405A point mutation should disrupt the most important residue in a putative Ca^{2+}/phospholipid nucleation site of the 'C2-like' domain III (Strobl et al., 2000; Alexa et al., 2004). A number of site-directed mutagenesis studies with the C2 domains of PKCα (Bolsover et al., 2003), cPLA (Bittova et al., 1999), synaptotagmin (Fernandez-Chacon et al., 2002) and other C2 domain proteins have shown that neutralising mutations of the acidic residues in the Ca^{2+}-binding loops of C2 domains usually result in an important loss of affinity for membrane lipids. Our data suggest that the contribution of the single acidic-loop residue mutated here is minimal in the context of a heterodimeric calpain.

Conclusions

Ca^{2+}-dependent binding of recombinant μ-calpain to the model membranes studied in this work seems to be due to the exposure of a hydrophobic surface upon activation, and thus exhibits a similar Ca^{2+} requirement as the activation process in the absence of lipids *in vitro*. It remains open to what extent these findings are relevant *in vivo*, because the Ca^{2+} concentrations used in our experiments were far above the physiological Ca^{2+} levels that have been shown to activate calpain in cells (Matsumura et al., 2001). However, Friedrich (2004) recently argued that 'the unphysiologically high Ca^{2+} demand for activation may turn out to be an evolutionary adjusted safety device', and the local activation of only a small portion of total calpain 'working at the initial, ascending limb of its Ca^{2+} saturation curve' would meet best its roles in signal transduction. We hypothesise that binding of calpain to membrane lipids during or after activation may be a biologically relevant mechanism that helps to 'concentrate' and stabilise the few active molecules, thus enabling them to cleave substrates associated with the plasma membrane or membranes of subcellular organelles. Hitherto not well understood mechanisms, such as phosphorylation of the calpains by membrane-associated protein kinases and/or interactions with membrane proteins, may lower the Ca^{2+} demand for activation and locally increase the number of active molecules binding to and acting at the cellular membranes.

Materials and methods

Materials

Expand™ High Fidelity DNA polymerase, restriction endonucleases and other DNA modifying enzymes were purchased from Roche (Mannheim, Germany). Oligonucleotides were synthesised by Roth (Freiburg, Germany). Protocols, media and reagents for heterologous protein expression in *Spodoptera frugiperda* (*Sf*) insect cells using the baculovirus system were from Pharmingen (BD Biosciences, Heidelberg, Germany) and Invitrogen (Groningen, The Netherlands). Heterodimeric full-length C115A μ-calpain variants were expressed and purified as previously described (Fernandez-Montalvan et al., 2004). 2-*p*-Toluidinylnaphthalene-6-sulfonate (TNS) and *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (dansyl-DHPE) were purchased from Molecular Probes (Leiden, The Netherlands). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PE), 1-palmitoyl-2-oleoyl-*sn*-glyce-ro-3-[phospho-L-serine] (PS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (PG), L-α-phosphatidylinositol (PI), L-α-phosphatidylinositol-4-phosphate (PIP), L-α-phosphatidylinositol-4,5-bisphosphate (PIP_2) and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). CM5 and L-1 Pioneer sensor chips and other SPR-related reagents were purchased from Biacore (Uppsala, Sweden). Reagent-grade chemicals were obtained from Merck (Darmstadt, Germany) or Sigma-Aldrich (Taufkirchen, Germany) unless otherwise indicated.

Expression and purification of L+ΔVS μ-calpain

The N-terminally truncated small subunit (Δ1–85)S of human calpain had been previously cloned in the pET-22b(+) vector with a C-terminal hexahistidine (His_6) tag for bacterial expression (Diaz et al., 2001). For expression in insect cells, the sequence was subcloned into the baculovirus transfer plasmid pVL1392 by double digestion with XbaI/BamHI and subsequent ligation. Generation of a recombinant AcNPV-hCAPN4-(Δ1–85)S-His_6 baculovirus expressing the truncated small subunit was performed as described for the full-length variant (Fernandez-Montalvan et al., 2004). Production of the heterodimeric L+(ΔV)S

μ-calpain was achieved by co-infection of *Sf*21 cells with this new viral vector and the vector coding for the 80-kDa L subunit, AcNPV-hCAPN1-80K(C115A), as previously reported (Fernandez-Montalvan et al., 2004). Heterologous expression was followed by one-step purification on a calpastatin BC-peptide (subdomains B+C of calpastatin domain 1) affinity column as previously described (Fernandez-Montalvan et al., 2004).

Expression and purification of μ-calpain small-subunit variants

To produce the full-length small subunit (S, 28K), *Sf*21 insect cells were infected with the AcNPV-hCANPN4-28K viral vector described by Fernandez-Montalvan et al. (2004) at an MOI (multiplicity of infection) of 1. Protein purification was performed on the BC-calpastatin affinity column using the same protocol as for the heterodimeric molecule. Bacterial expression induced by isopropylthiogalactoside (IPTG) and affinity purification of the truncated (Δ1–85)S small subunit (21K) on a Ni-NTA column were performed as described by Diaz et al. (2001).

Ca^{2+}-dependent changes in TNS fluorescence

TNS fluorescence probing Ca^{2+}-dependent conformational changes of the heterodimeric L+S μ-calpain variants was measured as previously described (Fernandez-Montalvan et al., 2004).

Preparation of liposome vesicles

Phospholipids were purchased in their powder form and resuspended in chloroform to create stock solutions of defined concentrations. After mixing different species in the desired molar ratios (as listed below under FRET and SPR measurements) to a total concentration of 10 mM, chloroform was evaporated from the samples under a stream of nitrogen. The mixtures were further desiccated for 2 h and the resulting lipid film was resuspended in the appropriate assay buffer by vigorous vortexing. The vesicles were downsized to small unilamellar vesicles of uniform size using a LiposoFast™ mini-extruder (Avestin Inc., Ottawa, Canada) equipped with 50-nM polycarbonate membranes following the manufacturer's guidelines.

Ca^{2+}-induced protein-to-lipid FRET

Binding of (C115A) μ-calpain variants to dansyl-labelled liposomes was studied using the method described by Nalefski and Falke (2002). Different dansyl-labelled small unilamellar vesicles containing PC/PE, PC/PS, PC/PG or PC/PI in a molar ratio of 70:30 supplemented with 5% dansyl-DHPE were prepared in a buffer containing 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 5 mM 2-mercaptoethanol. FRET measurements (excitation at 280 nm) were performed in this buffer with and without $CaCl_2$ at 22°C in a Spex FluoroMax fluorescence spectrophotometer (Jobin Yvon, Edison, NJ, USA) equipped with a stirrer-adapted, temperature-controlled cuvette holder set. Ten μM of dansyl-labelled vesicles were mixed with 0.1 μM protein solution (based on absorbance at 280 nm) to a final volume of 3 ml. Small aliquots of a $CaCl_2$ stock solution were added to the continuously stirred reaction volume every 2 min and the fluorescence intensity was recorded after 1.5-min incubation until saturation was reached. The increment in reaction volume was kept to <1% of the total. Control measurements were performed with $MgCl_2$ instead of $CaCl_2$. The normalised fluorescence intensity data (emission at 510 nm) were fitted to a modified Hill equation $y=F_0+F_m\times[x^n/(x^n+[Ca^{2+}]_{0.5}{}^n)]$, where y is the fraction of maximum protein-to-membrane FRET, F_0 represents the protein-to-lipid FRET in the absence of Ca^{2+}, F_m is the maximum protein-to-lipid FRET achieved by Ca^{2+} saturation, x is the actual free Ca^{2+} concentration (μM), n is the Hill coefficient (Weiss, 1997), and $[Ca^{2+}]_{0.5}$ represents the calcium concentration for half-maximal protein-to-lipid FRET.

Surface plasmon resonance (SPR) measurements

SPR sensorgrams were collected using a Biacore 2000 instrument. CM5 chips were activated as described by Diaz et al. (2001) and recombinant calpastatin domain 1 (ABC), a fragment containing subdomains B+C (BC) (Fernandez-Montalvan et al., 2004) and chicken cystatin (CC) as control were immobilised via amine groups. Binding experiments with full-length S subunit, (ΔV)S subunit, and heterodimeric L+S μ-calpain variants were performed in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.015% Brij-35, with or without 1 mM free Ca^{2+} at a flow rate of 20 μl/min. After each experiment the surface was regenerated with 10 μl of 3 M guanidine hydrochloride, pH 6.5.

Small unilamellar vesicles for SPR measurements (10 mM) were prepared in running buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl) as described above with the various different lipid compositions listed in Table 1. Pioneer L1 sensor chips were coated with 30 μl of a 0.1 mM liposome suspension (2 μl/min) followed by several washing steps with 50 μl of 50 mM NaOH (100 μl/min). Interaction experiments were performed at 20 μl/min. Initially, a 4-min pulse of 0.05–1 μM of C115A μ-calpain variant or small subunit (1 mM $CaCl_2$) was injected, followed by intensive washes with regeneration solutions (see below) to block residual unspecific binding.

For Ca^{2+} titration experiments, solutions of the calpain variants in non-saturating concentrations [20–50 nM for full-length heterodimer, 100–200 nM for L+(ΔV)S heterodimer or small subunit, all based on 280-nm absorbance] were adjusted to increasing free Ca^{2+} concentrations between 0 and 2 mM immediately before sample injection. After injection, the association phase was monitored over 4 min, followed by a dissociation of 8 min. At the end of the interaction experiment, the immobilised vesicles were washed at 100 μl/min with two consecutive 2-min pulses of 100 mM EDTA and 50 mM NaOH. After each set of experiments, the lipids were removed from all four flowcells by injection of 10 μl of 40 mM octyl glycoside and/or 10 μl of 20 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) at 20 μl/min, and the L1-chip was coated with fresh vesicle solution. For evaluation, the increase in resonance units (RU) during the nearly linear initial phase of association (0.1–1 or 0.1–2 min) was plotted against the free Ca^{2+} concentration and fitted to a modified Hill equation: $y=R_0+R_m\times[x^n/(x^n+[Ca^{2+}]_{0.5}{}^n)]$, where R_0 is the response at $[Ca^{2+}]=0$, R_m is the maximum response achieved by Ca^{2+} saturation, x is the free Ca^{2+} concentration (μM), n is the Hill coefficient, and $[Ca^{2+}]_{0.5}$ is the calcium concentration for half-maximal binding (μM).

To analyse the membrane binding kinetics of calpain variants, Pioneer L1 sensor chips were coated and pretreated as described above. The running buffer (20 μl/min) was the same as before but supplemented with 1 mM $CaCl_2$. Solutions containing seven to ten increasing concentrations (based on 280-nm absorbance) of the calpain variants [0.001–0.1 μM of the full-length heterodimeric variants, 0.01–0.700 μM of the L+(ΔV)S heterodimeric variants or of the small subunit, 1–20 μM of the truncated (ΔV)S subunit] were injected in running buffer containing 1 mM free Ca^{2+}. Association was followed over 4 min and dissociation over 12 min. The membranes were regenerated as described above after each set of variant injections. A control lane loaded with aged vesicles of PC/PG (60:40) or PC, which showed virtually no protein binding, was subtracted to correct for bulk effects of buffer and/or sample and for non-specific binding. Association and dissociation phases of all sensorgrams were globally fitted to a 1:1 Langmuir binding model (pro-

tein+protein-binding site on the vesicle↔complex) using the Biaevaluation 3.0 software (Biacore). The dissociation phase was analysed using the equation:

$$R = R_0 e^{-k_d (t-t_0)}$$

where k_d is the dissociation rate constant (s^{-1}) and R_0 is the initial response. The association phase was evaluated using the equation:

$$R = [k_a C/(k_a C + k_d)] R_{max} [1 - e^{-(k_a C + k_d)(t-t_0)}] + RI$$

where R_{max} is the maximum analyte binding capacity, C is the analyte concentration, k_a is the association rate constant ($M^{-1} s^{-1}$), k_d is the dissociation rate constant, set to the value obtained from the dissociation phase, t_0 is the start time for the data fitting, and RI is the change in refractive index of the bulk. The equilibrium dissociation constant (K_d) was then calculated as $K_d = k_d/k_a$.

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