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TbRab2p, a marker for the endoplasmic reticulum of *Trypanosoma brucei*, localises to the ERGIC in mammalian cells

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SUMMARY

The Rab family of small GTPases is a subset of the Ras superfamily. Rabs regulate the flux through individual steps of the intracellular membrane trafficking pathway, such as ER-to-Golgi transport, probably by controlling SNARE complex assembly. In *Trypanosoma brucei* a number of Rab proteins have been isolated by EST analysis; here we characterise one of these, TbRab2p (originally designated Trab1p), which is a member of the Ypt1p subfamily of Rab proteins. Recombinant TbRab2p is capable of hydrolysing GTP and is post-translationally modified in vitro by addition of a geranylgeranyl prenyl group, properties of an authentic Rab GTPase. Antibodies against recombinant TbRab2p show that in trypanosomes TbRab2p is localised primarily to the endoplasmic reticulum (ER) and colocalises with BiP in wild-type trypanosomes. Over expression of TbRab2p in procyclic form *T. brucei* results in a cell population having a 40-fold increase in TbRab2p expression. In these cells biosynthesis of procyclin, a secretory pathway glycoprotein, is decreased, accompanied by an increase in general protein biosynthesis, suggesting that excess TbRab2p affects ER function. Heterologous expression of TbRab2p in COS cells resulted in targeting to the pre-Golgi transport intermediate (ERGIC), indicating that the targeting information is conserved between mammals and trypanosomes. Clustal and phylogenetic analyses support assignment of TbRab2p as a Rab2 homologue. In addition, over expression of TbRab2p in trypanosomes results in membrane reorganisation and formation of opaque vesicular structures visible by phase contrast microscopy, consistent with accumulation of ER-derived vesicular structures in cells highly overexpressing TbRab2p. Ultrastructural examination by electron microscopy confirmed the presence of a tubulo-vesicular membrane bound compartment in close proximity to the *cis*-Golgi, probably equivalent to the ERGIC. TbRab2p is therefore a new ER/ERGIC marker for *T. brucei*.

Key words: Rab protein, Small GTPase, Endoplasmic reticulum, BiP, Prenylation

INTRODUCTION

The protozoan parasite *Trypanosoma brucei*, the causative agent of African sleeping sickness is an important human pathogen with a digenetic life cycle. In both the mammalian host form (bloodstream form) and insect form (procyclic) the parasite produces ~10% of total biosynthesised protein as glycosylphosphatidylinositol (GPI)-anchored surface coat protein trafficking via the secretory pathway. By characterising components of the secretory system at the molecular level we are seeking to gain mechanistic insights into the vesicle transport system of this organism. The interface between host and parasite is provided by the trypanosome plasma membrane and associated structures; study of the process by which molecules are delivered to this system, i.e. vesicle transport, is predicted to contribute to understanding virulence and host:parasite interactions.

Rab proteins are p21 ras-related small GTPases with highly restricted subcellular localisations and are believed to regulate specific membrane transport steps (Nuoffer and Balch, 1994). Rabs are essential for fusion of transport vesicles with the target organelle; activated Rab•GTP proteins prime SNAREs prior to complex formation and vesicle docking (Lupashin and Waters, 1997; Pfeffer, 1996). The requirement for GTP hydrolysis is temporally separated from the fusion event itself (Rybin et al., 1996). The lifetime of Rab•GTP in the fusion complex defines the period when fusion may occur (Aridor and Balch, 1996). After fusion to the target organelle the Rab is deactivated by the GTPase activating protein before recycling to the donor organelle as Rab•GDP complexed with GDP dissociation inhibitor (Novick and Brennwald, 1993). When an excess of a mutant Rab is present, the process regulated by that Rab is inhibited (Gruenberg and Clague, 1992).

In mammals at least three Rabs are required for ER-to-Golgi transport, Rabs 1a, 1b and 2. Yeast apparently requires only a single Rab, Ypt1p (Lazar et al., 1997). Multiple Rabs probably reflect the existence of more than one population of pre-Golgi

transport vesicles, including tubulo-vesicular exit sites and the ER-Golgi intermediate compartment (ERGIC) in anteriograde transport in higher eukaryotes. During mitosis, Rab1 is phosphorylated whilst Rab2 is not, suggesting alternate controls during progression through the cell cycle (Bailly et al., 1991). While the subcellular locations of these Rab proteins overlap, Rab2 is restricted to the ER-exit sites and ERGIC, whilst Rab1 is also found in the ER and the Golgi stack. Rabs 1a and 1b act both in ER-to-Golgi transport, focused at an initial step in export from the ER, and in transport between Golgi subcompartments. Both Rab1b and Rab2 are required for delivery to the Golgi (Plutner et al., 1991; Tisdale et al., 1992). Microinjected mutant Rab1a causes Golgi fragmentation as well as a transport block (Wilson et al., 1994; Nuoffer et al., 1994).

Several sequences for Rab proteins from kinetoplastida parasites have been identified (Mendonca et al., 1993; Cappai et al., 1993; El-Sayed et al., 1995; Field and Boothroyd, 1995; Field et al., 1998; Bringaud et al., 1998), illustrating that protozoa utilise a system of exocytotic and endocytotic Rab proteins similar to higher eukaryotes. We previously reported the sequence of the gene for TbRab2p (previously Trab1p; Field and Field, 1997). *TbRAB2* is found immediately downstream of a second gene encoding a divergent Rab, *TbRAB8* (previously *Trab7*): the two genes are the result of a tandem duplication, and their products have distinct subcellular locations. Here we demonstrate that TbRab2p may be a Rab2 homologue that localises to the ER exit region/ERGIC in trypanosomes. Overexpression of TbRab2p in procyclic trypanosomes alters trafficking of the secreted surface glycoprotein procyclin, and results in the formation of abnormal membrane structures.

MATERIALS AND METHODS

Materials and molecular biology

Molecular biology materials and manipulations were performed as described; TbRab2p antibodies were raised in rabbits and affinity purified (Field and Field, 1997). BiP rabbit antiserum was from University of Wisconsin, Madison, USA, Procyclin antibodies were from University of Victoria, British Columbia, Canada; ERGIC-p53 antibodies were from Basel University, Basel, Switzerland; and mannosidase II antibodies were from University of Georgia, Georgia, USA. Secondary antibodies were supplied by Sigma, Jackson Laboratories or Molecular Probes. Purified recombinant (r-) Rac protein and an expression plasmid for GST-Ras were from Ludwig Institute, London. Rhizoxin was from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland, USA.

Southern blotting was on genomic DNA purified from logarithmic phase procyclic *T. brucei*, strain 427 (Medina-Acosta and Cross, 1993). To examine the tubulin locus, DNA was restricted with *Eco*RV, blotted and probed (Field and Field, 1997). The tubulin probe was a *Mlu*I-*Not*I fragment from plasmid pXS219myc9 (Bangs et al., 1996; and below). Hybridisation was in 6× SSPE/BLOTTO (Sambrook et al., 1989) at 65°C/16 hours, washed in 2× SSC/0.1% SDS/42°C. Autoradiography was at –85°C with an intensifying screen. Plasmid DNA was prepared using a Qiagen column.

Cell culture

COS cells were grown in DMEM and pretreated with nocodozole or brefeldin A (BFA) by adding 10 μg/ml to the medium for 1-3 hours. For expression in COS cells, the ORF of *TbRAB2* was inserted into pcDNAI (InVitrogen), and sequenced. COS cells were transiently transfected with 1 μg DNA as described (Wainwright and Field, 1997).

Procyclic *T. brucei brucei* strain 427 was grown in SDM79 (Field and Field, 1997). Pretreatment with rhizoxin at 5 nM and 100 nM was as described (Matthews et al., 1995). Cell numbers were determined with a Coulter Z1 Counter (Coulter Electronics Ltd). For transfection of trypanosomes, the ORF of *TbRAB2* was inserted into the vector pXS219myc9, constructed by placing the pUC19 polylinker into *Hin*dIII/*Eco*RI sites of pXS2 and adding a myc tag into the *Hin*dIII/*Bam*HI sites; construct pXS219myc•*TbRAB2* was verified by sequencing. Procyclic trypanosome cells were stably transformed with 100 μg of purified DNA, selected with 25 μg/ml geneticin (G418) and single cell clones derived as described (Bangs et al., 1996). Of 60 wells plated two produced clones; one clone, 2.1, is described here.

Immunofluorescence microscopy

16 hours after transfection, COS cells were processed for immunofluorescence assay (IFA; Wainwright and Field, 1997). Staining of trypanosomes was performed at room temperature: trypanosomes washed in phosphate buffered saline (PBS, Sigma) were applied to poly-lysine coated slides (Sigma) for 2 minutes (procyclics) and processed at ambient temperature, as described (Sherwin and Read, 1993). Where two rabbit primary antibodies were used, application of the first antibody and the first secondary (and washes) was followed by incubation with non-immunised rabbit serum diluted 1:100, followed by the second rabbit antibody (at 4°C overnight) and then the second secondary. Cells were examined on a Leica DMRXA epifluorescence microscope fitted with a Photometrics CH250 Slow Scan CCD camera or on a Nikon Microphot II fitted with a light camera (Field and Field, 1997). Digital images were captured using IP Lab spectrum 3.1 software and merged and assembled into figures using Adobe Photoshop 3.0.5 (Adobe Systems, Inc.).

Western blots

5×10^6-2×10^7 cells per lane were electrophoresed on 15% SDS-polyacrylamide gels and blotted onto 45 μm nitrocellulose by wet transfer. Filters were processed as described (Field and Field, 1997).

GTP-binding studies and characterisation of recombinant protein

GTP overlay assay was performed on 10^7 trypanosomes per lane electrophoresed on 15% SDS-polyacrylamide gels and blotted (Wilson et al., 1996). GTP hydrolysis assay was performed in solution with r-TbRab2p purified from *Escherichia coli* expressing glutathione-S-transferase (GST) fusion protein and cleaved with thrombin to release r-TbRab2p as described (Field and Field, 1997). For the solid phase assay, GST•TbRab2p fusion protein was bound to reduced glutathione beads (Pharmacia) as described by Foster et al. (1996). The solution assay was performed as described (Tavitian and Zahrouhi, 1992) using the buffer from Foster et al. (1996) as the loading buffer and adding 10 mM $MgCl_2$ to initiate the reaction.

Analysis of endogenous biosynthetic processes

For PP1 biosynthesis, 2×10^8 logarithmic phase cells were washed in SDM79 and labelled in 100 μl medium containing 100 μCi [^{3}H]ethanolamine (Amersham) for 1 hour. Cells were extracted in chloroform/methanol/water 10:10:3 (v/v/v) twice. Organic extracts were prepared for thin layer chromatography as described (Field and Menon, 1992). For procyclin biosynthesis, cells were starved for 1 hour in glutamate-free RPMI medium, labelled with [^{3}H]glutamate, and hypotonically lysed to remove cytoplasmic material; procyclin was extracted using 1% Triton X-100/10 mM EDTA/PBS, and acetone precipitated for SDS-PAGE. Gels were processed with En^{3}Hance (Dupont NEN) and autoradiographed for 2-7 weeks at –85°C. Protein synthesis was analysed by labelling 10^6 trypanosomes washed twice in methionine free RPMI (Gibco-BRL) then starved in methionine free

RPMI 1 hour, and labelled with 175 μCi [^{35}S]methionine (ICN) 1 hour. Cells were washed once in methionine free RPMI, lysed in boiling SDS sample buffer, electrophoresed on a 13% SDS-PA gel and visualised by Phosphorimager (Molecular Dynamics) after 16 hours.

FT and GGT II assays

Farnesyl and geranylgeranyl transferase assays were performed on r-TbRab2p. The method for Rab modification by GGT II was as described (Seabra et al., 1992): the reaction in a final volume of 25 μl contained: 50 mM Na^+ Hepes, pH7.2, 5 mM $MgCl_2$, 1 mM DTT, 1 mM Nonidet P-40, 0.15μCi [^{3}H]GGPP (geranylgeranyl pyrophosphate; 21.5 Ci/mmol, Dupont NEN) and r-TbRab2p, and was initiated by the addition of 25 mg rat brain cytosol (Reiss et al., 1990) then incubated at 37°C for 0-90 minutes. Half the mixture (12.5 μl) was boiled in sample buffer and electrophoresed onto 15% SDS-PAGE gels. The gels were incubated in En^{3}Hance, dried and exposed to Kodak BioMAX film at −85°C. Farnesyltransferase assay was performed as above but using the appropriate buffer and [^{3}H]farnesyl pyrophosphate (Reiss et al., 1990).

Electron microscopy

Procyclic cells were grown to logarithmic phase, washed in serum free SDM79 medium prior to fixing with 3% glutaraldehyde and processing for EM as described (Field et al., 1996).

Computing

Sequence data were retrieved from GenBank using Netscape Navigator (V4.01) and alignments were performed using Clustal W (V1.6) on a MacOS (V8.1) computer (Thompson et al., 1994). Phylogenetic analysis was done with PAUP (V4.0) as described (Field and Field, 1997). SeqVu (Garavan Institute of Medical Research, Sidney, Australia) was used to present alignments.

RESULTS

Trab1p is a Rab2 sequence homologue

We previously isolated the gene encoding Trab1p. Trab1p is 65% similar to the yeast Ypt1p subfamily, which is comparable to the 75% similarity between functional homologues Ypt1p and mammalian Rab1. Microscopic analysis using antibodies raised to r-Trab1p showed a diffuse reticular staining suggesting an ER localisation and indicating that Trab1p had functional similarity with Ypt1p, an ER-Golgi protein, but not Sec4p, a secretory vesicle protein (Field and Field, 1997). We aligned Trab1p with human Rab1a, rat Rab1b and human Rab2 (the rat sequence chosen because the human Rab1b sequence was unavailable; comparison of human and rat Rab1a and Rab2 sequences indicates strong conservation, e.g. human and Rat Rab2 differ by only three amino acids). Phylogenetic reconstruction using PAUP V4.0 followed by heuristic bootstrapping (1000 replicates) was used to determine that Trab1p was monophyletic with Rab2, at 100% confidence (data not shown). Significantly, we took the C-terminal regions alone (from and including the SAK consensus box, Fig. 1) which contain targeting signals (Chavrier et al., 1991); again, Trab1p and human Rab2 were monophyletic (confidence 97%). The C-terminal sequences of Rab2 and Trab1p both contain a 10 amino acid insertion. However, the effector loop sequence of Trab1p is more like that of a Rab1: the sequence STIAID is better conserved, at 4 out of 6 amino acids, with Rab1 (STIGVD), compared to 2 out of 6 positions with Rab2 (LTIGVE). We concluded that Trab1p is probably a trypanosome Rab2 homologue, although the effector loop homology indicates possible Rab1-like properties. Based on the phylogenetic analysis, we propose that Trab1p (an arbitrary designation) is a Rab2 homologue and rename it TbRab2p.

TbRab2p is a GTPase

Enzymatic activity (GTP hydrolysis) has not been directly tested in a *T. brucei* G protein. We purified r-TbRab2p from the N-terminal glutathione-S-transferase (GST) fusion protein overproduced in *Escherichia coli*, by cleaving the purified fusion protein with thrombin (Fig. 2A,B), and analysed for intrinsic GTPase activity. We used identically prepared human Rac, a highly active member of the ras superfamily, as a positive control. To ensure that activity was representative of the native protein, and not artefactual due to removal of critical residues by thrombin digestion, we determined the precise molecular mass of the purified, cleaved r-TbRab2p by MALDI TOF mass spectrometry. A single peak was detected at m/z 23512 Daltons corresponding to r-TbRab2p missing five N-terminal residues (not shown). Since this does not affect the GTP binding motifs, the GTPase activity seen in vitro is predicted to be a close approximation of the activity of the native protein.

GTP was hydrolysed to GDP following addition of Mg^{2+} to the reaction mixture containing r-TbRab2p. Over 120 minutes TbRab2p had similar activity to Rac, indicating that TbRab2p is an active GTPase (Fig. 2C, top). The GST-TbRab2 fusion protein also hydrolysed GTP; GST-TbRab2p bound to glutathione beads was loaded with [α-^{32}P]GTP and excess radiolabel removed by extensive washing at 0°C in the presence of Mg^{2+} and catalysis was initiated by incubation at 37°C. Whilst the return of total radioactivity was variable, bound nucleotide was completely hydrolysed over 60 minutes (Fig. 2C, bottom). Quantitation indicated that the proportion of GDP increased with time (data not shown). In contrast, no GTP-binding was seen using glutathione beads (Fig. 2C, bottom, left) or r-GST bound to beads (not shown). We conclude that TbRab2p binds GTP, hydrolyses it in a time- and Mg^{2+}-dependent manner, and is therefore an authentic GTPase. We also tested for GTP hydrolysis at 27°C, the optimal growth temperature of procyclic trypanosomes. GTPase activity was also seen at 27°C, but at 60% of the activity at 37°C (data not shown).

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TbRab2   1  SAKEKENVQSAFQKIAQEIFEDMKERTGKGLSPSGGAGGAGNGVRLAGNEGQKG-S---------KRGGCC
HsRab2   1  SAKTASNVEEAFINTAKEIYEKIQEGVFDINNE--------ANGIKIGPQHAATNATHAGNQGGQQAGGGCC
HsRab1a  1  SAKNATNVEQSFMTMAAEIKKRMGPGATAGGAE--------KSNVKIQSTPVKQ-----------SGGGCC
RnRab1b  1  SAKNATNVEQAFMTMAAEIKKRMGPGAASGGE--------RPNLKIDSTPVKS-----------ASGGCC
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Fig. 1. TbRab2p is a Rab2 homologue. A portion of the Clustal W alignment of TbRab2p with mammalian Rab1 and Rab2 sequences, corresponding to the C-terminal hypervariable region, including the SAK homology box is shown. Most significantly, the C-termini of TbRab2p and human Rab2 are several amino acids longer than the Rab1 proteins. Hs; *Homo sapiens*, Rn; *Rattus norvegicus*, Tb; *Trypanosoma brucei*. Identical residues conserved in three or more sequences are boxed, conservative substitutions (top sequence as reference) are shaded.

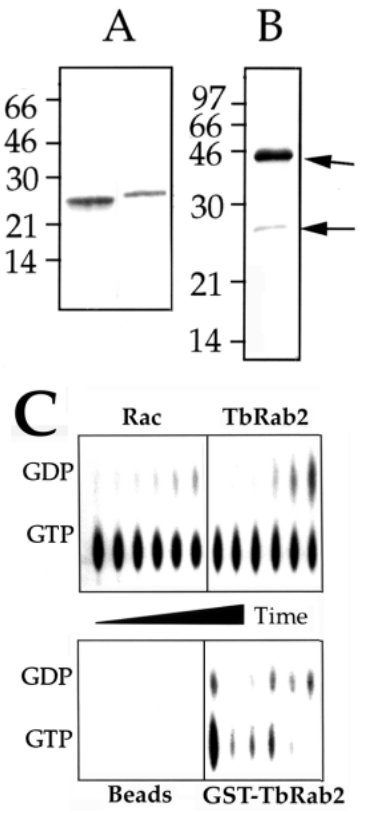


Fig. 2. TbRab2p is an authentic GTPase. (A and B) Coomassie-stained SDS gels of purified proteins r-TbRab2p (A, left) and r-Rac (A, right) and GST-TbRab2p (B) as used in GTPase assays. The migration positions of molecular mass standards are indicated in kDa. The upper arrow in B indicates the position of GST-TbRab2p and the lower arrow GST. (C) Thin layer chromatography and autoradiography of [α-^{32}P]GDP products generated at 37°C from [α-^{32}P]GTP. Top panel: products from aqueous phase assay, catalysed by r-Rac or r-TbRab2p at 0, 5, 15, 30, 60, 120 minutes. Bottom panel: products from GTPase assay in the presence of beads alone or GST-TbRab2 fusion protein bound to beads. Similar data were obtained with two separate preparations of TbRab2p and GST-TbRab2p.

TbRab2p is geranylgeranylated

Most small GTPases are modified at C-terminal cysteine residues by prenylation, prerequisite for membrane association and function. Farnesyl or geranylgeranyl groups are added to protein in both mammals and *T. brucei* in a ratio of ~20% farnesyl:80% geranylgeranyl (Field et al., 1996). C-termini with sequence CAAX, e.g. Ras, are farnesylated by farnesyltransferase, whereas Rab C-termini (CCXX, CXC, XXCC) are normally modified with two geranylgeranyl groups by geranylgeranyltransferase type II (GGT II). TbRab2p has the sequence GGCC, in common with human Rab1 and Rab2, and would be expected to be modified by two geranylgeranyl groups.

We have detected prenyltransferase activity in trypanosome lysates using highly sensitive peptide-based assays, but have not been able to reconstitute detectable levels of activity against protein due to the presence of high levels of a phosphatase that degrades the prenylpyrophosphate donor (BRSA and MCF, unpublished). An in vitro assay using mammalian cytosol as a source of prenyltransferase showed that under conditions suitable

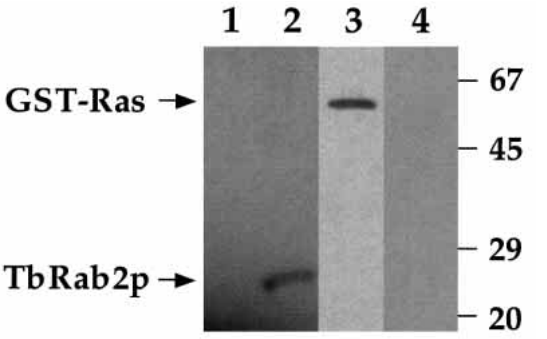


Fig. 3. TbRab2p is geranylgeranylated. Autoradiograph of an SDS-PAGE gel separating r-TbRab2p and GST-Ras modified with [^{3}H]prenyl groups from low molecular mass [^{3}H]isoprene pyrophosphate donor (not seen). Lanes 1 and 2, reactions with TbRab2p as acceptor and GGPP as donor. Lane 1, zero time incubation; lane 2, 90 minutes incubation. Lanes 3 and 4, reactions with GST-Ras (lane 3) or TbRab2p (lane 4) as acceptors and FPP as donor. The reaction in lane 3 was incubated for 60 minutes, that in lane 4 for 90 minutes.

for GGT II, [^{3}H]geranylgeranyl moieties were transferred to TbRab2p (Fig. 3, lane 2), whilst under conditions suitable for farnesyl transferase, as indicated by successful modification of Ras (Fig. 3, lane 3), farnesylation was not detected (Fig. 3, lane 4). Therefore TbRab2p is a substrate for the GGT II enzyme and is most likely geranylgeranylated in vivo, consistent with its C terminus. Although not directly determined, it is likely that TbRab2p was geranylgeranylated on both cysteines, since GGT II is capable of acting catalytically and producing detectable product in the assay only when adding two geranylgeranyl groups to a Rab protein (Shen and Seabra, 1996): in support of this, using a small G protein with a single cysteine in the C terminus, no prenylation was detected (unpublished observations).

Localisation of TbRab2p in trypanosomes

TbRab2p has a diffuse, reticular cytoplasmic location distinct from that of the cocistronic protein, TbRab8p (Trab7p in Field and Field, 1997). We examined TbRab2p staining at higher resolution using an improved IFA procedure to provide a clearer definition of the TbRab2p compartment. TbRab2p staining was uniform in all cells, with highly evident reticular, lightly stranded cytoplasmic staining and in particular, a nuclear rim staining presumably corresponding to the nuclear envelope (Fig. 4B), suggesting that TbRab2p is localised on the ER.

Rab proteins are located on the cytoplasmic face of organellar membranes. *T. brucei* BiP is an ER-lumenal protein (Bangs et al., 1993): however, at the resolution afforded by light microscopy, significant colocalisation would be expected. We costained cells for TbRab2p and BiP (Fig. 4C-F); both proteins localised to reticular material throughout the cytoplasm. BiP, like TbRab2p, showed perinuclear staining as well as particulate staining throughout the cytoplasm and the majority of the BiP colocalised with TbRab2p, whilst significant amounts of TbRab2p staining had no underlying BiP.

Heterologous expression of TbRab2p in COS cells

The evolutionary distance between trypanosomes and higher mammals makes primary structure an incomplete basis for functional assignment; moreover, the presence of

subcompartments of the trypanosome ER or an ERGIC have so far not been documented. We expressed TbRab2p in COS cells where the ERGIC is well defined and can be discriminated from Golgi stacks and ER by differential drug sensitivity using brefeldin A and nocodozole. Neither compound is effective on trypanosomes, ruling out the use of this approach in the homologous system. However, rhizoxin, an alternative microtubule disrupter effective on trypanosomes has very little effect on the distribution of TbRab2p, consistent with an ER location, even in cells highly overexpressing TbRab2p where staining is enhanced (data not shown).

TbRab2p was transiently over expressed in COS-7 cells. Metabolic labelling with [^{35}S]methionine, immunoprecipitation with anti-TbRab2p antibodies and resolution on SDS-PAGE identified a specific band at 27.5 kDa, confirming both expression of TbRab2p and recognition by the polyclonal antibody (data not shown). By IFA, TbRab2p appeared at a juxtanuclear location similar to the ERGIC and Golgi apparatus (Fig. 5; Schweizer et al., 1988; Presley et al., 1997). We treated the cells with brefeldin A (BFA) or nocodozole; BFA to fragment the Golgi apparatus and leave the ERGIC intact, and nocodozole to disrupt the microtubule network affecting both ERGIC and Golgi. We found that the juxtanuclear localisation of TbRab2p was dispersed by nocodozole, consistent with either an ERGIC or a Golgi structure, but was unaffected by BFA, ruling out a Golgi location. BFA treatment in our hands effectively disrupted the Golgi as indicated by mannosidase II staining (Fig. 5). Therefore, the ER localisation signal present on TbRab2p is recognised by mammalian delivery systems as Rab2.

The possibility of subcompartmentalisation of the trypanosome ER is consistent with incomplete colocalisation of BiP and TbRab2p and indicates that an ERGIC or similar structure may be present in the trypanosome secretory system. Examination of the trypanosome Golgi area by electron microscopy showed tubulo-vesicular structures at the *cis*-face of the Golgi apparatus: such structures were clearly present on both faces of the Golgi stacks, corresponding to the *trans*-Golgi network and the ERGIC. Interestingly, the Golgi complex was stacked parallel to the normal to the flagellar pocket (FP) membrane in most of our sections, which made assignment based on orientation to the FP, the site of exocytosis, difficult. However, in most eukaryotes with a stacked Golgi the *trans* face structures tend to have thicker membranes. In addition the Golgi stack is most frequently concave around the *trans* face (below Golgi, Fig. 6). Tubular thin membrane bound structures are visible at the other face of the stack which we suggest represent closely juxtaposed ER transitional elements. Our observations concur with those of Duszenko and Seyfang (1992). Immuno electron microscopy has proved difficult in wild-type cells due to the low abundance of TbRab2p in these cells and further work will be required to explore the ERGIC in *T. brucei* more closely. Interestingly, the major region of noncoincident staining between BiP and TbRab2p was in the area close to the FP (Fig. 4) which correlates well with the proposed ERGIC region observed by electron microscopy.

Stable overexpression of TbRab2p

Rabs are expressed at extremely low levels. To provide initial information on the function of TbRab2p we stably overexpressed wild-type protein in procyclic form parasites with a construct allowing homologous recombination into the tubulin locus, and containing the *TbRAB2* gene under the control of the strong procyclin promoter. Clones were selected by limiting dilution in the presence of geneticin. Insertion of the gene in the tubulin locus was demonstrated by Southern

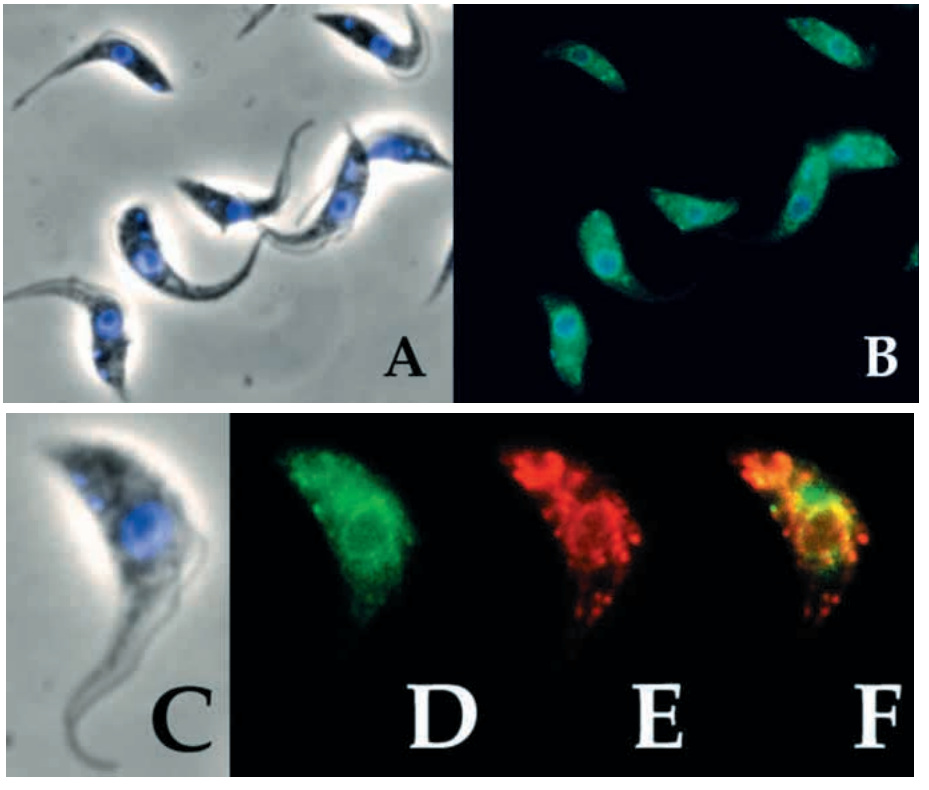


Fig. 4. Localisation of endogenous TbRab2p in procyclic *T. brucei*. Cells were fixed in paraformaldehyde, methanol treated and stained with antibodies raised against r-TbRab2p (which recognise a 27.5 kDa band from *T. brucei* by western blotting; Field and Field (1997). (A and B) Field of procyclic *T. brucei* stained with anti-r-TbRab2p antibodies. (A) Phase contrast with DNA, (B) TbRab2p and DNA. (C-F) Colocalisation of TbRab2p and BiP in a single cell. (C) Phase and DNA, (D) TbRab2p, (E) BiP, (F) merge, TbRab2p and BiP. DNA is blue, TbRab2p green and BiP red.

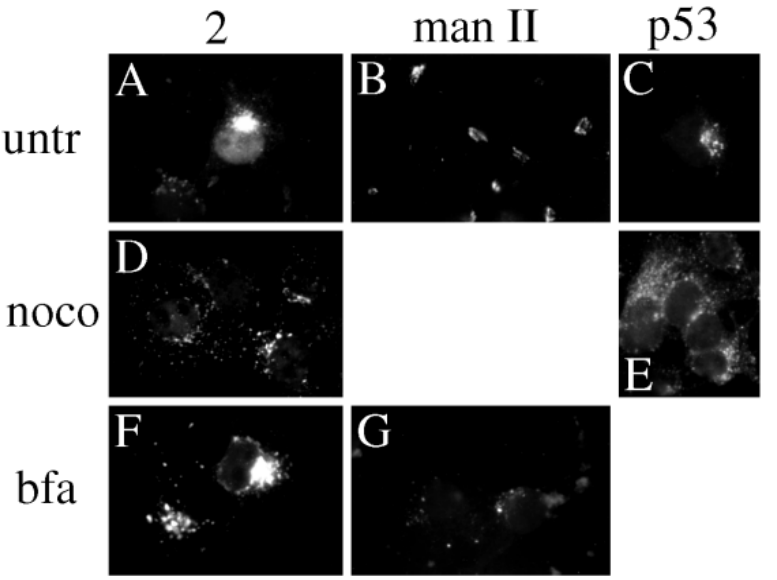


Fig. 5. TbRab2p expressed in COS cells localises to the ERGIC. TbRab2p transiently overexpressed in COS cells, either untreated (A-C) or nocodozole- (D,E) or BFA-treated (F,G) prior to fixation and staining. Cells were stained for TbRab2p (A,D,F), the Golgi marker mannosidase II (B,G) or ERGIC-p53 (C,E). There is no cross-reactivity of anti-TbRab2p antibodies with mammalian proteins and no signal in untransfected cells (not shown). TbRab2p localises to a juxtanuclear structure (A) close to the Golgi apparatus (B). ERGIC (C) was distinguished from Golgi (B) by disruption of the Golgi with BFA (G), when TbRab2p was unaffected (F). When the ERGIC was fragmented with nocodozole (E), TbRab2p was also dispersed (D).

blotting and over expression of the protein was examined by western blotting; ~40-fold more TbRab2p was expressed in clone 2.1 compared with wild-type cells (Fig. 7A and B). To ensure that this level of overexpression did not result in failure to insert into membranes we hypotonically lysed 2.1 cells and extracted the pellet with 0.2% Triton X-100. By western blotting, essentially no TbRab2p was recovered from the cytosolic fraction (data not shown), but >90% of TbRab2p was observed in the detergent soluble fraction of the post-lysis pellet, indicating that the r-TbRab2p, like the native protein, was predominantly associated with membranes (Fig. 7C).

Total GTP-binding ability of trypanosome lysates assessed by [α-^{32}P]GTP overlays of protein extracts from 10^7 overexpressing 2.1 cells appeared identical to the wild type

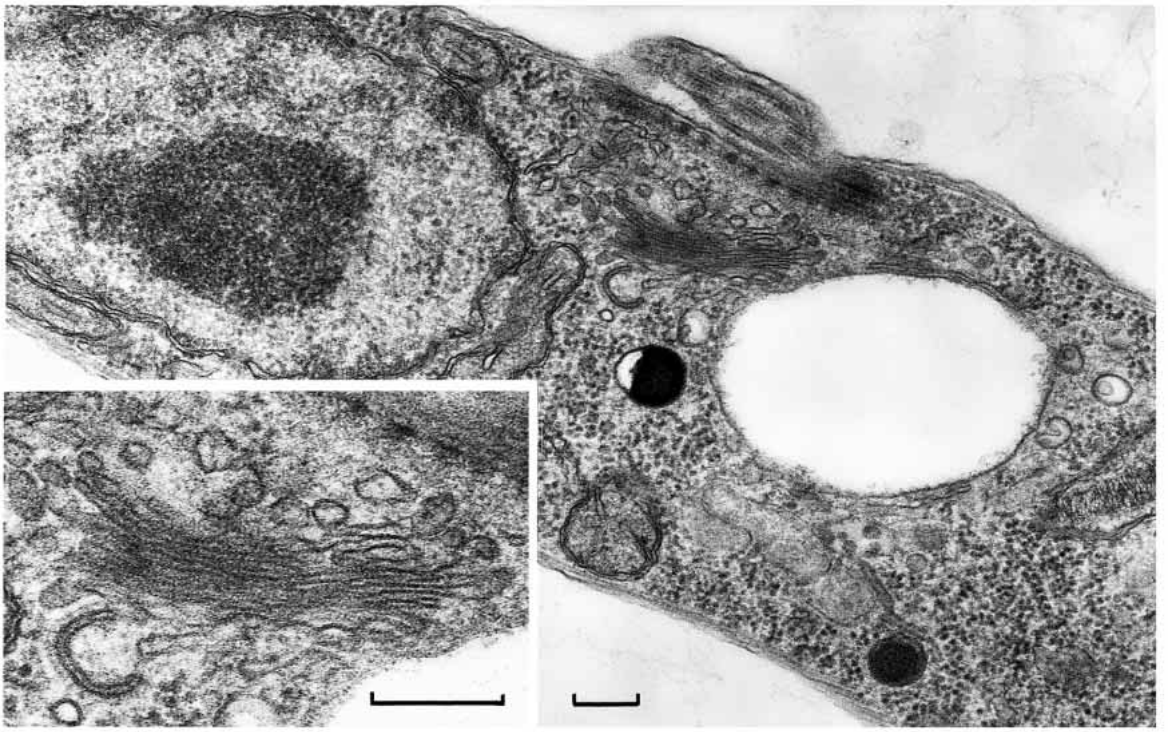
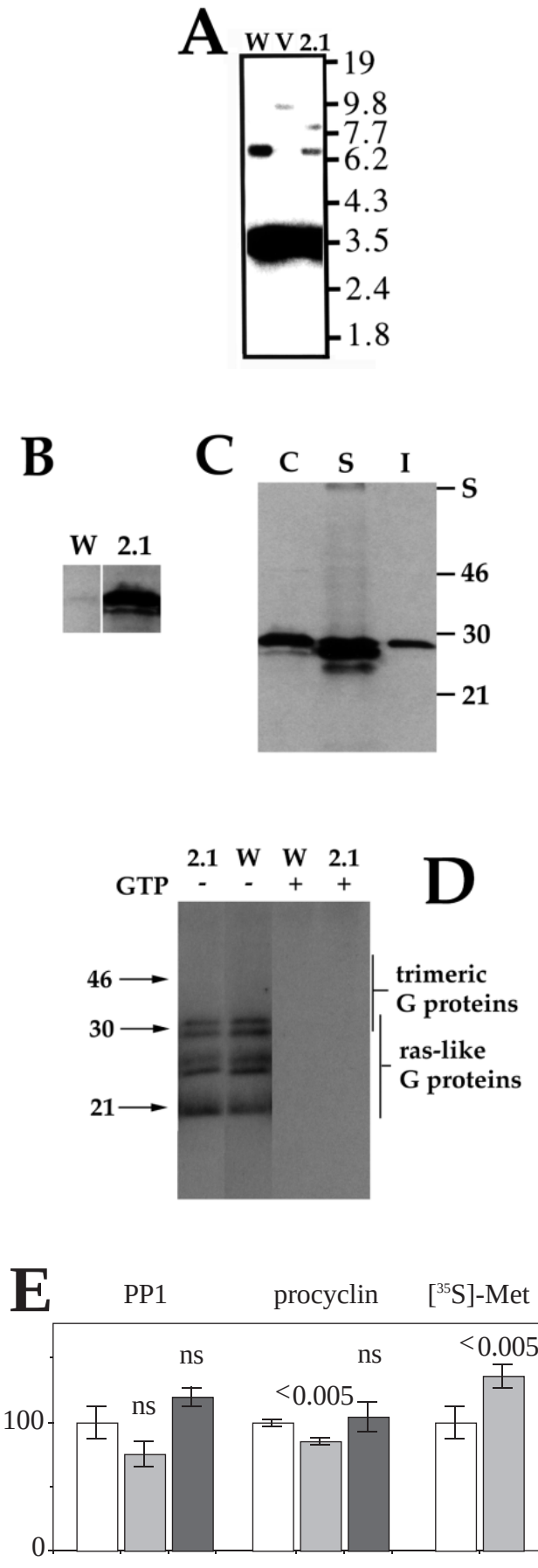


Fig. 6. Ultrastructural evidence for an ERGIC in the trypanosome. Electron micrograph of a procyclic form trypanosome showing the FP region together with a Golgi complex profile. Note that there are tubulo-vesicular structures associated with both faces of the Golgi complex indicating the presence of extended networks from both the *trans* and *cis* sides. The presence of these structures on both sides is indicative of the existence of an extended *cis*-Golgi network or ERGIC. The network closest to the cell membrane contains structures typical of higher eukaryote *trans*-Golgi network morphologies, whilst the less pronounced membrane system on the obverse face of the stack is typical of ERGIC/*cis*-Golgi membranes. Inset shows the Golgi region in greater detail. Bars, 200 nm.



(Fig. 7D). This is consistent with the extremely low abundance of individual Rab GTPases and may also reflect that fact that r-TbRab2p does not bind GTP efficiently when renatured on nitrocellulose (data not shown). In this experiment we were unable to detect significant binding of GTP to bands migrating larger than ~30 kDa, i.e. in the region where G_α subunits of heterotrimeric G proteins would migrate, and therefore only selected trypanosome G proteins were sampled.

Levels of the GPI-anchor precursor PP1 (Field et al., 1991), synthesised in the ER, were unaltered in 2.1 cells (Fig. 7E). As the major protein cargo of the secretory pathway we analysed the synthesis of procyclin in 2.1 and wild-type cells. Procyclin, mainly the EP form, was detected as a strongly labelled band at ~45 kDa in trypanosome lysates (Clayton and Mowatt, 1989). Predominance of the EP form is to be expected as the strain used here has been extensively cultured (Treumann et al., 1997). A ~15% decrease in the amount of procyclin synthesised was observed with high statistical significance ($P<0.005$). A cloned procyclic cell line over expressing another *T. brucei* Rab showed no change in either procyclin or PP1 biosynthesis therefore these effects are specific to TbRab2p. Interestingly, [^{35}S]methionine incorporation into 2.1 cells was increased (Fig. 7E).

We observed a transient ~10% increase in growth rate of the 2.1 cells, evident soon after transfection and geneticin selection which diminished following prolonged culturing. Increased

Fig. 7. Characterisation of procyclic *T. brucei* overexpressing TbRab2p. (A) Southern blot of genomic DNA restricted with *Eco*RV and probed for the *T. brucei* tubulin locus. *T. brucei* were transformed with: nothing (Lane W, wild type); vector alone (clone V-1 lane V); plasmid pXS219myc•*TbRAB2* (clone 2.1, lane 2.1). Molecular mass markers are in kbp (right). (B) Western blot of protein from 10^7 wild-type cells (W) or TbRab2p-overexpressing cells (2.1) lysed in boiling SDS-PAGE sample buffer, blotted and probed with anti-TbRab2p antibodies. TbRab2p was estimated from these data to be overexpressed ~40-fold in 2.1 cells: both lanes were exposed on the same filter, but cut and pasted for presentation. (C) Western blotting indicated that TbRab2p is associated with membranes in overexpressing cells. Protein from 2.1 cells was fractionated: the whole cells (C), the detergent soluble fraction (S) and the detergent insoluble pellet (I) blotted and probed with anti-TbRab2p antibodies. Most TbRab2p associated with the detergent-soluble fraction. Some variance in the molecular mass is observed in the S fraction, probably due to interactions with the detergent and proteolysis. Lane 2.1 in B corresponds to an overexposure of lane C in panel C. Molecular mass markers are in kDa, and the position of the stacking gel indicated (S, right). (D) [α-^{32}P]GTP overlay of trypanosome proteins blotted onto nitrocellulose. 10^7 wild-type or 2.1 cells were lysed in boiling SDS sample buffer and run on reducing SDS-PAGE. After renaturation, proteins were transferred to a filter which was incubated with a buffer containing [α-^{32}P]GTP. Molecular weight markers (in kDa) and the expected migration positions of ras-like and the GTP-binding subunits of heterotrimeric G proteins are indicated. (E) Quantitation of metabolic labelling of 2.1 and wild-type cells for PP1 and procyclin biosynthesis and for [^{35}S]-Methionine incorporation (see Materials and Methods). A cell line overexpressing TbRab8p was used as a control (HF and MCF, in preparation). Data are normalised to 100% for the wild type. Determinations were performed in triplicate with the standard deviation indicated. Significance (two-tailed unpaired *t*-test) is indicated above the bars; the difference in metabolic capacity is highly significant for procyclin. Experiments were performed twice with similar results. White bars: wild-type procyclics; grey bars, 2.1 cells; dark bars, procyclic clone overexpressing TbRab8p.

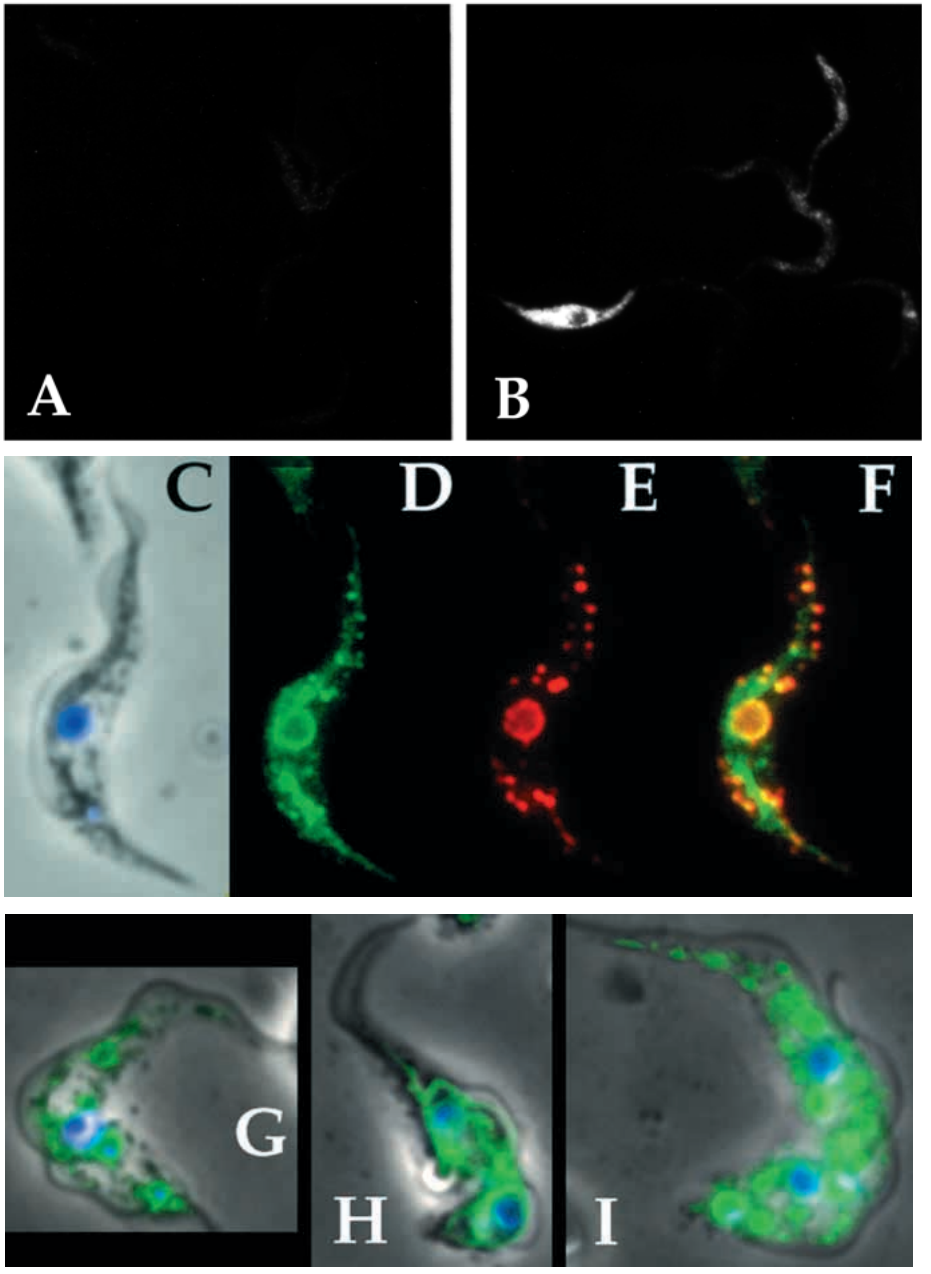


Fig. 8. Immunofluorescence analysis of procyclics overexpressing TbRab2p. (A and B) comparison of staining for TbRab2p in wild-type cells (A) and clone 2.1 (B). In the overexpressor, staining intensity ranges from close to wild-type levels to very intense. (C-F) Colocalisation of TbRab2p and BiP in a single cell. (C) Phase and DNA (blue), (D) TbRab2p (green), (E) BiP (red), (F) Merge of TbRab2p and BiP (costaining is yellow). BiP is more punctate in 2.1 than wild-type cells, and there are heavy strands of TbRab2p-staining that do not contain BiP. (G-I) 2.1 cells stained for TbRab2p, showing abnormal morphologies corresponding to large vacuolar structures with TbRab2p rim staining. Images are phase contrast merged with DNA (blue) and TbRab2p stain (green). Although the intensity of TbRab2p staining is greater for 2.1 cells than for wild-type cells, the gain in panels C-F and in Fig. 4 have been adjusted for comparison and therefore the differences are not apparent.

growth rates have been observed in transformed *T. brucei* by others (McCulloch et al., 1997) and also by us with different inserts and selection conditions. Subsequent normalisation of growth rate may be a manifestation of epigenetic control mechanisms which may also minimise the effects of overexpressed TbRab2p by selection for long-term survival rather than short-term growth rate increases.

Reorganisation of membranes by overexpression of TbRab2p

By IFA, staining for TbRab2p was significantly stronger in 2.1 cells compared to wild type and cell-to-cell variation in the intensity of TbRab2p stained was observed (Fig. 8A,B). This variability did not correlate with cell cycle (data not shown). At high resolution, perinuclear staining and a distinct reticular pattern were seen in clone 2.1 similar to the wild type (Fig. 8C-F) although thickened strands staining with TbRab2p indicated a morphology intermediate between normal TbRab2p distribution and the extreme patterns seen at the highest expression levels (Fig. 8D,H-I). Costaining for *T. brucei* BiP and TbRab2p in 2.1 cells demonstrated that TbRab2p still largely colocalised with BiP, especially in the perinuclear location (Fig. 8E,F). A minor population of cells (~5% of the total) had a strongly abnormal morphology with TbRab2p forming large round vesicles visible as vacuoles by phase contrast; these cells were also expressing the most TbRab2p as judged by IFA, so abnormal phenotype correlates with expression level (Fig. 8G-I). Therefore in the presence of excess TbRab2p ER assumes abnormal properties, such as excess vacuolisation, or excess TbRab2p localises to membraneous structures other than the ER. Levels of BiP staining was uniform in 2.1 cells.

DISCUSSION

TbRab2p is a small GTPase of the Rab family, which partially colocalises with *T. brucei* BiP, an ER-lumenal protein. It is present at similar levels in procyclic and BSF cells (Field and Field, 1997), and appears to be expressed uniformly throughout the cell cycle, consistent with a constitutive role. The protein is membrane bound, associated with the insoluble fraction of hypotonically lysed cells (Field and Field, 1997) and with the detergent-extractable fraction. Because TbRab2p can be geranylgeranylated by mammalian cytosol, and trypanosome G proteins are modified in vivo by prenylation, it is likely that in common with mammalian rabs the isoprene moieties are embedded in the membrane and are responsible for membrane association.

It was previously unclear which mammalian Rab protein was most homologous to TbRab2p (Trab1p in Field and Field, 1997), since Blast searches of GenBank revealed homology to pre- and post-Golgi rabs of both mammals and yeast. However, prompted by our preliminary observations that TbRab2p gave an ER-like staining pattern in IFA, we specifically examined the relationship of TbRab2p to ER-Golgi mammalian Rabs, 1a, 1b and 2. The most significant homology was found to Rab2 and included the important C-terminal hypervariable region which plays a primary role in membrane targeting. The higher level of homology suggested that the C terminus of TbRab2p may be recognised by the mammalian Rab targeting system; transfection of TbRab2p into mammalian cells resulted in an ERGIC localisation, where the major proportion of Rab2 is found (a small amount also being localised to the ER exit regions) and confirmed the assignment. The ERGIC localisation of TbRab2p in mammalian cells suggested that ERGIC-like membraneous tubulovesicular structures may also exist in *T. brucei* cells. We observed candidate structures in close proximity to the trypanosome *cis*-Golgi highly suggestive of an ERGIC-like compartment.

These observations raise the question of whether there is a true Rab1 homologue in *T. brucei*. A kinetoplastida Ypt1p homologue was recently isolated from *Leishmania* (Cappai et al., 1993), but the homology between LmYpt1 and TbRab2p is not striking, suggesting that a protein similar to LmYpt1 may exist in *T. brucei*. The distinction between mammalian Rab1 and Rab2 cannot be assumed in protozoa, and similarities in the effector loop sequences of human Rab1 and TbRab2p would be consistent with a single protein having multiple roles. In this regard it is interesting to note that a recently reported possible TbRab1p sequence (Bringaud et al., 1998) increases the likelihood that TbRab2p is a Rab2 functional homologue and a second Rab is involved in trypanosome ER-to-Golgi transport.

In cells overexpressing TbRab2p (2.1 clone) by IFA ~15% of cells have extremely high levels of the transgene product. In the cell population overall, there is a small decrease in procyclin biosynthesis, whilst by contrast, there is an increase in overall protein synthesis. These data suggest that TbRab2p has an affect on ER function, namely on procyclin processing and export. The increased general protein synthesis may be due to rapid turnover of abnormal ER or associated membrane structures, as seen in the fraction of cells most highly expressing TbRab2p. However, as the 2.1 clone is no longer homogeneous, it is not possible to determine if decreased procyclin synthesis and increased protein synthesis occur in the same or distinct cell populations.

Different levels of TbRab2p staining were observed in individual 2.1 cells. The mechanism by which a cloned cell can give rise to apparent variable levels of expression of a transgene is not completely clear. 2.1 cells were derived from a single cell by limiting dilution cloning: only 2 of 60 wells produced cultures, indicating that less that one cell per well was plated. The expression level is a continuum rather than bimodal, as predicted for two cells cloned into the same well. During extended culture, additional adaptions may occur, involving genetic alterations within the target locus or elsewhere, so that 2.1 cells have ceased to be a true clone, but rather are a population derived from a clone. Asymmetric partitioning of ER/ERGIC may occur during mitosis in 2.1 cells. It may be advantageous to the cell population to maintain some cells highly overexpressing TbRab2p, creating a reproducible diversity in the population which might be maintained even after recloning, an effect seen in B cells (Taylor et al., 1997). It may be necessary to use an inducible expression system to avoid clonal heterogeneity observed here.

Over expression of TbRab2p led to the production of abnormal membrane structures in a subpopulation of cells, and the abnormalities increased with the amount of over expression in individual cells. As well as stranded structures, this effect was visible as large vacuolar structures which were stained positively for TbRab2p, most prominent in the population of cells expressing the highest levels of TbRab2p. Inhibition of ER-to-Golgi trafficking has been observed to cause swelling of rough ER in *Drosophila* (Satoh et al., 1997). Such perturbations suggest that the small G protein has a role in control of ER/ERGIC membrane dynamics; increased vacuolar structures may result from augmented production of ER transport vesicles and/or structures within the transit zone. Levels of mammalian Rab2 are quite low at the ER, and as TbRab2p is expressed at low levels in wild-type trypanosomes (Field and Boothroyd, 1995), increased expression of TbRab2p could be expected to result in amplified vesicle production by Rab1/Rab2 budding activity and is consistent with data from the mammalian system indicating that Rab2 is required for maturation of pre-Golgi intermediates (Tisdale and Balch, 1996). In fact an excess of a peptide derived from Rab2 recruits coatomer to and prevents uncoating of pre-Golgi intermediates, inhibiting fusion to the Golgi, causing accumulation of pre-Golgi intermediates, and wild-type Rab2 may have similar action (Tisdale and Jackson, 1998). Accumulation of structures may be due to limiting amounts of a further component, for example a coat protein, preventing completion of e.g. bud formation or maturation and fusion with distal compartments.

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