The membrane-bound histidine acid phosphatase TbMBAP1 is essential for endocytosis and membrane recycling in Trypanosoma brucei

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Summary
In the parasitic protozoan Trypanosoma brucei, endocytosis and exocytosis occur exclusively at an invagination of the plasma membrane around the base of the flagellum, called the flagellar pocket, which actively communicates by vesicular membrane flow with cisternal/tubulovesicular endosomes. The division of the cell surface into three morphologically distinct sub-domains and the rapid plasma membrane turnover establishes T. brucei as an interesting model for investigations on the sorting and recycling of membrane proteins. In this study we show that the type I membrane protein TbMBAP1, an L-(+)-tartrate-sensitive acid phosphatase, is present in all endosomal membranes but is virtually absent from the lysosome membrane (where this type of protein is mainly found in other organisms) and is not detectable at the cell surface. The endosomal localization of TbMBAP1 is a function of protein abundance. Moderate overexpression (three- to fourfold) leads to an increased appearance within the flagellar pocket membrane. At higher levels the protein is found in the flagellum, and routing to the pellicular plasma membrane is observed at levels 10- to 25-fold above that of wild type. In other organisms L-(+)-tartrate-sensitive acid phosphatases appear to be dispensable but TbMBAP1 is essential, as shown by RNA interference, which causes growth arrest followed by cell death. Comparison of the phenotype of TbMBAP1-depleted cells with that of cells in which endocytosis or exocytosis has been specifically inhibited by RNAi against clathrin of RAB11, reveals that TbMBAP1 is essential for both incoming and recycling membrane traffic. During differentiation of the organism from bloodstream to insect stage, TbMBAP1 is down-regulated and differentially modified in parallel with a 10-fold decrease in the rate of endocytosis.

Introduction
How membrane proteins are sorted between different cellular compartments or between domains of continuous membranes has been actively investigated in representatives from bacteria to mammalian cells. Among the protists, specific ultrastructural features and recent progress in genetic manipulation techniques make the mammalian stage of the parasitic protozoan Trypanosoma brucei an interesting model for the elucidation of both basic and unique aspects of protein sorting. Most studies on the trafficking and sorting in this organism have concentrated on proteins modified by glycosylphosphatidylinositol (GPI) residues, e.g. the highly abundant coat protein known as the variant surface glycoprotein (VSG) either in its native form [see summary in Overath and Engstler (Overath and Engstler, 2004)] or modified versions (Böhme and Cross, 2002; Wang et al., 2003; Triggs and Bangs, 2003) and the transferrin receptor (Mußmann et al., 2004; Mußmann et al., 2003). In contrast, little is known about the sorting of transmembrane proteins. Here we describe a type I membrane protein that can be used for investigating mechanisms of intracellular sorting as well as the molecular traffic between different surface domains.

The bloodstream form of T. brucei is an elongated and highly polarized cell, in which the Golgi complex and all endosomal compartments are compactly organized in the posterior region of the cell behind the centrally located nucleus (Fig. 1). Endocytosis and exocytosis are restricted to the flagellar pocket (FP) membrane, which is formed by an invagination of the plasma membrane at the base of the flagellum and constitutes, together with its carbohydrate-rich matrix, a concealed yet extracellular compartment. The flagellar pocket membrane is continuous with both the membrane of the flagellum (F) and the pellicular cell surface (PM). Therefore, the surface is divided in three sub-domains. The mechanisms regulating the composition and dynamics of membrane components between the flagellar pocket membrane
and either the flagellum or the cell body remain poorly defined (Landfear and Ignatushchenko, 2001).

Endocytosis at the flagellar pocket membrane occurs by way of clathrin-coated vesicles (CCVs) with an average diameter of 135 nm that have been designated class I CCVs (Fig. 1). After internalization, these vesicles rapidly shed their clathrin coat and fuse with cisternal and vesicular structures located mainly close to the lysosome, and the presence of the small GTPase Rab5 defines these structures as early endosomes (EE). The posterior region of T. brucei contains abundant extended sheet-like cisternae and at least half of these are decorated with Rab11, an established marker for recycling endosomes (RE). Budding from early and recycling endosomes produces a second smaller type of clathrin-coated vesicles (class II CCVs, average diameter 50-60 nm), which have been proposed to be directed to Rab7-positive late endosomes and/or the lysosome. The recycling endosomes give rise to Rab11-positive disc-like structures, designated EXCs for exocytic carrier vesicles, which fuse with the flagellar pocket membrane, thus closing the endocytic cycle. Kinetic experiments have shown that endocytosis occurs at the very high rate of 6-7 class I CCVs per second and that the endosomal pool of the GPI-anchored VSG is recycled once per minute (Grünfelder et al., 2003; Engstler et al., 2004; Overath and Engstler, 2004).

In searching for a marker of the flagellar pocket membrane of T. brucei, we previously purified a 70 kDa glycoprotein with an activity of an L- (+)-tartrate-sensitive acid phosphatase (Schell et al., 1990). Here, we demonstrate that this enzyme is an endosomal type I membrane protein, which has essential functions in the endocytic cycle. Furthermore, we present a detailed analysis of the cellular distribution of the phosphatase as a function of the expression level. The studies suggest that this system will be useful for further analyses of sorting mechanisms between endosomes and the flagellar pocket membrane as well as for the trafficking between the pocket, the flagellum and the pellicular cell surface.

Materials and Methods

Parasites and bacterial cells

Bloodstream form Trypanosoma brucei (strain 427, variant MITat 1.2) cells were grown in HMI-9 + 10% foetal bovine serum (Hirumi and Hirumi, 1989). Cloning was performed in the Escherichia coli strain XL-1-Blue.

DNA techniques

Restriction digestions, ligations and transformations into E. coli as well as DNA gel electrophoresis were performed as described previously (Sambrook et al., 1989). Isolation of plasmid DNA was performed using a commercial kit (Qiagen, Hilden, Germany); T. brucei DNA was isolated according to the method of Medina-Acosta and Cross (Medina-Acosta and Cross, 1993).

Cloning and sequencing of the membrane-bound acid phosphatase genes TbMBAP1 and TbMBAP2

A BLAST search using the protein sequence of Leishmania mexicana membrane-bound acid phosphatase [LmxMBAP, accession number Z46971, cf. (Wiese et al., 1996)] against the TIGR T. brucei database revealed two homologous sequences, GATSF78TF and AQ941274. Two pairs of primers were used to generate probes: Tb1. for (5′-ACCTTTGKCAGCTKGTSCA-3′) and Tb1.rev (5′-GGCACACA- TACCCTCATTG-3′) matching positions 67-85 and 554-536, respectively, of GATSF78TF, and Tb2. for (5′-ATAAAAGCACAA ACAGGGTG-3′) and Tb2.rev (5′-GTGTATAGCTGGAA AGAXT-3′) matching positions 264-282 and 686-668, respectively, of AQ941274.

DIG-labelled probes generated by using these primers on genomic DNA were obtained using a commercial protocol (Roche, Mannheim, Germany). These probes were then used to identify positive clones from the T. brucei 927 P1 library (Cambridge University, Laboratory for Parasite Genome Analysis, Cambridge, UK). Subfragments derived from digestion of clone 927-9G8 with NruI (for TbMBAP1) and of clone 927-13G2 with HincII (for TbMBAP2) were sub-cloned into pBS (Stratagene, La Jolla, CA, USA) and linearized with EcoRV, resulting in plasmids pTb1NruI and pTb2HincII, respectively. Filter-lifts of the resulting E. coli clones were probed with the DIG-labelled probes (see above) to identify positive clones for sequencing. Plasmid DNA from positive clones was isolated and sequenced by primer walking. The DNA sequences were deposited in the EMBL/GenBank/DBJ databases under the accession numbers AJ303100 (TbMBAP1) and AJ303101 (TbMBAP2).

Generation of constructs for protein amplification and knockdown by RNAi

For moderate overexpression of TbMBAP1, the 1609 bp insert was released from pTb1NruI with HincIII (partial) and XhoI, and ligated into plasmid pLew20 (Engstler et al., 1997) cut with the same enzymes. For transfection of T. brucei, pLew20MBAP1 was linearized with NotI. For high-level overexpression the HincIII/XbaI fragment released from pTb1Nru1 was ligated to the plasmid pLew82 (Wirtz

Fig. 1. Schematic view of the posterior part of the bloodstream stage of T. brucei. CCV I, class I clathrin-coated vesicles; CCV II, class II clathrin-coated vesicles; FP, flagellar pocket; EE, early endosomes; RE, recycling endosomes; LE, late endosomes; EXC, exocytic carrier vesicles; L, lysosome; ER, endoplasmic reticulum; G, Golgi complex; N, nucleus; PM, plasma membrane; F, flagellum. See text for explanations. Modified from Engstler et al. (Engstler et al., 2004).
et al., 1999) cut with BamHI, filled with Klenow and cut with HindIII. The resulting pLew82MBAP1 was linearized with NolI for transfection of T. brucei. For inducible RNAi a 739 bp fragment was released from pLew20MBAP1 by cleavage with PvuII and HindIII and pasted into the vector p2T7STT3 (LaCount et al., 2000) (M.E., unpublished results) cut with the same enzymes. For stable insertion into the T. brucei genome the resulting plasmid p2T7MBAP1 was cut with NolI.

For inducible RNAi of clathrin heavy chain an 842 bp PCR-product was amplified using the primers CLHU (5′-AAT AAG CCT CAA CTT GCA GAT ATT CGA CGT GGA C-3′) and CLHL (5′-TGT AGA TCT CAC CTC CCA GGT TAG CAG AGC C-3′), cut with HindIII and BamHI and ligated into p2T7 cut with the same enzymes. Primers RAB11U (5′-TAA CCT GCT GCT ACA CAG CCG ATG AGT TCA GTC-3′) and RAB11L (5′-TGG TTA GCC TGG CTG GTT TGA ATT GGA AG-3′) were used to amplify a 563 bp RAB11-PCR product. Following restriction with HindIII and BamHI, the PCR product was cloned into p2T7, yielding the inducible RAB11-RNAi construct.

Transfection of T. brucei
Culture conditions and transfection protocols for T. brucei are given in detailed elsewhere (McCulloch et al., 2004). For selection of recombinant trypanosomes, antibiotics were added 18 hours post-transfection (2 μg/ml phleomycin for pLew20; 4 μg/ml phleomycin for pLew82 and p2T7). Antibiotic-resistant recombinant trypanosome pools were obtained after 5-7 days. For constitutive overexpression, pLew20MBAP1 was transfected into wild-type T. brucei MITat1.2. For tetracyline-inducible overexpression or RNAi, pLew20MBAP1, pLew82MBAP1 and p2T7MBAP1 were transfected into the T. brucei MITat1.2 line 13.90 (Wirtz et al., 1999).

Generation of anti-peptide antibodies
Peptides consisting of the 16 C-terminal amino acid residues of TbMBAP1 (NH2-CGRANRAREGSDGPVP-COOH) and of TbMBAP2 (NH2-CGRANRAREGSDGPVP-COOH) were synthesized (cf. Fig. 1A and supplementary material), coupled to keyhole limpet haemocyanin, and used for the immunization of rabbits (Eurogentech, Seraing, Belgium). The antibodies were designated anti-TbMBAP1 and anti-TbMBAP2, respectively.

Immunoprecipitation and determination of acid phosphatase activity
1.2×10⁶ cells grown in vitro were harvested in the cold by centrifugation for 10 minutes at 200 g, washed once with PBS, resuspended to 3×10⁶ cells/ml in lysis buffer (5 mM Hapes, 2.5 mM EDTA, 2 mM EGTA, 1 mM PMSF; 100 μM TLCK, 10 mM 1,10-phenanthroline) and lysed by sonication in a Branson Sonifier at grade 4 for 20 seconds. The lystate was centrifuged for 1 hour at 100,000 g in a Beckman TLA 100.3 rotor, the supernatant was discarded and the pellet was suspended in 1.5 ml of extraction buffer (PBS, 150 mM KCl, 0.9% octyl-β-D-glucopyranoside, 1 mM PMSF, 100 μM TLCK, 10 mM 1,10-phenanthroline). Extraction was performed by end-over-end rotation for 18 hours at 4°C. Centrifugation for 1 hour at 100,000 g in a Beckman TLA 100.3 rotor yielded a supernatant (detergent extract) and a particulate fraction, which was discarded. The extract (100 μl) was added to 400 μl of Protein G-Sepharose beads (Amersham Biosciences, Uppsala, Sweden) in binding buffer (TBS, 0.5% Triton X-100, 1 mg/ml BSA), which had previously been incubated with either anti-TbMBAP1 serum or supernatant of hybridoma cells expressing monoclonal antibody mAT502 (Schell et al., 1990). Binding was allowed while rotating end-over-end for 2 hours at 4°C; the beads were pelleted by centrifugation in a table top centrifuge for 30 seconds at 3000 rpm. The supernatant (unbound fraction) was set aside, the beads were washed twice with binding buffer and suspended to a volume of 0.5 ml (bound fraction).

The bound and unbound fractions (100 μl each) were incubated for 1 hour at 37°C with 900 μl of substrate buffer (5 mM p-nitrophenyl phosphate, 100 mM sodium acetate, pH 5.0) in the absence or the presence of 40 mM sodium L-(–)-tartrate. A sample of 90 μl was drawn, neutralized by the addition of 10 μl of 1 M NaOH, and the amount of p-nitrophenol generated was estimated by the absorbance at 405 nm.

Immunoblot analysis
For the results shown in Fig. 1C, 50 μl of the bound and unbound fractions from the immunoprecipitation experiment described above, and 10 μl of the detergent extract, corresponding to approx. 2×10⁶ cells, were subjected to SDS-PAGE on a 12.5% polyacrylamide gel. The proteins were transferred to an Immobilon P membrane (Millipore, Bedford, MA, USA). The blot was developed using anti-TbMBAP1 antisera (1:500) as primary antibody and goat-anti-rabbit horseradish peroxidase (1:20,000, Dianova, Hamburg, Germany) as secondary antibody. For detection, a commercial kit (ECL Western Blot Analysis, Amersham Pharmacia, Little Chalfont, UK) was used.

For protein quantification, blots containing 10⁶ cell equivalents per lane were probed with a mixture of rabbit anti-TbMBAP1 antibodies (1:5000) and a mouse anti-T. brucei HSP 60 monoclonal antibody (1:1000; a kind gift from F. Bringaud, Bordeaux, France) and subsequently with a mixture of Alexa Fluor™ 680-conjugated or IRDye 800-conjugated goat anti-rabbit + goat anti-mouse IgG (H+L) antibodies (all diluted 1:5000; Molecular Probes). The fluorescence signals were quantified by laser scanning using the Odyssey System (LI-COR). Following automatic subtraction of the background values (Median Left/Right method) the TbMBAP1 signals were normalized to the HSP60 loading control using the Odyssey software (LI-COR).

3D-Immunofluorescence and immunoelectron microscopy
Immunofluorescence was performed as described previously (Grünfelder et al., 2003; Engstler et al., 2004). For immunodetection of TbMBAP1, the corresponding rabbit antisera (1:200 in PBS, 1% BSA) and Alexa Fluor™ 488-conjugated goat anti-rabbit antibodies (1:2000; Molecular Probes) were used. Images were acquired using a motorized Axioskop 2 microscope controlled by the IPLab 3.9 software (Scanalytics, Fairfax, USA). For digital deconvolution of 3D-images the Huygens System (SVI, Hilversum, Netherlands) was used. Image processing and colocalization analysis was conducted with the Imaris Surpass software (Bitplane, Zurich, Switzerland) (for details, see Engstler et al., 2004; Mußmann et al., 2004).

For colocalization of intracellular markers with TbMBAP1, rabbit anti-MBAP1 IgG purified via Protein G Sepharose chromatography was fluorescently labelled using the Zenon™ Alexa Fluor 488 Rabbit IgG Labeling Kit (Molecular Probes). T. brucei MITat1.2 bloodstream forms were harvested at a density of 5×10⁵ cells/ml, washed twice in PBS and fixed overnight in 4% paraformaldehyde in PBS at 4°C. Following two washes in PBS, the cells were incubated in PBS, 1% BSA with the respective marker antibody for 1 hour at room temperature following treatment with species-specific Alexa Fluor 594 secondary antibodies (1:1000). The cells were then incubated in PBS/BSA containing 2 μg/100 μl anti-MBAP1 IgG Zeon. Colocalization was quantified as described previously (Engstler et al., 2004; Grünfelder et al., 2003). In order to restrict the analysis to non-dividing forms (1K1N), the cells were stained with DAPI and a threshold segmentation was conducted prior to the colocalization analysis (Engstler et al., 2004).

Immunoelectron microscopy of Tokuyasu cryosections was performed as described previously (Grünfelder et al., 2003) using rabbit anti-TbMBAP1 antibodies (1:100) or pre-immune serum
Kinetics of fluid phase uptake

The kinetics of fluid-phase endocytosis was measured as described by Engstler et al. (Engstler et al., 2004). Briefly, freshly harvested trypanosomes were resuspended in HMI-9 at a density of 4×10⁸ cells/ml and kept on ice. Aliquots of 20 µl of the cell suspension were transferred to 180 µl of prewarmed (37°C) HMI-9, containing 5 mg/ml Alexa Fluor 488-conjugated dextran (M₄10,000; Molecular Probes). At different times the endocytosis was stopped by the addition of 200 µl of ice-cold 8% paraformaldehyde. Following fixation overnight, the cells were washed twice, stained with DAPI, and the relative dextran fluorescence intensity of non-dividing trypanosomes was determined by quantitative fluorescence microscopy.

Results

TbMBAP1 encodes the Trypanosoma brucei tartrate-sensitive acid phosphatase

We have identified and cloned two genes, called TbMBAP1 and TbMBAP2, present in the T. brucei genome databases, which show high homology to Leishmania membrane-bound acid phosphatase genes. TbMBAP1 and TbMBAP2 are 58% identical, and have a 40% and 41% amino acid identity, respectively, to the well-characterized L. mexicana membrane-bound acid phosphatase LmxMBAP (Wiese et al., 1996) (see additional information and alignment in supplementary material). TbMBAP1 and TbMBAP2 possess the two regions involved in the formation of the active centre of histidine acid phosphatases: the N-terminal RHGXRXP element (InterPro Entry IPR000560) containing the histidine residue that forms a phosphohistidine intermediate, and the HD motif in the C-terminal section containing the histidine residue that possibly acts as a proton donor (Fig. 2A,B) (Ostanin et al., 1992; Schneider et al., 1993; van Etten et al., 1991). Hydrophobicity plots of both proteins predict stretches of hydrophobic amino acids close to their C termini, which may form transmembrane α-helices. Therefore, these proteins have the typical features of Type I membrane proteins and are similar to their close relative LmxMBAP. After cleavage of the putative 27-residue signal sequence, TbMBAP1 is predicted to have a molecular mass of 56.1 kDa, implying that the mature protein (72 kDa; Fig. 2C) is likely to be modified extensively by N-glycosylation. Homology-based modelling of the three-dimensional structure of the TbMBAP1 catalytic domain reveals a well-conserved core of anti-parallel β-sheets that centres on the catalytically important histidines 42 and 292 (Fig. 2B). The protein core is surrounded by solvent-exposed α-helices. All eight asparagine residues predicted to act as N-glycosylation sites are exposed on the surface of the molecule.

Approximately 40% of the total acid phosphatase activity was found to be tartrate-resistant and 60% tartrate-sensitive in a cell lysates of T. brucei bloodstream forms (Table 1), which was in agreement with previous estimates (Schell et al., 1990). In the detergent extract of two catalytically important histidine residues are shown in red and putative N-glycosylation sites in green. Numbers indicate amino acid positions. (C) Monoclonal antibody mAT502 binds to TbMBAP1. An aliquot of a detergent extract from the total membrane fraction of T. brucei bloodstream forms was immunoprecipitated using the mouse monoclonal antibody mAT502. Samples of the precipitate (lane 3) and the supernatant (lane 2) of the immunoprecipitation as well as the detergent extract (lane 1), each corresponding to the equivalent of 2×10⁶ cells, were subjected to immunoblotting. The blot was probed with rabbit anti-TbMBAP1 and horseradish peroxidase coupled secondary antibodies. The molecular mass of standard proteins in kDa is indicated.

Fig. 2. (A) Schematic representation of the TbMBAP1 protein of T. brucei. The putative signal sequence is highlighted in red, the catalytically active regions appear in blue and the predicted transmembrane region in grey. The C-terminal amino acids recognized by an anti-peptide antibody appear as a black box. Green lines denote potential N-glycosylation sites. The PFAM domain PF00328 (histidine acid phosphatase) is highlighted in yellow. Numbers indicate amino acid positions. (B) Predicted structure of the TbMBAP1 core domain (amino acids 32-326). The coordinates are based on three homology models that were independently generated with CPHmodels-2.0 (http://www.cbs.dtu.dk/services/CPHmodels/), 3D-JIGSAW-2.0 (http://www.bmm.icnet.uk/servers/3djigsaw/) and SDSC1 (http://cl.sdsc.edu/hm.html). The three sets of coordinates were analysed, optimized and visualized with the Deep View software (http://www.expasy.org/spdbv/). The images show a ribbon representation of the integrated model. Helices appear in yellow and sheets in blue. The
TbMBAP1 localizes to endosomes

The intracellular distribution of TbMBAP1 was investigated by a colocalization analysis using a panel of organelle-specific antibodies (Fig. 3A, compare Fig. 1 for relevant structures). Nearly all (78-92%) of the early (RAB5-positive), the recycling (RAB11-positive) and the late (RAB7-positive) and the recycling (RAB11-positive) endosomes contained TbMBAP1. In addition, most of the total cellular staining (~66%) was associated with these endosomal sub-compartments. While fluorescence microscopy suggested that roughly half of the intracellular TbMBAP1 was associated with recycling endosomes, only a small fraction of the phosphatase colocalized with clathrin-containing structures. Interestingly, TbMBAP1 was essentially absent from the lysosome that was identified as a p67-positive structure (Kelley et al., 1999).

We also compared the endosomal distribution of TbMBAP1 with that of VSG, which covers the entire cell surface as a dense coat and rapidly cycles through the endosomal system (Fig. 3B). The cell surface VSG was visualized by the blue fluorescence of the coumaryl dye AMCA (VSGAMCA), while endocytosed biotinylated VSG (VSGbiotin) was detected by the red fluorescence of bound Alexa Fluor™ 594-conjugated Streptavidin. TbMBAP1 was visualized by rabbit antibodies against the C terminus and Alexa Fluor™ 488-conjugated goat anti-rabbit antibodies. These antibodies specifically recognized intracellular structures located between the flagellar pocket and the nucleus. Thirty randomly chosen cells were analyzed by quantitative colocalization analysis after digital deconvolution. No significant colocalization of cell surface-associated VSGAMCA and TbMBAP1 was detectable. In contrast, 46±8.2% of endocytosed VSGbiotin colocalized with TbMBAP1 and 69±7.3% of TbMBAP1 colocalized with VSGbiotin. A careful inspection of a series of cells suggested that some TbMBAP1-containing structures were located close to the flagellar pocket but the protein was essentially absent from the membrane of the pocket.

The ultrastructural localization of TbMBAP1 was determined by immunoelectron microscopy of cryosections, which is shown in Fig. 3C-E [see Fig. 1 and previous studies (Grünfelder et al., 2002; Grünfelder et al., 2003; Overath and Engstler, 2004) for relevant structures and details]. Specific immunolabelling was observed on endosomal cisternae (Fig. 3C; EC) as well as on exocytic carrier vesicles (EXCs; Fig. 3D), which are known to fuse with the flagellar pocket membrane (Fig. 3D, inset). Together these structures accounted for 80% of the specific intracellular gold label (Table 2). As shown before (Grünfelder et al., 2003), the EXCs and about half of the endosomal cisternae were positive for RAB 11. Remarkably, only a small amount of the protein appeared to be

Table 2. Cellular distribution of TbMBAP1 determined by immunoelectron microscopy

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Number of PAG-6 gold grains*</th>
<th>Difference</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Surface+mitochondrion</td>
<td>45</td>
<td>17</td>
<td>28</td>
</tr>
<tr>
<td>Flagellar pocket membrane</td>
<td>12</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Flagellum</td>
<td>39</td>
<td>35</td>
<td>4</td>
</tr>
<tr>
<td>Kinetoplast</td>
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<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Nucleus</td>
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<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Cytoplasm+ER</td>
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</tr>
<tr>
<td>Golgi complex</td>
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<td>3</td>
</tr>
<tr>
<td>Small vesicles</td>
<td>41</td>
<td>53</td>
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<td>94</td>
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<tr>
<td>Exocytic carrier vesicles</td>
<td>175</td>
<td>26</td>
<td>149</td>
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<tr>
<td>Class I clathrin-coated vesicles</td>
<td>0</td>
<td>0</td>
<td>0</td>
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*The numbers refer to gold complexes counted on a total of 103 randomly chosen trypanosome profiles for pre- and immune sera. The data are the sum of one labeling experiment using anti-TbMBAP1 antiserum (50 profiles) and another (53 profiles) with antibodies obtained from this antiserum by affinity chromatography on immobilized C-terminal peptide. The purification did not significantly alter the extend of labeling or its specificity.
specifically associated with the flagellar pocket membrane while the protein was absent from the class I clathrin-coated vesicles. It was also clear that the gold label was mainly associated with the cytoplasmic face of the endosomal cisternae (Fig. 3C), which was consistent with the fact that the antibodies were directed against the cytosolic C-terminal domain of \textit{TbMBAP1}. In contrast, immunogold label specifically associated with the variant surface glycoprotein is mostly found in the lumen of the cisternae (Grünfelder et al., 2002).

In summary, \textit{TbMBAP1} is located in intracellular compartments that have been defined as early, late and recycling endosomes by the presence of small GTPases. Very little antigen is associated with the cell surface, class I clathrin-coated vesicles and the lysosome.

The expression level determines the cellular localization of \textit{TbMBAP1}

The presence of a large fraction of \textit{TbMBAP1} in RAB11-
Fig. 3. Analysis of the cellular distribution of TbMBAP1 in bloodstream stage trypanosomes. (A) Quantitative colocalization analysis of TbMBAP1 and organelle marker proteins. Nuclear and mitochondrial DNA was stained with DAPI (blue). TbMBAP1 was visualized with Zenon™-Alexa Fluor 488-labeled anti-TbMBAP1-specific rabbit IgG (middle panel, green). The respective marker proteins were detected with specific antibodies and Alexa Fluor 594 secondary antibodies (right panel, red). The generation of the antibodies against marker molecules has been described previously: clathrin heavy chain (Morgan et al., 2001); RAB5 (Field et al., 1998); RAB11 (Jeffries et al., 2001); p67 (Kelley et al., 1999); BiP (Bangs et al., 1993). The merged colour channels are shown in the left panels. All images are representative examples from a 3D quantitative colocalization analysis (Imaris Surpass Colocalisation software, Bitplane, CH). Sampling numbers (n) are given in the left panels. The numbers in the middle panels indicate the percentage of TbMBAP1 colocalizing with the respective marker proteins, and in the right panel the percentage colocalization of markers with TbMBAP1 is given [for details see Materials and methods and Engstler et al. (Engstler et al., 2004)]. (B) Colocalization of TbMBAP1 with endocytosed VSG. Cells were labelled on ice with sulfo-NHS-SS-biotin and AMCA-sulfo-NHS. Following endocytosis to the steady state (5 minutes at 37°C), cell surface biotin was removed with glutathione. Red colour: Endocytosed VSGbiotin detected with Alexa Fluor™ 594 conjugated Streptavidin. VSGAMCA fluorescence (blue) is exclusively visible on the cell surface because the intracellular fluorescence is quenched. TbMBAP1 visualized with anti-TbMBAP1-specific rabbit antiserum and Alexa Fluor™ 488-conjugated goat anti-rabbit IgG is in green. The representative image (left panel, top) shows a maximum intensity projection of the corresponding deconvolved 3-channel 3D data set [see Materials and Methods]. (B) Detection of overexpressed TbMBAP1 in bloodstream form trypanosomes by immunoelectron microscopy. Cryosections were marked by an open arrowhead. N, nucleus; FP, flagellar pocket. Bars: 3 µm. (C-E) Detection of TbMBAP1 in bloodstream form trypanosomes by immunoelectron microscopy. Cryosections were labelled with anti-TbMBAP1 antibodies and PAG-6 (C,D). For improved visibility the gold grains of the images were digitally enlarged. The protein can be detected in the Golgi complex (G), on its cis-side in the adjacent budding zone (BZ, in C), and in many profiles of endosomal cisternae (ECs). Specific labelling is also observed on disk-like exocytic carrier vesicles (EXC), which are abundant near the flagellar pocket (FP in D). The inset in D shows top views of EXCs in the process of fusion with the flagellar pocket membrane. The arrow in C points to a type II clathrin-coated bud at the rim of an endosomal cisterna. (E) In the control with pre-immune serum, EXCs are not labelled. cEC, circular endosomal profile; L, lysosome; MVB, multi-vesicular body; SC, surface coat; FL, flagellum.

In the second approach, we attempted to achieve a broader range of cellular TbMBAP1 levels by expressing the protein under the control of a constitutive (K) or tetracycline-inducible (Ti) procyclin promoter. Quantification of TbMBAP1 on immunoblots shows a moderate overexpression of three to fourfold compared to the wild type (wt). The red colour indicates the TbMBAP1 protein detected with anti-TbMBAP1-specific rabbit antiserum and IRDye 800 second reagent. A loading control, a mouse anti-HSP60 antibody was detected with Alexa Fluor™ 680-conjugated second antibody. Fluorescence quantification was done with the LiCor Odyssey system (see Materials and Methods). (B) Detection of overexpressed TbMBAP1 in the flagellar pocket. Cells were surface-labelled with AMCA-sulfo-NHS (blue). Detection of TbMBAP1 (green) and image processing were as in Fig. 3A. The lysosomal membrane protein p67 was visualized with a specific monoclonal antibody and Alexa-594-conjugated second antibody. While in wild-type cells TbMBAP1 fluorescence is absent from the flagellar pocket (see Fig. 3A), the protein can be detected in flagellar pocket (FP) membrane upon fourfold overexpression of the protein. The overall localization of TbMBAP1 between flagellar pocket and nucleus (N) is not affected.

motility. The degree of overexpression was stable, even after repeated depletion of tetracycline and subsequent re-induction. As shown by 3D-fluorescence microscopy, the majority of the overexpressed protein remained associated with endosomal structures. Interestingly, a clear signal was detected in the flagellar pocket membrane, while the cell surface remained negative (Fig. 4B). Furthermore, the lysosome remained free of TbMBAP1, even in the presence of the cysteine proteinase inhibitor Z-Phe-Ala-diazomethyl ketone (data not shown) (cf. Scory et al., 1999).
The strong overexpression of *Tb*MBAP1 was compatible with cell growth, albeit at a reduced rate (generation time 8 hours) compared to the background strain 13-90 (generation time 6 hours). No apparent change in the morphology or motility of the fully induced cells was detectable.

We have analysed the localization of *Tb*MBAP1 at 1-hour intervals during the time-course of inducible overexpression (Fig. 5C). Uninduced cells had the same phenotype as wild-type trypanosomes: the protein was restricted to endosomes and was absent from the flagellar pocket and lysosome. Within 1 hour of induction, the total *Tb*MBAP1 fluorescence intensity increased about threefold and the protein was clearly visible in the flagellar pocket. Interestingly, after 2-3 hours the overexpressed protein gradually appeared on the flagellar membrane. The most distinctive flagellar staining was visible at 5 hours post-induction (Fig. 5C) when the summed fluorescence intensity/cell was 8- to 10-fold higher than in wild-type trypanosomes. No protein was detectable on the pellicular cell surface suggesting a preferential and specific routing of *Tb*MBAP1 from the flagellar pocket to the flagellar membrane. The fluorescence signal on the flagellar surface was at least 30-fold over background. After 8-9 hours of induction the protein progressively started to distribute over the pellicular cell surface. As determined by quantitative fluorescence microscopy, the overexpression threshold for cell surface appearance of *Tb*MBAP1 was 12- to 13-fold above the wild-type level. The amount of surface protein increased with time and reached a maximum at about 20-24 hours post-induction. However, even at this very high expression level (>25-fold) the flagellar membrane contained about eightfold more *Tb*MBAP1 than the pellicular cell surface. This observation suggests there was a continuous, preferential trafficking of the protein from the pocket to the flagellum. It may be noteworthy that even at maximum induction no *Tb*MBAP1 protein was detectable within the lysosome.

Taken together these results demonstrated a connection between the expression level and the localization of *Tb*MBAP1. Interestingly, this link was not continuous, but rather involves distinct steps marked by thresholds: a low 3- to 4-fold overexpression saturates endosomal retention and the enzyme is detected in the flagellar pocket. A moderate 6-fold increase over wild-type leads to routing of phosphatase to the flagellar membrane. For entry onto the pellicular cell surface overexpression levels of at least 12-fold are required.

*Tb*MBAP1 is essential for endocytosis and membrane recycling

The reduction in the growth rate of cells expressing high levels of *Tb*MBAP1 suggested that the protein might serve a critical function in the trypanosome cell. This possibility was tested by inducible RNA interference (RNAi). Part of the *Tb*MBAP1-sequence was inserted into the RNAi-vector p2T7 (LaCount et al., 2000) and the resulting construct was transfected into a derivative of the MITat1.2 13-90 line, termed 13-90/EP:GFP (M.G. and M.E., unpublished). In addition to the tetracycline repressor and the T7-polymerase, this cell line constitutively expresses a green fluorescent protein (GFP)-procyclin fusion protein that localizes to the endosomal compartment and to the flagellar pocket (Engstler and Boshart, 2004) and shuttles in an infinite loop between endosomes and the flagellar pocket. The resulting cell line allowed the tetracycline-inducible expression of *Tb*MBAP1-specific dsRNA. Remarkably, upon induction, the cells continued to grow for only half a generation and subsequently died (Fig. 6A). This behaviour correlated with the decay in the cellular *Tb*MBAP1 levels, which had a half time of 5-6 hours.
Acid phosphatase of Trypanosoma brucei

Fig. 6. Tetracycline-inducible knockdown by RNAi shows that TbMBAP1 is essential for growth of T. brucei bloodstream cells. (A) Growth in the presence (black circles), absence (white circles) of TbMBAP1-dsRNA. (B) Immunoblot showing the decrease in TbMBAP1 within 20 hours of RNAi. HSP60 was used as a loading control. (C) 3D-Fluorescence microscopy of cells before (0) and after 4, 7 and 12 hours of induction. Upper panel: The cell surface is visualized by AMCA-sulfo-NHS staining (grey), TbMBAP1 by anti-C-terminus antibodies and Alexa Fluor™-594-labeled secondary antibodies (red), and endosomes (green) by the autofluorescence of the marker protein EP:GFP (Engstler and Boshart, 2004). The yellow colour indicates colocalization of GFP and TbMBAP1. The arrows point to the flagellar pocket. In the lower panels only TbMBAP1 is shown. For each time point, images of representative cells were chosen. For the ease of visualization the deconvolved 3D data were processed by isosurface rendering.

As shown by immunoblots (Fig. 6B), and a corresponding reduction of enzyme activity (data not shown) as well as with the loss of the protein at the single-cell level as judged by 3D-immunofluorescence (Fig. 6C, lower panels). While more than 60% of TbMBAP1 colocalized with endosomal GFP in the uninduced cells, colocalization was lost within 3-4 hours after induction with tetracycline (Fig. 6C, upper panels). During knockdown of TbMBAP1 the EP:GFP fluorescence decreased with much slower kinetics and even in cells induced for 12 hours, 72% of the GFP was still visible between the flagellar pocket and nucleus (Fig. 6C). Remarkably, the GFP-fluorescence within the flagellar pocket was lost very rapidly, within 2 hours post-induction, but the size of the flagellar pocket remained constant.

In order to analyse the effect of TbMBAP1RNAi on endosome function, we generated transgenic cell lines, in which clathrin heavy chain or RAB11, a key-protein on recycling endosomes, were down-regulated. Three independent RNAi-cell lines were tested for their ability to endocytose and to exocytose. These processes were assessed in three ways: firstly, by measuring fluid phase uptake (Fig. 7A); secondly, by analysing the localization of the fluorescent endosomal, GPI-anchored membrane protein EP:GFP (Fig. 7B); thirdly, by monitoring the uptake and recycling of plasma membrane in living cells stained with the membrane dye FM2-10 (Fig. 7C). The TbMBAP1RNAi cells were induced with tetracycline for 6 hours, followed by 0-60 minutes incubation at 37°C in culture medium containing 5 mg/ml Alexa Fluor 488-conjugated dextran (Fig. 7A). Uptake was quantified by fluorescence microscopy of 80 automatically selected cells for every time point. While the fluid phase marker was internalized with fast kinetics in the uninduced cells, the TbMBAP1-depleted trypanosomes showed only basal levels of cell-associated fluorescence, which did not increase with time. Essentially the same phenotype was observed in clathrinRNAi cells, induced with tetracycline for 6 hours. We and others have previously shown that clathrin is essential for endocytosis from the flagellar pocket (Allen et al., 2003; Engstler and Overath, 2004). Thus, an early consequence of the knockdown of TbMBAP1 was that the cells ceased to endocytose. As a control, we attempted to interrupt specifically the exocytic arm of endosomal traffic by knocking down RAB11, a protein found abundantly on recycling endosomes. When induced for 10 hours with tetracycline, RAB11RNAi cells only showed a minor decrease in dextran uptake but the parasites died 4-8 hours later, demonstrating that RAB11 is essential for trypanosome survival.

We next compared the localization of the fluorescent endosomal reporter EP:GFP after induction of RNAi (Fig. 7B). Interestingly, in this experiment the phenotype of TbMBAP1RNAi did not resemble that of clathrinRNAi, but instead was indistinguishable from the RAB11RNAi phenotype. While wild-type cells had strong green fluorescence in both endosomes and the flagellar pocket, the EP:GFP was absent from the pocket in TbMBAP1 and RAB11 knockdowns, indicating that exocytosis to the flagellar pocket was blocked. The size of the flagellar pocket remained unaffected. In contrast, clathrin-depleted cells had relocalized the protein to an enlarged flagellar pocket, which demonstrated that the flow of membrane from the pocket to endosomes was arrested even though endosomal membrane was still being delivered by exocytosis to the flagellar pocket. Thus, the above experiments suggested that a knockdown of TbMBAP1 affects both directions of endosomal membrane flow. This was supported by monitoring plasma membrane uptake and recycling (Fig. 7C). Cells were incubated on ice with the hydrophobic dye FMR2-10, which readily inserted into the entire cell surface. Endocytosis rapidly resumed in the wild-type when the cells were warmed to 37°C as indicated by an intense intracellular membrane labelling. In TbMBAP1RNAi cells induced with tetracycline for 6 hours prior to the FMR2-10 treatment no such endosomal staining was detectable, not even after incubation at 37°C for more than 1 hour. Similarly, the clathrin-depleted cells did not endocytose the fluorescent surface membrane but showed a marked increase in flagellar pocket size, which was not observed in TbMBAP1-depleted trypanosomes. Knockdown of RAB11 did not affect membrane uptake but it quantitatively blocked the recycling of the internalized surface membrane. This block was
demonstrated by washing cells that had endocytosed the fluorescent plasma membrane in FM R2-10-free buffer. In wild-type cells the fluorescence vanished from the cell surface and endosomes within 5-10 minutes. In RAB11RNAi trypanosomes, however, even after 1 hour of incubation at 37°C a significant amount of endosomal fluorescence was visible. In conclusion, our analyses support the view that TbMBAP1 is essential for both routes of endomembrane traffic, endocytosis and exocytosis.

The expression of TbMBAP is developmentally regulated
The development of T. brucei from the bloodstream stages to the procyclic insect stage is accompanied by marked changes in cell surface protein expression and metabolism (Matthews et al., 2004). We have analysed the influence of developmental stage transition on the abundance and enzymatic activity of TbMBAP1. Trypanosome differentiation was induced as described by a temperature shift from 37°C to 27°C, and by the addition of 6 mM cis-aconitate (Brun and Schönenberger, 1981). As shown by immunoblotting, the amount of TbMBAP1 protein decreased within 72 hours to about 20-30% of the bloodstream stage level (Fig. 8A). Established procyclic cells contained about 6-fold less TbMBAP1 than bloodstream trypanosomes. The decline in TbMBAP1 expression was accompanied by a corresponding loss in TbMBAP1 enzyme activity (Fig. 8B). Remarkably, between 24 and 72 hours of differentiation the electrophoretic mobility of the protein was reduced from 72 kDa to about 65 kDa (Fig. 8A), which is caused by changes in N-glycosylation (D. Nolan and M.E., unpublished result). The decline in TbMBAP1 expression occurred with similar kinetics as the decrease in the rate of fluid phase endocytosis (Fig. 8C). Trypanosomes were harvested at different times during transformation, and endocytosis of fluorescent dextran was allowed to proceed for 15 minutes. Within the first 20 hours of differentiation, the endocytic rate did not change dramatically, but dropped to about 25% of the bloodstream stage level after 48 hours and to 10% after 3 days (Fig. 8C). In conclusion, these data demonstrate that, during development from the bloodstream to the procyclic insect stage of T. brucei, the downregulation of TbMBAP1 occurs parallel with a dramatic reduction in the rate of endocytosis.

Discussion
Here we report several remarkable findings related to a tartrate-sensitive histidine acid phosphatase of Trypanosoma brucei (TbMBAP1). First, TbMBAP1 is an endosomal membrane protein that changes its cellular localization stepwise as a function of protein abundance. Second, this protein has an essential function in the maintenance of endocytosis and exocytosis in mammalian stage trypanosomes. Finally, the significant decrease in endocytic activity that occurs during
glycoprotein (VSG117) with the transmembrane domain of the flagellar pocket membrane (Coppens et al., 1988). Böhme and Cross 1992). In contrast, ESAG 4, a type I transmembrane protein specific routed to the flagellum. This routing contrasts with overexpressed TfR, which distributes over the flagellum and the pellicular cell surface. In carefully controlled experiments, we could not detect TbMBAP1 on the cell body of trypanosomes, which express the protein in a range of concentrations as high as 90,000 molecules per cell, an expression level 10-fold higher than described for TfR (Engstler and Boshart, 2004). Flagellar pocket retention of all these proteins may involve common features such as protein modifications by N-linked glycans containing linear poly-N-acetyl-lactosamine (Nolan et al., 1999) or phosphodiester-linked glycans (Nozaki et al., 1996), which are recognized by lectins residing in the dense matrix of the flagellar pocket. Remarkably, when expressed at higher levels, TbMBAP1 is specifically routed to the flagellum. This routing contrasts with overexpressed TfR, which distributes over the flagellum and the pellicular cell surface. In carefully controlled experiments, we could not detect TbMBAP1 on the cell body of trypanosomes, which express the protein in a range of 80,000-160,000 molecules per cell. When we raised the expression level to above 160,000 molecules per cell, the phosphatase spread all over the plasma membrane but even at these high levels the enzyme predominantly localized to the flagellum.

Other type I transmembrane proteins, e.g. the invariant surface glycoprotein ISG65 and ISG75, are distributed over the whole cell surface in the wild-type situation [65,000 and 50,000 molecules per cell, respectively (Ziegelbauer et al., 1992)]. In contrast, ESAG 4, a type I transmembrane protein that functions as an adenylate cyclase, is exclusively found on the flagellar surface of the parasites (Paindavoine et al., 1992), while the LDL-receptor appears to be restricted to the flagellar pocket membrane (Coppens et al., 1988). Böhme and Cross replaced the GPI-anchor signal sequence of a variant surface glycoprotein (VSG117) with the transmembrane domain of

hypothesized, considering that TbMBAP1 lacks tyrosine- and di-leucine-based sorting motifs in its C terminus that are involved in membrane protein uptake in mammalian cells (Bonifacino and Traub, 2003; Heilker et al., 1999). In addition, genes corresponding to the adaptor complex, AP2, are absent from the trypanosome genome database. Whether the potential C-terminal acidic cluster sorting signal K\textsuperscript{R305}EEREEEPVIEEGV\textsuperscript{N325} is involved in TbMBAP1 trafficking between the flagellar pocket and endosomes is also questionable, because this type of motif is mainly found in transmembrane proteins that cycle between the trans-Golgi network (TGN) and endosomes (Bonifacino and Traub, 2003). Therefore, a systematic mutational analysis of the C terminus would be required, which may reveal novel, trypanosome-specific sorting motives required for concentrative uptake in clathrin-coated vesicles.

A three- to fourfold overexpression (40,000 molecules per cell) resulted in the appearance of TbMBAP1 in the flagellar pocket membrane, but, even at about 80,000 molecules per cell, the protein was undetectable on the flagellar and pellicular membranes. In contrast, the GPI-anchored transferrin receptor (TfR), which is retained in endosomes and the flagellar pocket at wild-type levels (about 3000 molecules per cell), escapes to the cell surface even at a modest (threefold) over-production. Mußmann et al. (Mußmann et al., 2003; Mußmann et al., 2004) have postulated a saturable flagellar pocket retention mechanism for TfR. Such a mechanism may involve recognition of structural determinants of the protein or covalently bound carbohydrate chains. The GPI-anchored, insect-stage-specific procytofin is retained in the flagellar pocket and in endosomes of bloodstream cells, at concentrations as high as 90,000 molecules per cell, an expression level 10-fold higher than described for TfR (Engstler and Boshart, 2004). Flagellar pocket retention of all these proteins may involve common features such as protein modifications by N-linked glycans containing linear poly-N-acetyl-lactosamine (Nolan et al., 1999) or phosphodiester-linked glycans (Nozaki et al., 1996), which are recognized by lectins residing in the dense matrix of the flagellar pocket. Remarkably, when expressed at higher levels, TbMBAP1 is specifically routed to the flagellum. This routing contrasts with overexpressed TfR, which distributes over the flagellum and the pellicular cell surface. In carefully controlled experiments, we could not detect TbMBAP1 on the cell body of trypanosomes, which express the protein in a range of 80,000-160,000 molecules per cell. When we raised the expression level to above 160,000 molecules per cell, the phosphatase spread all over the plasma membrane but even at these high levels the enzyme predominantly localized to the flagellum.

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ISG65 (Böhme and Cross, 2002). The recombinant protein was expressed at a concentration of about 50,000 molecules per cell, a similar level to the wild-type ISG65. We found that the VSG117-TM protein is located in endosomes, the flagellar pocket and, remarkably, the flagellum, while the native ISG65 is detectable all over the cell surface (M.E., U. Böhme and G. A. M. Cross, unpublished observation). Thus, two proteins carrying the same cytoplasmic tail and expressed at similar levels show a different localization. Finally, in an elegant demonstration, the differential sorting between the flagellum and the pellicular plasma membrane has been shown to depend on the structure of the cytosolic N-terminal domains for two related multi-membrane-spanning glucose transporters in Leishmania enrietti (Nasser and Landfear, 2004).

In summary, protein sorting in bloodstream forms of T. brucei between the endosomal system and the membranes of the flagellar pocket, the flagellum and the pellicular cell surface, depends on a multitude of parameters. These are (1) structural motifs in the cytoplasmic tails of transmembrane proteins and their interaction with adaptor complexes; (2) the structure of the extracellular domains (Wang et al., 2003) and, most likely, associated carbohydrates; (3) components such as lectins in the lumen of the flagellar pocket, which may serve as trapping devices of limited capacity; (4) machineries such as intra-flagellar transport particles or other components, which specifically regulate the communication between the flagellar pocket and the flagellum (Rosenbaum and Witman, 2002); (5) transport to the pellicular cell surface. The latter has been proposed to occur by default in promastigotes (Landfear and Ignatushchenko, 2001).

What is the function of the T. brucei acid phosphatase and homologous enzymes in other organisms? Knockout mice of a lysosomal tartrate-sensitive phosphatase have a very minor phenotype (Saftig et al., 1997). In L. mexicana, LmxMBAP has been shown to be neither required for the growth of the insect stage Leishmania promastigotes nor for infection of cultured macrophages or of mice (Benzel et al., 2000). Therefore, it was a surprise that TbMBAP1 is required for growth of bloodstream stage T. brucei. As the protein is unlikely to have a structural role, the phosphohydrolase activity itself is probably critical for function. Possible substrates could be phosphoproteins or lipids with a messenger function, such as lysophosphatidic acid (Moolenaar, 1995a; Moolenaar, 1995b).

The plasma membrane covering the flagellum is tightly associated with that of the cell body and with the sub-pellicular microtubule corset. In contrast, the membrane of the flagellar pocket is support-free on the cytosolic side. This unique situation implies that imbalances in the rates of endocytosis and exocytosis (ratio, r=1 under normal growth conditions) manifest themselves in changes in the size of the pocket, because the rest of the surface cannot readily contract or expand (Fig. 9). In fact, a dramatic increase in the size of the flagellar pocket occurs upon downregulation of clathrin or actin by RNAi (Allen et al., 2003; García-Salcedo et al., 2004) and, concomitantly, all endosomal TIR moves to the flagellar pocket membrane [see Fig. 3B in Overath and Engstler (Overath and Engstler, 2004)]. The same is also true for the EP:GFP reporter (Engstler and Boshart, 2004), which in wild-type cells shuttles between endosomes and flagellar pocket but accumulates in the flagellar pocket upon downregulation of clathrin. This behaviour is explained by an r <1, i.e. a shutdown of endocytosis with ongoing exocytosis. On the other hand, in RAB11 RNAi trypanosomes all EP:GFP is shuttled from the flagellar pocket to endosomes, suggesting an r >1, i.e. ongoing endocytosis but a decrease in the rate of exocytosis. The size of the pocket did not significantly shrink under these conditions.

wild type

TbCLH RNAi

TbRAB11 RNAi

TbMBAP RNAi

**Fig. 9.** Schematic representation of membrane flow between endosomes and cell surface. Membrane areas are drawn as spheres and are approximately to scale (based on Grünfelder et al., 2002). The green colour indicates the localization of the EP:GFP reporter. Dark arrows indicate membrane flow and light grey arrows symbolize the impaired flow of membrane caused by RNAi. F, flagellum; FP, flagellar pocket; E, endosomes. The dotted lines mark membrane compartments that are connected to either the pellicular microtubule cytoskeleton or the flagellar axoneme. In wild-type cells endo- and exocytic traffic are balanced. The EP:GFP reporter is shuttled between endosomes and the flagellar pocket. In trypanosomes depleted of clathrin heavy chain (TbCLH) endocytosis halts while exocytosis continues. This results in a specific enlargement of the flagellar pocket. The EP:GFP reporter is found exclusively in the flagellar pocket. After downregulation of TbRAB11 endocytosis continues while exocytosis is blocked, resulting in the clearance of EP:GFP from the flagellar pocket. Knockdown of TbMBAP1 affects both endocytosis and exocytosis.
conditions. There are two possible reasons for this apparent anomaly. Firstly, this is a circular process and interruption of membrane flow at any point will eventually halt the entire cycle. Secondly, the flagellar pocket is filled with a dense matrix that contains glycoproteins with giant poly-N-acetyl-lactosamine carbohydrate chains (Atrih et al., 2005), which may hinder a significant contraction of the pocket. Interestingly, the phenotype of the TbMBAP1 RNAi cells combines features from the RAB11 and clathrin knockdowns. As observed in RAB11-depleted cells, the EP:GFP reporter protein rapidly disappears from the flagellar pocket and the pocket size remains constant in the TbMBAP1 knockdown. In addition the loss of endocytic activity precisely mirrors that observed for clathrin-depleted cells. Therefore, we would like to suggest that TbMBAP1 has dual functions in endocytosis and in membrane recycling.

For almost 30 years, it was taken for granted that insect stage T. brucei ingest proteins to a lesser extent than mammalian bloodstream stages (Langreth and Balber, 1975). More recently, it was shown that endosomal marker proteins are down-regulated in the insect procyclic stage (Alexander et al., 2002; Jeffries et al., 2001; Morgan et al., 2001). However, a quantification of the changes in the abundance of endosomal proteins, coupled with measurements of cargo uptake during differentiation, was missing. We have compared protein abundance and enzyme activity of TbMBAP1 with the endocytic capacity during developmental transformation. Interestingly, the downregulation of endocytosis was accompanied not only by a loss of the acid phosphatase activity, but also by a marked change of the electrophoretic mobility of the TbMBAP1 protein. The nature of this putative protein modification and any functional implication remain to be elucidated. Investigations of the coordination of events that lead to the shut-down of endocytosis during T. brucei development appear to be an interesting prospect. TbMBAP1 may become an important tool in this endeavour.

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References


LmxMBAP2  ~~~MPYACLH GGSLCLLISL L....SCLGA QAVQTLHVLQ LVHRHGRASP
LmxMBAP MLRGGFLYR CVVVVVVAVV VSA..AVVSA APMYKVELVQ VVHRHGRASP
LmxSAP ~~~~~~~~~M ASRLVRVLAAMLVAAAVSV fårFVVRMVQ VVHRHGRASP

L271912  ~~~~~~~~~MRQ VLPFLLLAGL LCCPALVTA APDMKLVMVQ LIHRHGRATTA
LmxMBAP2 ~~~~~~~~~MRQ VLPFLLLAAP LCCPVALVTA APDMKLVMVQ LIHRHGRATTA
TbMBAP2  ~~~MPYACLH GGSLCLLISL L....SCLGA QAVQTLHVLQ LVHRHGRASP
TbMBAP1  MKERKGSWVN LAVCVFFFFT LHI....HSYAA QPTKTDLVQ LVHRHGRASP
LmxMBAP  MLRGGFLYR CVVVVVVAVV VSA..AVVSA APMYKVELVQ VVHRHGRASP
LmxSAP ~~~~~~~~~M ASRLVRVLAAMLVAAAVSV fårFVVRMVQ VVHRHGRASP

L271912  EPSYNKTQIC G.DTPCGYLT WSGIEMLSKT CAFLRSHYNT DASVSEPMF
LmxMBAP2 EPSYNKTQIC G.DTPCGYLT WSGIEMLSKT CAFLRSHYNT DASVSEPMF
TbMBAP2  KVKNQSQIC GEV.PCGYLN AACKMMLVNA GEFLRNHAYS NASE...PFF
TbMBAP1  LVPHNATEIC GGE.PCGSLT REGTLMLINT GKFRLHYNZ. NASSV...PFF
LmxMBAP LVDDNHTLCI GTFEPQCFGL YEQAMLVNL GKYLHHRYTE NPVSVPKFY
LmxSAP  LIDDNTTEIC GTLYPCGELT GCGVEMVRAI GEFARASYNN .LSLVESPLF

L271912  PSEDYLDVPAF SRTSTDVLRT LQSAESFLRG FPNPNTSLLP AIHTAPEQDD
LmxMBAP2 PSEDYDVDA YRSTDQVLR LQSAESFLRG FPNPNTSLLP AIHTAPEQDD
TbMBAP2  PESYNCSVT YRSTDQVRT LQSAEGLMRG MPUASFEFP AIHTADVSTD
TbMBAP1  PSTSYNLSVS HTESTVNRIT IQSAGELLGK LPFDENTFPP VVYTRYDRGN
LmxMBAP  PSWYNLISYT IRFSTDVLRT LQSANGLLOQ LPFNSTMFFP AIHAVGKED
LmxSAP  PSTRYNSSV LHRSTHTQRT IQSATAFLRG LFQD.DYFPP VVYSTNRTE

L271912  YILYTNYVPQ FPQFYSWLDMA GVRAVCNPVV DRNPFPDNTL TTIAQEVEYSE
LmxMBAP2 YILYTNYVPQ FPQFYSWLDMA GVRAVCNPVV DRNPFPDNTL TTIAQEVEYSE
TbMBAP2  WLLRYDVIPQ AYAFHSHELDE WWRNVCNPKL DT.LIDTNTL LSVSREVFSE
TbMBAP1  VLQQRYSPNY TAFLNLDVE WWRNVCNPNT D.IKYDTL LSISKEVSEF
LmxMBAP  VLLHNSYMVP IRARFNYAKE ELRAVCDEVIL DR.LMSFQKL QAARAVIHSQ
LmxSAP  TLLSTDAPVS VVGRSWLDNP ALHAALNPIV DEHL.SWDAI QSAAKDAWVE

L271912  GCYNFTTRRT DISAFTLDIAR VRSEKAIEGD NYPKLLKTRNR KLTQVAREHF
LmxMBAP2 GCYSDFTTRT DISAFTLDIAR VRSEKAIAGLE NYPKLLKTRNR RLSQVAREHF
TbMBAP2  GPCADPNQRC HCAMTFIDIA VAQSMGRID HPPLRTENLG LRLEDKFFED
TbMBAP1  GMCANPEDR HCQATLFDIG ASMEADEGRIA KHPLLQHVK QLRNVTEFCF
LmxMBAP  RFCANYTLSR RCAFRLCDGVR YEPTRGLLE SLPILLSRHD DVCAMTAMSS
LmxSAP  GLCADYNAAR NCVIDMYDVA AAFEAAGRLD NATLKLAVYP GLQEVAEEAF

L271912  ARQYYNRSD TRCFQQGSSG QPILEQEFVKN IGAAMAGSSR ..YKLYHYS
LmxMBAP2 ARQYYNRSD TRCFQQGSSG QPILEQEFVKN IGAAMAGTSS ..YKLYHYS
TbMBAP2  SHRFVYNASD RTHAKMGLG QHQAQELKDN AENHMGLTS ..YKLYHYS
TbMBAP1  REEFYNSSD KTHVNMSQGG QDLAQRILFN AESRANGTGT ..LKLYHYS
LmxMBAP  YFYFAYNASN PVQHKQGAPF YHLAACKVSN MVHAQQRETA PYPKLYEYSA
LmxSAP  KYVFSWNHNTS KDLTQTGSAS QNLAVTVLAN INAHRL...LS PSYNMQYSA

L271912  ADY...LGRGQGGSQ GPPQKQQVKN IGAAMAGSSR ..YKLYHYS
LmxMBAP2 ADY...LGRGQGGSQ GPPQKQQVKN IGAAMAGTSS ..YKLYHYS
TbMBAP2  SHRFVYNASD RTHAKMGLG QHQAQELKDN AENHMGLTS ..YKLYHYS
TbMBAP1  REEFYNSSD KTHVNMSQGG QDLAQRILFN AESRANGTGT ..LKLYHYS
LmxMBAP  YFYFAYNASN PVQHKQGAPF YHLAACKVSN MVHAQQRETA PYPKLYEYSA
LmxSAP  KYVFSWNHNTS KDLTQTGSAS QNLAVTVLAN INAHRL...LS PSYNMQYSA