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TbRAB18, a developmentally regulated Golgi GTPase from *Trypanosoma brucei*☆

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Abstract

The trypanosomal secretory system is broadly similar to that of higher eukaryotes as proteins enter the system via the endoplasmic reticulum and are transported to the Golgi complex for elaboration of glycan chains. Importantly *N*-glycan processing is stage specific with only the bloodstream form (BSF) processing beyond the oligomannose form. Increased complexity of the BSF Golgi apparatus, as evidenced by morphological studies, may underpin this higher activity, but few trypanosome-specific Golgi proteins have been described that may play a role in this developmental alteration. Here we describe a novel member of the *T. brucei* Rab family, TbRAB18, which is stage-regulated and highly expressed in the BSF whilst barely detectable in the insect stage. This stage-specific expression suggests the presence of a TbRAB18-dependent transport pathway required for survival in the mammalian host. Furthermore, data indicate that TbRAB18 localises to membranes in close juxtaposition to structures stained with BODIPY-ceramide, a Golgi marker. Wild type TbRAB18, ectopically expressed in insect stage cells colocalises with TbRAB31, and hence is targeted to the Golgi complex, consistent with the location of the endogenous protein in the bloodstream form, whilst GTP and GDP-locked mutant isoforms demonstrate distinct localisations, suggesting that Golgi-targetting of TbRAB18 is nucleotide-state dependent. We also find that ectopic expression of TbRAB18 wild type and mutant isoforms has no detectable effect on the synthetic anteriograde trafficking probe, TbBiPN. Finally, the location, and hence function, of TbRAB18 are distinct from the closest metazoan homologue, murine Rab18; the latter protein is involved in endocytic transport pathways whilst clearly TbRAB18 is not. Our data indicate further complexity in the evolution of small GTPases, and highlight the need for robust functional data prior to assignment of members of complex gene families.

Keywords: Golgi; Small GTPase; *Trypanosoma brucei*; Rab

1. Introduction

Trypanosoma brucei, has a digenetic life cycle alternating between a mammalian host and the Tsetse fly insect vector. *T. brucei* evades the mammalian host immune response principally through antigenic variation mediated via the variant surface glycoprotein (VSG) [1]. However additional virulence mechanisms also appear to operate, including rapid degradation of surface antibody bound to VSG via traffic through endosomal compartments facilitating cleavage of anti-VSG IgG/IgM [2]. A dramatic increase in the endocytic flux in BSFs compared with the insect stage, potentially controlled by increased expression of the major endocytic coat protein clathrin, may have a prominent role in this process [3].

Small GTPases of the Rab family are master regulators of intracellular vesicle trafficking [4]. In brewer's yeast the family is limited to 11 members but in mammals a more extensive family containing over 50 distinct proteins has been described. Rabs function as

Abbreviations: BSF, bloodstream form; GPI, glycosylphosphatidylinositol; GST, glutathione *S*-transferase; ORF, open reading frame; PBS, phosphate-buffered saline; PCF, procyclic culture form; pNAL, polylactosamine; PNS, post nuclear supernatant; VSG, variant surface glycoprotein.

☆ *Note:* Nucleotide sequence data reported in this paper have been submitted to the GenBank™, EMBL and DDBJ databases with the accession number AF131291.

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molecular switches and by direct and indirect interaction with a large number of proteins control vesicle fusion, cargo selection, cytoskeletal transport and integrate intracellular transport with signal transduction pathways [5–7]. Rab function includes controlling the binding of transport vesicles to their intracellular destination in a dilution resistant manner by recruitment of tethering factors in addition to the assembly of protein complexes which provide fusion competency to trafficking vesicles with their target membranes [5,8]. The intrinsic enzymatic activity of Rab proteins is modulated by ancillary factors which stimulate the rate of nucleotide hydrolysis or exchange. Importantly, Rabs localise to specific endomembrane structures indicating that members of this protein family act in part as specificity determinants in vesicular trafficking [9]. We have exploited this property to dissect vesicle trafficking pathways in *T. brucei* [10].

Our laboratory has identified 18 *T. brucei* Rab proteins (TbRAB) at the sequence level. Studies indicate significant complexity in the regulation of trafficking processes in *T. brucei*, for example, four TbRABs involved in endosomal dynamics have been described [11,12]. Of the six TbRABs characterised in detail, five are expressed constitutively suggesting that the majority of TbRAB regulated transport processes are required throughout the life cycle. TbRAB11, a TbRAB localised to endosomal regions containing recycling cargo, is upregulated in the BSF and is implicated as playing a role in virulence mechanisms in this stage [12]. Despite constitutive expression, TbRAB31, TbRAB5A and TbRAB5B exhibit stage-specific alterations to their location, and hence their precise role in BSFs may be nonequivalent in the PCF [11,13]. In all cases, we have observed that the BSF has additional compartmental complexity compared with the insect stage. A number of additional alterations to BSF secretory protein processing and organisation of the endomembrane system, including increased *N*-glycan processing and greater complexity in Golgi elements also indicate that there is a stage-specific role for posttranslational protein modification [13,14]. Here we describe a further trypanosomal Rab, TbRAB18, which is upregulated in the BSF stage and localised predominantly to elements of the Golgi complex.

2. Materials and methods

2.1. Maintenance and manipulation of Trypanosoma brucei

Culture-adapted MiTAT bloodstream form expressing VSG 221 and procyclic form parasites, strain 427, were grown and transformed as previously described [11].

2.2. Nucleic acids and recombinant DNA methods

Standard molecular biology methods were carried out as described [15] unless otherwise indicated. Nitrocellulose membranes for Western blotting and nylon membranes for Northern blotting were from Amersham Pharmacia. Plasmids were propagated in *Escherichia coli* XL1-Blue (Stratagene), unless otherwise mentioned. PCR was performed using a 480 Thermal Cycler (Perkin Elmer) with *Pfu* polymerase (Stratagene) and gel embedded DNA purified using Geneclean (BIO101). Mini prep plasmid isolations were conducted using Spin kits from Qiagen.

2.3. Library screening and cloning of the TbRAB18 ORF

An EST potentially encoding a trypanosomal small GTPase (Rtb5) [10] was used to screen a λFIX library prepared with Sau3A digested *T. brucei* genomic DNA (kind gift of Dr John Mansfield, Madison). Two distinct clones were isolated and phage DNA prepared from both. Inverse PCR using the primer combination IPRtb5f (TGGTGGATTCGATGGGGAGGCAGAAGAAAC) and IPRtb5r (GACAGGACTGTTGTCACCTTGCGCCATTAC) after digestion of the phage DNA with PstI followed by dyedeoxynucleotide sequencing on an ABI 377 sequencer permitted isolation of the sequence for the entire Rtb5-containing ORF. Sequences were compared against the nonredundant database at NCBI with BLAST. Based on this analysis the ORF was designated TbRAB18.

2.4. RNA isolation and Northern blotting

Total RNA was isolated from 10^8 PCF and BSF parasites as described previously [12]. RNA was resolved through a denaturing formaldehyde gel [16] and blotted onto nylon membranes. Subsequent to blotting the membrane was cross-linked using a UV Stratalinker (Stratagene) and hybridised overnight with a probe generated by a random priming method [16] using the full TbRAB18 nucleotide sequence as a template. Blots were washed three times for twenty minutes in 0.2 × SSC/0.1% SDS before exposure against X-ray film (Kodak).

2.4.1. Generation of TbRAB18 constructs

The TbRAB18 ORF was ligated into pGEX-2TK in frame by PCR amplification of purified phage DNA using the 5′ primer TbRAB18Xf, which contains a BamHI site, (GACGTGGGATCCATGGVGCAAGGTGACAACAGTCCTGTCAAGATC) and the 3′ primer TbRAB18Xb, which contains an EcoRI site GTTGTCATGAATTCGCTAACAGCACACCGCGCCGCTTGTGGGGTCACT) followed by restriction digestion with BamHI and EcoRI. Amplification of

the TbRAB18 ORF using the 5′ primer Tb18Gf, containing a HindIII site (TGCAAGCTTATGGCG-CAAGGTGACAACAGT) and the 3′ primer Tb18Gr, which contains a BamHI site (CTGGGATCCCTAA-CAGCACACCGCGCCGCT), followed by restriction digestion with HindIII and BamHI allowed subcloning of the gene into the PCF trypanosomal expression vector pXS219 [17].

2.5. Generation of TbRAB18 mutants

Mutagenesis of the TbRAB18 ORF to generate TbRAB18^{SN} and TbRAB18^{QL} was performed by mega-primer PCR. Amplification of the TbRAB18 ORF using the 5′ primer Tb18Gf and the 3′ primer Tb18GD, containing an AcsI site (CGAGAGAAGCAGT-GAATTCTTTCCCACG) yielded a 76bp product which was purified and used in a second PCR in conjunction with Tb18Gb to yield full length mutant TbRAB18 ORF. After confirming incorporation of the mutant site by AcsI restriction the mutant ORF was subcloned into pXS219 and sequenced by dyedeoxy sequencing. TbRAB18^{SN} was inserted into pGEX-2TK by amplification using the 5′ primer TbRAB18Xf and the 3′ primer TbRAB18MXr, which contains a SmaI site (TATCCCGGGCTAACAGCA-CACCGCGCCGCTTGT) followed by restriction digest with BamHI and SmaI. Amplification of the TbRAB18 ORF using the 5′ primer Tb18Gf (see above) and the 3′ primer Tb18GT, which contains an XbaI site (CCGGAACCGCTCTAGACC CGCTGTGTCCCA) generated a 231 bp oligonucleotide, which was used in a second PCR with the 3′ primer Tb18Gr to produce TbRAB18^{QL}. TbRAB18^{QL} was subcloned into pXS219 and verified by sequencing. TbRAB18^{QL} was inserted into pGEX-2TK by amplification of the mutant ORF with the 5′ primer TbRAB18Xf and the 3′ primer TbRAB18X.

2.6. Recombinant protein expression and generation of antibodies

TbRAB18 was expressed as GST-fusion protein from pGEX-2TK in the *E. coli* strain BL21 (Stratagene). Wild type fusion proteins were purified using Glutathione Sepharose 4B (Pharmacia) and cleaved to release TbRAB18 by incubation at room temperature with Thrombin (New England Biolabs) and used to raise polyclonal antibodies in a rabbit and ice. Affinity purified antibodies were isolated using recombinant protein coupled to CNBr-activiated Sepharose 4B (Pharmacia, UK) by standard methods. Recombinant TbRAB11 was prepared as described previously [12].

2.7. Western blotting

Cell pellets were resuspended in boiling SDS sample buffer containing 10mM DTT and resolved by SDS–PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes (Amersham) by semidry electrophoresis. Blots were blocked in BLOTTO (0.2% Tween20, 5% Milk, PBS) for at least one hour and antibodies diluted in BLOTTO. Rabbit and mouse anti-TbRAB18 was diluted 1:1000 for blotting and detected using a horseradish peroxidase conjugate second antibody (Sigma). Immunoreactivity was detected by the luminol reaction and visualised by fluorography.

2.8. Immunofluorescence microscopy

Immunofluorescence microscopy was essentially as described previously [11] with minor variations. Anti-TbRAB18 was used at 1:100 with wild type BSF/PCF parasites and 1:400 with overexpressing PCF parasites. Secondary antibodies, anti-Rabbit Cy3 (Sigma) and anti-mouse FITC (Sigma), were used as according to the manufacturers instructions. The trypanosomal Golgi complex was stained using BODIPY-TR-ceramide (Molecular Probes) as previously described [18,13]. Cells were observed either on a Nikon Microphot-FX epifluorescent microscope attached to a Photometrics CH350 CCD camera or with a Zeiss laser scanning confocal microscope. Images were prepared for presentation using Adobe Photoshop.

2.9. GTPase assays

Activity was monitored essentially as described [19]. Briefly, GST fusion proteins expressed in *E. coli* were purified using Glutathione-agarose and checked by SDS–PAGE. Approximately 20 μg of protein was loaded with [α-^{32}P]-GTP (10 μCi; 400 Ci mmol^{-1}, Amersham) in 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 5 mM EDTA, 0.1 mM EGTA, 0.1 mM dithiothreitol, 10 mM ATP at 37 °C for 10 min in a total volume of 100 μl. Subsequently, beads were washed three times at 4 °C in a wash buffer consisting of 50 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM DTT and 1mg/ml bovine serum albumin (BSA). Samples were resuspended in wash buffer prewarmed to 37 °C and hydrolysis allowed to occur. At various time points samples were taken and added to an equal volume of ice cold quench buffer (5 mM EDTA, 50 mM GTP, 50 mM GDP). Bound nucleotides were eluted by incubation at 65 °C for 5 min in elution buffer (1% SDS, 20 mM EDTA). Eluted samples were spotted onto polyethyleneimine–cellulose plates and the released nucleotides resolved by thin layer chromatography (TLC) in 0.75 M KH_2PO_4 (pH 3.4) and visualised on a phos-

phorimager screen (Molecular Dynamics). Following conversion of the data to a TIFF format, GDP:GTP ratios were calculated using NIH Image 1.59.

2.10. Metabolic labeling of PCF T. brucei

PCF parasites were grown to mid-log phase, pelleted and washed three times with PBS. Cells were resuspended at a density of 10^8 parasites ml^{-1} in DMEM supplemented with 1% BSA but lacking methionine and cysteine (ICN). After one hour of starvation Trans[^{35}S]-Label (ICN) was added to a specific activity of 200 μCi ml^{-1} and cells incubated at 27 °C. For pulse labeling cells were pulsed with label for 15 min before being diluted 1 in 10 with SDM79 for initiation of the chase.

2.11. Immunoprecipitation

Labeled cells (10^7) were centrifuged and the supernatants reserved. Cells were washed once in PBS and then lysed in lysis buffer (50 mM Tris–HCL, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet-P40, 0.5% Deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Complete Mini, Roche) for 5 min on ice. A post-nuclear supernatant (PNS) was obtained by centrifugation at 14 000 rpm in an Eppendorf centrifuge 5417C. The detergents present in lysis buffer were added to the supernatant to the same final concentration. Both the PNS and supernatant were precleared using Pansorbin (Calbiochem) and subsequently antibodies were added (rabbit anti-TbBiP was used at 1:100 dilution). Samples were mixed overnight at 4 °C. 100 μl of a 10% protein A-agarose (Sigma) solution, washed with lysis buffer, was added to each sample which were incubated one hour at 4 °C with mixing. Samples were washed four times in lysis buffer and twice with TEN buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA). Final precipitates were suspended in 50 μl Protein Sample Buffer, fractionated by 12% SDS–PAGE and analysed by autoradiography.

2.12. Bioinformatics

BLAST searches were conducted using the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/blast), the *T. brucei* database at TIGR (http://www.tigr.org/tdb/mdb/tbdb/index.html) and at the PSU Sanger (http://www.sanger.ac.uk). Sequence alignments were performed using ClustalX with default parameters on a Macintosh computer.

3. Results

3.1. Cloning of a Rab18 homologue in Trypanosoma brucei

Several ESTs encoding partial open reading frames (ORF) corresponding to small GTPases were isolated by degenerate PCR [10]. One, Rtb5, was used to screen a *T. brucei* genomic library; two λ clones were isolated and by inverse PCR nucleotide sequences encoding a complete GTPase ORF derived. Both inverse PCR products had identical sequence (GenBank™ accession AF131291). Hypothetical translation of the nucleotide sequence predicted a 648bp ORF encoding a protein with a molecular weight of ~23.5kDa and terminating in a prenylation signal. Southern analysis indicated the gene was single copy in 427 *T. brucei* (data not shown). By BLAST the translated ORF was most similar to murine Rab18 (41% identical, 55% similar), a protein enriched on apical endocytic domains of mouse kidney cortex [20] and hence we designated the ORF TbRAB18. The closest yeast homologue was Ypt1p (36% identity, 51% similarity) whilst mouse Rab1A was 36% identical and 52% similar, both of which are implicated in ER to Golgi transport [4]. Therefore TbRAB18 falls into the Ypt1/Sec4 Rab subfamily [21]. TbRAB18 possesses all the typical features of Rab GTPases; four GTP binding motifs, an effector domain and a canonical C-terminal geranylgeranylation signal, but none of the closest homologues shared a fully conserved effector region, suggesting nonidentical function. In addition several short insertions predicted to lie between conserved α-helical and β-sheet elements are present (Fig. 1) [22]. Whilst not anticipated as affecting catalytic function, these insertions may provide TbRAB18-specific binding sites for accessory factors.

3.2. TbRAB18 encodes a developmentally regulated GTP-hydrolysis competent Rab protein

Expression of TbRAB18 was first examined at the mRNA level. Northern analysis using full length TbRAB18 as a probe revealed a single transcript of approximately 1.3 kb that was significantly more abundant in BSF than in PCF (Fig. 2A). The size of this mRNA species is consistent with that detected for other TbRABs [12,23].

Antibodies were raised in a rabbit and mice using recombinant TbRAB18 as immunogen and affinity purified. The antibodies recognised recombinant TbRAB18 but not recombinant TbRAB11 demonstrating specificity for TbRAB18 (data not shown). Additionally, reactivity towards TbRAB18 was abrogated by prior incubation of the antibodies with GST-TbRAB18, but not GST-TbRAB11 (data not shown). BSF and PCF lysates were resolved by 1D SDS–PAGE, trans-

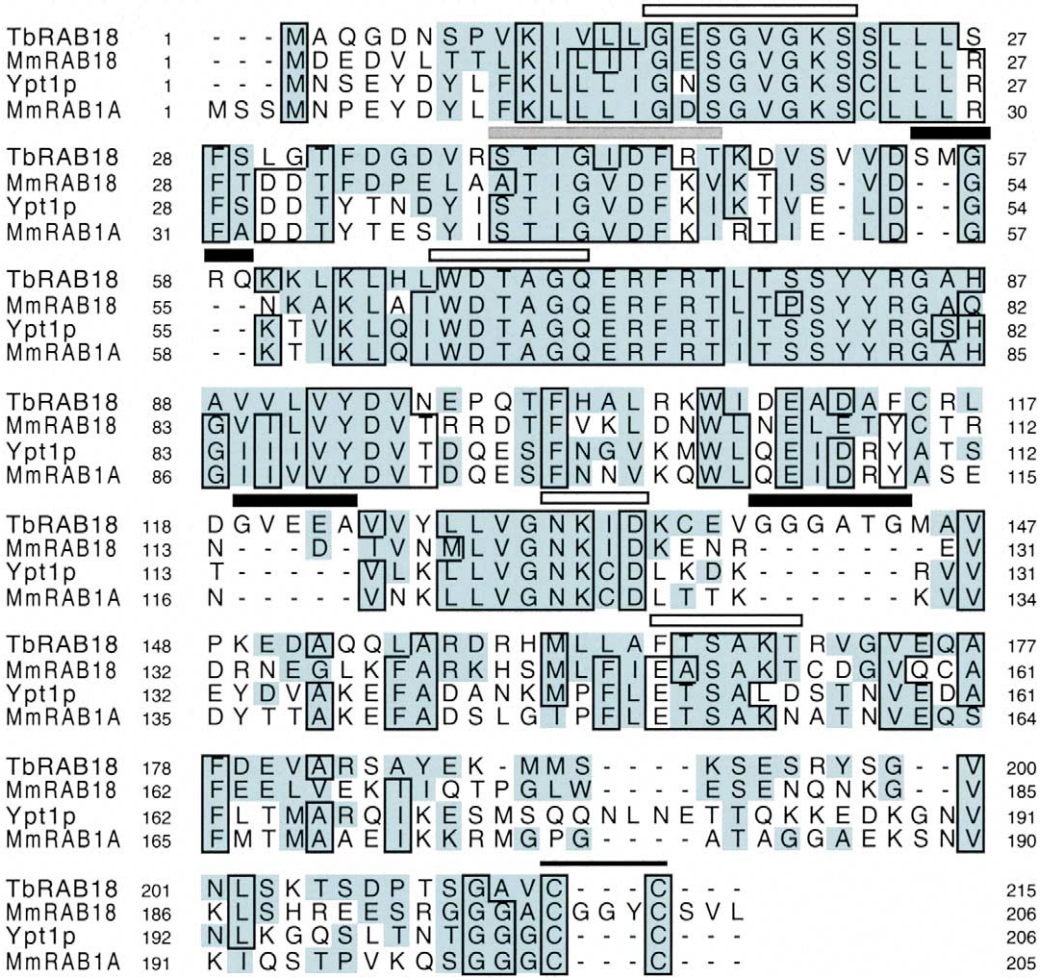

Fig. 1. Alignment of TbRAB18 with its three closest homologues: ClustalX alignment of the predicted translation of the ORF of TbRAB18 with its closest mammalian and yeast orthologues identified by BLAST at GenBank. *Mm*, *M. musculus*; *Ypt*, *S. cerevisiae*. Open bars above the alignment indicate GTP-binding motifs, grey bar represents effector domain and black bars indicate amino acid insertions in TbRAB18 absent in the three homologues. The dicysteinyl C-terminal prenylation signal is indicated by an overline. Identical residues are boxed, and similar residues are shaded; dashes indicate gaps introduced to optimise the alignment.

ferred to nitrocellulose membranes and probed with either the rabbit or murine anti-TbRAB18 antibodies. In each case a single band of immunoreactivity at approximately 23 kDa was observed which was significantly more abundant in the BSF than PCF (Fig. 2B and C). Immunoreactivity was again blocked by preincubation of antibodies with GST-TbRAB18 and not with GST-TbRAB11 (data not shown). Therefore, by both Northern and Western analyses, expression of TbRAB18 is developmentally regulated with a significant increase in protein levels in the BSF.

3.3. Subcellular localisation of TbRAB18

We chose to investigate the function of TbRAB18 further by immunofluorescence microscopy. The developmental regulation of TbRAB18 expression indicated that comparison of the subcellular location of TbRAB18 in BSF and PCF parasites was of interest, whilst the primary structure suggested that TbRAB18 may encode a protein involved in endosome dynamics [20]. In *T. brucei* endosomal regions are located between the nucleus and kinetoplast [11,12]. However, the similarity with Ypt1 and Rab1A suggested alternatively that TbRAB18 could be involved in exocytosis.

Staining PCF parasites with anti-TbRAB18 antibodies resulted in no detectable signal above background levels (data not shown). BSF parasites stained with anti-TbRAB18 antibodies possessed immunoreactivity predominately situated in a position posterior of the nucleus (Fig. 3). Preincubation of the mouse antibody with recombinant TbRAB18 abrogated reactivity (Fig. 3B). Whilst this location was consistent with an endosomal role for TbRAB18, the Golgi complex and other elements of the exocytic pathway are also located in this region of the cell, and hence we could not determine the location of TbRAB18 from these data alone. Hence, we next chose to determine if the

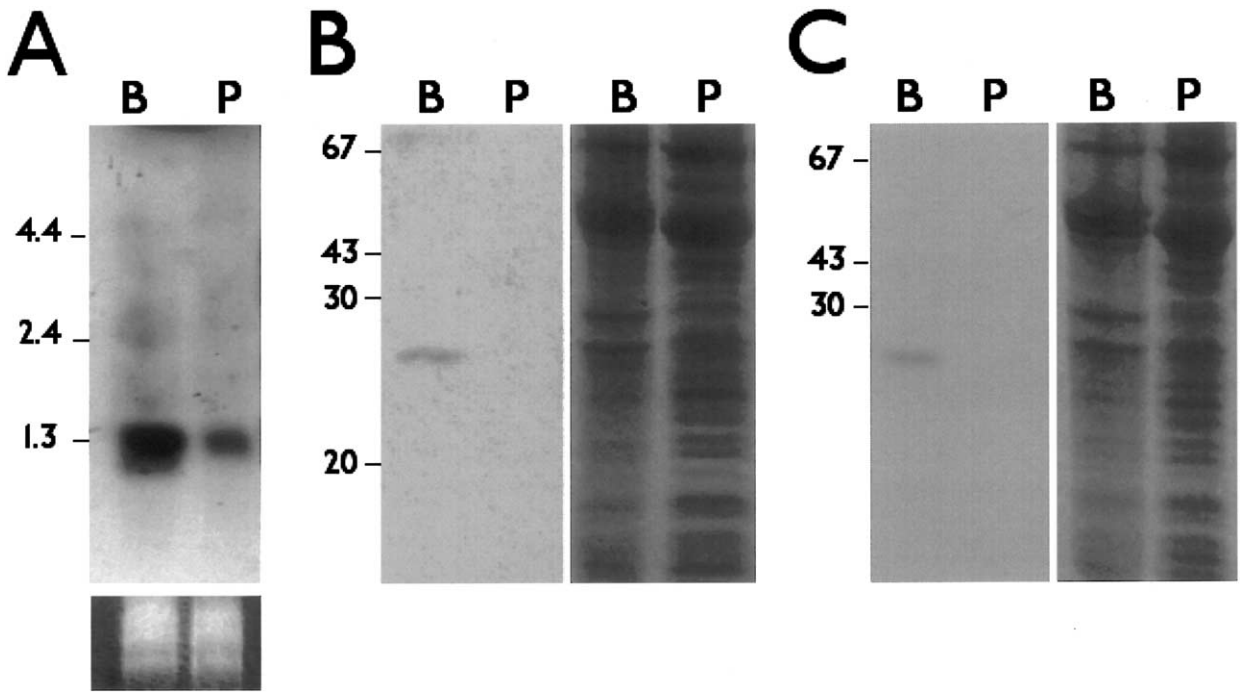

Fig. 2. TbRAB18 is developmentally regulated at the RNA and protein level. (A) Northern blot analysis demonstrating bloodstream form (BSF) parasites express TbRAB18 mRNA at a higher level than procyclic form parasites (PCF). Total RNA from 10^7 cells was probed with radiolabelled full length TbRAB18. Positions of RNA standards in kb are shown to the left and ethidium bromide stain of the rRNA bands for loading equivalence at the base of the figure. (B and C) Western blot analyses of trypanosome lyastes probed with affinity purified anti-TbRAB18 antibodies generated in rabbit (B) or mice (C). 10^7 parasite equivalents were loaded in each lane. Loading controls (Coomassie stained gels run in parallel) demonstrating the presence of protein in each lane are shown to the right of each blot. Molecular weight standards are indicated to the left in kDa. B, BSF; P, PCF.

TbRAB18 compartment corresponded to a previously described subcellular structure.

The trypanosomal Golgi is located close to the posterior face of the nucleus and can be specifically stained with the fluorescent lipid BODIPY-TR-Ceramide [13]. The relationship between the Golgi and TbRAB18 was examined by allowing BSF parasites to accumulate BODIPY-TR-ceramide in the Golgi prior to fixation for microscopy and staining with mouse anti-TbRAB18. The Golgi regions defined by BODIPY-TR-ceramide can be seen to partially colocalise with murine anti-TbRAB18 immunoreactivity suggesting that the Rab protein may be binding a Golgi-associated element or subcompartment of the Golgi, but which is nonequivalent to the BODIPY-TR-ceramide region (Fig. 4). A similar close configuration but nonidentical staining between TbRAB31, a Golgi Rab protein, and BODIPY-ceramide, has been reported previously [13].

The proximity of the TbRAB18-containing structures with the Golgi prompted an examination of the relationship between the TbRAB18 positive region with the subcellular localisation of another Golgi-associated trypanosomal protein. TbβAP1 is a recently described trypanosomal β-adaptin implicated in membrane dynamics at late Golgi structures [3,24]. Costaining parasites for TbβAP1 and TbRAB18 demonstrated partial colocalisation and tight juxtaposition of the two structures (Fig. 4). These results strongly suggest that TbRAB18 localises to a Golgi element.

3.3.1. *Mutagenesis of TbRAB18*

The nucleotide state of Rab proteins correlates with their activity and defined mutations within the conserved GTP binding motifs allows construction of Rab protein isoforms deficient in nucleotide hydrolysis or exchange of GDP for GTP [25]. Hence, TbRAB18^{SN} has an S to N mutation in the first GTP-binding motif, predicted to prevent release of GDP following hydrolysis of the nucleotide, whilst TbRAB18^{QL} has a Q to L substitution in the WDTAGQE box, a mutation predicted to ablate GTPase activity and lock the protein in the GTP-bound conformation (Fig. 5).

To examine the ability of the TbRAB18 isoforms to hydrolyse GTP, TbRAB18, TbRAB18^{SN} and TbRAB18^{QL} were expressed as GST-fusion proteins in *E. coli*. GST-TbRAB18 and GST-TbRAB18^{QL} could be isolated (data not shown, Fig. 5) but GST-TbRAB18^{SN} was unstable in *E. coli* (data not shown). Hence we tested the GTPase activity of GST-TbRAB18 and GST-TbRAB18^{QL} only. Whilst GST-TbRAB18^{QL} bound GTP to a similar level as the wild type protein, the

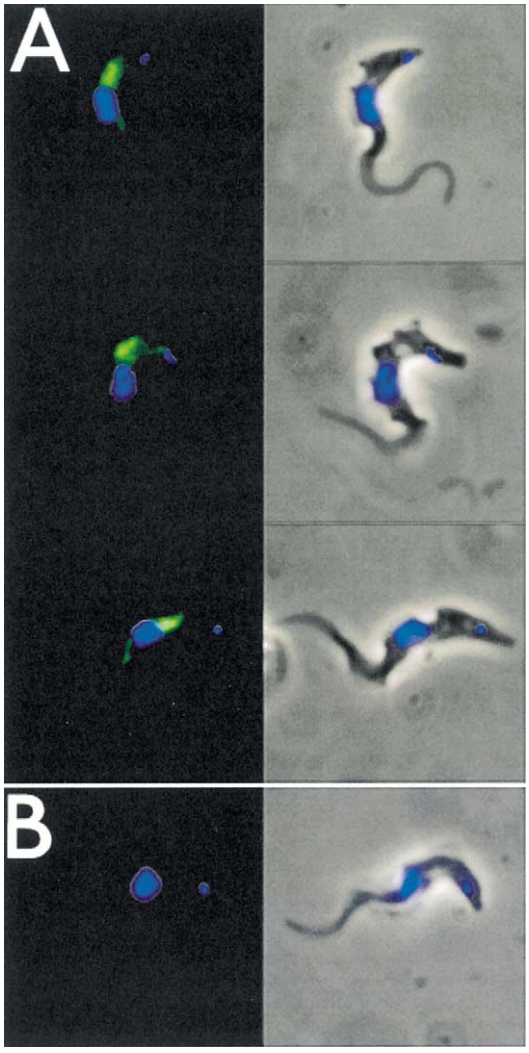

Fig. 3. TbRAB18 localises to the inter-nuclear-kinetoplast region of the cell. (A) Gallery of BSF parasites stained with murine anti-TbRAB18 followed by an anti-mouse FITC conjugate (green). (B) BSF parasites stained with murine anti-TbRAB18 after preincubation with recombinant TbRAB18. Each panel is comprised of a fluorescent image (left) and the corresponding phase contrast image (right) of the same field with DAPI (blue) overlaid.

ability of the mutant to hydrolyse nucleotide was decreased by fivefold (Fig. 5).

3.4. Expression of wild type and mutagenised TbRAB18 isoforms in PCF parasites

PCF parasites were transfected with wild type and mutagenised versions of TbRAB18 in pXS219 [26]. In each case three independent clones were selected for each isoform by limiting dilution. Integration of the correct isoform of TbRAB18 into the trypanosomal tubulin locus was confirmed by Southern blot (data not shown) and Western analysis of overexpressing clones demonstrated that in all cases there was a ~20-fold increase in anti-TbRAB18 immunoreactivity compared to parental parasites (Fig. 6).

The subcellular location of the ectopic TbRAB18 isoforms was examined by immunofluorescence microscopy (Fig. 6). We chose to do these analyses in PCFs as the endogenous level of TbRAB18 was undetectable and hence all immunoreactivity could be ascribed to the ectopic copy alone. Cell lines overexpressing wild type TbRAB18 had a distinct focus of anti-TbRAB18 immunoreactivity situated close to the posterior face of the nucleus, and very similar to the location in the BSF. The identity of the punctate focus of anti-TbRAB18 immunoreactivity observed in PCF parasites overexpressing TbRAB18 was examined further by colocalisation studies. TbRAB31, a Golgi Rab protein, was localised to the same perinuclear structure defined by TbRAB18^{WT}. Significantly, no significant colocalisation of TbRAB18 with TbBiP (the ER) was seen, but the morphology of the ER was unaltered. Additionally, TbRAB18 was juxtaposed to, but distinct from lysosomal elements as determined by costaining for p67, a lysosomal protein [28] (Fig. 7). Overall, TbRAB18^{WT} appears correctly targetted to the Golgi complex.

By contrast to TbRAB18^{WT}, the mutant TbRAB18 isoforms exhibited altered distribution, indicating nucleotide state-specific targetting of TbRAB18. Clones expressing TbRAB18^{QL} possess anti-TbRAB18 immunoreactivity situated at the posterior face of the nucleus, plus a considerable level of anti-TbRAB18 located at the nuclear rim (Fig. 6). In contrast the vast bulk of TbRAB18^{SN} reactivity was localised to punctata situated between the nucleus and kinetoplast. The highly distinctive nature of the location of the TbRAB18 isoforms, plus the well documented specificity of Rab targetting, argues that the mutant TbRAB18 isoforms are specifically localised.

3.5. Expression of TbRAB18, TbRAB18^{QL} or TbRAB18^{SN} in PCF does not compromise secretion of BiPN

The data above indicate that TbRAB18 is localised to the trypanosomal Golgi complex. We asked if an estabished anteriorgrade secretion marker, the ATPase domain of the *T. brucei* BiP (BiPN) was affected by the presence of the mutant isoforms of TbRAB18 [26,27].

PCF parasites transfected with BiPN secrete BiPN with a $t_{1/2}$ of ~60 minutes [26]. Recombinant parasites were pulsed with ^{35}S-Met and chased for 4 h: at 0.0, 0.5, 1.0, 2.0 and 4.0 h, cells and supernatant were harvested and a rabbit anti-TbBiP antibody used to immunoprecipitate endogenous BiP and BiPN. The kinetics of export of BiPN in wild type parasites and PCF transfected with the TbRAB18 isoforms was determined by densitometry. There was no significant alteration in transport kinetics of BiPN to the cell surface in the

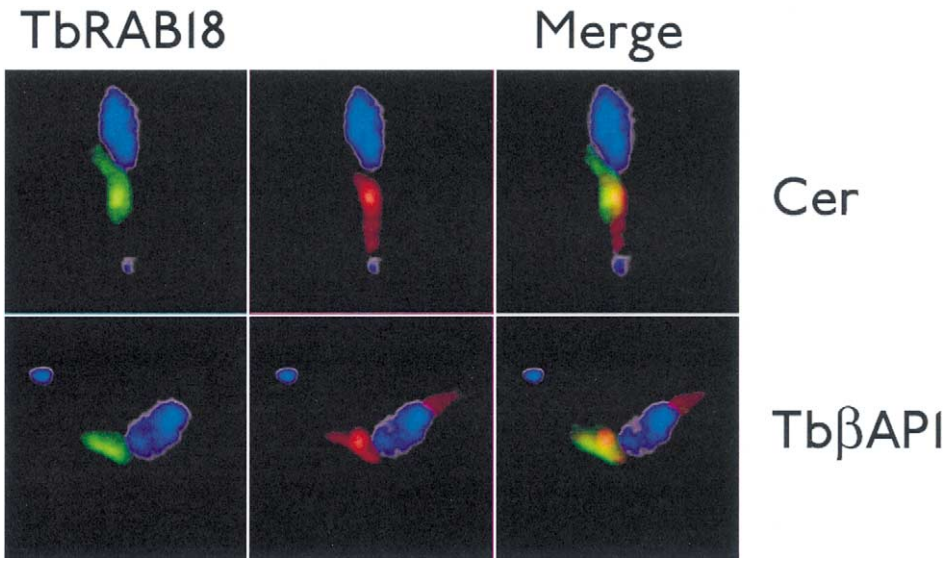

Fig. 4. TbRAB18 partially colocalises with the Golgi complex and TbβAP1. Epifluorescent microscopy was used to examine the relationship of TbRAB18 with the trypanosomal Golgi. BSF parasites were fed BODIPY-TR-Ceramide to permit visualisation of the Golgi complex (top panels, Cer; red). Cells were costained with anti-TbRAB18 (green) and DNA with DAPI (blue). The TbRAB18-positive compartment partially colocalises with the fluorescent lipid stain. BSF parasites were also stained with anti-TbβAP1 (red) and DNA with DAPI (blue).

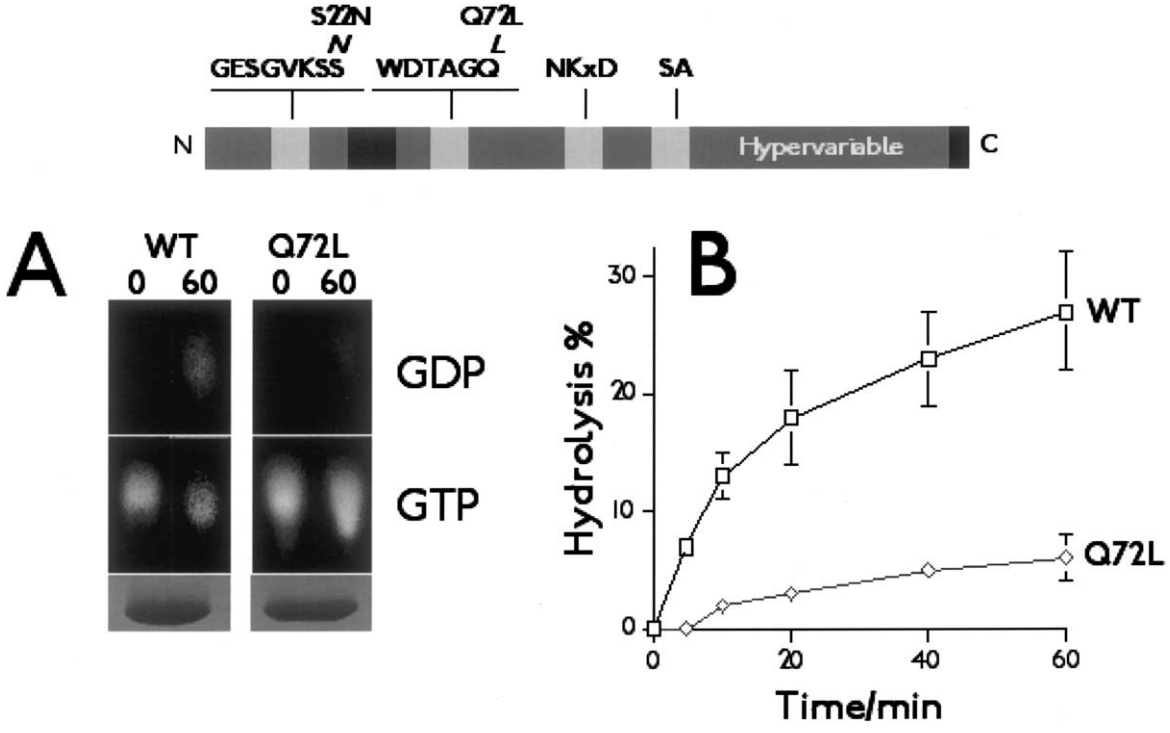

Fig. 5. Mutagenesis of TbRAB18: Schematic representation of TbRAB18 domain structure indicating sites of mutagenesis. GTP-binding motifs (open), effector domain (dark grey) and predicted site of geranylgeranylation (black). The hypervariable C-terminal domain is indicated. The wild type sequence (GESK…, etc.) is indicated in the lower line, and the positions of the altered amino acids shown. (A) Autoradiograms of products produced from [α-^{32}P]GTP after incubation with TbRAB18 or TbRAB18^{QL} GST-fusion proteins at 37 °C, resolved by thin layer. Regions corresponding to GTP and GDP are shown; no other radioactivity was detected on the TLC plate. 0, 0-min incubation; 60, 60-min incubation. Lower panel: Coomassie stained SDS–PAGE after resolution of GST-TbRAB18 and GST-TbRAB18^{QL} at the amounts used in the GTPase assay to demonstrate equivalent levels of protein as used for each assay. (B) Kinetics for hydrolysis of [α-^{32}P]GTP to [α-^{32}P]GDP by GST-TbRAB18 (squares) and GST-TbRAB18^{QL} (diamonds). The data are representative of three independent experiments.

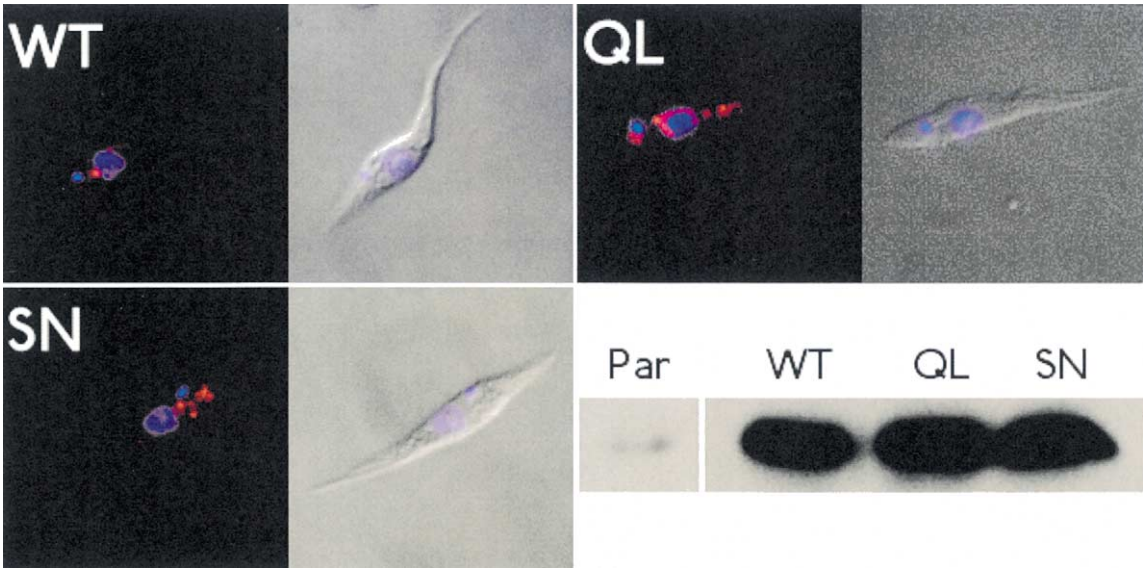

Fig. 6. Analysis of cloned cell lines overexpressing isoforms of TbRAB18: PCF parasites transfected with TbRAB18 (WT), TbRAB18^{QL} (QL) or TbRAB18^{SN} (SN) were stained with anti-TbRAB18 antibodies (red). DNA was visualised using DAPI (blue). The anti-TbRAB18 immunoreactivity can be seen to be organised as the endogenous proten in WT, but not in the SN and QL transformants. Inset: after transformation and single cell cloning recombinant trypanosomes were analysed for overexpression by Western blotting. Membranes were probed with rabbit anti-TbRAB18 followed by an anti-rabbit horseradish peroxidase conjugate. Par, parental 427; WT, cells expressing TbRAB18; QL, cells expressing TbRAB18^{QL}; SN, cells expressing TbRAB18^{SN}. Note: the parental lane has been exposed for three times as long as the transgenic parasite lanes. Data were identical for three independent clones (data not shown).

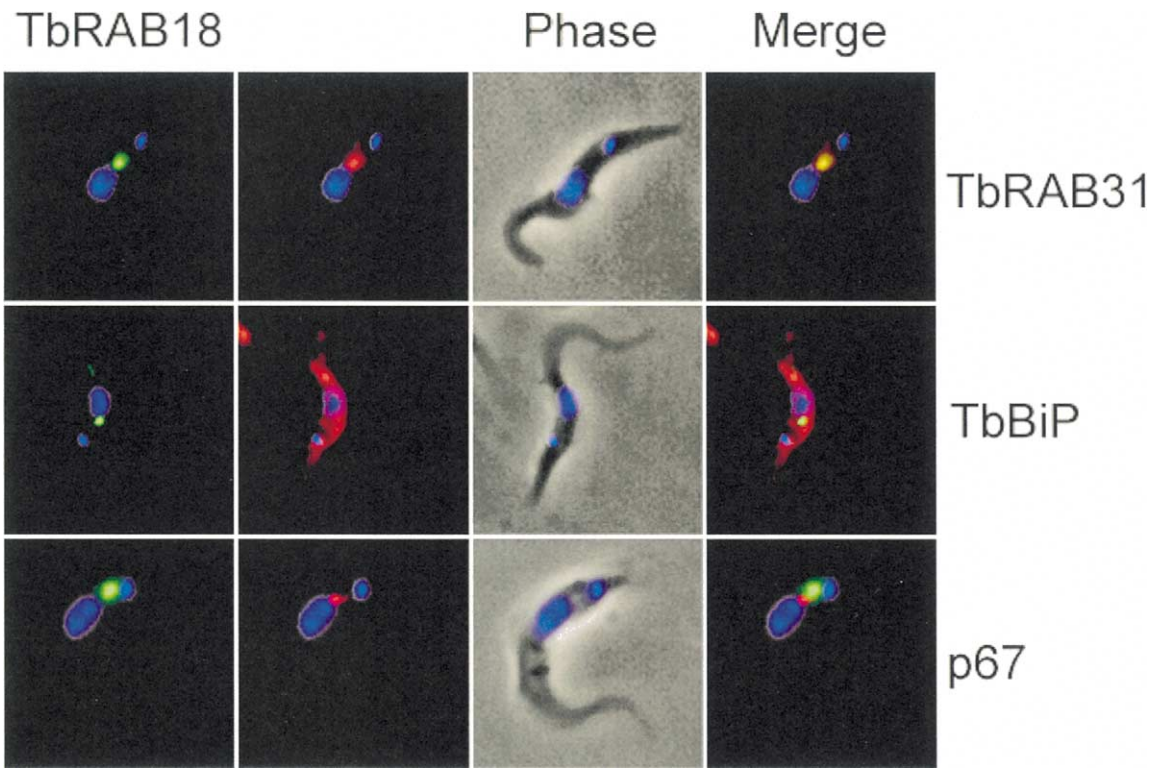

Fig. 7. TbRAB18 overexpressed in procyclics localiseds to the Golgi complex. Parasites transfected with TbRAB18^{WT} were stained with mouse anti-TbRAB18 (green) and rabbit antibodies against TbRAB31, TbBiP and p67 (red). DNA was visualised with DAPI (blue). Note the colocalisation between TbRAB18 and TbRAB31, but not p67 or TbBiP

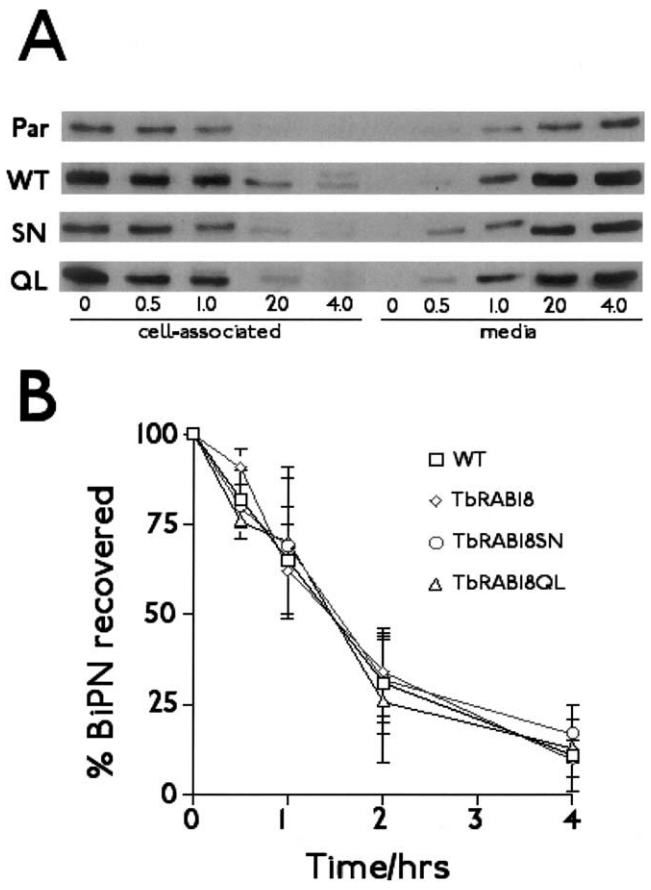

Fig. 8. Expression of TbRAB18 isoforms does not perturb anterograde secretory processes in procyclic parasites. PCF parasites transfected with BiPN and wild type or mutagenised isoforms of TbRAB18 were assayed for BiPN secretion. (A) Representative data set of immunoprecipitated radiolabeled BiPN. Par, 427 parental cells; WT, 427 PCFs expressing TbRAB18; QL, 427 PCFs expressing TbRAB18^{QL}; SN, 427 PCFs expressing TbRAB18^{SN}. (B) The kinetics of export of BiPN, i.e. loss of cell-associated material, for each cell line was determined in three independent experiments. Data show the mean and standard deviations. Kinetics of appearance of BiPN in the media was the inverse of the cell-associated kinetics shown here. All three cell lines transfected with TbRAB18 ectopic copies exhibit kinetics of BiPN secretion indistinguishable from the parental.

mutant PCF parasites compared to wild type PCFs (Fig. 8).

4. Discussion

Here we describe the characterisation of a new developmentally regulated trypanosomal Rab protein, TbRAB18. By both Northern and Western analyses TbRAB18 exhibits stage-specific expression and is considerably more abundant in BSF parasites than in PCFs. Several lines of evidence indicate that TbRAB18 localises to elements of the Golgi complex: (i) the position of the TbRAB18 compartment is consistent with the position of the Golgi; (ii) in BSF the protein partially colocalises with BODIPY-TR-Ceramide and TbβAP1 [3]; and (iii) wild type TbRAB18 overexpressed in PCF parasites colocalises with TbRAB31, a previously documented Golgi-associated TbRAB [13]. We believe that targetting of TbRAB18^{WT} in the PCF is correct as, firstly, it is as consistent with the BSF location and secondly, as Rabs interact with a large number of proteins, many of which have multiple roles within the cell, it is likely that the PCF Golgi complex expresses TbRAB18-binding sites. This is also supported by the fact that TbRAB18 is expressed in this life stage at the protein level. TbRAB18 hence represents the second trypanosomal Golgi Rab protein to be identified, and interestingly both TbRAB18 and TbRAB31 exhibit developmental regulation, TbRAB18 in expression level and TbRAB31 in morphology [13]. In each case, the BSF demonstrates increased complexity over the insect stage. It should also be noted that the the Golgi complex is rather more extensive in the BSF [13], which accounts for the differences in TbRAB18 staining between the two life stages.

By BLAST the closest mammalian homologue to TbRAB18 is murine Rab18 which localises to apical endosomal regions in epithelial cells [20]. The Golgi localisation of TbRAB18 is more consistent with that of its closest yeast homologue Ypt1p, a *S. cerevisiae* Rab protein involved in modulating ER-to-Golgi traffic. TbRAB18 is rather less closely homologous to Rab1A, a mammalian Rab also involved in ER-to-Golgi traffic. Phylogenetic analysis of human Rab GTPases clusters Rab18 closer to Rab proteins involved in ER and Golgi trafficking processes (e.g. Rab1A, Rab8) than to those involved in the regulation of endosomal dynamics (e.g. Rab4, Rab5, Rab11) [21]. The disparate locations and hence functions of TbRAB18 and mammalian Rab18 provide clear evidence that inference of cellular role from sequence homology is unreliable and is likely to be particularly difficult for multigene families, as is the case for Rab proteins. Additionally, sequence analysis also revealed the presence of a number of short insertions in TbRAB18 but absent in close homologues. The functional relevance of these insertions has not been determined experimentally, but alignment with three-dimensional G protein structures [22] indicates that these additional elements most likely form loops between the α-helical and β-sheet elements of the folded protein. These loops potentially facilitate trypanosome-specific protein–protein interactions which are not conserved with the higher eukaryote homologous proteins.

In PCF parasites TbRAB18^{WT} localised to the Golgi complex, but for TbRAB18^{QL} a significant level of anti-TbRAB18 immunoreactivity was also located around the nuclear rim and for TbRAB18^{SN} several punctate cytoplasmic structures were observed. These data indicate that TbRAB18 localises to the Golgi complex by a nucleotide state-dependent mechanism. Constitutively

activated Rab proteins typically localise to the acceptor compartment of the trafficking pathway modulated by a particular Rab, and $Rab5^{QL}$ is found principally on early endosomes in mammalian systems, the acceptor compartment for endocytic material [25]. When strongly overexpressed, Rab proteins can be found in a cytoplasmic location as well as at their correct site; membrane mistargetting is rare for these proteins as a specific recognition system is an important component of the membrane attachment process. The presence of $TbRAB18^{QL}$ on the nuclear rim may suggest that TbRAB18 is involved in regulating a retrograde trafficking route leading from the Golgi to nuclear rim regions of the ER; in the absence of a plausible assay for retrograde transport in the trypanosome system this hypothesis must remain untested. Overexpressing wild type or mutagenised isoforms of TbRAB18 did not affect secretory pathway transit for TbBiPN; however it is possible that this substrate is not transported by a TbRAB18-dependent pathway [29] or that TbRAB18 is redundant in the PCF, as it is normally expressed at a low level. The TbRAB18 expression profile suggests that the TbRAB18-mediated pathway is more active in the BSF, and probably reflects specific requirements in this life stage for folding and processing of surface proteins in the Golgi complex.

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