The predominant calcimedins from *Trypanosoma brucei* comprise a family of flagellar EF-hand calcium-binding proteins

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The cellular complement of calcimedins was identified in *Trypanosoma brucei* by Ca^{2+} -dependent association with phenyl-Sepharose. Predominant calcimedins with molecular mass of 23–26 kDa and 44 kDa, along with minor calcimedins of 96, 120 and 230 kDa, were obtained. The trypanosome calcimedins were unrelated to vertebrate annexins, based upon antibody cross-reactivity and an inability to associate in a Ca^{2+} -dependent way with phospholipid vesicles comprised of phosphatidylserine or phosphatidylethanolamine/phosphatidylcholine (1:1, w/w). Partial sequence analysis demonstrated that 44 kDa calcimin (Tb-44) contained an EF-hand calcium-binding loop. Five CNBr/tryptic fragments exhibited a total of 93% similarity with Tb-17, a 23 kDa EF-hand protein in *T. brucei*. The trypanosome calcimedins appeared to comprise a family of proteins, based on sequence similarities and antibody cross-reactivity of affinity-purified anti-Tb44 with the 23–26 kDa cluster. No evidence was found for Tb-44 in the related species *T. cruzi*, *Leishmania tarentolae* or *Crithidia fasciculata*. Antibodies against Tb-44 were localized by immunofluorescence along the flagellum of *T. brucei*. Immunoblot analysis of flagella-enriched preparations demonstrated that Tb-44 and the 23–26 kDa cluster were present in this structure. We conclude that annexin family members are not among the predominant trypanosome proteins that associate with phenyl-Sepharose in a Ca^{2+} -dependent way. Instead, the major trypanosome calcimedins comprise a family of flagellar EF-hand calcium-binding proteins.

INTRODUCTION

Calcium is a universal intracellular messenger that modifies cellular activities by virtue of its interaction with specific Ca^{2+} -binding proteins. The present study was undertaken to systematically characterize Ca^{2+} -binding proteins in the pathogenic protozoan *Trypanosoma brucei*. Proteins which exposed hydrophobic residues in the presence of calcium were sought. Such proteins have been previously identified in vertebrate tissues based upon their Ca^{2+} -dependent association with fluphenazine-Sepharose [1], membranes [2] or phenyl-Sepharose [3]. The complement of proteins that associate with these hydrophobic matrices have been termed calcimedins [4]. These proteins actually belong to three general families: (1) EF-hand structures [5], (2) annexins [6,7], and (3) protein kinase C isoenzymes [8]. The EF-hand proteins, such as parvalbumin and calmodulin, are characterized by a repeated helix-turn-helix Ca^{2+} -binding motif. Annexins differ from EF-hand family members in that they contain an array of some 70 residues tandemly repeated four or eight times as a membrane-binding core, and a 17-residue consensus sequence within each array. Protein kinase C isoenzymes contain a regulatory domain that binds to calcium and phospholipids, and a catalytic domain. Their structural diversity is generated by multiple genes and alternative splicing of a single gene.

While EF-hand proteins, annexins and protein kinase C isoenzymes have been extensively studied in vertebrate systems, little is known of their role in protozoa. African trypanosomes are flagellated protozoa that spend part of their life in the blood, lymphatics and cerebral spinal fluid of susceptible mammalian hosts and part of their life in the insect host. The parasite adapts to changes within and between hosts, suggesting a possible regulatory role for calcium signals. However, the role of calcium in mediating life cycle events is poorly understood. Two pools of

exchangeable calcium have recently been described in *T. brucei* which may serve to trigger calcium signals [9,10]. Proteins which are activated by elevated intracellular Ca^{2+} concentration have also been identified. Calmodulin [11], protein kinase C-like activity [12], calcium-sensitive adenylate cyclase [13] and a 22 kDa calcium-binding protein [14] have been partially characterized from *T. brucei* homogenates. The present study extends this earlier work by employing a systematic approach in order to characterize the cellular complement of calcimedins from *T. brucei*. Special emphasis has been placed on the relationship between trypanosome calcimedins and annexin family members. We report here that the predominant trypanosome calcimedins are flagellar EF-hand Ca^{2+} -binding proteins.

MATERIALS AND METHODS

Organisms

Trypanosoma brucei brucei antigenic type M110 [15] was used throughout this study. Storage of stabiles, infection in mice and rats, and harvesting of the cells from blood were as described previously [16]. Cultured procyclic forms of YTat1.1 were maintained in Cunningham's medium.

Purification of trypanosome calcimedins

Trypanosome calcimedins were obtained by a combination of DE-52 and phenyl-Sepharose chromatography. Frozen cells were suspended at a density of 5×10^{11} cells in 75 ml of 50 mM-Tris/HCl, 3 mM-EDTA, 0.5 mM-dithiothreitol (DTT), 1 mM-phenylmethanesulphonyl fluoride (PMSF), and 10 μg of leupeptin/ml (pH 7.5). Cells were disrupted at 4 °C with a Braun sonifier set at 250 W full power for 7 cycles of 15 s intervals. The homogenate volume was then brought to 150 ml with homogenization buffer and centrifuged at 100 000 g for 1 h. The supernatant was applied to a DE-52 anion-exchange column

Abbreviations used: DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline (3 mM- NaH_2PO_4 /47 mM- Na_2HPO_4 /70 mM- NaCl , pH 8.0); DMSO, dimethyl sulphoxide; FCaBP, flagellar Ca^{2+} -binding protein.

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(3 cm × 9 cm; 60 ml) pre-equilibrated with 50 mM-Tris/HCl, 1 mM-EDTA and 0.5 mM-DTT, pH 7.5 (buffer A) followed by extensive washing with the same buffer. The column was then eluted with buffer A containing 150 mM-NaCl. The Ca²⁺ content of the eluate was adjusted to 5 mM and the sample was loaded on to a phenyl-Sepharose CL-4B column (1 cm × 15 cm, 12 ml) pre-equilibrated with 50 mM-Tris/HCl, 150 mM-NaCl, 0.2 mM-Ca²⁺ and 0.5 mM-DTT, pH 7.5. The column was washed with 30 ml of equilibration buffer, followed by equilibration buffer containing 500 mM-NaCl, until the *A*₂₈₀ returned to baseline. Finally, the column was eluted with 50 mM-Tris/HCl, 500 mM-NaCl, 2 mM-EGTA and 0.5 mM-DTT, pH 7.5, and 1.2 ml fractions were collected. Elution was monitored with a Single Path Monitor UV-1 (Pharmacia) at 280 nm.

Bovine liver calcimedins

Calcimedins from bovine liver were isolated by phenyl-Sepharose chromatography as described by Südhof *et al.* [17].

Electrophoresis and immunoblot analyses

Immunoblot analyses, including protein separation by SDS/PAGE, electrophoretic transfer of proteins to nitrocellulose or Immobilon (Millipore) membranes, and incubation with primary and secondary antibodies were performed as described previously [16]. Coomassie Blue-stained gels and alkaline phosphatase-stained immunoblots were quantified with a Zeineh soft laser scanning densitometer (model SL-2DUV).

Protein chemistry

Isolated trypanosome calcimedins were separated by SDS/PAGE and transferred to Immobilon membranes. The protein band migrating on SDS/PAGE at 44 kDa, that we called Tb-44, was excised and digested with CNBr and trypsin as described [18]. The fragments were separated on a C18 reverse-phase column (Vydac), and selected fragments were sequenced with a model 470A amino acid sequencer (Applied Biosystems) according to the manufacturer's programming and chemicals.

Antibodies

Purified calcimedins were separated by SDS/PAGE, transferred to nitrocellulose membranes, and Tb-44 was identified with Ponceau S. The excised membrane containing 4 µg of Tb-44 was dissolved in dimethyl sulphoxide (DMSO), resuspended in phosphate-buffered saline (PBS) and emulsified with Freund's adjuvant. Antibodies were raised in mice by intraperitoneal injection at 14-day intervals. The antisera were harvested and affinity-purified by the method of Olmstead, using pure Tb-44 as absorbant [19]. Monoclonal antibodies against annexins I, II, IIP11 subunit, IV and VI [20] were purchased from Zymed Laboratories. Polyclonal antibodies against 35 kDa calcimedin [21] were kindly provided by J. R. Dedman, Department of Physiology and Biophysics, Cincinnati College of Medicine, Cincinnati, OH, U.S.A. Homogenates of *T. cruzi* and polyclonal antibodies against *T. cruzi* flagellar Ca²⁺-binding protein [22] were generously supplied by L. V. Kirchhoff, Department of Internal Medicine, University of Iowa, Iowa City, IA, U.S.A.

Ca²⁺-dependent phospholipid vesicle binding assay

Vesicles were prepared from phosphatidylcholine/phosphatidylethanolamine (PC/PE, 1:1, w/w) and phosphatidylserine (PS) by the method of Reeves & Dowben [23]. Assays were performed with calcimedins from trypanosomes (14.4 µg) or bovine liver (15.3 µg). Samples were incubated with vesicles (112 µg) for 10 min in buffer P (20 mM-Hepes, 100 mM-NaCl, 2 mM-MgCl₂ and 2 mM-NaN₃, pH 7.4) in the presence of 4 mM-Ca²⁺ or 4 mM-EGTA and the mixtures were centrifuged at

100000 *g* for 45 min. The pellets and supernatants were analysed by SDS/PAGE.

Fractionation of *T. brucei* flagella

A flagella-enriched fraction was obtained by modifications of previously described methods [24,25]. These modifications included disruption of cells by N₂ cavitation at 4140 kPa (600 lb/in²) for 30 min, and centrifugation on step gradients consisting of 1.11 M-, 1.52 M- and 1.90 M-sucrose at 130000 *g* for 4 h. An Olympus BH2 microscope was used to monitor the flagella-enriched fractions throughout the procedure.

Immunolocalization

Immunolocalizations were done as previously described [16]. Briefly, slender and procyclic forms of *T. brucei* were ethanol-fixed and incubated on cover slips with affinity-purified anti-Tb-44 at 37 °C for 1 h. After extensive washing with PBS, the cells were incubated for 1 h with goat anti-mouse conjugated to fluorescein isothiocyanate (FITC) (Fisher). The cover slips were washed and set in mounting medium [1.1 ml of 10% (w/v) polyvinyl alcohol, 30 mM-Tris/HCl, pH 8.5, 2 mg of *p*-phenylenediamine, 400 µl of DMSO and 500 µl of methanol] for phase contrast and fluorescence microscopy (Nikon Microphot-FX).

RESULTS

Purification of trypanosome calcimedins

To obtain the cellular complement of calcium-binding proteins from *T. brucei*, we applied the 100000 *g* EGTA supernatant of whole-cell homogenates on to a DE-52 anion-exchange column and eluted the column with buffer containing 0.15 M-NaCl. The DE-52 eluate was further fractionated by hydrophobic interaction chromatography (Fig. 1a). A phenyl-Sepharose column was initially washed with buffer containing 0.2 mM-Ca²⁺ and 0.15 M-NaCl, followed by extensive washing with buffer containing 0.2 mM-Ca²⁺ and 0.5 M-NaCl until the eluate absorbance had returned to background levels (Fig. 1a and Fig. 1b, lanes d–f). The column was then eluted with 2 mM-EGTA, and proteins that eluted from the column in a Ca²⁺-dependent manner included a cluster with a molecular mass of 23–26 kDa and proteins of 44, 96, 120 and 230 kDa (Fig. 1b, lanes 7–10 and g). We designated these proteins as trypanosome calcimedins since they bound to the hydrophobic matrix in a Ca²⁺-dependent manner [4]. To verify whether these proteins in the 0.15 M-NaCl eluate from the DE-52 column represented the majority of calcimedins in *T. brucei*, the 100000 *g* EGTA supernatant from 4 × 10¹¹ cells was brought to 2 mM-Ca²⁺ and loaded directly on to the phenyl-Sepharose column without prior fractionation by DE-52 chromatography. The EGTA eluate contained the same predominant calcimedins as before, along with an extra band of molecular mass 17 kDa (Fig. 1c). This latter protein was identified as calmodulin. Significant quantities of non-specific proteins continuously leached off the phenyl-Sepharose column (Fig. 1c, lane b). We conclude that calcimedins in the 0.15 M-NaCl eluate from DE-52 are the predominant calcimedins in *T. brucei*.

Comparison of trypanosome calcimedins with vertebrate annexins by immunoblot analyses

The predominant calcimedins from vertebrate tissues include calmodulin and annexin family members. To determine whether trypanosome calcimedins are related to annexins, immunoblot analyses were performed with monoclonal antibodies against annexin I (35 kDa lipocortin I or calpactin II), annexin II (36 kDa lipocortin II or calpactin I), annexin IIP11 (11 kDa subunit of annexin II), annexin IV (32.5 kDa calelectrin or

Fig. 1. Purification of trypanosome calcimedins

Trypanosome calcimedins were obtained by a combination of DE-52 anion-exchange and Ca^{2+} -dependent hydrophobic interaction chromatography. *T. brucei* whole-cell homogenate was centrifuged at 100000 *g* and the supernatant was passed over a DE-52 anion-exchange column. The 0.15 M-NaCl eluate from the column was made 5 mM in Ca^{2+} and loaded on to a phenyl-Sepharose column pre-equilibrated with buffer containing 0.2 mM- Ca^{2+} . (a) Chromatogram of Ca^{2+} -dependent elution from the phenyl-Sepharose column. The column was successively washed with equilibration buffer and the same buffer containing 0.5 M-NaCl, as indicated by the arrows. EGTA elution started at 310 min and 1.2 ml fractions were collected. (b) SDS/PAGE analysis of aliquots of each purification step. Individual gel lanes contain: a, whole cell homogenate; b, 100000 *g* supernatant; c, 0.15 M-NaCl DE-52 eluate; d, phenyl-Sepharose column flow-through; e, peak of 0.5 M-NaCl wash; f, end of 0.5 M-NaCl wash; 6–11, peak fractions from the EGTA elution; g, concentrated peak. (c) Trypanosome calcimedins isolated directly from the unfractionated 100000 *g* supernatant by phenyl-Sepharose chromatography. Lane a, concentrated calcimedins; lane b, concentrated pre-elution wash. The arrows indicate trypanosome calcimedins with molecular mass of 230, 120, 96, 44 and 26–23 kDa cluster respectively. The positions of molecular mass standards in kDa are shown.

endonexin I) and annexin VI (67 kDa calelectrin, calcimedin or lipocortin VI) [20]. Polyclonal antibodies against 35 kDa liver calcimedins (P35) [21] and antibodies that we raised against 44 kDa trypanosome calcimedin (Tb-44) were also tested (Fig. 2). The monoclonal and polyclonal antibodies against annexins detected less than 3 ng of purified protein (Fig. 2a,b,c, lanes d) along with annexins in the preparation of bovine liver calcimedins (Fig. 2a,b,c, lanes c). However, none of these antibodies reacted with trypanosome whole-cell homogenates or purified trypanosome calcimedins (Fig. 2a,b,c, lanes a and b). For the sake of clarity, only immunoblots with monoclonal anti-(annexins II and IV), and polyclonal anti-(P35 calcimedins) are shown. Conversely, affinity-purified polyclonal antibodies raised against Tb-44 detected proteins in the trypanosome calcimedin pre-

Fig. 2. Relationship between trypanosome calcimedins and mammalian annexins

Immunoblot analyses were performed with monoclonal antibodies against (a) annexin II and (b) annexin IV, (c) polyclonal antiserum against 35 kDa liver calcimedin (anti-P35) and (d) affinity-purified antiserum against 44 kDa trypanosome calcimedin (anti-Tb44) as shown. Individual gel lanes contained: a, *T. brucei* whole-cell homogenate (60 µg of total protein); b, trypanosome calcimedins (4.2 µg of total protein); c, bovine liver calcimedins (3.1 µg of total protein); d, authentic annexins mixture containing annexin I, annexin II and its 11 kDa subunit, annexin VI and annexin IV (15 ng of total protein). Arrowheads indicate the positions of cross-reacting antigens. The positions of molecular mass standards are shown at the left.

Fig. 3. Ca^{2+} -dependent interaction of calcimedins with phospholipid vesicles

Calcimedins from (a) trypanosomes or (b) bovine liver were incubated with vesicles comprised of PC/PE (1:1, w/w) in the presence of either Ca^{2+} (A) or EGTA (B). Control incubations were conducted in the presence of Ca^{2+} , without vesicles (C). The incubation mixtures were centrifuged at 100000 *g* and aliquots of the supernatant (S) or pellet (P) were separated by SDS/PAGE followed by Coomassie Blue staining. At the left margin are shown the positions of molecular mass standards in kDa.

paration (Fig. 2d, lane b), but did not recognize liver calcimedins or the mixture of authentic annexins. Interestingly, anti-Tb-44 cross-reacted with the 23–26 kDa calcimedin cluster from *T. brucei*, suggesting a structural relationship between these proteins. Taken as a whole, these data demonstrate that trypanosome calcimedins do not share extensive sequence similarity with vertebrate annexins, but appear to share identity with each other.

Ca^{2+} -dependent membrane-binding properties of trypanosome calcimedins

Another distinguishing feature of annexin family members is

Table 1. Purification of calmedins from *T. brucei*

Specific activity was calculated from densitometry of immunoblots with anti-Tb-44, and expressed as the area under the peak per μg of protein applied to the gel.

Fraction	Total protein (mg)	Recovery (%)	Tb-44 activity	Purification (-fold)
Whole cell (5×10^{11} cells)	2530	100	16.9	1
100 000 g supernatant	1280	51	8.7	0.52
DE52 eluate (0.15 M-NaCl)	216	8.5	59.8	3.5
Phenyl-Sepharose (EGTA) eluate	0.630	0.025	2660	160

Fig. 4. Sequence similarity between Tb-44 and Tb-17

Sequences of random CNBr/tryptic fragments of Tb-44 were compared with that of Tb-17, a 23 kDa putative flagellar calcium binding protein in *T. brucei*. Boxes show regions of variability between sequences. Numbers above the sequence indicate the positions of residues within Tb-17. The bar indicates the putative EF-hand Ca^{2+} -binding loop. The Tb-44 fragment number (#) refers to retention time on reverse-phase h.p.l.c. after the double digestion.

appeared in the particulate fraction. By contrast, the majority of liver calmedins associated with vesicles in the presence of calcium (Fig. 3, columns A). None of the proteins bound to vesicles in the presence of EGTA (Fig. 3, columns B). Similar results were obtained with vesicles composed of PS (results not shown). The small amount of Tb-44 in the particulate fraction was independent of the lipid composition or the amount of vesicles added (results not shown). The same amount of Tb-44 was pelleted even in the absence of vesicles (Fig. 3, columns C). No precipitation with liver calmedins was observed in the absence of vesicles (Fig. 3, columns C). We conclude that, after compensating for vesicle-independent precipitations, trypanosome calmedins do not bind to phospholipid vesicles in a Ca^{2+} -dependent manner. In order to determine whether any trypanosome proteins associate with vesicles in a Ca^{2+} -dependent manner, the experiments were repeated with 100 000 g supernatants instead of purified calmedins. Again, no proteins were identified that bound to vesicles in the presence of calcium (results not shown).

Partial amino acid sequencing of Tb-44 and comparison with *T. cruzi* flagellar Ca^{2+} -binding protein (FCaBP)

Tb-44 was chosen for further study since it is among the predominant trypanosome calmedins and is well resolved by SDS/PAGE. Densitometry of Coomassie Blue-stained trypanosome calmedins indicated that Tb-44 represented approx. 30 % of the calmedins. Anti-Tb-44 was used to quantify fold-purification during the isolation protocol (Table 1). Cell fractions were subjected to immunoblot analysis and quantified by densitometry. A substantial loss of Tb-44 was observed during the initial 100 000 g centrifugation step, suggesting that Tb-44 was retained in the particulate fractions. Overall, Tb-44 was purified 160-fold.

To better evaluate the relationship between Tb-44 and other Ca^{2+} -binding proteins, partial amino acid sequence analysis was performed. Calmedins were transferred to Immobilon and the band corresponding to Tb-44 was excised and digested with CNBr/trypsin. Fragments were separated by reverse-phase h.p.l.c. and each of the sequenced fragments (indicated by peak number) exhibited extensive similarity with Tb-17, a putative flagellar Ca^{2+} -binding protein from *T. brucei* (Fig. 4) [27]. The presence of Tb-17 was originally deduced from a cDNA clone, and the translation product was not sought. Its Ca^{2+} -binding properties and flagellar location were inferred from its similarity to a previously identified 24 kDa flagellar Ca^{2+} -binding protein (FCaBP) in *T. cruzi* [22]. Two EF-hands were indicated for both Tb-17 and FCaBP. The EF-hand Ca^{2+} -binding loop 1 was identified in Tb-44 (bar in Fig. 4).

To determine the relationship between Tb-44 and FCaBP, immunoblot analyses were performed using polyclonal antiserum against FCaBP and affinity-purified anti-Tb-44 (Fig. 5). Antibodies against Tb-44 did not cross-react with any components from *T. cruzi* homogenates (Fig. 5a). However, antibodies against the *T. cruzi* FCaBP did cross-react with lower intensity with Tb-44 and the 23–26 kDa cluster (Fig. 5b). Interestingly, the antibodies against FCaBP did not identify a homologue of Tb-44 in *T. cruzi* homogenates (Fig. 5b, lane b). Non-immune serum reacted with neither trypanosome calmedins nor FCaBP (arrows). Taken as a whole, these data suggest that Tb-44 does not occur in *T. cruzi*. Alternatively, sequence divergence between *T. cruzi* and *T. brucei* proteins may prevent the cross-reactivity.

Tb-44 fragments which exhibited sequence similarities to each other were also found. For example, peaks 41 and 51 differed from each other and Tb-17 by a single amino acid. Peaks 83 and 84 differed from each other by two amino acids. It is possible that Tb-44 resulted from gene duplication of a structure

Fig. 5. Relationship between Tb-44 and *T. cruzi* FCaBP

Immunoblot analysis was used to compare trypanosome calmedins (lane a) and *T. cruzi* cell lysate (lane b). Affinity-purified antiserum against Tb-44 (a) and antiserum against *T. cruzi* FCaBP (b) were used. The control blot (c) was incubated with non-immune serum. Arrows indicate the positions of major trypanosome calmedins recognized by both antisera but not by non-immune serum. Positions of molecular mass standards in kDa are shown at the left.

their ability to bind phospholipid vesicles in a Ca^{2+} -dependent manner [26]. To test if trypanosome calmedins possess this property, phospholipid vesicles were prepared. Proteins that associated with vesicles were detected by SDS/PAGE following centrifugation. Bovine liver calmedins were used as control. In the presence of calcium and vesicles (PC/PE, 1:1, w/w), 96 and 120 kDa calmedins along with small quantities of Tb-44

Fig. 6. Flagellar localization of trypanosome calcimedins

(a) Immunolocalization of Tb-44 was performed with bloodstream and cultured procyclic forms of *T. brucei* with indirect-immunofluorescent microscopy. Arrows indicate the positions of fluorescent flagella. (b) A flagellar-enriched fraction was obtained by discontinuous sucrose gradient centrifugation. Aliquots during the fractionation were separated by SDS/PAGE. Gels were either stained with Coomassie Blue or analysed for the presence of Tb-44 by immunoblot procedures using antiserum against Tb-44. Key to the gel lanes: a, *T. brucei* whole-cell lysate; b, 2500 g supernatant; c, flagella-enriched interface between 1.51 M- and 1.90 M-sucrose; d, 15000 g supernatant after Triton X-100 treatment of the flagella; e, 15000 g pellet; f, trypanosome calcimedins. Positions of molecular mass standards in kDa are indicated.

very similar to Tb-17. The 23–26 kDa cluster is not likely to result from proteolysis of Tb-44, since a separate gene encoding a homologous 23 kDa protein has been cloned (Tb-17). Altogether, these data suggest that a family of structurally related EF-hand calcium-binding proteins occur in *T. brucei*. Gene duplication may contribute towards the diversity of this protein family.

Immunolocalization and flagellar fractionation

Immunolocalization was performed with affinity-purified anti-Tb-44 to determine whether the trypanosome calcimedins are localized to the flagella, as predicted from the original paper on Tb-17 [27]. Flagella of both slender and procyclic forms were recognized by the antibodies (Fig. 6a), while flagella from *Leishmania tarentolae* and *Crithidia fasciculata* were not recognized (results not shown). No other localization was observed for trypanosome calcimedins. However, since anti-Tb-44 cross-reacted with the 23–26 kDa cluster, this experiment could not determine which of the trypanosome calcimedins associated with *T. brucei* flagella.

To address this problem, *T. brucei* cells were lysed by N_2 cavitation and flagella-enriched fractions were prepared by sucrose gradient centrifugation. The cell fractions were analysed by immunoblot analysis using anti-Tb-44 (Fig. 6b). The flagella-enriched fraction contained both Tb-44 and the 23–26 kDa cluster (Fig. 6b, lane c). These data are consistent with the immunofluorescent localization which showed no calcimedins in regions other than the flagellum. Flagella were further fractionated by addition of 1% Triton X-100 followed by centrifugation. Flagellar soluble components such as variant surface glycoprotein (Fig. 6b, lane d), and insoluble components including tubulin and paraxial rod proteins (Fig. 6b, lane e), were obtained. Tb-44 and the 23–26 kDa cluster appeared in both fractions, demonstrating that they are partially released by detergent.

DISCUSSION

The present study was undertaken to further define how calcium is used in signal transduction pathways in the parasitic protozoan *T. brucei*. Towards that goal, we have characterized a sub-set of Ca^{2+} -binding proteins from *T. brucei* homogenates. Our strategy was to purify proteins which bound to phenyl-Sepharose in a Ca^{2+} -dependent way. We have named these proteins trypanosome calcimedins. Calcimedins from vertebrate tissues have been identified by using similar hydrophobic interaction chromatography procedures [17]. In the present study, trypanosome calcimedins were obtained from the 0–0.15 M-NaCl DE-52 eluate. This approach probably did not underestimate the cellular complement of calcimedins. Previous studies with *T. brucei* employed a ^{45}Ca -gel overlay procedure to detect Ca^{2+} -binding proteins following fractionation of trypanosome homogenates by DE-52, phenyl-Sepharose, Mono-Q and Superose 12 chromatography [14]. In these studies, no calcimedins were identified in the DE-52 eluate between 0.15 and 0.4 M-NaCl. Nonetheless, non-calcimin Ca^{2+} -binding proteins with molecular masses of 22, 24 and 38 kDa were purified and characterized. In the present study, when unfractionated 100000 g supernatant was passed directly through a phenyl-Sepharose column without a prior DE-52 step, no other calcimedins (except calmodulin) were detected. Therefore the proteins described in this report represent the major polypeptides in *T. brucei* capable of associating with phenyl-Sepharose in a Ca^{2+} -dependent way.

In vertebrate tissues, proteins that exhibit Ca^{2+} -dependent conformational changes include annexin family members [7]. Annexins are conserved proteins that share repeated domain structures, bind to phospholipid, and mediate vesicle interactions [26], cell secretion [28], Ca^{2+} -channel activity [29] and phosphoinositide metabolism [30]. Conserved structural features of annexin family members have allowed their detection by antibody cross-reactivity in plants [31], insects [32] and different vertebrate species [21,32,33]. However, monoclonal antibodies against annexins I, II, IIp11, IV and VI, or polyclonal antibodies against 35 kDa calcimin, were not able to cross-react with any trypanosome calcimedins. Results with the polyclonal antibodies were especially important, since these antibodies recognized several members of the annexin family. Moreover, none of the trypanosome calcimedins or soluble trypanosome proteins bound to phospholipid vesicles in a Ca^{2+} -dependent way. Lack of identity with annexins was confirmed when partial sequence analysis of Tb-44 revealed that CNBr/trypsin fragments of this protein exhibited a total similarity of 93% with Tb-17, a 23 kDa EF-hand family member [27]. Cross-reactivity of the other calcimedins with Tb-44 suggested that they were also part of an EF-hand family. These data indicate that, unlike the situation in

vertebrate tissues, annexins are not a major component of the trypanosome calmodulins.

Tb-44 was found to share sequence similarities with *T. brucei* Tb-17 [27] and *T. cruzi* FCaBP [22]. Both Tb-17 and FCaBP were originally identified in expression libraries as predominant targets of antisera from infected hosts. FCaBP was expressed and shown to bind ^{45}Ca in a gel overlay procedure, while antibodies localized FCaBP to flagella. By contrast, Tb-17 was not evaluated for Ca^{2+} -binding properties or its distribution in the cell. We show here that Tb-44 and the 23–26 kDa cluster undergo hydrophobic conformational changes, indicating their ability to bind calcium. Moreover, both Tb-44 and the 23–26 kDa cluster are associated with flagella.

The requirement for multiple EF-hand Ca^{2+} -binding proteins for flagellar motility in trypanosomatids is not understood. Aside from the calmodulins described in this report, calmodulin has also been localized to the flagellum from trypanosomes [34] and mammalian sperm [35], and cilia from *Tetrahymena* [36]. Ca^{2+} ions have been shown to change the waveform and frequency of flagellar beating in the related trypanosomatid *Crithidia* [37]. The Ca^{2+} -calmodulin complex may affect flagellar beating by regulating guanylate cyclase [38], protein phosphatase [39] and K^+ channels [40]. In *Tetrahymena*, a family of EF-hand proteins (calmodulin, TCBP-25 and TCBP-23) have been isolated from cilia [41]. These ciliary EF-hand proteins differ from Tb-17 and FCaBP in primary sequence. Their role in ciliary function is not understood. However, the presence of multiple EF-hand proteins in protozoa cilia and flagella suggest a greater complexity of calcium control than had previously been considered. Whether similar proteins are found in mammalian cilia or flagella has yet to be determined.

The flagellum of *T. brucei* is unusual since it attaches to the cell surface at regularly spaced intervals by means of junctional complexes [42]. The flagellum also contains an accessory structure known as the paraxial rod [43]. The paraxial rod proteins have been cloned, but their contribution to force generation by the flagellum is not known. However, co-ordinated bending of the axonemal complex and paraxial rod is required for motility. Immunogold labelling has localized calmodulin to the paraxial rod, suggesting that calcium may help to co-ordinate this bending (L. Ruben, P. Webster, T. Burrage and C. L. Patton, unpublished work). Since flagella from *T. cruzi* and *T. brucei* are structurally similar, it was surprising that Tb-44 and members of the 23–26 kDa cluster appeared to be absent from *T. cruzi* homogenates (Fig. 5). These data suggest that some of the non-shared EF-hand Ca^{2+} -binding proteins in *T. brucei* flagella may contribute to specialized functions other than motility. For example, the flagellum of *T. brucei* serves as a specialized region where signal/recognition reactions occur. Attachment of the parasite by the flagellum to the insect host or an artificial surface signals progression of the trypanosome life cycle [44]. The base of the flagellum forms the flagellar pocket, which may also be a site of signal transduction. This region appears to be the sole site of endocytosis in *T. brucei*. [45]. Localization of some receptors to the flagellar pocket and along the flagellum has been reported [46–47].

In conclusion, we have used Ca^{2+} -dependent association with phenyl-Sepharose as a criterion for identifying the cellular complement of calmodulins from *T. brucei* homogenates. The predominant calmodulins comprise a family of flagellar EF-hand Ca^{2+} -binding proteins. These proteins may help to co-ordinate flagellar bending during cell motility or contribute towards the signal/recognition function of the trypanosome flagellum.

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