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TbRAB1 and TbRAB2 mediate trafficking through the early secretory pathway of *Trypanosoma brucei*

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Abstract

The African trypanosome possesses a total of 16 small GTPases of the Rab family, which are involved in control of various membrane transport events. Recently the roles of these proteins in the endocytosis and recycling of the major surface antigen of the bloodstream form, the variant surface glycoprotein (VSG), have been described but little has been reported on the roles of Rab proteins in exocytic pathways in trypanosomatids. Whilst phylogenetic analysis based on sequence similarity indicates a comparatively well conserved core set of Rab proteins, the evolutionary distance of the trypanosome lineage from crown eukaryote model systems requires direct experimental evidence to support these sequence data. By database searching we identified two further Rab genes, TbRAB1 and TbRAB2, which are the trypanosome sequence orthologues of mammalian Rab1 and Rab2, important mediators of ER to Golgi and *intra*-Golgi transport processes. A remarkably high level of sequence conservation is retained between the trypanosome and higher eukaryote orthologues. By immunolocalisation we find that both TbRAB1 and TbRAB2 reside on membranes in intimate association with the Golgi complex. By heterologous expression in mammalian cells we also demonstrate conservation of targeting information in the TbRAB1 and TbRAB2 proteins, whilst TbRAB1, but not TbRAB2, can complement a Ypt1^{ts} conditional mutant in *Saccharomyces cerevisiae*. The roles of TbRAB1 and TbRAB2 in exocytosis were examined using RNAi. Suppression of TbRAB1 or TbRAB2 was strongly inhibitory to growth and most importantly both TbRAB1 and TbRAB2 were required for normal progression of VSG through the early secretory pathway. These data indicate conservation of function for these proteins between trypanosomes and crown eukaryotes.

Keywords: Trypanosoma; Small G protein; Vesicle trafficking; Exocytosis; Variant surface glycoprotein; Rab; Golgi complex

1. Introduction

The majority of proteins that enter the secretory pathway can be considered to belong to one of three distinct topological classes; soluble, transmembrane domain (TMD) anchored or glycosylphosphatidylinositol (GPI)-anchored. Study of exocytic systems in higher eukaryotes suggests specific transport requirements exist for each class and in particular in the early exocytic system. For example, GPI addition is essential for export of GPI proteins to the Golgi complex; only after the GPI-anchor has been attached are proteins allowed to exit the ER [1]. The specificity of this event has been studied intensively in *Saccharomyces cerevisiae*, where GPI-anchored proteins are transported from the ER to the Golgi complex via a distinct subset of vesicular carriers [2]; sorting is mediated by the Rab GTPase Ypt1, the tethering factor Uso1 and the Sec34/35 complex [3]. In mammalian cells sorting of GPI-anchored and TMD proteins occurs later in the Golgi complex [4].

Whilst in higher eukaryotes GPI-anchored proteins are a minority of the surface protein complement, in

Abbreviations: FP, flagellar pocket; FITC, fluorescein isothiocyanate; GPI-PLC, GPI-specific phospholipase C; GST, glutathione *S*-transferase; IFA, immunofluorescence analysis; ORF, open reading frame; PBS, phosphate buffered saline; TMD, transmembrane domain; VSG, variant surface glycoprotein

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trypanosomatids the GPI-anchor represents a dominant mode of membrane protein attachment. For example, the infective bloodstream form stage (BSF) of *Trypanosoma brucei* expresses at its surface 5×10^6 copies of the GPI-anchored protein variant surface glycoprotein (VSG) [5]. Because VSG is at a higher concentration in the Golgi complex and the cell surface compared to the ER, *T. brucei* must have efficient mechanisms for the sorting of GPI-anchored proteins [6].

It is not known if trafficking or sorting mechanisms of GPI proteins are identical throughout the eukaryotic lineage or if the bias in membrane attachment reflects distinct adaptations to protein transport systems. VSG is endocytosed from the cell surface via a clathrin-dependent mechanism that does not involve a concentration step [22]. This is an unusual mode of internalisation as in most organisms incorporation into clathrin-coated pits concentrates the protein via interaction with cytoplasmic receptors whilst many GPI-anchored proteins are internalised by clathrin-independent pathways. Endocytosis and recycling of VSG is dependent on TbRAB5A and TbRAB11 [20–22,27] but the roles of Rab proteins in the early secretory pathway has not been evaluated. Addition of the GPI-anchor is clearly important for efficient exocytosis of VSG [23–25].

Small GTPases of the Rab family play central roles in the regulation of vesicular trafficking and are involved in vesicular targeting, tethering and fusion [7–9]. The family is extensive with at least 60 members identified in mammalian cells, 11 in *S. cerevisiae* [10,11] and 16 in *T. brucei*. Rab proteins can be subdivided into clades, one of which is involved in early exocytic events, principally exit from the ER and progress through the Golgi complex [12]. In metazoans this group consists of Rab1 (A and B isoforms) and Rab2 (also A and B), suggesting a potentially complex network of pathways at the earliest stages of exocytosis [13–15]. In yeast a single Rab protein, Ypt1, is involved in trafficking between the ER and Golgi complex [16]. Additional to their roles in trafficking, mammalian Rab1A, Rab1B and Rab2A are also required for maintenance of Golgi structure [17–19]. A recent *in silico* analysis of the Rab GTPase complement of *T. brucei* using the completed trypanosome genome indicates a conserved core of Rab proteins that are likely involved in mediating the major exocytic and endocytic pathways (Ackers et al., in press). To date, only one GTPase, TbRABX1, has been implicated with a putative role in early steps of exocytosis [26]; however, re-examination with the complete *T. brucei* genome sequence indicates that TbRABX1 (formerly designated TbRAB2 [26], systematically named by Ackers et al., in press) is not a true orthologue of mammalian or yeast Rab proteins involved in ER to Golgi transport. Hence essentially no information on the role of Rabs in ER exit is available and the level of complexity associated with Rab function in early secretory events is currently unknown for trypanosomatids. Here we investigate two new trypanosome Rab proteins that are orthologous to Rab1 and Rab2 from mammals in order to gain insight into early protein export events.

2. Experimental procedures

2.1. Bioinformatics

DNA and protein sequences were aligned using default parameters in Clustal X and presented using SeqVu. Phylogenetic trees were constructed with PAUP 4.0*b10 using the heuristic search option and resultant trees subjected to 1000 replicate bootstrap analysis. The *T. brucei* genome database (http://www.sanger.ac.uk/Projects/T_brucei/) was screened by tBLASTx using HsRab1, HsRab1B, HsRab2, HsRab2B, ScYpt1, ScYpt31, ScSec4, TbRABX1 and TbRABX2 using the BLOSUM62 matrix.

2.2. Recombinant DNA manipulation

TbRAB1 and TbRAB2 were amplified from 427 *T. brucei* genomic DNA using primer combinations FwdRab1:RevRab1 and FwdRab2:RevRab2 (Table 1) and cloned into pGEX2TK (Pharmacia). RNAi p2T7 plasmids were constructed by amplifying the open reading frames (ORFs) for TbRAB1 and TbRAB2 and a 440 bp fragment of TbRABX1 from the respective pGEX2TK vector (pGEXT2TK.TbRABX1 is described in ref. [26]) with the primers FwdXSRab1:RevRNAiRab1, FwdRNAiRabX1:RevRNAiRabX1 and FwdXSRab2:RevRNAIRab2 and cloned into p2T7 using *Hin*dIII and *Xho*I sites. For yeast expression ORFs were excised from their respective pGEX2TK constructs using *Bam*HI and *Eco*RI and subcloned into pYES2 (Invitrogen). For expression in COS cells TbRAB1 and TbRAB2 ORFs were amplified from pGEX2TK constructs using the primer combinations FwdXSRab1:RevXSRab1 and FwdXSRab2:RevXSRab2 and cloned in frame with an N-terminal GFP into pEGFP-C3 (Clontech) using *Hin*dIII and *Bam*HI. pXS5:TbGRASP:GFP was generated by the excision of a TbGRASP:GFP fragment by partial digestion, from pXS2:TbGRASP:GFP (kind gift from Graham Warren and Cynthia He) with *Bam*HI and

Table 1
Sequences of synthetic oligonucleotides used in the constructs described here

Name	Sequence
FwdRAB1	GG**GGATCC**ATGTCTACAGAGTAC
RevRAB1	GG**GAATTC**TTAGCAACATCCACTC
FwdRAB2	GG**GGATCC**ATGCAGCAGCACCC
RevRAB2	GG**GAATTC**TCAGCAGAAGCAGCC
FwdXSRAB1	CCC**AAGCTT**ATGTCTACAGAGTA
RevXSRAB1	GG**GGTACC**TTAGCAACATCCACTC
RevRNAiRAB1	CCG**CTCGAG**TTAGCAACATCC
FwdRNAiRABX1	GG**CTCGAG**ATGATCACAGCAGCT
RevRNAiRABX1	GG**AAGCTT**TTAACAGCAAACACC
FwdXSRAB2	CCC**AAGCTT**ATATGCAGCAGCACC
RevXSRAB2	GG**GGTACC**TCAGCAGAAGCAGCC
RevRNAiRAB2	CCG**CTCGAG**TCAGCAGAAGC

Restriction sites are indicated in bold. Typically the sites are *Bam*HI (GGTACC), *Eco*RI (GAATTC), *Hin*dIII (AAGCTT) and *Xho*I (TCGAGA). Sequences are written 5′ to 3′.

*Hin*dIII and direct cloning into *Bam*HI and *Hin*dIII digested pXS5.

2.3. Cell culture

PCF 29-13 and BSF 13-90 *T. brucei* Lister 427 strain cell lines were used throughout. Cells were grown in SDM79 or HMI9 media as appropriate [29], and cultured in the continuous presence of 25 $\mu g\,ml^{-1}$ G418 and 25 $\mu g\,ml^{-1}$ hygromycin (PCFs) or 2 $\mu g\,ml^{-1}$ G418 and 5 $\mu g\,ml^{-1}$ hygromycin (BSFs). Cell number was determined with a Z2 Coulter Counter, averaging at least three measurements. COS cells were grown in DMEM (Gibco) supplemented with 10% FCS at 37 °C/5% CO_2 using vented tissue culture flasks (Corning). *S. cerevisiae* were grown at 25 °C on a shaking platform (250 rpm) in YPD (1% yeast extract, 2% peptone, 2% dextrose) or SC media (synthetic minimal defined media); 0.67% yeast nitrogen base (without amino acids), 2% carbon source (glucose or galactose), supplemented with the desired amino acids or drop out media to maintain the pYES2 plasmid. To grow yeast on plates, 2% agar was added to the media.

2.4. Transfection

Trypanosomes were transfected as described [29]. Selection was applied (Zeocyin 2.5 $\mu g\,\mu l^{-1}$ for p2T7 constructs or 2 $\mu g\,\mu l^{-1}$ G418 for pXS5 constructs) after 16 h (PCFs) or 6 h (BSFs). COS cells were transfected using FuGENE.

2.5. Yeast complementation

Yeast were grown to late log phase in SC (containing glucose) supplemented with drop out media (omitting Uracil to maintain the pYES2 construct) overnight on a shaking platform (250 rpm) at 25 °C. Protein expression was induced by diluting the culture into induction media (SC media containing Galactose as a carbon source, supplemented with drop out media as above) and growing for 16 h at 25 °C on a shaking platform (250 rpm). Functional complementation was assayed by diluting the cells to an OD_{600} of 1 into either inducing or non-inducing media. The cells were grown for 8 h before serial dilutions (10×) were made and 20 μl of each dilution spotted onto appropriate media plates. Plates were grown at the permissive temperature (25 °C) or at the non-permissive temperature (37 °C). Complementation was analysed by comparing growth of induced and non-induced colonies at the non-permissive temperature (37 °C).

2.6. Polyclonal antisera production

TbRAB1 and TbRAB2 were expressed as GST-fusion proteins from pGEX-2TK in the *E. coli* strain BL21 (Stratagene). Fusion proteins were purified using glutathione-Sepharose 4B (Pharmacia). Purified recombinant protein was used to raise polyclonal antibodies in rabbits as described [28]. Affinity purified antibodies were isolated using recombinant protein coupled to CNBr-activiated Sepharose 4B (Pharmacia) by standard methods.

2.7. Immunochemistry

Trypanosome cell pellets (1×10^7 cells) were resuspended in 100 μl of 4× sample buffer, boiled and resolved by reducing SDS-PAGE. Proteins were transferred to nitrocellulose membranes by wet transfer under standard conditions for 16 h. Nonspecific binding sites were blocked in BLOTTO (0.1% Tween-20, 5% freeze dried milk, phosphate buffered saline pH 7.6 (PBS)). Antibodies were also diluted in BLOTTO. Rabbit anti-TbRAB1, anti-TbRABX1 [26], anti-TbRAB2 and anti-TbBIP (kind gift from Jay Bangs) were used at dilutions of 1:1000, 1:200, 1:500 and 1:5000, respectively, and detected with goat anti-rabbit horseradish peroxidase conjugate (Sigma). Bound conjugate was detected with ECL reagent (Sigma).

2.8. Immunofluorescence microscopy

Trypanosomes were harvested by centrifugation for 10 min at 800 × *g*, washed once in Voorheis's modified PBS (vPBS; PBS supplemented with 10 mM glucose and 79 mM sucrose, pH 7.6) and fixed with 3% paraformaldehyde (w/v) (in vPBS) for 1 h on ice (PCFs) or 10 min (BSFs). After fixation cells were applied to poly-lysine slides for 20 min (Sigma), permeabilised in 0.1% Triton X-100 for 10 min and then blocked in 20% FCS (in PBS) for an hour. Primary antibodies were used at the following concentrations: anti-TbRAB1 (1:100), anti-TbRAB2 (1:100), and secondary antibodies following the manufacturer's instructions. Slides were washed and mounted in Mowiol (Calichem) containing 1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI) to visualise DNA. COS cells were grown on coverslips, washed three times in PBS, fixed in methanol at −20 °C for 5 min and blocked (20% FCS in PBS) at room temperature. Primary antibody was diluted in block (mouse monoclonal anti-Golgin-97, 1:200; anti-PDI, 1:200; Molecular Probes) and incubated with the cells at room temperature; secondary antibodies were used according to the manufacturer's instructions. The cells were washed and mounted in Mowiol. Cells were examined using a Nikon ECLIPSE E600 microscope and images captured using a Photometrics CoolSNAP FX camera and false coloured using MetaMorph 5.0 software (Universal Imaging Corporation). Note that the different fluorescent channels were shown to be in register by double staining for the same antigen with two different secondary antibodies (data not shown).

2.9. Transmission electron microscopy

Thin section and cryo-electron microscopy were performed as described [29].

2.10. Concanavalin A endocytosis

Parasites were harvested at mid-log phase, washed once and resuspended at a density of 1×10^7 parasites ml^{-1} in serum free media pre-equilibrated at 4 °C. Hundred micrograms of ConA-FITC (Vector Labs) was added per 10^7 cells and parasites incubated for 10 min on ice. Subsequently, parasites were washed at 4 °C in serum-free media, resuspended in media pre-equilibrated at the desired temperature and incubated for 30 min. Parasites were then washed in PBS at 4 °C and fixed with 3% paraformaldehyde (w/v) (in vPBS) for 10 min on ice and processed for immunofluorescence as above.

2.11. VSG export assay

VSG transport was monitored exactly as described [29].

3. Results

3.1. In silico identification of candidate Rab proteins of the exocytic pathway of trypanosomes

The *T. brucei* genome was screened with Rab sequences with known roles in transport from the ER to the Golgi complex; HsRab1, HsRab1B, HsRab2, HsRab2B, Scypt1, Scypt31 and ScSec4. TbRABX1 and TbRABX2 were also used as prior work has shown that these two proteins associate with the trypanosome ER and Golgi complex, respectively [30]. Note that the ORF originally designated as TbRAB2 [26] has recently been systematically renamed TbRABX1 and the new nomenclature will be used throughout this report (Ackers et al., in press).

These efforts yielded two new open reading frames (ORFs) for trypanosome Rabs possibly involved in exocytosis. The human and yeast orthologues were identified by reverse-BLAST at NCBI (www.ncbi.nlm.nih.gov/BLAST/) which demonstrated that one ORF was a Rab1 orthologue and the second ORF a Rab2 orthologue. These new members of the trypanosome Rab family were designated TbRAB1 and TbRAB2.

The predicted protein sequences were aligned with selected higher eukaryote orthologues (Fig. 1A and B). TbRAB1 and TbRAB2 have extremely high homology with their mammalian orthologues; TbRAB1 is 73% identical and 81% similar to *Homo sapiens* Rab1 and TbRAB2 is 67% identical and 79% similar to HsRab2 (Fig. 1C). This is the highest level of sequence conservation observed for trypanosome Rabs compared to their mammalian orthologues and suggests particularly strong selective pressure. This conclusion is further supported by inspection of the alignments; all domains characteristic of Rab proteins and in particular the GTP-binding motifs and the effector regions, are extremely well conserved. By contrast TbRABX1 is only 40% identical and 60% similar to HsRab2 whilst the effector domains of TbRAB2 and TbRABX1 are divergent indicating that they likely interact with distinct downstream molecules, and hence have distinct functionality.

The evolutionary relationships between TbRAB1, TbRAB2, TbRABX1 and their mammalian homologues were analysed by phylogenetic reconstruction (Fig. 1D). The Rab1 and Rab2 sequences are monophyletic, indicating that these Rab subfamilies arose prior to the speciation event separating trypanosomes from higher eukaryotes and implies conservation of function for Rab1 and Rab2 throughout the eukaryotic lineage. By contrast, TbRABX1 does not share an ancestral relationship with either TbRAB2 or HsRab2. Overall, these data indicate that TbRAB1 and TbRAB2 represent evolutionarily conserved members of the Rab1 and Rab2 family.

3.2. Expression of TbRAB1 and TbRAB2

To investigate the expression patterns of TbRAB1 and TbRAB2 both ORFs were expressed in *E. coli* as GST-fusion proteins that were then used to raise polyclonal rabbit antisera. By western blot affinity purified antibodies from these sera recognised bands of 25 kDa in both life stages, consistent with the predicted molecular weights of TbRAB1 and TbRAB2 (Fig. 2A and data not shown). These bands could be competed with recombinant GST-TbRAB1 and TbRAB2, respectively, but not with the non-cognate TbRAB fusion protein (data not shown). When the signal intensity of TbRAB1 and TbRAB2 immunoreactivity in PCF and BSF life stages was normalised to a loading control (TbBIP), similar intensities were obtained for both TbRAB1 and TbRAB2 (data not shown). Therefore TbRAB1 and TbRAB2 are not subject to major developmental regulation.

Immunofluorescence microscopy confirmed that both TbRAB1 and TbRAB2 are expressed in BSF and PCF parasites. Both proteins were predominantly detected on compartments between the nucleus and the kinetoplast, suggestive of localisation to a subcompartment of the trafficking system (Fig. 2B). TbRAB1 exhibited a more extensive staining pattern in BSF than in PCF life stages and conversely TbRAB2 immunoreactivity was more extensive in the PCF. Both TbRAB1 and TbRAB2 show a staining pattern significantly distinct to that of TbRABX1 [26], indicating that TbRAB1 and TbRAB2 define separate endomembrane microdomains to TbRABX1.

3.3. Subcellular localisation of TbRAB1 and TbRAB2

Staining of cryogenic sections of BSF parasites with anti-TbRAB1 antibodies followed by gold-labelled secondary antibodies identified reticular membranes and a stacked set of cisternae as the major organelles labelled with gold (Fig. 3A and B). These are most likely to be components of the ER and Golgi complex, which is consistent with the location as determined by IFA and also as predicted based on homology

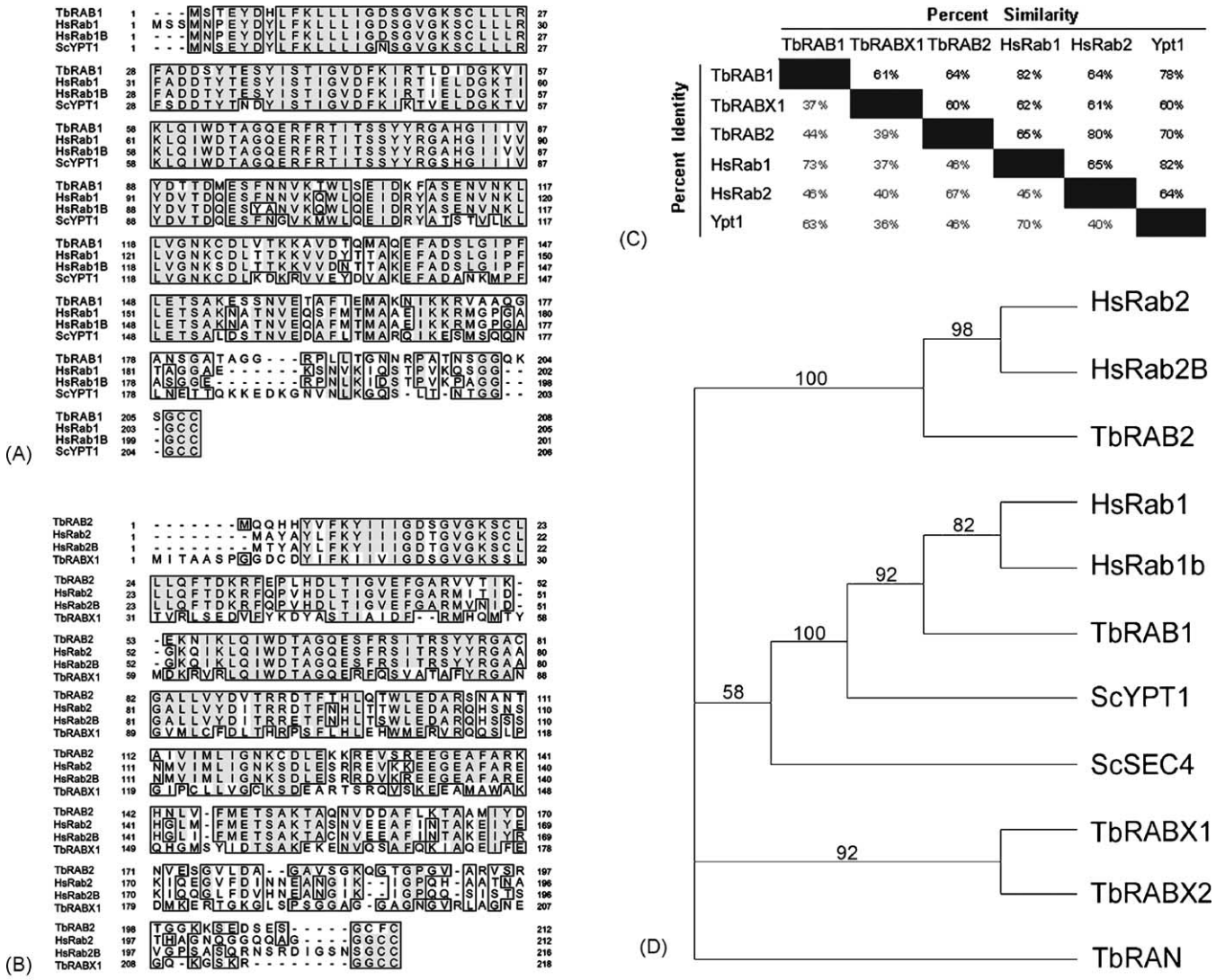


Fig. 1. TbRAB1 and TbRAB2B are highly conserved with their higher eukaryote orthologues. (Panel A) Clustal X alignments of hypothetical translations of TbRAB1, HsRab1, HsRab1B and Scypt1, demonstrating the similarity within this group of proteins. TbRAB1 has a consensus sequence for prenylation at its carboxyl terminus. Note that all the GTPase-binding motifs are conserved and the only region of significant divergence is within the C-terminal hypervariable region. (Panel B) Clustal X alignments of hypothetical translations of TbRAB2, HsRab2, HsRab2B and TbRABX1, demonstrating a number of conserved features in this group of proteins. TbRABX1 shows divergence within the effector domain. Again for TbRAB2, divergence between the higher eukaryote and trypanosome sequences resides mainly within the C-terminus. Similarities are shown boxed, identities shaded. (Panel C) Comparison of the similarities and identities between TbRAB1, TbRAB2, TbRABX1, HsRab1, HsRab2 and ScYpt1. (Panel D) Phylogenetic reconstruction of the Rab1 and Rab2 family using sequences from *T. brucei* and crown eukaryotes. Analysis was done with PAUP 4.0* [33], using the heuristic algorithm with 1000 bootstraps (values shown on the internodes). TbRAB1 and TbRAB2B share common ancestry with their mammalian orthologues, indicating an ancient origin.

to HsRab1, itself located mainly at ER-exit sites and on the Golgi stack. We also detected TbRAB1 reactivity on a number of additional tubular structures; these cannot be unambiguously assigned based on the data presented here, but based on their morphology and position they are most probably ER-associated membranes. Unfortunately, multiple attempts to obtain immunoEM images with antibodies to TbRAB2 were uniformly unsuccessful; this likely reflects the low abundance of some Rab proteins and specific loss of reactivity for TbRAB2 in the cryoEM procedure in particular.

In order to compare the locations of TbRAB1 and TbRAB2 with the position of the Golgi complex we exploited a recently identified Golgi structural protein, TbGRASP. This protein has been characterised in detail as a GFP-fusion protein and faithfully targets to the stacked cisternae of the trypanosome Golgi complex [34]. GRASP proteins are also effectors of Rab proteins in mammalian cells and involved in early steps in exocytosis [18,19]. A BSF strain expressing TbGRASP-GFP was counterstained with anti-TbRAB1 antibodies. TbRAB1 was localised to structures in very close juxtaposition with TbGRASP, suggesting association with membranes close to and including part of the Golgi complex (Fig. 4). In higher eukaryotes Rab1 is found predominantly on the *cis*-face of the complex associated with

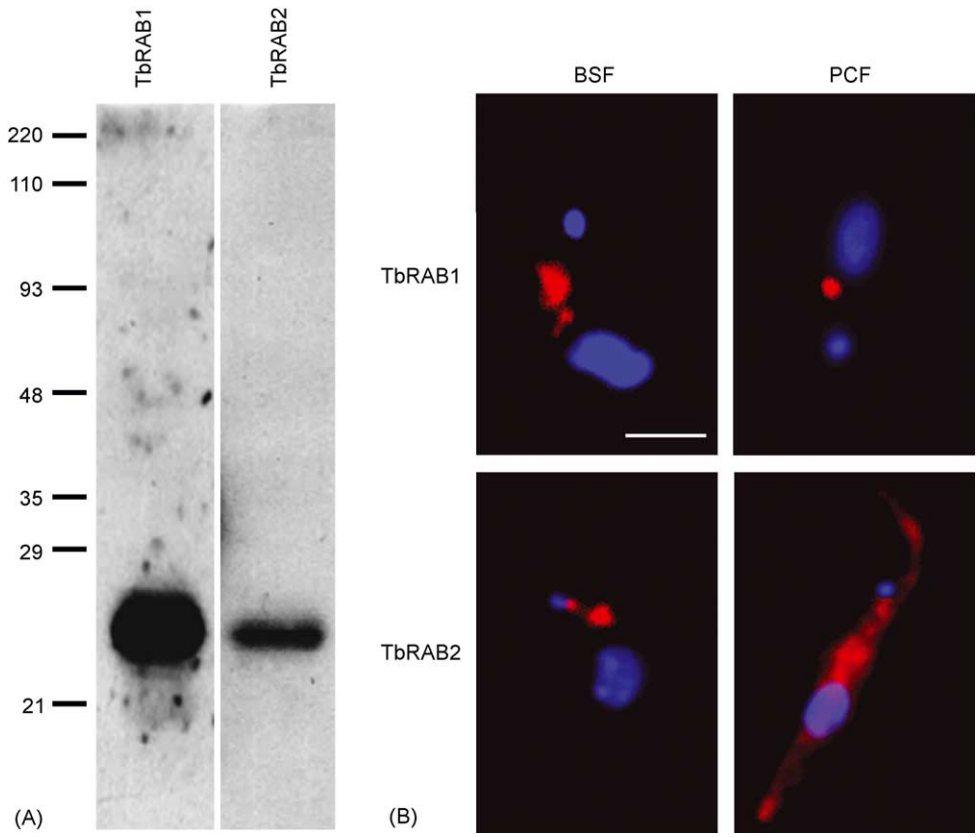


Fig. 2. TbRAB1 and TbRAB2 are constitutively expressed proteins. (Panel A) Western blot analysis of TbRAB1 and TbRAB2 expression in BSF parasites using affinity purified antibodies. TbRAB1 and TbRAB2 migrate at ∼25 kDa on reducing SDS-PAGE gels. (Panel B) Immunofluorescence microscopy of TbRAB1 and TbRAB2. Cells were fixed, permeabilised and then stained with primary and secondary antibodies. Left panels show BSF parasites and the right panels show PCF parasites. DNA is visualised with DAPI (blue), TbRAB1 (Top panels) or TbRAB2 (bottom panels) are in red. Note the clear staining of TbRAB1 and TbRAB2 in regions between the kinetoplast and the nucleus. Scale bar = 2 μm.

transport structures originating from the ER, and is consistent with the localisation obtained for TbRAB1. TbRAB2 was also in close juxtaposition to TbGRASP (Fig. 4). Hence both TbRAB1 and TbRAB2 are located on membranes close to the Golgi complex, whilst our immunoEM data indicate that the location of TbRAB1 extends into the Golgi stack itself.

To eliminate the possibility that the tubular structures identified by electron microscopy were part of the endocytic machinery rather than the exocytic system, BSF parasites were assayed using the lectin Concanavalin A (ConA) as a probe for general membrane uptake [21,29]. Neither TbRAB1 or TbRAB2 co-localised with ConA that had accumulated in the lysosome; some overlap in signal for TbRAB1 and ConA was not coincident in the *z*-plane and it was clear that the structures were fully distinct (Fig. 4 and data not shown). In addition the location of TbRAB1 and TbARB2 immunoreactivity did not resemble that for authenticated early endosome markers. These observations suggest that neither TbRAB1 nor TbRAB2 participate in endocytic trafficking, again consistent with the function predicted by sequence homology.

3.4. Evolutionary conservation of TbRAB1 and TbRAB2 targeting

The precise mechanism of Rab protein targeting remains unknown but likely requires information from several regions of the proteins. Due to the very high levels of sequence identity of TbRAB1 and TbRAB2 with their mammalian orthologues, we asked if these evolutionary divergent Rab family members were correctly targeted in a higher eukaryote system. This approach would indicate if the targeting regions of TbRAB1 and TbRAB2 were included in the conserved or non-conserved regions of the protein, and also provide insight into the locations of targeting information within TbRAB proteins. Previous work demonstrated a location at the ER–Golgi intermediate compartment for TbRABX1 when expressed in COS cells [26].

Both TbRAB1 and TbRAB2B were tagged at the amino terminus with GFP and transiently transfected into COS cells and analysed by immunofluorescence. Similar to overexpression of mammalian Rab1, TbRAB1 is found to localise on the same structures as Golgin-97, a marker of the Golgi complex (Fig. 5) and not with PDI, a marker for ER exit sites. Only a

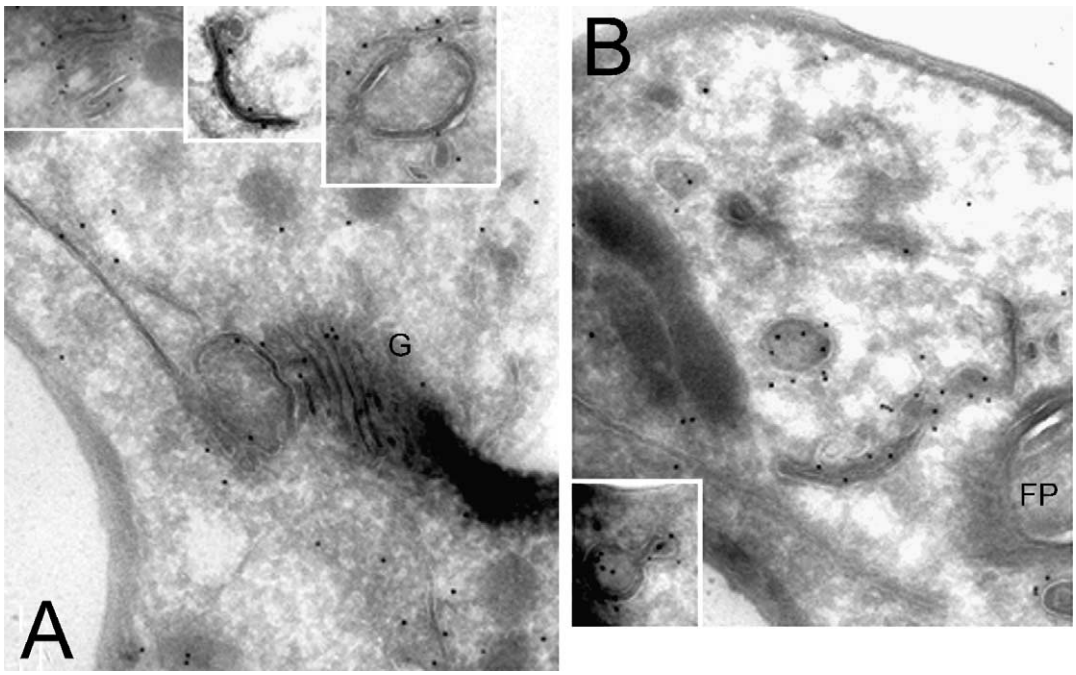


Fig. 3. TbRAB1 associates with compartments of the early secretory pathway. Cryo immunoEM analysis of BSF parasites with affinity purified anti-TbRAB1 followed by gold-conjugated secondary antibodies. TbRAB1, visualised by gold particles, is seen in close association with the Golgi complex and reticular structures (A), and in close association with vesicles and tubular compartments (B). Insets show additional membrane figures associated with TbRAB1 immunoreactivity. G, Golgi complex; FP, flagellar pocket.

small fraction of TbRAB2 co-localised with Golgin-97; instead TbRAB2 had a reticular distribution, including some localisation to the nuclear envelope, consistent with the ER plus a small population on Golgi-associated membranes. Some colocalisation with PDI was also obtained; as the Rab protein is cytosolic and PDI luminal, this is consistent with an ER location.

These data suggest that TbRAB1 and TbRAB2 are targeted to membrane components of the early exocytic system in mammalian cells. For TbRAB1, the location in the COS cells was highly similar to the endogenous protein in the trypanosome and is consistent with the extremely high level of conservation of primary structure (Fig. 1). In the case of TbRAB2 the extensive ER location in COS cells is at some variance with the more restricted location of the protein in the homologous trypanosomes system. Hence targeting is likely only partially conserved between mammalian and trypanosome Rab2 and the ∼30% of divergent residues between the human and trypanosome orthologues presumably contribute to intracellular targeting. Significantly, the greatest region of divergence is the C-terminus, which is considered to be the primary determinant for specifying location.

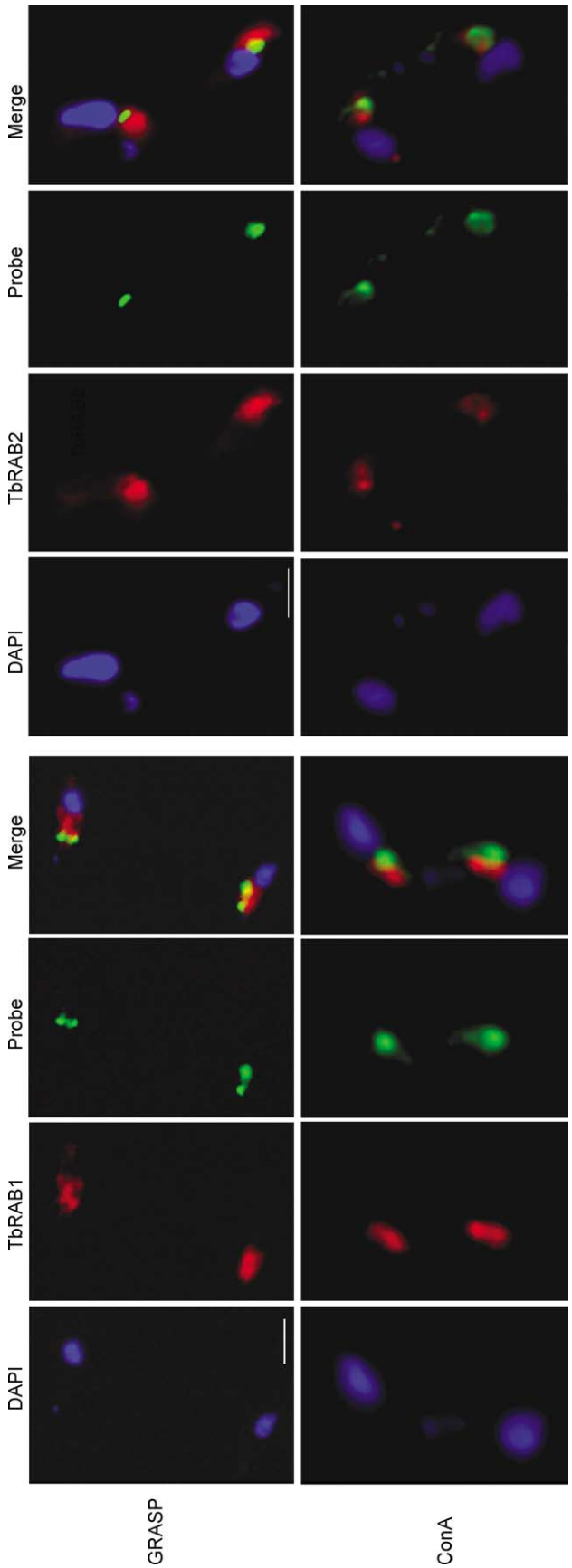
3.5. TbRAB1 is a functional homologue of Ypt1

To further define the functions of TbRAB1 and TbRAB2, yeast complementation studies were performed. Ypt1 is the *S. cerevisiae* orthologue of Rab1. Yeast strain Ypt1–3 contains a $Ypt1^{ts}$ mutant allele, with a severe growth defect at the non-permissive temperature. Strain Ypt1–3 was transformed with yeast expression constructs (pYES2) containing the TbRAB1, TbRAB2 or TbRABX1 ORFs and restoration of growth at the nonpermissive temperature analysed following induction. All transformed strains were able to propagate normally at the permissive temperature but under non-permissive conditions only TbRAB1 was able to partially restore growth (Fig. 6). These observations indicate that TbRAB1 is a functional homologue of Ypt1 whilst TbRAB2 and TbRABX1 are not. These data further confirm the assignments based on primary structure.

3.6. TbRAB1 and TbRAB2 are required for normal growth and morphology

To directly analyse the functions of TbRAB1, TbRAB2 and TbRABX1 we used RNAi. dsRNAs corresponding to portions of each of the three ORFs were expressed from the inducible p2T7 vector in BSF cells harbouring the Tet repressor protein and T7 polymerase. Growth curves were obtained by counting cells at intervals in both induced and non-induced cultures in three separate experiments. Suppression of the expression of each TbRAB protein was achieved by this procedure and verified by Western analysis (Fig. 7).

Reduced levels of TbRAB1 in PCFs resulted in growth inhibition after 48 h. By contrast, severe growth inhibition was observed as early as 24 h post induction in BSF cells. A reduction in growth and cell death was also observed in TbRAB2 RNAi cells; 24 h post induction in BSFs and 4 days post



induction in PCFs. In PCFs we also observed a reproducible slow growth in the uninduced cells; this may be the result of leakiness in this particular cell line, but at 7 days a clear and specific growth defect was apparent in the induced cells. TbRABX1 suppression showed no significant alterations to growth in BSF, but had a delayed and minor effect on PCF growth at 5 days post induction. Overall, these data indicate that TbRAB1 and TbRAB2 are required for normal cell growth in both major life stages, whereas TbRABX1 does appear to be required for normal cell proliferation in vitro.

By electron microscopy suppression of TbRAB1 was found to generate a distended Golgi complex with the *trans*-most cisternae engorged (the *trans*-face is the concave face of the structure) (Fig. 8A). The Golgi stack was unaffected by TbRAB2 RNAi, indicating that the effect was specific (Fig. 8B). These observations provide evidence for the involvement of TbRAB1 in the maintenance and/or the assembly of the trypanosome Golgi complex. The TbRAB2 RNAi cells accumulated vesicular structures, which may be transport intermediates but with the current rudimentary state of knowledge of the secretory system in trypanosomes cannot be assigned further. Significantly the ER and Golgi complex remained unaffected (Fig. 8B). No morphological defects were seen in the TbRABX1 cells (data not shown).

3.7. TbRAB1 and TbRAB2 are required for export of VSG

VSG is an excellent marker for monitoring protein export to the cell surface. The VSG polypeptide can be rapidly released by cleavage of the GPI-anchor by an endogenous GPI-phospholipase C (GPI-PLC), a reaction that occurs only at the cell membrane following lysis and does not affect molecules in transit to the cell surface [30]. VSG may be quantitatively recovered using the mannose-binding lectin Concanavalin A [29].

BSF TbRAB RNAi mutants were induced to allow suppression of protein but prior to the onset of secondary alterations. TbRAB1 mutants were induced for 12 h, TbRABX1 for 16 h and TbRAB2 for 20 prior to analysis. Parasites were pulse-labelled with ^{35}S Met/Cys for 5 min and then chased for up to 1 h. ConA was used to specifically isolate glycoproteins present in the supernatant and membrane fractions at various time points. For each cell line the ratio at each time point between the band intensities of VSG in the supernatant and in the pellet fraction was calculated and plotted relative to

Fig. 4. TbRAB1 and TbRAB2 are in close association with Golgi membranes. In each gallery, the extreme left panels show DNA, visualised with DAPI (blue), middle left panels show TbRAB1 (red), middle right panels show either TbGRASP or ConA (green) and extreme right panels are a merged image. TbRAB1 and TbRAB2 immunoreactivity is found in close juxtaposition with Golgi membranes as visualised with TbGRASP. Neither TbRAB protein has any association with endosomal compartments as defined by accessibility to ConA. Scale = 2 μm.

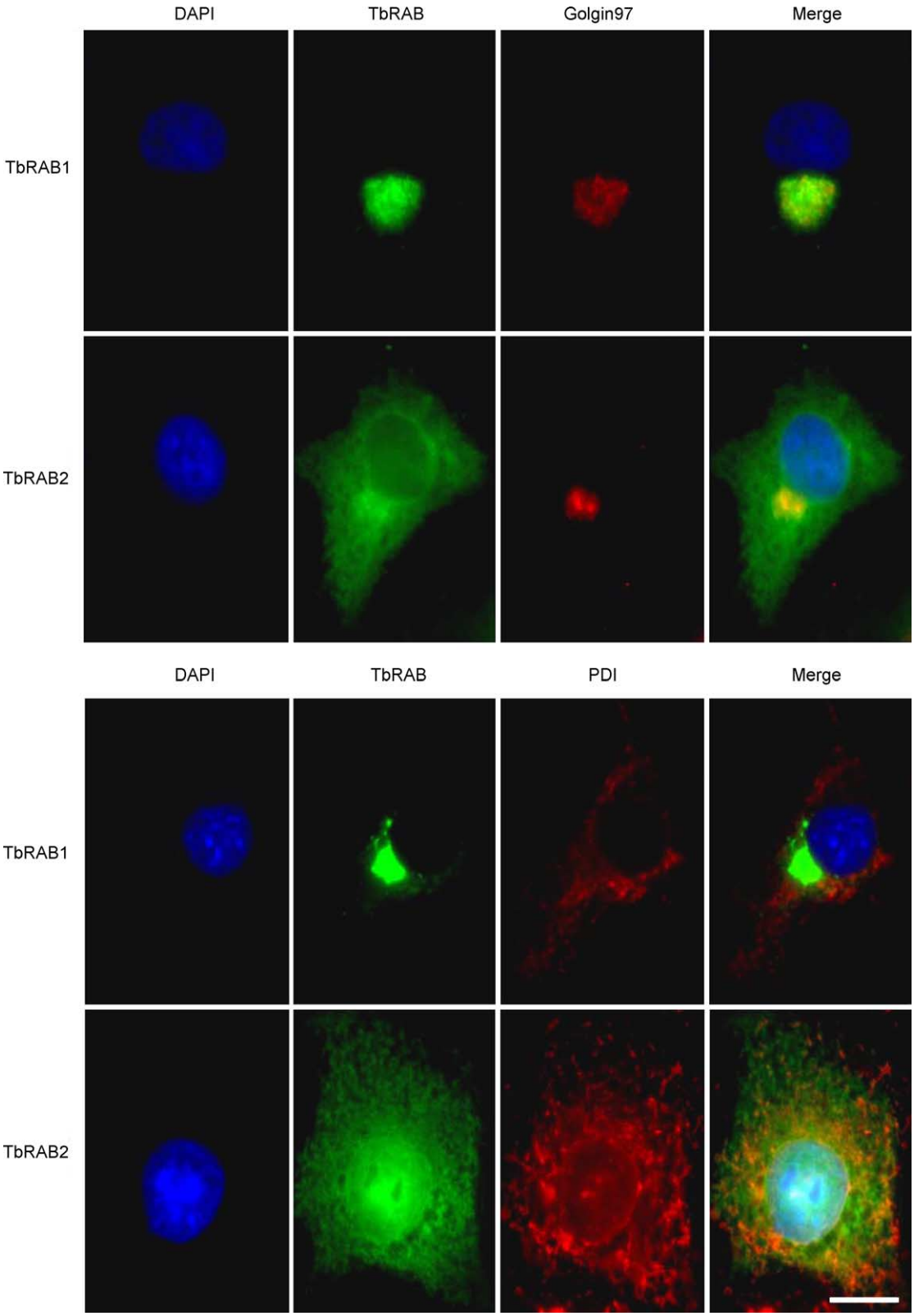


Fig. 5. TbRAB1 and TbRAB2 are differentially targeted in mammalian cells. COS cells were transfected with N-terminally GFP-tagged TbRAB1 or TbRAB2 (green). Transfected cells were processed for IFA and counterstained with anti-Golgin 97 (red, upper panels) or anti-PDI (red, lower panels). DNA is in blue. TbRAB1 shows colocalisation with Golgin 97, consistent with localisation at the Golgi complex (top of upper set of panels). TbRAB2 has a reticular staining and partial overlap with PDI, indicative of a predominantly ER location (bottom of lower set of panels). Scale = 15 μm.

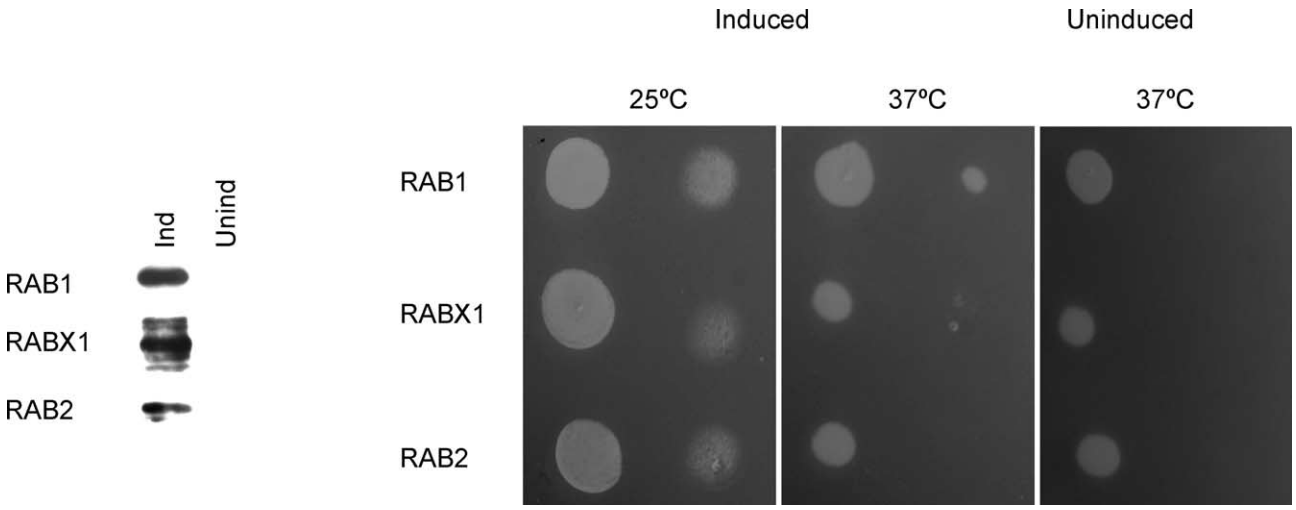


Fig. 6. TbRAB1 complements *Saccharomyces cerevisiae* Ypt1. Expression of TbRAB proteins were induced in *S. cerevisiae* and cell lysates (1×10^7) analysed by Western blot. Effective induction of TbRAB1, TbRAB2, and TbRABX1 was seen (Ind: induced, Unind: uninduced). Complemented *S. cerevisiae* strains were induced at 25 °C (the permissive temperature) and not diluted or diluted 10-fold (left to right), plated and grown at 25 °C (left panel) or at 37 °C (middle panel). An uninduced control was grown at 37 °C (right panel). TbRAB1 can partially complement the $Ypt1^{ts}$ as seen by a restoration to the growth deficit at 37 °C. Neither TbRAB2 nor TbRABX1 could restore growth. The small artefact in the induced TbRABX1 image is a bubble in the agar.

the ratio at time 0 (Fig. 9). At later times the soluble fraction approaches 100% but due to small levels of residual VSG in the cell pellet, does not reach it.

Overall, the time taken for 50% of labelled VSG to reach the surface was 4.1 (±0.3) min in the control cell line, similar to that in TbRABX1 RNAi, which took 4.3 (±0.4) min and broadly consistent with published values [29,30]. However VSG export had a halftime of 7.8 (±0.1) and 7.1 (±0.3) min in TbRAB1 and TbRAB2 RNAi cells, respectively. These observations show a reduction of ∼50% in the rate of VSG transport to the surface indicating that export of VSG through the exocytic pathway requires both TbRAB1 and TbRAB2. By contrast, this analysis suggests that TbRABX1 has no or only a minimal role in the trafficking of VSG. It is possible that VSG export could be altered once a cell begins to die; however prior work has demonstrated that no significant alterations to

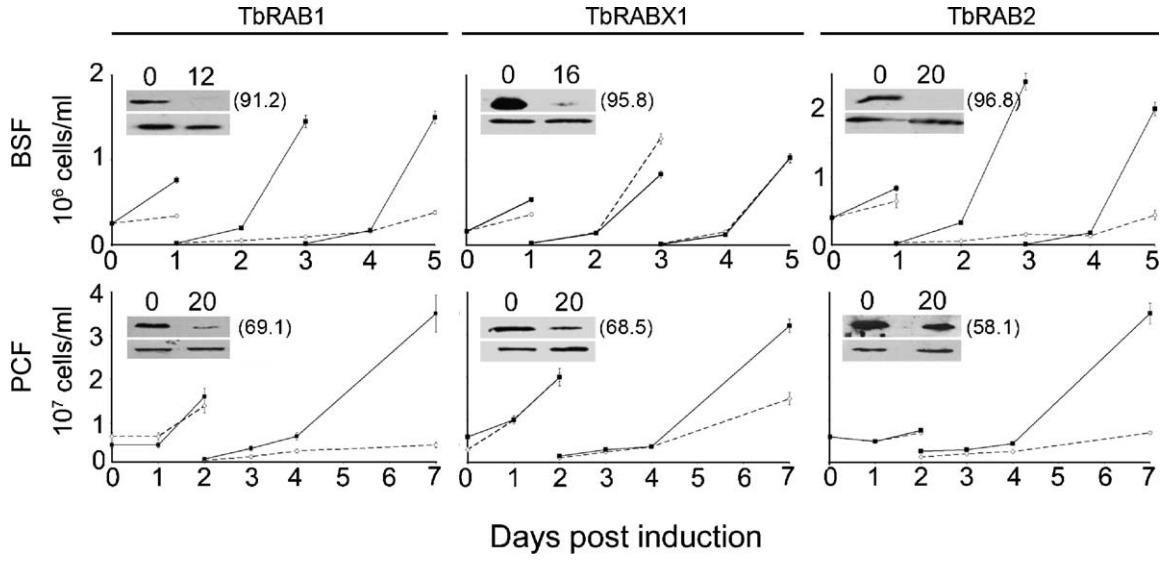


Fig. 7. RNAi indicates that TbRAB1 and TbRAB2 are essential. Growth curves of bloodstream and procyclic form cultures following induction of TbRAB1, TbRAB2 and TbRABX1 RNAi with tetracycline. Cultures were monitored daily for cell number. Noninduced cultures are shown in solid lines, and induced in broken lines. Error bars indicate the standard error and in some cases are obscured by the plot symbol. TbRAB1 suppression results in growth inhibition after 24 h in BSF and 48 h post induction in PCF, TbRABX1 has no significant effect on growth in BSF or PCF and TbRAB2 suppression results in growth inhibition after 24 h in BSF and 4 days in PCF. Insets show western blots demonstrating decreased levels of various Rab proteins (top panel of each inset; Rab protein, bottom panel; TbBiP, loading control); note that suppression is better than 90% for BSFs where the subsequent analysis has been performed. The figure in parenthesis is the percent suppression, normalised to the loading control.

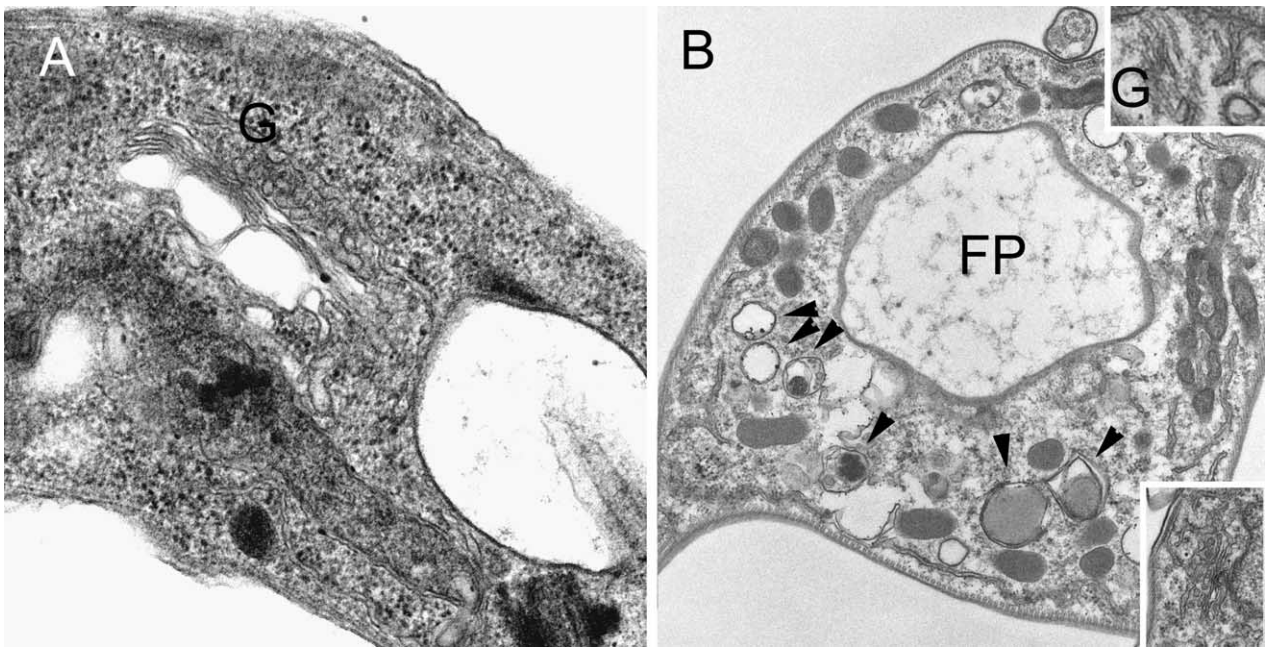


Fig. 8. TbRAB1 and TbRAB2 are required for normal endomembrane morphology. Ultrastructural analysis of TbRAB1 and TbRAB2 RNAi mutants was carried out using TEM. Suppression of TbRAB1 or TbRAB2 in BSF parasites was induced with tetracycline (1 μg/ml). (Panel A) 12 h following TbRAB1 suppression, *trans*-cisternae within the Golgi complex become expanded. (Panel B) 20 h following TbRAB2 suppression large vesicular structures are observed (arrowheads), whereas the Golgi complex (insets) remains normal.

VSG transport occur on suppression of clathrin (a protein that is involved in endocytosis) despite the rapid lethal phenotype observed [29]. The overall efficiency of VSG export to the cell surface was similar in all the cell lines, suggesting that once VSG has passed the transport step requiring TbRAB1 or TbRAB2B, then exocytosis is completed normally.

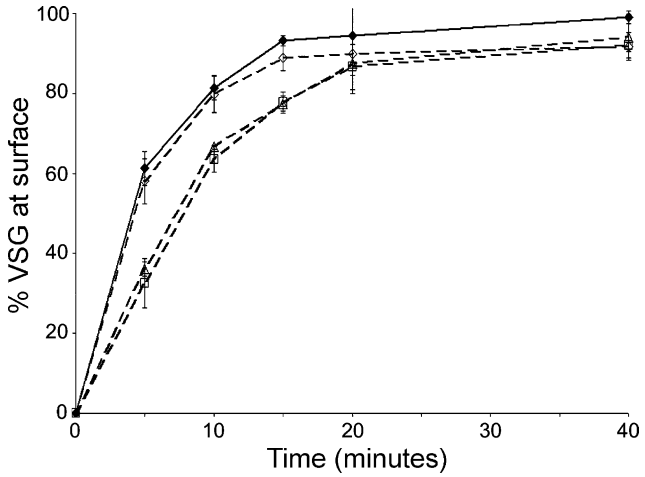


Fig. 9. Both TbRAB1 and TbRAB2B are required for VSG transport. 5 × 10^7 BSF cells were pulse-labelled with ^{35}S Met/Cys. The half-time for VSG to reach the surface was 4.1 (±0.3) min in the 427 cell line (♦), 4.3 (±0.4) min in TbRABX1:RNAi cells (◊), 7.8 (±0.1) min in TbRAB1:RNAi (□) and 7.1 (±0.3) min in TbRAB2:RNAi cells (△). Data are the result of three independent determinations. Zero percent is defined as the ratio obtained at time = 0, and 100% is the ratio when all VSG is in the soluble fraction, i.e. at the surface; for technical reasons, this latter value is not fully reached.

4. Discussion

Our major findings reported here are that the trypanosome orthologues of Rab1 and Rab2 are remarkably highly conserved, being the most conserved of all trypanosome Rab proteins in the genome. Further, TbRAB1 and TbRAB2 are expressed in both major life stages, and found in the Golgi complex region of the cell. RNA interference indicates that TbRAB1 and TbRAB2 are required for normal growth and both proteins are required for efficient export of VSG. Suppression of TbRAB1 results in abnormal Golgi morphology suggesting a role in maintenance of the Golgi complex. TbRAB1 and TbRAB2 are localised to the exocytic system in COS cells, indicating partial maintenance of Rab1 and Rab2 targeting information from trypanosomes to mammals and TbRAB1 can complement *S. cerevisiae* Ypt1 indicating functional conservation with higher eukaryotes. Overall, our data argue that TbRAB1 and TbRAB2 have conserved functions with Rab1 and Rab2 of crown eukaryotes.

In mammalian cells Rab1 interacts with several proteins, including GRASP65, GM130, p115 and Golgin-84 [31] and may also mediate COP II recruitment. By contrast Rab2 interacts with Golgin-45 and GRASP55 [17–19]. BLAST analysis using TbGRASP, HsGRASP55 and HsGRASP65 failed to identify further GRASP sequences in the trypanosome genome, indicating that there is likely only one GRASP protein in trypanosomes, which is more similar to GRASP65 (data not shown), consistent with the finding that TbRAB1 is found close to structures containing TbGRASP. The absence of a GRASP55 homologue suggests that maintenance

of the Golgi is simpler in trypanosomes compared with mammalian cells. At early stages of TbRAB1 suppression the Golgi complex was perturbed, and in particular the *trans*-most cisternae became engorged and swollen, indicating that TbRAB1 expression is required to retain normal Golgi architecture, potentially via TbGRASP recruitment. Mammalian Rab1 and Rab1B have known effectors important in Golgi complex assembly, including GM130 and Golgin-84 [17] whilst over-expression of an inactive Rab1B results in mislocation of Golgi proteins to the ER, consistent with a major blockade to anteriograde transport [19]. It is likely that the absence of TbRAB1 results in a failure in the recruitment of Golgi maintenance proteins, resulting in destabilisation of the Golgi complex. Suppression of TbRAB2 resulted in the accumulation of vesicles, which are possibly transport intermediates, and implies a role similar to that performed by mammalian Rab2 where a similar phenotype is observed [32].

TbRAB1 and TbRAB2 theoretically could mediate fully separate functions, act sequentially or be redundant. Retention of TbRAB1 and TbRAB2 orthologues through evolutionary time argues strongly against redundancy, whilst our data clearly indicate distinct locations, phenotypes and by expression in heterologous systems, distinct functions for these two proteins. Export of VSG is delayed by reducing levels of either TbRAB1 or TbRAB2, suggesting that both proteins are required for normal VSG trafficking, a protein which represents the significant bulk of plasma-membrane destined cargo within the trypanosome; hence fully separate functions can also be ruled out. The most probable relationship between TbRAB1 and TbRAB2B is therefore acting sequentially within the same pathway and is fully consistent with the data here and with studies in higher eukaryotes.

Reduced levels of TbRABX1 did not significantly alter the kinetics of VSG export, and therefore although this protein localises to the ER TbRABX1 is functionality distinct from TbRAB1 and TbRAB2. Interestingly, TbRABX1 is one of a subgroup of Rab proteins that appear to be highly conserved in the kinetoplastida but which are absent from the crown eukaryotes and hence may be specific to the parasite life style (Ackers et al., in press). In summary, in *T. brucei* trafficking through the secretory pathway is dependent on two members of the TbRAB family, a configuration conserved with the mammalian system and not with the simpler situation found in yeast.

Acknowledgements

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References

[1] Doering TL, Schekman R. GPI anchor attachment is required for Gas1p transport from the endoplasmic reticulum in COP II vesicles. EMBO J 1996;15:182–91.
[2] Muniz M, Morsomme P, Riezman H. Protein sorting upon exit from the endoplasmic reticulum. Cell 2001;104:313–20.
[3] Morsomme P, Riezman H. The Rab GTPase Ypt1p and tethering factors couple protein sorting at the ER to vesicle targeting to the Golgi apparatus. Dev Cell 2002;2:307–17.
[4] Benting JH, Rietveld AG, Simons K. *N*-Glycans mediate the apical sorting of a GPI-anchored, raft-associated protein in Madin–Darby canine kidney cells. J Cell Biol 1999;146:313–20.
[5] Cross GA, Wirtz LE, Navarro M. Regulation of vsg expression site transcription and switching in *Trypanosoma brucei*. Mol Biochem Parasitol 1998;91:77–91.
[6] Grunfelder CG, Engstler M, Weise F, et al. Accumulation of a GPI-anchored protein at the cell surface requires sorting at multiple intracellular levels. Traffic 2002;3:547–59.
[7] Simonsen A, Lippe R, Christoforidis S, et al. EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature 1998;394:494–8.
[8] Smythe E. Direct interactions between rab GTPases and cargo. Mol Cell 2002;9:205–6.
[9] Waters MG, Pfeffer SR. Membrane tethering in intracellular transport. Curr Opin Cell Biol 1999;11:453–9.
[10] Pereira-Leal JB, Seabra MC. Evolution of the Rab family of small GTP-binding proteins. J Mol Biol 2001;313:889–901.
[11] Bock JB, Matern HT, Peden AA, Scheller RH. A genomic perspective on membrane compartment organization. Nature 2001;409:839–41.
[12] Pereira-Leal JB, Seabra MC. The mammalian Rab family of small GTPases: definition of family and subfamily sequence motifs suggests a mechanism for functional specificity in the Ras superfamily. J Mol Biol 2000;301:1077–87.
[13] Tisdale EJ, Bourne JR, Khosravi-Far R, Der CJ, Balch WE. GTP-binding mutants of rab1 and rab2 are potent inhibitors of vesicular transport from the endoplasmic reticulum to the Golgi complex. J Cell Biol 1992;119:749–61.
[14] Wilson AL, Erdman RA, Maltese WA. Association of Rab1B with GDP-dissociation inhibitor (GDI) is required for recycling but not initial membrane targeting of the Rab protein. J Biol Chem 1996;271:10932–40.
[15] Ni X, Ma Y, Cheng H, et al. Molecular cloning and characterization of a novel human Rab (Rab2B) gene. J Hum Genet 2002;47:548–51.
[16] Jedd G, Richardson C, Litt R, Segev N. The Ypt1 GTPase is essential for the first two steps of the yeast secretory pathway. J Cell Biol 1995;131:583–90.
[17] Satoh A, Wang Y, Malsam J, Beard MB, Warren G. Golgin-84 is a rab1 binding partner Involved in Golgi structure. Traffic 2003;4:153–61.
[18] Short B, Preisinger C, Korner R, Kopajtich R, Byron O, Barr FA. A GRASP55-rab2 effector complex linking Golgi structure to membrane traffic. J Cell Biol 2001;155:877–83.
[19] Alvarez C, Garcia-Mata R, Brandon E, Sztul E. COPI recruitment is modulated by a Rab1b-dependent mechanism. Mol Biol Cell 2003;14:2116–27.
[20] Pal A, Hall BS, Nesbeth DN, Field HI, Field MC. Differential endocytic functions of *Trypanosoma brucei* Rab5 isoforms reveal a glycosylphosphatidylinositol-specific endosomal pathway. J Biol Chem 2002;277:9529–39.

[21] Jeffries TR, Morgan GW, Field MC. A developmentally regulated rab11 homologue in *Trypanosoma brucei* is involved in recycling processes. J Cell Sci 2001;114:2617–26.
[22] Grunfelder CG, Engstler M, Weise F, et al. Endocytosis of a glycosylphosphatidylinositol-anchored protein via clathrin-coated vesicles, sorting by default in endosomes, and exocytosis via RAB11-positive carriers. Mol Biol Cell 2003;14:2029–40.
[23] Bangs JD. Surface coats and secretory trafficking in African trypanosomes. Curr Opin Microbiol 1998;1:448–54.
[24] Triggs VP, Bangs JD. Glycosylphosphatidylinositol-dependent protein trafficking in bloodstream stage *Trypanosoma brucei*. Eukaryot Cell 2003;2:76–83.
[25] McDowell MA, Ransom DM, Bangs JD. Glycosylphosphatidylinositol-dependent secretory transport in *Trypanosoma brucei*. Biochem J 1998;335:681–9.
[26] Field H, Ali BR, Sherwin T, Gull K, Croft SL, Field MC. TbRab2p, a marker for the endoplasmic reticulum of *Trypanosoma brucei*, localises to the ERGIC in mammalian cells. J Cell Sci 1999;112:147–56.
[27] Field H, Farjah M, Pal A, Gull K, Field MC. Complexity of trypanosomatid endocytosis pathways revealed by Rab4 and Rab5 isoforms in *Trypanosoma brucei*. J Biol Chem 1998;273:32102–10.
[28] Jeffries TR, Morgan GW, Field MC. TbRAB18, a developmentally regulated Golgi GTPase from *Trypanosoma brucei*. Mol Biochem Parasitol 2002;121:63–74.
[29] Allen CL, Goulding D, Field MC. Clathrin-mediated endocytosis is essential in *Trypanosoma brucei*. EMBO J 2003;22:4991–5002.
[30] Bangs JD, Andrews NW, Hart GW, Englund PT. Post-translational modification and intracellular transport of a trypanosome variant surface glycoprotein. J Cell Biol 1986;103:255–63.
[31] Tisdale EJ, Balch WE. Rab2 is essential for the maturation of pre-Golgi intermediates. J Biol Chem 1996;271:29372–9.
[32] Weide T, Bayer M, Koster M, Siebrasse JP, Peters R, Barnekow A. The Golgi matrix protein GM130: a specific interacting partner of the small GTPase rab1b. EMBO Rep 2001;2:336–41.
[33] Swofford DL. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4b10. Sunderland, Massachusetts: Sinauer Associates.
[34] He CY, Ho HH, Malsam J, et al. Golgi duplication in *Trypanosoma brucei*. J Cell Biol 2004;165:313–21.