ELSEVIER

Molecular & Biochemical Parasitology 116 (2001) 25–34

MOLECULAR & BIOCHEMICAL PARASITOLOGY

www.parasitology-online.com.

CAP5.5, a life-cycle-regulated, cytoskeleton-associated protein is a member of a novel family of calpain-related proteins in *Trypanosoma brucei*☆

Christiane Hertz-Fowler, Klaus Ersfeld, Keith Gull *

School of Biological Sciences, University of Manchester, 2.205 Stopford Building, Oxford Road, Manchester M13 9PT, UK

Received 26 February 2001; received in revised form 20 April 2001; accepted 2 May 2001

Abstract

The cell shape of African trypanosomes is determined by the presence of an extensive subpellicular microtubule cytoskeleton. Other possible functions of the cytoskeleton, such as providing a potential framework for signalling proteins transducing information from the intracellular and extracellular environment, have not yet been investigated in trypanosomes. In this study, we have identified a novel cytoskeleton-associated protein in *Trypanosoma brucei*. CAP5.5 is the first member of a new family of proteins in trypanosomes, characterised by their similarity to the catalytic region of calpain-type proteases. CAP5.5 is only expressed in procyclic, but not in bloodstream, trypanosomes. Furthermore, CAP5.5 has been shown to be both myristoylated and palmitoylated, suggesting a stable interaction with the cell membrane. A bioinformatics analysis of the trypanosome genome revealed a diverse family of calpain-related proteins with primary structures similar to CAP5.5, but of varying length. We suggest a nomenclature for this new family of proteins in *T. brucei*.

Keywords: Acylation; Calpains; Cytoskeleton; Differentiation; Protease; Trypanosome

1. Introduction

One of the most noticeable ultrastructural features of trypanosomatids is the dense corset of microtubules underlying the plasma membrane [1,2]. Transverse electron microscope sections show that the individual microtubules of this subpellicular corset exhibit a very precise and uniform spacing between each other but also to the plasma membrane [3,4].

A number of proteins have been described that associate with the subpellicular cytoskeleton and some reveal in vitro microtubule-binding activity [2]. In some instances, electron microscope immunolocalisation studies suggest that these proteins localise to cross-linking structures that appear to cross-link between microtubules, or between microtubules and the plasma membrane. None of the cytoskeleton-associated proteins described in *Trypanosoma brucei* have been suggested to play a role other than being a structural component of the cytoskeleton [2].

In higher eukaryotes, it is now evident that the cytoskeleton and associated proteins are involved in diverse cellular functions. Beside the established roles of the cytoskeleton in cell shape and motility, it is now recognised that the cytoskeleton can provide an intracellular relay system for signal transduction. This is achieved by linking the intracellular components of the cytoskeleton to the membrane and membrane receptor proteins [5,6]. Such functions for the cytoskeleton of trypanosomes are so far completely unexplored. However, the complex life cycle of the parasite, which involves exposure and adaptation to very different environments, suggests the presence of complex signalling systems transducing and converting extracellular stimuli into a physiological response.

Abbreviations: ORF, open reading frame; UTR, untranslated region.

☆ *Note*: Nucleotide sequence data reported in this paper have been submitted to the GenBank™ database with the accession number AF321838.

* Corresponding author. Tel.: +44-161-2755108; Fax: +44-161-2755763.

E-mail address: k.gull@man.ac.uk (K. Gull).

0166-6851/01/$ - see front matter
PII: S0166-6851(01)00296-1

In this study, we present the characterisation of a protein, CAP5.5 (also labelled *Tb*CALPI, see Note at the end of the paper), which is specifically associated with the subpellicular cytoskeleton of trypanosomes. Its expression is tightly regulated in a life-cycle-dependent manner. It is not expressed in bloodstream trypanosomes and first appears during differentiation to the procyclic form. In the absence of any notable differences between the cytoskeletal organisations in the different life cycle forms, it is unlikely that CAP5.5 only plays a structural role as a cytoskeleton associated protein. Protein sequence analysis of CAP5.5 suggests a more complex function for this protein, which represents the first characterised member of a novel family of calpain-related genes in *T. brucei*.

2. Materials and methods

2.1. Cell culture

Procyclic *T. brucei* 427 were grown in SDM 79 medium containing 10% calf serum at 27 °C [7]. Pleomorphic *T. brucei rhodesiense* GUP 2962 were isolated from infected mice and differentiated in vitro to procyclic forms as described previously [8].

2.2. Metabolic labelling

Procyclic trypanosomes were labelled with [^{3}H]-myristic acid and [^{3}H]-palmitic acid as described previously [9]. Briefly, 200 μCi of radiolabelled fatty acids (Amersham) in 20 μl of ethanol were mixed with 14 μ1 of 1.8% (w/v) defatted bovine albumin (Sigma). Five millilitres of a trypanosome culture at a density of 1.5×10^7 cells/ml in SDM-79 medium supplemented with 7.5 μg/ml of hemin and 0.5 mg/ml of bovine transferrin were added to the labelled fatty acids and incubated overnight at 27 °C.

2.3. Immunoprecipitation

Cytoskeleton fractions of cells were prepared as previously described [10]. Pellets of cytoskeletons derived from 1 ml of metabolically labelled cells (see above) were dissolved in 100 μl of RIPA-buffer (1% Triton X-l00, 0.2% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM β-mercaptoethanol, 15 mM Tris–Cl, pH 7.4). Lysates were precleared by incubation with 20 μl of a 50% suspension of Protein G-sepharose (Sigma) for 30 min. All incubation steps were carried out at 4 °C. The cleared lysates were then incubated with 1.5 μg of purified anti-CAP5.5 monoclonal antibody for 2.5 h. Twenty microlitres of a 50% Protein G sepharose bead suspension were then added and incubation continued for a further hour. Beads were washed four times with RIPA buffer. The antibody–antigen complex was dissolved by boiling the drained beads in 50 μl of SDS-PAGE sample buffer for 10 min. Samples were analysed by SDS-PAGE as described previously [11]. Gels were stained with Coomassie, destained, incubated in Amplify (Amersham), dried onto filter paper and exposed to autoradiography film (Kodak BioMax-MS) at −80 °C.

2.4. DNA techniques

A 379 bp cDNA clone encoding the partial *CAP5.5* gene was isolated from a *T. brucei* λgt11 cDNA expression library as described previously [16]. A filter containing a P1 plasmid library of genomic DNA from *T. brucei* strain 927 (average insert size 60 kb, kindly provided by S. Melville, University of Cambridge) was screened with the digoxigenin-labelled 379 bp cDNA fragment using the digoxigenin filter hybridisation system (Roche Diagnostics). Purified P1 plasmid DNA from a positive clone was separately digested with *Eco*RV and *Hin*dIII. Using a Southern blot analysis of the restriction patterns, a 2.7 and 6 kb fragment were isolated from the *Eco*RV and *Hin*dIII digest, respectively. These fragments were isolated from agarose gels, cloned into pBluescript plasmid vector (Stratagene) and sequenced using a primer walking approach and the BigDye cycle sequencing protocol (Perkin-Elmer). The *Eco*RV-derived clone was labelled clone 10-7, and the *Hin*dIII-derived clone was labelled clone 6.

2.5. Protein analysis

Cells were fractionated into a detergent-soluble and cytoskeleton fraction by extraction with 0.5% NP-40 exactly as described previously [10]. SDS-PAGE and Western blotting were performed using standard techniques. Chemiluminescence was used for blot development (ECL, Amersham). Two-dimensional gel electrophoresis was performed as described previously [12,13].

2.6. Monoclonal antibodies

The 379 bp DNA fragment was used to express a recombinant β-galactosidase-CAP5.5 fusion protein in *E. coli*. The affinity-purified protein was injected into Balb/c mice, and monoclonal antibodies were obtained using PEG-induced fusion of spleen cells with P3X63-Ag8.653 myeloma cells according to standard methods [14].

Hybridoma cells were selected in OptiMEM plus 5% fetal calf serum and HAT (Gibco-BRL). The clone finally isolated was of the IgG1 subtype. Cell-culture supernatants were used for most experiments. For im-

munoprecipitations, Protein G-purified antibodies were used (HiTrap, Pharmacia).

2.7. Immunolocalisation studies

Trypanosomes were settled onto poly-lysine-coated slides, briefly washed with PBS and fixed in 100% methanol at −20 °C for 30 min. For the preparation of cytoskeletons, cells were extracted prior to fixation for 2 min with cold 0.5% NP-40 in 100 mM PIPES, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$. Cells were briefly rehydrated in PBS and incubated with anti-CAP5.5 antibody for 1 h and developed with a FITC-conjugated secondary antibody (Dako). Cells were embedded in Vectashield (Vector Laboratories) containing 100 ng/ml of DAPI. Images were digitally recorded on a Leica DMR epifluorescence microscope using a cooled CCD-camera (Roper Scientific, UK) analysed with IP-Lab software (Scanalytics, USA) and pseudo-coloured in Adobe Photoshop.

Electron microscope immunolocalisation was conducted with the affinity-purified polyclonal anti CAP5.5 antiserum and the monoclonal anti-tubulin antibody (TATi) as described previously [15].

3. Results

3.1. Identification and cloning of CAP5.5

In an attempt to identify novel cytoskeletal proteins in *Trypanosoma brucei*, cells extracted with 0.5% Triton X-100 and 0.6 M NaCl were injected into rabbits, and the resulting polyspecific/polyclonal antiserum was used to screen an λgt11-cDNA expression library [16]. Several clones were isolated, and the rabbit antiserum was affinity-purified on filter lifts of individual IPTG-induced λ-clones. Individual affinity-purified, clone-specific antisera were then used for immunofluorescence microscopy on trypanosomes to investigate the intracellular localisation of the proteins encoded by the cDNAs. One antiserum was found that reacted with the whole cell body of cells but excluded the flagellum. The cell-body staining was retained when cells were extracted in situ with non-ionic detergents, and therefore, the antigen was defined as cytoskeleton-associated. The protein was named CAP5.5 (for cytoskeleton-associated protein). In accordance with the suggested nomenclature (see Note at the end of this paper), the alternative label is *Tb*CALP1. The initial cDNA clone represented only a partial sequence of 379 bp. The nature of the cDNA library (dT-primed) and sequence features suggested that it coded for the C-terminus of a protein and included a portion of the 3′-UTR. To confirm the identity of this antigen, we produced a monoclonal antibody against a purified GST/CAP5.5 recombinant protein. The CAP5.5 portion corresponded to the original 379 bp fragment. The resulting mouse monoclonal antibody showed an immunofluorescence-labelling pattern identical to that of the affinity-purified polyclonal antiserum (Fig. 1). In methanol-fixed whole cells, a diffuse staining of the whole cell body with the exception of the flagellum was observed. In Triton X-100 extracted cells, CAP5.5 followed the helical organisation of the subpellicular cytoskeleton. Also, a single or double dot was stained intensely. Staining of the cellular DNA with DAPI showed that these dots were close to the kinetoplast and could colocalise either with the basal body or with the flagellar pocket. The exclusive localisation of CAP5.5 to the subpellicular cytoskeletal microtubules was also observed by immuno-electron microscopy on whole-mount cytoskeletons (Fig. 2). The antiserum to CAP5.5 did not label the axonemal microtubules. In contrast, labelling with the anti-tubulin antibody TAT1 occurred on both subpellicular and axonemal microtubules. The biochemical fractionation of cells into a Triton X-100 soluble and insoluble fraction showed that the Western blotting signal was completely restricted to the insoluble cytoskeletal fraction (Fig. 3a). This distribution is identical to that observed for acetylated tubulin using the monoclonal antibody C3B9. Acetylated tubulin is exclusively associated with assembled microtubules and not the soluble tubulin pool. Tyrosinated tubulin was detected in both the detergent-soluble and -insoluble fraction using the antibody YL1/2 since this post-translational modification occurs within the soluble tubulin pool of the cell, and tyrosinated tubulin is subsequently incorporated

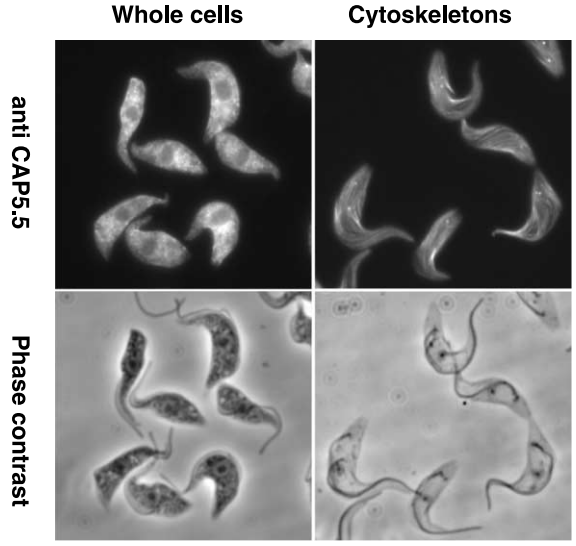

Fig. 1. Immunofluorescence localisation of CAP5.5 in whole cells and cytoskeletons (upper panel) using the monoclonal anti CAP5.5 antibody. Note the absence of labelling on the flagellum and the helical arrangement of CAP5.5 on cytoskeletons. The lower panel shows the corresponding phase-contrast images.

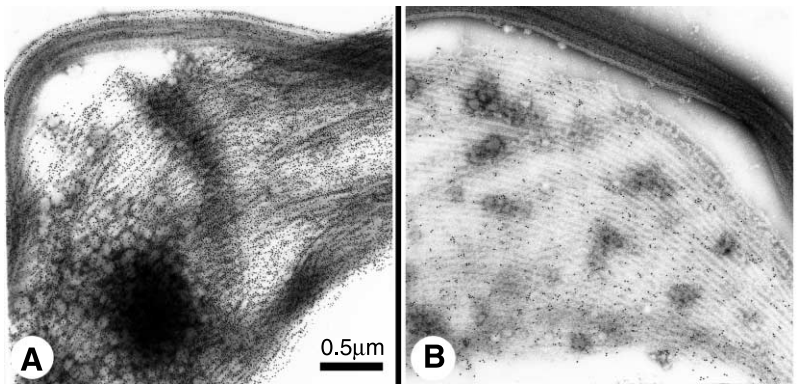

Fig. 2. Electron microscopic localisation of the anti-tubulin antibody TAT1(A) and the anti-CAP5.5 antibody (B) on whole-mount cytoskeletons. TAT1 labels the cell body and the axoneme, whereas anti-CAP5.5 labels only the cell body.

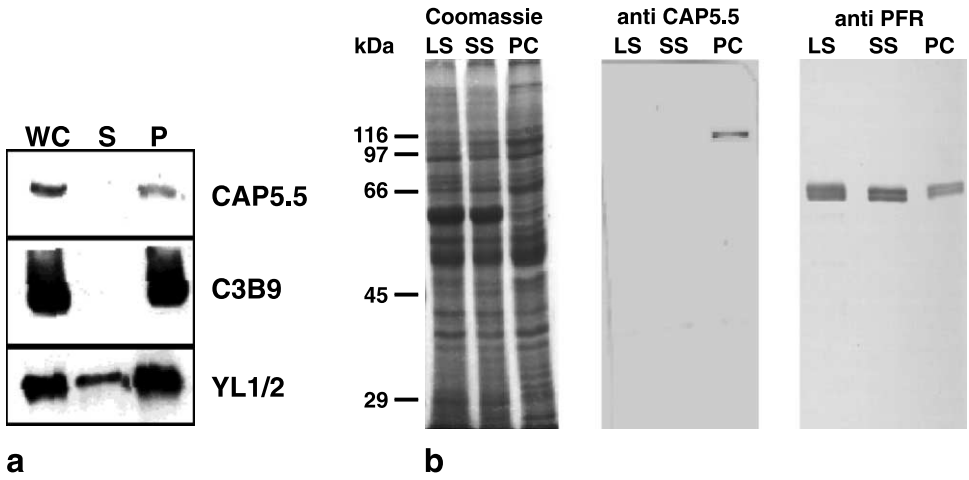

Fig. 3. (a) Biochemical fractionation of CAP5.5. The protein is detectable in whole cell extracts (WC) and in the cytoskeletal fraction of Triton X- 100 extracted cells (P) but not in the soluble fraction (S). Acetylated tubulin, detected with the monoclonal antibody C3B9, shows the same distribution, whereas tyrosinated tubulin, detected with YLI/2, is found in both the detergent-soluble and -insoluble fractions. (b) Differential expression of CAP5.5 in bloodstream and procyclic trypanosomes. Using a chemiluminescent detection system CAP5.5 is not detectable in long slender (LS) and short stumpy (SS) bloodstream forms but is present in procyclic (PC) forms. PFRA/C proteins are expressed in all life-cycle stages and were detected with an appropriate antibody. The Coomassie-stained gel illustrates an even loading of all lanes.

into microtubules. On Western blots, the antibody detected a single band of approximately 120 kDa. To isolate the full-length gene for *CAP5.5*, a genomic P1 library was screened with the 379 bp cDNA fragment. Two clones were isolated and one (12A11) used for further analysis. Two restriction fragments of 2.7 and 6 kb, respectively, were isolated, subcloned and sequenced. The 2.7 kb fragment contained the 379 bp sequence of the original λ-clone and additional sequence extending towards the 5′-end of the *CAP5.5* gene. An open reading frame of 2558 bp was identified (Fig. 4). The calculated molecular mass of the encoded protein was 94772 Da and contained 853 amino acid residues with a statistical p*I* of 4.6.

This acidic p*I* was confirmed by two-dimensional electrophoresis with subsequent Western blotting detection of CAP5.5 using the monoclonal antibody (Fig. 5). The low p*I* of CAP5.5 is significant because it is also characteristic of many other microtubule-associated proteins in various organisms [17]. Furthermore, it argues against the possibility of a basic protein attaching non-specifically to the exposed acidic projections of microtubules. The calculated molecular mass was approximately 20 kDa smaller than the observed mass of the protein on Western blots. A bioinformatics analysis of the sequence revealed a number of interesting motifs (Fig. 4). The N-terminus contained a consensus motif for myristoylation and palmitoylation. Near the N-terminus was a proline-rich region that corresponded to consensus motifs found in many ligands of SH3-type proteins. The central domain of CAP5.5, comprising approximately 30% of the protein, showed a significant degree of similarity to the catalytic domain of calpain-like cysteine proteases. Details of these sequence features are discussed below.

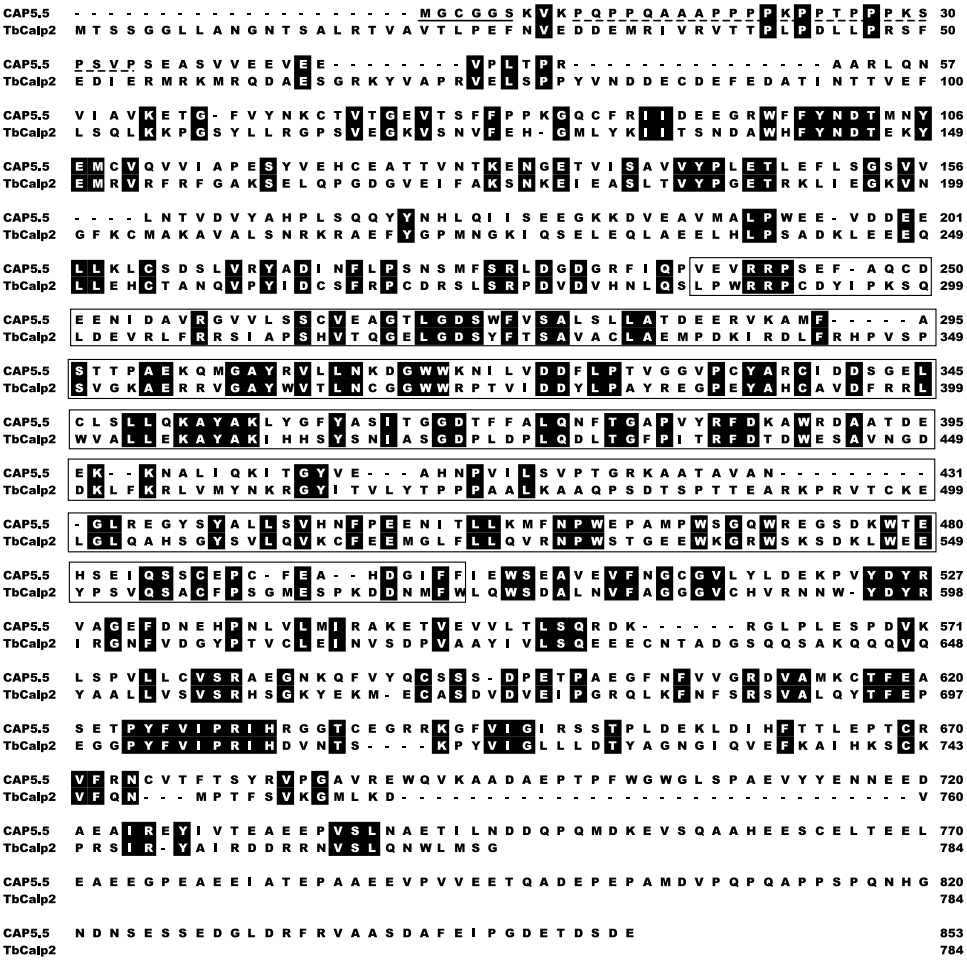

Fig. 4. Alignment of CAP5.5 (or *Tb*CALP1) with *Tb*CALP2. Identical amino acid residues are highlighted. The N-terminal dual acylation motif of CAP5.5 is underlined, the proline-rich motif is underlined with a broken line, and the region of similarity of CAP5.5 and *Tb*CALP2 with typical calpains is boxed.

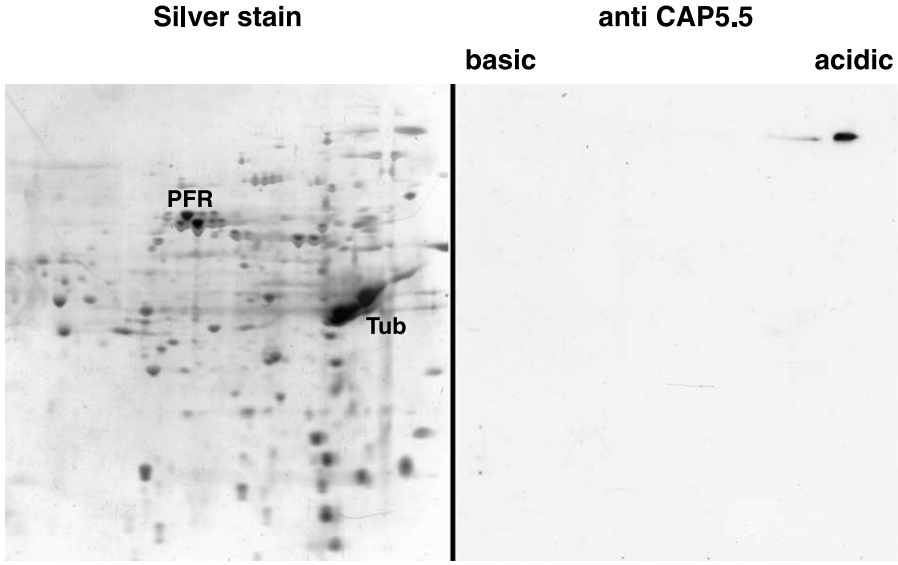

Fig. 5. Two-dimensional gel electrophoresis of cytoskeletal proteins and Western blot detection of CAP5.5. The position of the paraflagellar rod protein complex (PFRA/C, 69–72 kDa, p*I* 6.5) and the tubulins (51–53 kDa, p*I* 5.5) are indicated on the silver-stained gel.

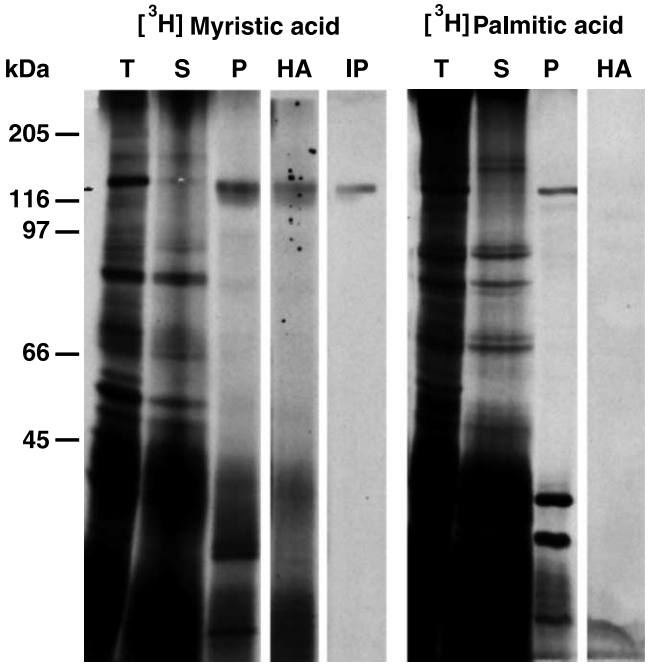

Fig. 6. In-vivo acylation of CAP5.5 with myristic and palmitic acid. Cells were metabolically labelled and then extracted with Triton-X100 and separated into a soluble (S) and insoluble cytoskeletal (P) fraction. Total cellular extracts are also shown (T). In both experiments, CAP5.5 is the major high-molecular weight protein that is labelled with myristic and palmitic acid. In the cytoskeletal fraction, it is the only detectable acylated protein above 45 kDa. The identity of the 120 kDa band as CAP5.5 is confirmed by immunoprecipitation of labelled cellular extracts with the monoclonal CAP5.5 antibody (IP). Only palmitoylation is sensitive to treatment with hydroxylamine; myristoylation is not affected (HA).

3.2. Expression pattern of CAP5.5 during the life cycle

Western blot analysis of the expression pattern of CAP5.5 showed that the protein is detectable in procyclic, but not in long-slender and short-stumpy bloodstream form trypanosomes (Fig. 3b). A previous account of differentiation in *T. brucei* used the original polyclonal antibody to CAP5.5 as a marker that appears late in the differentiation of bloodstream to procyclic cells [8].

3.3. Acylation of CAP5.5

The N-terminal amino acid sequence of CAP5.5 contains a motif (MGCGGS) that corresponds to a consensus sequence often found in proteins that are dually acylated with myristic acid at the glycine residue and palmitic acid at the cysteine residue. To test this possibility, we labelled trypanosomes with the two tritiated fatty acids in separate experiments. After 16 h of labelling, cells were lysed in RIPA buffer and CAP5.5 immunoprecipitated with the monoclonal antibody. In both labelling reactions, a positive band of the correct molecular mass was observed (Fig. 6). In the case of palmitic acid, the labelling reaction was sensitive to subsequent incubation with hydroxylamine, indicating the presence of a thioester bond characteristic of N-palmitoylation of proteins. The amide bond between myristate and glycine was resistant to hydroxylamine treatment. The dual acylation goes some way toward explaining the apparent larger molecular mass on SDS-gels in relation to the calculated mass based on the primary sequence.

3.4. Genomic organisation of CAP5.5 reveals a related protein

In order to complete the molecular analysis of the *CAP5.5* gene, we obtained the sequence of the 3′-untranslated region (UTR). To achieve this, we sequenced the second P1 subclone containing a 6 kb insert. The 5′-end of this clone overlapped with the 3′-end of the coding region of *CAP5.5*, therefore providing a continuous segment of the genome. The 3′-UTR was A/T-rich (63%), mainly due to the presence of several homopolymeric A or T tracts. Comparison with other life-cycle-regulated genes revealed no significant similarities that might be related to mRNA stability and life-cycle regulation [18]. However, after approximately 2.8 kb of non-coding sequence, the 6 kb fragment contained an open reading frame of 2352 bp corresponding to a putative protein of 784 amino acid residues (Fig. 4). This putative protein (*Tb*Calp2) shares a significant degree of similarity with CAP5.5/*Tb*Calpl. The central domains of both proteins (approximately 600 amino acid residues) are 30% identical. This region of similarity corresponds to the same part of the CAP5.5 sequence, which is related to calpain-like proteases. *Tb*Calp2 has a unique N-terminal extension of 20 amino acids, whereas CAP5.5 has a 108 amino acid extension of its C-terminus in comparison to *Tb*Calp2 (Fig. 4). A potential N-terminal dual acylation motif is not present in *Tb*Calp2. Northern blot analysis of the *Tb*Calp2 mRNA revealed single band signals of equally strong intensity from RNA preparations of both bloodstream and procyclic cells, suggesting that the transcript is, in contrast to CAP5.5, not life-cycle-regulated (data not shown). Restriction analysis of the *CAP5.5* and *TbCalp2* genes indicated that both are present as single copies in the diploid genome (data not shown). Currently, we have no information on the subcellular localisation of the putative *TbCalp2* gene product.

4. Discussion

4.1. Characterisation of CAP5.5

Here, we describe the characterisation of a novel cytoskeletal-associated protein, CAP5.5, in *T. brucei*.

Its association with the cytoskeleton has been shown using biochemical and structural criteria. Firstly, biochemical fractionation of cells with the non-ionic detergent Triton X-100 leaves the protein quantitatively in the insoluble fraction, which contains the cytoskeletal components of the cell (Fig. 3a). Secondly, immunofluorescence and electron microscopy localisation on cytoskeletons clearly show the association of CAP5.5 with the subpellicular microtubules (Figs. 1 and 2). The protein distribution follows the helical arrangement of the microtubules along the cell body. The protein is not found on the axonemal microtubules of the flagellum. The primary sequence itself reveals no features that relate CAP5.5 to other known microtubule-associated proteins in trypanosomes or other organisms. In particular, CAP5.5 has none of the repetitive elements that are typical for some other MAPs in trypanosomes [19].

The cortical microtubule cytoskeleton is in close (10 nm) proximity to the plasma membrane. The spacing between individual microtubules is 18–20 nm. Even using immunogold electron microscopy, it is difficult to define whether a protein is more likely to participate in membrane–microtubules interactions, in intermicrotubule connections or in other functions [2,20,21]. We have shown, however, that CAP5.5 is doubly acylated with myristic and palmitic acid, and dual acylation of this type is exclusively found in membrane-associated proteins [22]. Myristoylation is necessary for protein–membrane interactions because it targets proteins to hydrophobic lipid domains within the cell and is also a prerequisite for subsequent palmitoylation, which then establishes a high-affinity interaction with membranes [22,23]. Despite the strong possibility that CAP5.5 interacts with the plasma membrane via the acyl chains, the protein nevertheless sequesters completely into the Triton X-100 insoluble fraction of a cellular fractionation experiment. This indicates that CAP5.5 also interacts strongly with the subpellicular microtubule cytoskeleton. Therefore, a potential function of CAP5.5 could be the crosslinking of the plasma membrane with microtubules. Interestingly, one of the few other proteins in trypanosomes that has been shown to be modified with palmitic acid is p41, a protein that binds to microtubules in a Ca^{2+}-dependent manner and which might also play a role in microtubules–membrane interactions [24]. In higher eukaryotes, several proteins that are involved in membrane–cytoskeleton interactions, such as ankyrin, vinculin, spectrin and human erythrocyte protein 4.2, have been shown to be acylated [25–28]. It is, however, intriguing that CAP5.5 is strictly life-cycle-regulated and only expressed in procyclic forms but not in bloodstream-form trypanosomes. It has been characterised as a late marker of bloodstream to procyclic differentiation and is detectable by immunofluorescence microscopy approximately 8 h after the induction of an in vitro differentiation time course [8]. As there are no major ultrastructural differences between the subpellicular cytoskeleton of both forms, it appears unlikely that CAP5.5 merely fulfils a simple structural role in the organisation of the cytoskeleton.

Two further characteristics of the primary CAP5.5 sequence might give some indications as to the possible functions of this protein. Close to its N-terminus, CAP5.5 is rich in proline residues. The sequence APPPPKPP conforms to a sequence motif that is often found in the binding site of ligands that bind to proteins containing SH3-domains [29,30]. Proline-rich motifs in proteins are now recognised as a major interface for protein–protein interactions, particularly at the plasma membrane [31–33]. Furthermore, the motif contains both a lysine and a threonine residue, and phosphorylation is a common mechanism of regulating the interaction of proline motifs with their ligands [34,35].

A third remarkable feature of the CAP5.5 sequence is the high degree of similarity with calpain-type cysteine proteases. The region of similarity is confined to a central domain within CAP5.5, beginning at amino acid residue V240 and ending at residue F500 (Fig. 7). Classical calpains, such as m- or μ-calpain, can be divided into four distinct domains: an N-terminal-or prodomain, the protease domain, a linker domain and a Ca^{2+}-binding domain [36–38]. The similarity with CAP5.5 is restricted only to the protease domain of calpains. CAP5.5 does not possess EF-hand type domains typical for some other calpains. Moreover, although the overall similarity with the catalytically active domains of calpains is obvious, only one of the three amino acids constituting the active site in calpains is conserved (Fig. 8). Work done in several groups over the last few years has revealed the presence of large numbers of calpain-related proteins in various organisms, such as CAPN5 and CAPN6 in vertebrates [39]. As in CAP5.5, many of these proteins lack domain IV containing the Ca^{2+}-binding EF-hand motifs and show variation in the catalytic triad (Fig. 8). A protease activity has, in most cases, not been demonstrated. Very little is known about the identity of in-vivo substrates for calpains, and hence their physiological functions are ill defined. They are implicated in a wide range of activities such as regulatory functions during the cell cycle, apoptosis, cytoskeleton remodelling and environmental adaptation [40–46].

4.2. Genomic context of CAP5.5 as a calpain-related protein

When sequencing the 3′ untranslated region of *CAP5.5*, we identified an additional open reading frame of 2352 nucleotides. The putative protein *Tb*CALP2 encoded by this ORF revealed a significant similarity to

CAP5.5. This similarity is not restricted to the protease domain of calpains but extends over almost the entire length of the proteins. Only the C-terminal extension of 108 amino acid residues is unique to CAP5.5 and accounts for the higher molecular mass of CAP5.5. *Tb*CALP2 does not contain the N-terminal dual acylation motif found in CAP5.5, but amino acid residues 5–10 (GGLLAN) represent a potential myristoylation motif. A comparison of the proline-rich region of CAP5.5 and *Tb*CALP2 shows that, although *Tb*CALP2 does not contain as many proline residues as CAP5.5, the position of three prolines is conserved between CAP5.5 and *Tb*CALP2. Potential phosphorylation sites are also present in the vicinity of this proline motif.

As expected, a search of the available trypanosome databanks revealed a series of genomic shotgun sequences (GSS) identical to the CAP5.5 and *Tb*CALP2 sequence. However, in addition, we found a number of GSS showing significant similarity to CAP5.5 (*Tb*CALP1) and *Tb*CALP2 both on DNA and protein level. Interestingly, most of these latter GSS have been assembled into annotated contigs and are all clustered in a region of approximately 34 kb on the left side of the tubulin gene cluster on chromosome I. *CAP5.5* and *TbCALP2*, although present as gene fragments in the GSS databases, are not located on this chromosome. The 34 kb cluster on chromosome I contains 12 open

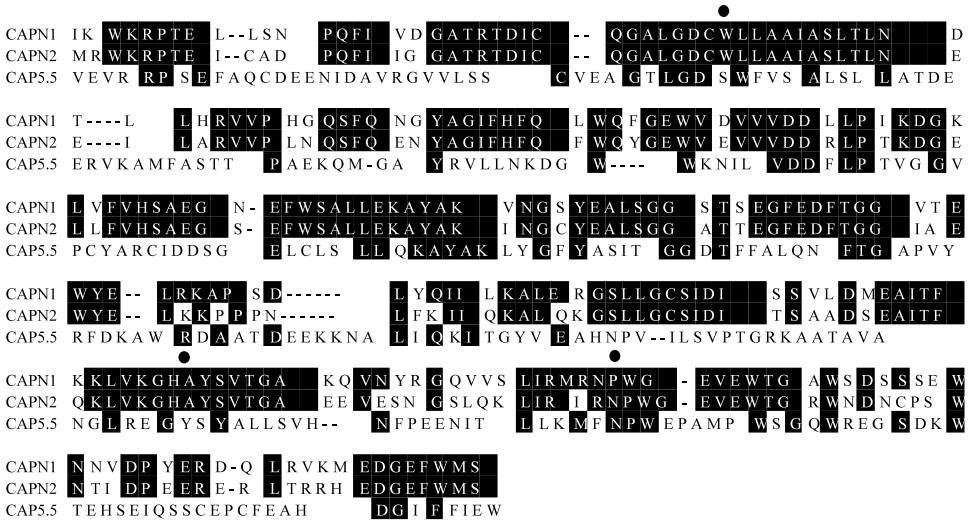

Fig. 7. Alignment of CAP5.5 with two human calpains (CAPN1/2, also known as m/μ calpains, EMBL accession number as in Fig. 8). These two calpains represent the best characterised calpains and have been shown to be catalytically active. Identical amino acid residues are highlighted, and the position of the three amino acid residues constituting the catalytic triad in CAPN1/2 is marked with black dots above the sequence. Only the catalytic domains of the calpains and the corresponding domain of CAP5.5, as indicated in Fig. 4, have been included in this alignment.

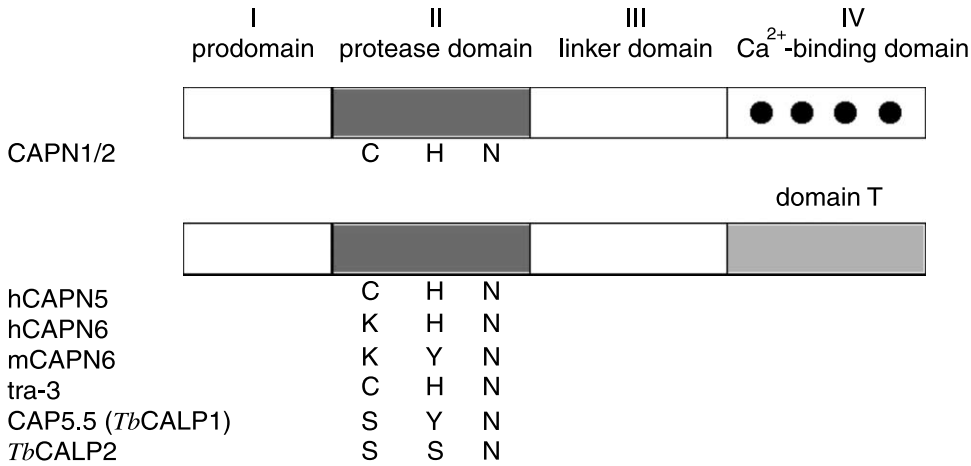

Fig. 8. Comparison of the domain structure and catalytic triad amino acid residues (in single-letter code) of *T. brucei* CAP5.5 (*Tb*CALP1) and *Tb*CALP2 with members of the calpain family from other organisms. Human CAPN1 and 2 (EMBL accession number X04366, M23254), human CAPN5 (Y10552), human CAPN6 (AJ000388), mouse CAPN6 (Y12582), *C. elegans* tra-3 (U12921). The domain description is adapted from Ref. [41]. CAPN5/6 and tra-3 represent calpain-like proteins lacking the Ca^{2+}-binding domains.

reading frames. Seven of these ORFs show significant degrees of similarity with calpains. We suggest that their putative protein products should be named *Tb*CALP3–9. In all cases, the region of similarity is, as with CAP5.5 (*Tb*CALPl) and *Tb*CALP2, restricted to the proteolytic domain of calpains. Only the *TbCALP3–5* ORFs of this cluster encode for proteins of a length comparable to CAP5.5 or other calpains (between 700 and 1100 amino acids). The remaining four ORFs *(TbCALP6–9)* represent putative proteins that are only approximately 120 amino acids in length. The absence of calcium-binding domains in all of the trypanosomal calpain-like proteins supports the hypothesis that the acquisition of this domain by gene fusion was a relatively late event in the evolution and diversification of this family of proteins [47]. As yet, we have no indication whether any of these genes are expressed, and hence, a comprehensive analysis of this calpain-like gene family is beyond the scope of this paper. An evolutionary perspective on the diversity of calpains has been presented by Jekely [37]. If, however, the *TbCALP3–9* ORFs, along with CAP5.5 (*Tb*CALP1) and *Tb*CALP2, are expressed then trypanosomes would possess one of the largest families of calpain-related proteins described for any organism, and it would surely be of great importance to investigate their physiological functions.

Note: Due to the potential presence of a large number of calpain-like proteins in *Trypanosoma brucei*, we suggest a unified nomenclature for these proteins. We have named the (putative) calpain-like proteins encoded by the ORFs on the 34 kb cluster on chromosome I *Tb*CALP3–9 (for *T. brucei* calpain-like protein). CAP5.5 will have the alternative label *Tb*CALP 1. The protein encoded by the ORF immediately downstream of CAP5.5 is labelled *Tb*CALP2.

Acknowledgements

The work in our lab is funded by a Wellcome Trust Programme and Equipment grant and a Wellcome Trust Prize Studentship (CHF). We are grateful to Drs. Neil Hall and Matthew Berriman (The Sanger Centre Pathogen Sequencing Unit, Cambridge) for communicating chromosome I contig sequence data. Sequencing of *T. brucei*: chromosome I was accomplished as part of the Trypanosoma Genome Network with support from the Wellcome Trust. We also acknowledge the scientific contributions of Drs R. Docherty and R. Gerke-Bonet in the early phase of this project.

References

[1] Vickerman K. Developmental cycles and biology of pathogenic trypanosomes. Br Med Bull 1985;41:105–14.
[2] Gull K. The cytoskeleton of trypanosomatid parasites. Annu Rev Microbiol 1999;53:629–55.
[3] Sherwin T, Gull K. The cell division cycle of *Trypanosoma brucei brucei*: timing of event markers and cytoskeletal modulations. Philos Trans R Soc London Ser B 1989;323:573–88.
[4] Bramblett GT, Chang SL, Flavin M. Periodic crosslinking of microtubules by cytoplasmic microtubule- associated and microtubule-corset proteins from a trypanosomatid. Proc Natl Acad Sci USA 1987;84:3259–63.
[5] Barth AI, Nathke IS, Nelson WJ. Cadherins, catenins and APC protein: interplay between cytoskeletal complexes and signalling pathways. Curr Opin Cell Biol 1997;9:683–90.
[6] Schoenwaelder SM, Burridge K. Bidirectional signalling between the cytoskeleton and integrins. Curr Opin Cell Biol 1999;11:274–86.
[7] Brun R, Schonenburger M. Cultivation and in vitro cloning of procyclic forms of *Trypanosoma brucei* in a semi-defined medium. Acta Trop 1979;36:289–92.
[8] Matthews KR, Gull K. Evidence for an interplay between cell cycle progression and the initiation of differentiation between life cycle forms of African trypanosomes. J Cell Biol 1994;125:1147–56.
[9] Field MC, Menon AK. In: Hooper NM, Turner AJ, editors. Lipid Modifications of Proteins. Oxford: Oxford University Press, 1992.
[10] Woods A, Baines AJ, Gull K. Evidence for a Mr 88000 glycoprotein with a transmembrane association to a unique flagellum attachment region in *Trypanosoma brucei*. J Cell Sci 1989;93:501–8.
[11] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[12] O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 1975;250:4007–21.
[13] Burland TG, Gull K, Schedl T, Boston RS, Dove WF. Cell type-dependent expression of tubulins in *Physarum*. J Cell Biol 1983;97:1852–9.
[14] Ersfeld K, Ogbadoyi E, Robinson D, Sherwin T, Gull K. The architecture of the *Trypanosoma brucei* nucleus during interphase and mitosis. Chromosoma 2000;108:501–13.
[15] Sherwin T, Gull K. Visualization of detyrosination along single microtubules reveals novel mechanisms of assembly during cytoskeletal duplication in trypanosomes. Cell 1989;57:211–21.
[16] Birkett CR, Parma AE, Gerke-Bonet R, Woodward R, Gull K. Isolation of cDNA clones encoding proteins of complex structures: analysis of the *Trypanosoma brucei* cytoskeleton. Gene 1992;110:65–70.
[17] Kreis T, Vale R. Guidebook to the Cytoskeletal and Motor Proteins. Oxford: Sambrook & Tooze Pub. at Oxford University Press, 1999.
[18] Hotz HR, Biebinger S, Flaspohler J, Clayton C. PARP gene expression: control at many levels. Mol Biochem Parasitol 1998;91:131–43.
[19] Affolter M, Hemphill A, Roditi I, Muller N, Seebeck T. The repetitive microtubule-associated proteins MARP-l and MARP-2 of *Trypanosoma brucei*. J Struct Biol 1994;112:241–51.
[20] Hemphill A, Seebeck T, Lawson D. The *Trypanosoma brucei* cytoskeleton: ultrastructure and localization of microtubule-associated and spectrin-like proteins using quick-freeze, deep-etch, immunogold electron microscopy. J Struct Biol 1991;107:211–20.
[21] Kohl L, Gull K. Molecular architecture of the trypanosome cytoskeleton. Mol Biochem Parasitol 1998;93:1–9.
[22] Resh MD. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochim Biophys Acta 1999;1451:1–16.

[23] Godsel LM, Engman DM. Flagellar protein localization mediated by a calcium-myristoyl/palmitoyl switch mechanism. EMBO J 1999;18:2057–65.
[24] Schneider A, Eichenberger W, Seebeck T. A microtubule-binding protein of *Trypanosoma brucei* which contains covalently bound fatty acid. J Biol Chem 1988;263:6472–5.
[25] Burn P, Burger MM. The cytoskeletal protein vinculin contains transformation-sensitive, covalently bound lipid. Science 1987;235:476–9.
[26] Mariani M, Maretzki D, Lutz HU. A tightly membrane-associated subpopulation of spectrin is 3H-palmitoylated. J Biol Chem 1993;268:12996–3001.
[27] Risinger MA, Dotimas EM, Cohen CM. Human erythrocyte protein 4.2, a high copy number membrane protein, is N-myristylated. J Biol Chem 1992;267:5680–5.
[28] Staufenbiel M, Lazarides E. Ankyrin is fatty acid acylated in erythrocytes. Proc Natl Acad Sci USA 1986;83:318–22.
[29] Ren R, Mayer BJ, Cicchetti P, Baltimore D. Identification of a ten-amino acid proline-rich SH3 binding site. Science 1993;259:1157–61.
[30] Yu H, Chen JK, Feng S, Dalgarno DC, Brauer AW, Schreiber SL. Structural basis for the binding of proline-rich peptides to SH3 domains. Cell 1994;76:933–45.
[31] Buday L. Membrane-targeting of signalling molecules by SH2/SH3 domain-containing adaptor proteins. Biochim Biophys Acta 1999;1422:187–204.
[32] Musacchio A, Wilmanns M, Saraste M. Structure and function of the SH3 domain. Prog Biophys Mol Biol 1994;61:283–97.
[33] Niebuhr K, Ebel F, Frank R, et al. A novel proline-rich motif present in ActA of *Listeria monocytogenes* and cytoskeletal proteins is the ligand for the EVH1 domain, a protein module present in the Ena/VASP family. EMBO J 1997;16:5433–44.
[34] Sanchez C, Diaz-Nido I, Avila I. Phosphorylation of microtubule-associated protein 2 (MAP2) and its relevance for the regulation of the neuronal cytoskeleton function. Prog Neurobiol 2000;61:133–68.
[35] Kay BK, Williamson MP, Sudol M. The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. FASEB J 2000;14:231–41.
[36] Hosfield CM, Elce IS, Davies PL, Jia Z. Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. EMBO J 1999;18:6880–9.
[37] Jekely G, Friedrich P. The evolution of the calpain family as reflected in paralogous chromosome regions. J Mol Evol 1999;49:272–81.
[38] Strobl S, Fernandez-Catalan C, Braun M, et al. The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. Proc Natl Acad Sci USA 2000;97:588–92.
[39] Dear N, Matena K, Vingron M, Boehm T. A new subfamily of vertebrate calpains lacking a calmodulin-like domain: implications for calpain regulation and evolution. Genomics 1997;45:175–84.
[40] Carafoli E, Molinari M. Calpain: a protease in search of a function? Biochem Biophys Res Commun 1998;247:193–203.
[41] Sorimachi H, Ishiura S, Suzuki K. Structure and physiological function of calpains. Biochem J 1997;328:721–32.
[42] Emori Y, Saigo K. Calpain localization changes in coordination with actin-related cytoskeletal changes during early embryonic development of *Drosophila*. J Biol Chem 1994;269:25137–42.
[43] Ono Y, Sorimachi H, Suzuki K. Structure and physiology of calpain, an enigmatic protease. Biochem Biophys Res Commun 1998;245:289–94.
[44] Potter DA, Tirnauer IS, Janssen R, et al. Calpain regulates actin remodeling during cell spreading. J Cell Biol 1998;141:647–62.
[45] Denison SH, Orejas M, Arst HN Jr. Signaling of ambient pH in *Aspergillus* involves a cysteine protease. J Biol Chem 1995;270:28519–22.
[46] Suzuki K, Sorimachi H. A novel aspect of calpain activation. FEBS Lett 1998;433:1–4.
[47] Ohno S, Emori Y, Imajoh S, Kawasaki H, Kisaragi M, Suzuki K. Evolutionary origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium-binding protein? Nature 1984;312:566–70.