A novel C-terminal kinesin is essential for maintaining functional acidocalcisomes in *Trypanosoma brucei*

by

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SUMMARY

Kinesins are cytoskeletal motor proteins which play roles in a variety of fundamental cellular processes including cell division and the anterograde transport of vesicles and organelles. We purified, cloned and functionally characterized in Trypanosoma brucei (T. brucei) a new member of the C-terminal kinesin family, TbKIFC1. Kinetic constants of the recombinant motor domain of TbKIFC1 were estimated at 0.56 µM for the microtubule dissociation constant (kd) with a Kcat of 0.2 s\(^{-1}\). Immunolocalisation analysis showed an association of TbKIFC1 with punctate structures. Because they were rapidly transported to the negative pole of the microtubule after NH\(_4\)Cl treatment, these structures were considered to be associated with acidic vesicles. To determine the role of the kinesin in vivo we produced an inducible kinesin deficient strain by dsRNA interference methodology. Mutant cells were loaded with the fluorescent reagent fura2-AM to measure intracellular free calcium ([Ca\(^{++}\)]\(_i\)). The resting [Ca\(^{++}\)]\(_i\) was unchanged in mutant cells, however alkalinisation of acidic vesicles induced by NH\(_4\)Cl or nigericin was not followed by release of Ca\(^{++}\). These data and the relative importance of the ionomycin-releasable and the ionomycin-plus-NH\(_4\)Cl-releasable Ca\(^{++}\) pools suggest a lower Ca\(^{++}\) content in acidocalcisomes and dysfunctional Ca\(^{++}\) release.
INTRODUCTION

African trypanosomes, the causative agents of sleeping sickness in humans and Nagana in cattle, are unicellular flagellated protozoa. In order to survive and develop in the distinctive environment of the mammalian host and the insect vector, *Trypanosoma brucei* (*T. brucei*) must undergo morphological, biochemical and physiological changes to adapt to the different environmental conditions. Cell viability requires mechanisms to control pH and Ca\(^{++}\) homeostasis (1,2). The level of endocytosis demonstrated by bloodstream forms (BF)\(^1\) is among the highest described so far for eukaryotic cells (3). Interestingly this endocytic pathway is associated with an unusual cytoskeleton comprised of a precisely ordered subpellicular microtubule array that confers a high polarity to the cell, defined by a positive pole at its posterior end (4). The flagellum exits the cell from this positive pole at the flagellar pocket, a surface membrane invagination specialized for endocytosis and exocytosis (3). These features make the trypanosome an interesting model for the study of vesicular biogenesis and communication.

Kinesin proteins constitute a super family, the kinesin family proteins (KIFs), also known as kinesin-like proteins (KLPs). Sequence differences between members of the family within the conserved motor domain of around 340 amino acids (5) are used to classify the kinesin proteins into at least 10 families (6). Regions outside the motor domain are family specific and share little, if any, sequence homology. This diversity suggests that different kinesins have distinct roles in many different cellular processes including cell division, signal transduction and microtubule dynamics (7). They also participate in the trafficking of macromolecular complexes (8) and organelles including mitochondria (9), lysosomes (10) (11) and synaptic vesicles (12). These cargoes are moved along microtubules (13) by the action of the
molecular motor domain. Kinesins are usually associated with plus-end transport while minus-end directed membrane organelle transport is generally attributed to the dynein protein family (14).

Since TbKIFC1 did not belong to any of the reported kinesin families with a known cellular function, we conducted a functional study designed to reveal the role of this kinesin in *T. brucei*, and provide insight into the mode of action of C-ter kinesins involved in minus-end transport. Results from these approaches have shown the essential role of TbKIFC1 for the biogenesis of acidocalcisomes and Ca^{2+} homeostasis in *T. brucei*. 
EXPERIMENTAL PROCEDURES

Strains used - *T. brucei brucei* monomorphic strain 427 from clone MITat 1.4 (15,16) was used for protein purification and gene cloning. For the protein expression study procyclics derived from 427, the pleomorphic *T. brucei* GUTat 3.1 (17) and *T. congolense* IL3000 clone 49 (provided by E.Authié) were used. The transgenic cell lines (gift from G.A.M. Cross) were created from a *T. brucei* 427 wild-type background (18). Procyclics were grown in SDM-79 (19) with 10% calf serum at 27°C. For IL3000 procyclic and epimastigote forms, and isolation of metacyclic forms from epimastigote cultures were performed as described (20). Bloodstream slender forms were obtained from infected rats for MITat and infected mice for GUTat and *T. congolense* and from infected immunosuppressed mice for GUTat stumpy forms. These bloodstream forms were subsequently purified from blood cells by DEAE-cellulose chromatography (21). Bloodstream forms were also cultured in vitro in modified MEM supplemented with 10% foetal calf serum (22).

*TbKIFC1* purification - Columns and Instruments: DEAE Sepharose Fast flow (Pharmacia, Saclay, France), was used for ion exchange chromatography. O-Phospho-L-Tyrosine (O-(P)Tyr) immobilized on cross-linked 4% beaded agarose from Sigma was used for affinity chromatography. The chromatographic system used throughout this study was the FPLC workstation from Pharmacia. All buffers contained a protease inhibitor cocktail with final concentrations of 1 µM Chemostatin; 1 µM Leupeptin; 1 µM Pepstatin and 10 µM PMSF (phenylmethylsulfonyl fluoride).

Purification procedure: The fraction corresponding to soluble protein obtained by hypotonic lysis (23) was equilibrated in buffer A (50 mM Tris HCl pH 7.0; 20 mM NaCl; 1 mM DTT; 1 mM EDTA; protease inhibitor cocktail) and loaded onto the DEAE fast flow column from Pharmacia preequilibrated in buffer A. The column was then washed with buffer B and the flowthrough collected. Elution was
performed by the discontinuous step gradient method. Fractions were collected at 50 and 100% buffer B (50 mM Tris HCl pH 7.0; 500 mM NaCl; 1 mM DTT; 1 mM EDTA; protease inhibitor cocktail).

The 50% eluted DEAE fraction was diluted and equilibrated with pre-equilibration buffer A and adsorbed onto a buffer A pre-equilibrated O-(P)Tyr agarose affinity column. The column was washed with buffer B and the flowthrough collected. The enzyme was eluted by a discontinuous step gradient at 50 and 100% of buffer B in a total volume of 20 ml. The flow rate was 2 ml/min for each step.

Immobilized Metal Affinity Chromatography (IMAC): NterKIFC and MDKIFC (the recombinant proteins expressed in E. coli) were purified from the His·Bind® column from Pharmacia which was used according to the manufacturer’s instructions (Novagen). Before performing the ATPase activity tests and the microtubule binding experiments we desalted the MD-Kin recombinant purified protein in PEM buffer (200 mM Pipes/NaOH pH 6.9, 2 mM EGTA, 2 mM MgSO4) on a PD10 column (Pharmacia).

Microsequencing - A purification procedure was performed starting with 1.5 x 10^{11} bloodstream form trypanosomes. Gel slices for sequencing were obtained from amidoblock stained SDS-polyacrylamide gels and sent for microsequencing to the "Laboratoire de Microsequençage des Protéines" Dr Dalayer, Institut Pasteur Paris (24).

Cloning by reverse transcriptase polymerase chain reaction (RT-PCR) and analysis of the TbKIFC1 gene - Total cell RNA and polyA+ RNA were prepared and purified according to Sambrook (25). cDNA was obtained by random priming with hexanucleotides and reverse transcription with the Stratagene reverse transcriptase RT II. The 90P26AS (5’-TTYTGCCANCCDATNAC-3’), 90P31AS (5’-TGYTGNGTNGCYTCYTG-3’) degenerate oligonucleotides were generated by the reverse translation of sequenced peptides, P26 (VIGWQK) and P31 (QEATQQ), respectively. A portion of the spliced leader sequence common to all T. brucei
mRNAs (5’-ACAGTTTCTGTACTATATTG-3’) was also used as a 5’ primer (MEX2). The cDNA was then used as a template for PCR amplification using the primer associations MEX2/90P26AS; MEX2/90P31AS. The amplified fragments were gel-isolated, cloned into the pT7blue T-vector (Novagen) and sequenced.

The amplified and cloned MEX2/90P26AS fragment was used as a probe to screen a T. brucei genomic DNA library (25) generated in the c2X75 cosmid vector (26) as previously described (27). BamHI/EcoRI fragments of the isolated 2.1 cosmid were subcloned into the pUC18 vector from Appligene and screened with the cDNA fragment MEX2/90P26AS. A BamHI/EcoRI fragment of 5,000 bp was isolated and sequenced using the AmpliTaq DNA polymerase, as described by the manufacturer (ABI PRISM™, Perkin Elmer).

Cloning, expression and purification of the N-and C-terminal domain of TbKIFC1 in E. coli - A 690 bp fragment comprising the N-terminal region and 1,550 bp fragment containing the C-terminus motor domain were generated by PCR. A 5 Kb BamHI/EcoRI genomic fragment from AnTat 1 subcloned into pBlueScript (Stratagene) was used as a template for both PCR reactions. For the kinesin N-terminal construction (NterKIFC) the 5’ primer (5’-TGCAGACATATGTCTGCGGAACAACCC) contained a 12 nucleotides linker with an NdeI restriction site to facilitate subcloning and 5 adjacent N-terminal residues (SAEQP). The 3’ primer, (5’-GCTTTCCGATCCTAATGGTGATGGTGATGGTGGCACTTCATCTTGTGATT) included a 12-nucleotide linker with a BamHI site for cloning, a stop codon, codons for 6 histidine tag residues to allow binding to the His®-Bind column, and codons for 6 C-terminal residues (NHKMKC). The PCR product was inserted into the NdeI/BamHI sites of the pET3a plasmid (Novagen). The C-terminal region of TbKIFC1 coding for the motor domain (MDKIFC) was obtained by using the 5´ primer (5’-GCTCAGGCTAGCCACCATCACCATCACCATCTGCGTAAGCAGTACTAC) and the 3´ primer (5’-TGGATTCGCGCGCTTAGCCAAGAGATACGCCAGCCACG). The PCR amplified DNA fragment encoded a region between the amino acids L475 and P820 of

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TbKIFC1. This product was cloned between the *NheI/NotI* sites of the pET23a plasmid (Novagen). The resulting recombinant NterKIFC and MDKIFC proteins were expressed in *E.coli* BL21 (DE3) from Novagen according to the manufacturer’s instructions. Cells were lysed in 1X binding buffer, containing the protease inhibitor cocktail, by three steps of freezing and thawing and brief sonication. The lysate was centrifuged for 30 min at 10,000 g and the resulting supernatant applied to a Ni²⁺ His-­­® column.

**Phylogenetic study** - DNA and amino acid sequences were analyzed by the DNA STRIDER software and database searches by the BLAST algorithm. Multiple alignment of amino acid sequences and hamming distances were determined from the CLUSTAL W version 1.6 (28) and the phylogenetic tree constructed from version 3.5c of the PHYLIP program package of J. Felsenstein (BLAST, CLUSTAL W and PHYLIP softwares were obtained through Bisance and Infobiogen facilities). The matrix of pair-wise sequence distances were calculated by the Dayhoff’s method using PRODIST. The phylogenetic trees were constructed from the distance matrix by the Neighbour or Fitch methods, and were rooted with the ScSmy1 sequence as an out-group. The phylogenetic tree was drawn with TREEVIEW version 1.3 (29).

**Production of a monoclonal antibody against TbKIFC1 and Western blot** - The NterKIFC recombinant protein was IMAC purified and electroeluted from a 10 % SDS PAGE gel. A mouse was injected with 20 µg of the purified recombinant protein in complete Freund’s adjuvant. A further two 20 µg samples in Freund’s incomplete adjuvant were injected 15 and 30 days later. After four weeks, a fourth aliquot of 20 µg of protein was injected and the hybridoma technique was begun 4 days later according to (30). One hybridoma, H3, was selected by ELISA screening against the recombinant NterKIFC protein. Antibodies were purified from the culture medium by a protein A affinity column according to the manufacturers instructions (Pharmacia).
**Protein Electrophoresis and Western blotting** - For Western blot analysis, proteins in sample buffer (2.2% SDS; 50 mM DTT, 90 mM Tris-HCl pH 6.8 and 10% glycerol, mass/vol) were boiled for 5 min and subjected to 10% polyacrylamide gel electrophoresis (31). Proteins were then transferred to polyvinylidene difluoride (Immobilon P, Millipore) membranes by semi-dry blotting (32). Filters were blocked for 15 min with PBS-Tween-milk (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na2HPO4; 1.4 mM KH2PO4; 0.005% Tween-20; 5% milk), incubated overnight at 4°C with a supernatant culture of the H3 hybridoma. A 1/1000 dilution in PBS-Tween-milk of goat anti-mouse IgG conjugated to horseradish peroxidase (Sanofi-Pasteur) was then added for 2 hrs. Immunoreactive bands were revealed by washing in 50 mM Tris-HCl pH 7.5; 20 mM NaCl and a solution containing 0.05% H2O2 and 2.8 mM 4-chloro-1-naphthol or DAB (3,3’-diaminobenzidine) for the peroxidase conjugate, and according to Manufacturers’ instructions for chemiluminescence (ECL™) revelation (Amersham)

**Immunofluorescence Assay (IFA)** - Bloodstream and procyclic forms were fixed on ice for 15 min in 2% formaldehyde and permeabilized in 0.1% Triton X-100 for 10 min at room temperature. The formaldehyde was neutralized for 10 min with 0.1 M glycine at room temperature. After centrifugation and resuspension in PBS, trypanosomes were transferred to a microscope slide and treated for 30 min at room temperature with either monoclonal antibodies or polyclonal serum diluted in PBS containing 0.1% BSA. Secondary antibodies were conjugated to fluorescein isothiocyanate (Pasteur Sanofi), Alexa fluor 568 (Molecular Probes). After washing, slides containing the treated trypanosomes were mounted with anti-fade Vectashield (Vector Laboratories). Cells were examined on a Zeiss UV microscope and images analyzed by the use of a camera (Photometrics) with Metaview software and Adobe Photoshop 5.1 (Adobe Systems).

**Lysosome motility** - Acidic vesicle motility was measured according to the method of Heuser (33). Cultured bloodstream forms were washed three times in Ringer’s
solution (155 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 2 mM NaH₂PO₄, 10 mM Heps buffer, pH 7.2, 10 mM glucose and 0.5 mg/ml BSA) and incubated for 30 min at 37°C in this buffer. For the nocodazole assay cells were preincubated 20 min at room temperature in Ringer’s solution in presence of 10 µM nocodazole. To induce retrograde transport, NH₄Cl (30 mM final concentration) was added to the previous buffer and cells incubated for 15 min. Parasites were then either formaldehyde fixed, as described for the IFA, or washed three times in Acetate-Ringer’s solution (80 mM NaCl, 70 mM Na-acetate, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 2 mM Heps/NaOH pH 6.9, and 10 mM glucose), incubated for 15 min and fixed.

**Determination of protein concentration** - Protein concentrations were estimated by the method of Bradford (34) and by Coomassie blue staining after SDS-PAGE. BSA was used as a standard. For microtubule binding assays, proteins were quantified by Western blotting. Gels and membranes were then scanned to produce digital images and protein bands were quantified using NIH image. BSA and MDKIFC were used as standards.

**Kinesin biochemical characterization** - Preparation of microtubules - Bovine tubulin was kindly provided by B. Goud (Institut Curie Paris). After parasite lysis, *T. brucei* tubulin was purified by chromatography on DEAE Q fast flow (Pharmacia) and two cycles of polymerisation (35,36).

**Microtubule-binding assays** - 100 µg of tubulin was polymerized in PEM buffer in the presence of glycerol 33 % (v/v), 1 mM GTP, 1 mM MgCl₂ for 10 min at 37°C. Taxol was then added to a final concentration of 40 µM and the polymerisation continued for 30 min. Microtubules were centrifuged at 40,000 g for 30 min at 22°C. In order to eliminate protein aggregates, MDKIFC was centrifuged at 40,000g for 30 min at 4°C. Binding assays were performed by incubating MDKIFC protein (0.1-1.7
µM) with 1 µM microtubule in PEM buffer supplemented with 1 mM GTP, 2.5 mM MgCl₂, 2 mM DTT, 20 µM taxol and 2.5 mM ATP for 15 min at room temperature. The supernatants and pellets were analysed on SDS-PAGE and by Western blotting.

ATPase activity test - ATPase activity was measured as described by Mitsui et al. (37). 1 µg of MDKin protein was incubated for 5 min at 22°C in 100 µl of PEM buffer containing 2 mM DTT, 1 mM GTP, 1 µM taxol. Then 1 mM, final concentration, of [γ-³²P]ATP (0.55x10⁸ cpm/µmole) was added and the incubation continued for 15 min at 22°C. The reaction was stopped by the addition of 1% final SDS, 100 µl of (5M H₂SO₄, 10% ammonium molybdate, 0.1 M silicotungstic acid, in a volume ratio of 2 :2 :1) and 1 ml of xylene/isobutanol (65 :35). The reaction mixture was vortexed for 15 sec and centrifuged for 5 sec at 5,000g. Released phosphate was measured by Cerenkov counting.

DsRNA expression and trypanosome transformation - The inductible T7 RNAP-based protein expression system developed by E. Wirtz was used in this study (18). The pLew 100 vector as well as the procyclic host cell line 29-13 co-expressing T7 RNAP and TetR, were gifts from G. Cross. For dsRNA expression the following construct was produced. DNA fragments corresponding to the coding regions of TbKIFC1 from nucleotides 1 to 694 and from nucleotides 1 to 645 were PCR amplified. The 694 pb fragment was amplified using the following set of primers : DB/KIN/01 (5’-GGCCGGAAGCTTATGTCTGCGGAACAACCC) and DB/KIN/02 (5’-GGCCGGGGATCCGCTTGAATTCGCTTTCATAGCTTCCTGA) and cloned between the BamH1/HindIII restriction sites of pLew100 giving the pLew100-SKIN construction. In a second step the 645 pb fragment was cloned in the opposite orientation of the 694 fragment in the pLew100-SKIN construction between the EcoRI/BamHI restriction sites. This later fragment was obtained by PCR amplification using the following primers DB/KIN/03 (GGCCGGGAATTCCATTCTTGCACTCCC TTGC) and DB/KIN/04 (GGCCGGGGATCCATGTCTGCGGAGCAACCC. A PCR
DNA fragment containing the total TbKIFC1 coding region was also cloned into the pLew 100 vector between the HindIII/BamHI restriction sites. For stable transformation T. brucei procyclic forms (29-13 cell line) were harvested from a log-phase culture (5x10^6 cells) and washed once in ZPFM (18) and resuspended to a cell density of 4x10^7 cells/ml in ZPFM. 2x10^7 cells were electroporated with 10 µg of DNA in the Eurogentech cellject machine at 1600 V, R inf, 40 µF. Parasites were then transferred to SDM-79 medium containing 10 µg/ml G418 and 5 µg/ml hygromycin and cultured overnight before the addition of phleomycin 2.5 µg/ml.

Spectrofluorometric determinations - The [Ca^{++}]_i and pHi were measured according to Scott et al (38) and Fraser-L’Hostis et al (2). Briefly, cells were loaded with either fura 2/AM or BCECF/AM (Molecular Probes) and resuspended in Buffer A containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4, 5.5 mM glucose, 50 mM Hepes 7.4 and 1 mM EGTA for [Ca^{++}]_i determination. The fura 2 fluorescence response to the [Ca^{++}]_i was calibrated from the ratio 330/380 nm fluorescence values for an emission at 510 nm. For pHi we used the wavelengths 490 and 440 nm for excitation and 535 nm for emission. The spectrofluorometer fluoromax (SPEX Instruments) and software DM 3000 (SPEX Instruments) were used for data analysis.
RESULTS

Purification, gene cloning and classification of TbKIFC1 - We previously described a two-step chromatographic process for the purification of a plasma membrane phosphatase (39) and used this procedure to purify soluble phosphatase. After hypotonic lysis, the soluble fraction was applied to a DEAE fast flow column at pH 7. VSG, the most abundant protein, was removed in the flowthrough and phosphatase activity was collected in the 250 mM NaCl eluate fraction (data not shown). Purification of the DEAE eluted fraction proteins on an O-phospho-L-tyrosine affinity column led to the partial purification of a 91-kDa protein (Fig. 1A) that eluted at 150 mM NaCl. Although only a minority of the phosphatase activity was present in that fraction (40), the 91-kDa polypeptide was microsequenced after isolation from a 10% reducing SDS-PAGE. Three peptide sequences were obtained: 1) IISTVIGWQK; 2) ESAYYSSLTSAIASIAAAA; 3) NAQQVMLQAQEATQQ (Fig. 1B). Oligonucleotides based on these sequences were used to identify genomic fragments corresponding to the gene and after screening of a T. brucei cosmid library, the complete gene sequence of the 91 kDa protein (AF319546) was obtained. The single copy gene (Southern blot, data not shown) encoded a protein of 841 amino-acids. The gene was entitled TbKIFC1. Its C-terminal domain (367 amino acids) was more than 60% similar to the motor domain of kinesin (6). The N-terminal region did not reveal overt similarity to known kinesins or other proteins in the database, however, analysis of its composition revealed a potential globular head domain, a coiled-coil stalk domain typical of those conserved among most kinesins and the kinesin neck consensus sequence (Fig 1B). Molecular phylogenetic analysis, supported by high bootstrap values (Fig. 1C), showed that TbKIFC1 diverged from the four originally described C-terminal kinesin classes. Specific functions or particular cargoes have been identified for class I, II and III C-terminal kinesins (6), but our phylogenetic analysis suggests a different cellular function for TbKIFC1.

Biochemical properties of TbKIFC1 - To determine if TbKIFC1 belongs to the motor protein family, we tested the recombinant C-terminal domain MDKIFC1 (Fig. 1B)
(41) for a variety of biochemical functions. The six-histidine tagged recombinant protein was purified by an IMAC procedure. The $K_m$ value for ATP was determined as 51 µM. The dissociation constant ($K_d$) from microtubules in the presence of ATP was calculated by incubating different concentrations of MDKIFC1 with 2 µM microtubules and quantitating the amount of microtubule-bound motor domain (Fig. 2A). The dissociation constant of MDKIFC1 from microtubules was determined to be 0.56 µM. Microtubules (2.5 µM) stimulated MDKIFC1 ATPase activity by about 8-fold to 2 pmol/s per µg of protein (Fig 2B). The kinetic parameters, $k_{cat}$, the steady state turnover number, and the $K_{0.5MT}$, corresponding to the concentration of microtubules required for half maximal stimulation of the ATPase activity, were estimated respectively at 0.1 s$^{-1}$ and 0.7 µM (Fig 2B).

**Differential expression and localisation of TbKIFC1 kinesin protein** - Relative expression level quantification of *Tb*KIFC1 between the mammalian host bloodstream form (BF) and the insect procyclic form (PF) of the parasite was estimated by immunoblotting with a specific monoclonal antibody (H3) directed against the N-terminal domain (aa 1 to 231). Expression in bloodstream forms was 1000-fold greater than in procyclins (Fig. 3A). Furthermore figure 3B shows that *Tb*KIFC1 was also expressed at similar levels in the non-dividing metacyclic insect form and the dividing slender trypomastigote form and that stumpies did not express the protein. The results suggested that the function of *Tb*KIFC1 was related to the adaptation of the parasite to its mammalian host.

The nature of the specific cargo to which *Tb*KIFC1 associates was also investigated. Immunolocalisation in slender bloodstream form trypanosomes using the monoclonal antibody H3 revealed that *Tb*KIFC1 was confined to punctate structures. The higher density of these structures around the nucleus of the parasite (Fig. 4a,b) suggests an association with a perinuclear acidic compartment (42). Lysosomes and acidic vesicles in general can be
distinguished from other organelles by the capacity to induce their own cellular redistribution through changes in cytoplasmic pH (33). Alkaline Ringer’s solution induced TbKIFC1 (Fig. 4c,d) to accumulate at the anterior extremity of the parasite, corresponding to the negative pole of the subpellicular microtubule corset within 15 min. Subsequent acidification caused rapid redistribution of TbKIFC1 to its original site (Fig. 4e,f). These observations indicate a retrograde movement towards the anterior extremity of the cell. Microtubule polarity of the subpellicular corset might be considered to support a retrograde transport to the anterior extremity of the parasite. To assess its role in this redistribution, parasites were treated with benzimidazole inhibitors of microtubules (5). Nocodazole (10 µM) inhibited the observed NH₄Cl induced movement indicating that motion involved a microtubule network (data not shown).

However colocalisation studies performed with antibodies directed against p67 (43) and the *T. brucei* plant vacuolar like proton pyrophosphatase (V-H⁺-PPase) (manuscript in preparation) did not show an association of TbKIFC1 with either lysosomes or acidocalcisomes (data not shown). Moreover p67 containing vesicles and acidocalcisomes did not respond to similar alkaline treatment.

*Kinesin is required for normal release of Ca++ from the acidocalcisomes* - To estimate the potential action of TbKIFC1 in retrograde transport of acidic vesicles of the late endosomal pathway, we used RNA interference methodology to silence TbKIFC1 expression (44). Procyclic and bloodstream form kinRNAi strains modified for the expression of TbKIFC1 (18) were constructed. Since the kinesin appears to be essential to bloodstream trypanosomes, we could not perform gene silencing experiments in this life cycle stage. However, we were able to turn to procyclic forms. Although TbKIFC1 was less abundant in procyclics than in bloodstream
forms, immunofluorescence studies performed on procyclins overexpressing *Tb*KIFC1 (Fig. 5A) also indicated an association of the kinesin with punctate structures (data not shown). RNA interference led to a loss of protein (Fig. 5A) and also induced several measurable phenotypes. In trypanosomatids, acidocalcisomes are considered to be the major acidic compartment (45) and they contain a considerable fraction of the intracellular stored Ca++ (46). As a first step toward the characterization of the effect of suppressing *Tb*KIFC1 expression, the Ca++ content of the acidocalcisome was analysed. The K+/H+ exchanger, nigericin, and the weak base, NH$_4$Cl, failed to cause Ca++ release from acidocalcisomes in mutant cells (Fig. 5B). A second rise in [Ca++]$_i$ that is usually observed after the subsequent addition of ionomycin did occur but the rise in [Ca++]$_i$ was lower for the induced kinRNA strain (Fig. 5B). The acidity of the compartment was maintained since addition of nigericin caused a significantly decreased pHi (Fig. 5C), while NH$_4$Cl rapidly increased it (Fig. 5C) (38,47). The amplitudes of the observed pH variations were the same for the non-induced, induced and a wild type strain (data not shown). The reverse addition of NH$_4$Cl and ionomycin confirmed the previous observations (Fig. 5B). The pHi and [Ca++]$_i$ values were estimated at 7.2 and 90 nM, respectively. These mean pHi and [Ca++]$_i$ levels were therefore stable and unchanged between the wild type (38), induced and non induced kinRNAi strains.
DISCUSSION

In this paper we describe the purification, cloning, biochemical and phenotypic characterization of a carboxy-terminal type kinesin from the parasitic protozoan *Trypanosoma brucei*.

Sequence comparison analysis showed the presence of a C-terminal motor domain containing the ATP binding motif and a microtubule binding consensus sequence. The relationship of *Tb*KIFC1 to the kinesin-like protein (KLP) family was confirmed by the determination of several steady-state kinetic parameters which were compared to three biochemically well characterized kinesin proteins Ncd (48,49) kar3 (50,51) and a conventional KHC (52-54). The dissociation constant (Kd) from microtubules for *Tb*KIFC1 in the presence of ATP was estimated at 0.56 µM suggesting that it binds more tightly to microtubules than Ncd, Kar3 and KHC for which the Kd values were estimated at, respectively, 4.1 µM, 1.7 µM and 4.2 µM at 25 mM NaCl. In the kinesin ATPase cycle, kinesin dissociation from microtubules is the slow and rate-limiting step (54). Consequently, this low rate of dissociation from microtubules would result in a low ATPase activity (48,54). The Km values for ATP indicated that *Tb*KIFC1 (51 µM), Ncd (190 µM) and KHC (100µM) all bound this nucleotide with similar affinity. The concentrations of microtubules required for half-maximal stimulation were also similar at 0.7 µM, 1 µM, 0.7 µM and 0.5 µM for *Tb*KIFC1, KHC, Ncd and Kar3 respectively. The kcat value for *Tb*KIFC1 was estimated at 0.2 s⁻¹ in the presence of 2.5 µM microtubules corresponding to an increase of ATPase activity of around 8-fold. By comparison the rate constant for ADP release by Kar3, Ncd and KHC were respectively of 0.037 s⁻¹, 5.4 s⁻¹ and 20 s⁻¹ corresponding to an ATP hydrolysis stimulation of between 6 fold for Kar3 to around 1000 fold for Ncd and KHC. According to
the suggested correlation between ATPase activity and the \textit{in vitro} velocity of motor proteins (50), the velocity of \textit{Tb}KIFC1 was inferred to be closest to the Kar3 kinesin (1-2 µM/min).

Phylogenetic analysis suggested the existence of a new subgroup within the originally described C-terminal kinesin group (Fig 1) (5). Expression analysis revealed that \textit{Tb}KIFC1 expression correlated with the host adapted stages rather than with the dividing forms of the parasite (Fig. 3). Immunofluorescence localized the protein within the cytoplasm associated with punctate structures. The higher density of these structures around the nucleus and the rapid cellular redistribution of \textit{Tb}KIFC1 to the anterior extremity of the parasite after \textit{NH}_4\text{Cl} treatment (Fig. 4) indicated the association of the kinesin with acidic organelles.

\textit{Tb}KIFC1 does not directly interact with the lysosomal or acidocalcisome compartments. These data suggest that the protein might be associated either with shuttle vesicles or macromolecular complexes moving to acidic compartments (8). However, association with different acidic vesicles cannot be ruled out and we are currently attempting to identify the nature of the vesicles to which \textit{Tb}KIFC1 is associated. It has to be noted that the absence of detectable movement in procyclics even when overexpressing \textit{Tb}KIFC1 (Fig. 5A) indicated that this motion was under the control of other, as yet undefined, factors.

Acidocalcisomes are considered to be the major acidic compartment that accumulates Ca++ in kinetoplastids (1). Therefore the effect of suppressing \textit{Tb}KIFC1 expression was analysed for \textit{[Ca++]}, Ca++ release from that compartment and for pH and pH variations induced by nigericin and \textit{NH}_4\text{Cl}. One consequence of the suppression of \textit{Tb}KIFC1 expression was that the synergistic action of either nigericin or \textit{NH}_4\text{Cl} and ionomycin led to a lower \textit{[Ca++]}, corresponding to 150 nM +/- 40 nM
and 340 nM +/- 30 nM for the induced and non induced kinRNAi strain, respectively (Fig. 5B). Similar results were observed when the order of reagent addition was reversed (Fig 5B). According to these data and the reported observation that ionomycin used alone may reflect a release of calcium from non acidic compartments (38), we concluded that the Ca++ storage capacity of the acidocalcisomes was significantly reduced.

It has been shown that acidity of acidocalcisomes is maintained by the combined activities of a bafilomycin A1-sensitive H+-ATPase and a V-H+-PPase (55) and is essential for Ca++ storage and release (56). Reduced expression of these two pumps could reduce the internal pH gradient and lead to a lower calcium content. However, the pH of mutant cells was not modified and nigericin and NH₄Cl induced the same pH variations as in the non-induced and wild-type strains (Fig. 5C) (38). These data favor the presence of equivalent internal acidocalcisome pH gradients in mutant and wild-type strains. Calcium release has been shown to occur via a Ca++/nH+ antiporter (46) that can be stimulated by Na+ via a Na+/H+ antiporter. Therefore an altered antiporter might abolish the release of Ca++ mediated by alkalisation of the compartment by either nigericin or NH₄Cl.

*Tb*KIFC1 may contribute to the transport, via macromolecular complexes, of components from their site of synthesis to the acidic compartments, to give functional acidocalcisomes. The kinesin-II holoenzyme complex in Chlamydomonas was shown to transport « rafts » composed of a 16S protein complex (57,58). Alternatively, although acidocalcisomes were shown not to belong to the endocytic
pathway in *T. cruzi* (59), it was suggested that in normal *Leishmania amazonensis* the endosomal/lysosomal system was connected with acidocalcisomes during an autophagic process (60). The association of *Tb*KIFC1 with vesicles involved in such a process would result in its higher expression in bloodstream forms where endocytosis is maximum. Therefore an interconnection, partially motorized by this protein, between residual bodies of the endocytic/autophagic lysosomal pathway and acidocalcisomes is proposed. Such a connecting pathway would be essential to production of functional acidocalcisomes for Ca++ storage and release.

Nonetheless procyclic cells lacking *Tb*KIFC1 are still viable, supporting the hypothesis that other Ca++ tranporting organelles can safeguard against limited disruption of acidocalcisomes (61,62). For further analysis of this phenotype and the modifications linked to Ca++ content and release, we are currently attempting to characterize acidocalcisome specific proteins such as V-H+-PPase. While it is possible that the function of the kinesin differ in bloodstream and procyclic forms, our studies strongly suggest that experimental modifications of *Tb*KIFC1 expression in bloodstream forms and identification of associated cellular factors will enhance our understanding of the effect of *Tb*KIFC1 regulation on the biogenesis of acidocalcisomes.

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FIGURE LEGENDS

Figure 1: Purification, structural characterization and phylogenetic analysis of *Tb*KIFC1.
(A) 10% SDS-polyacrylamide gel electrophoresis of the initial soluble fraction after hypotonic lysis (F1), the DEAE flowthrough (FT), the 250 mM NaCl, DEAE eluted fractions (ELD), the 250 mM NaCl, affinity O-(P)Tyr eluted fraction (ELA). The gel was silver stained.–
(B) Global structure of *Tb*KIFC1. The black, hatched, grey and white boxes correspond to the globular head domain (GD), the stalk coiled-coil region (SR), the neck consensus sequence (N) and the motor domain respectively (MD). Amino acid sequences obtained by protein microsequencing (P26, P31, P38) are indicated. Sequences located between the aminoacids M1 and S231 and between K474 and P821 represent the *E.coli* BL21 (DE3) expressed recombinant proteins NterKIFC and MDKIFC, respectively. These proteins were purified by Immobilized Metal Affinity Chromatography (IMAC) using the His-Bind® column and procedures from Novagen. A PD10 column from Pharmacia was used for buffer exchanges. The NterKIFC protein was utilized to develop a specific monoclonal antibody (H3) against *Tb*KIFC1.
(C) Phylogenetic analysis of kinesin superfamily proteins (6). The phylogenetic tree was constructed from the distance matrix by the Fitch method as described in Bringaud et al (63) and was rooted with Scmy. Accession numbers are mentioned in (6).

Figure 2: Biochemical and expression studies of *Tb*KIFC1
(A) MD-KIFC binding to microtubules. 0.1 to 1.7 µM MD-KIFC protein were incubated with 1 µM microtubule in the binding medium containing 2.5 mM ATP or AMP-PNP (ct) for 15 minutes at room temperature. The MD-KIFC-microtubules complexes were pelleted (100,000 g for 30 minutes) and analyzed by Western
Blotting. Protein separation was performed on 12% SDS-PAGE, transferred to a PVDF membrane and immunoblotted with anti-\(Tb\)KIFC1 monoclonal antibody (H3). DAB was used to reveal the protein bands. For quantification, purified MDKIFC was used to establish a standard concentration curve with the NIH image 1.52 software. Inset: Scatchard linearisation.

(B) Tubulin activation of the ATPase activity of \(Tb\)KIFC1. 1µg of \(Tb\)KIFC1 was incubated for 15 min. with 1 mM MgATP and microtubules in a concentration range of 0 to 2.5 µM. Inset : Hanes linearisation

Figure 3 : Protein expression-

(A) Quantification of the level of \(Tb\)KIFC1 expression between PF and BF. Soluble proteins were obtained by hypotonic lysis of \(10^7\) PF and \(10^7, 10^6,10^5,10^4\) BF. H3 was used to recognize \(Tb\)KIFC1 and the chemiluminescence ECL kit to reveal the bands. 

(B) Expression level of \(Tb\)KIFC1 in the different trypanosome life stages. Soluble proteins were obtained by hypotonic lysis of \(10^7\) cells of each stage. sl (slender forms) st (stumpy forms) mf (metacyclic forms). H3 was used to recognize \(Tb\)KIFC1, a monoclonal anti-aldolase to recognize the aldolase protein and chloronaphtol to reveal the bands.

Figure 4 : Characteristics of \(Tb\)KIFC1 localization and displacement in bloodstream forms.

a : Immunolocalization performed on non-treated BF using a deconvolution procedure for image analysis (24). c : Immunolocalization performed after \(NH_4Cl\) treatment (\(NH_4Cl\) 30 mM for 15 min). e : Immunolocalization performed after \(NH_4Cl\) treatment and subsequent reacidification by two washes in Ringer´s solution followed by 15 min incubation in acidic Ringer´s solution (70 mM sodium acetate,
pH 6.9). a,c,e: Immunolocalizations of *Tb*KIFC1 performed with the monoclonal antibody H3. b and d: dapi staining of a and c. f: Phase contrast of e.

Figure 5: Phenotype analysis of the procyclic kinRNAi+tet cell line.

(A) Soluble proteins were obtained by hypotonic lysis of 10^7 parasite cells. Lane 1: Non-induced Kin RNAi cell line. Lane 2: KinRNAi+tet induced with 100 ng/ml tetracycline. Lane 3: The KIN cell line in the procyclic *Tb*KIFC1 overexpressers. Lane 4: WT is the 427 procyclic strain modified by E.Wirtz and used as a control. Kinesin was identified using the H3 antibody and the chemiluminescence ECL" revelation kit.

Effect of nigericin and NH4Cl on Ca++ release (B) and intracellular pH (C).

The experiments were performed as described by Moreno (64). Cells (0.3 mg of protein/ml) were added to the standard reaction buffer A containing either 6 μM fura-2/AM and 1mM EGTA or BCECF/AM 9 μM. 2.5 μM nigericin, 1 μM ionomycin and 20 mM NH4Cl were added where indicated (arrow). The black dots correspond to the non-induced kinRNAi cells and the grey dots to the induced kinRNAi cells. [Ca++]i was determined as described by Negulescu and Machen (65) and (64) and pH according to Frazer-L’Hostis et al (2).
A

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B

C

**Graph B**

**Graph C**
A novel C-terminal kinesin is essential for maintaining functional acidocalcisomes in *Trypanosoma brucei*

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