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Cell-cycle and developmental regulation of TbRAB31 localisation, a GTP-locked Rab protein from *Trypanosoma brucei*

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Abstract

Rab proteins are small GTPases that control the direction and timing of vesicle fusion during intracellular trafficking between membraneous compartments. Genome sequencing and EST analysis of *Trypanosoma brucei* indicates that the trypanosome Rab (*TbRAB*) gene family, and hence complexity of intracellular transport pathways, is intermediate between *Saccharomyces cerevisiae* and mammals. TbRAB31 is a constitutively expressed *T. brucei* Rab protein (formerly Trab7p) and is the product of one of two closely linked *TbRAB* genes, the other being *TbRAB2* (TbRab2p, in: Field H, Ali BRS, 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;112:147–156), involved in ER to Golgi transport. TbRAB31 has high homology to members of the Sec4/Ypt1 subfamily of Rab proteins from *S. cerevisiae* and to Rab13 and Rab11 from higher eukaryotes. Recombinant TbRAB31 binds GTP but, unusually for a Rab protein, has undetectable GTPase activity resulting in a constitutively GTP-bound protein. Antibodies against TbRAB31 recognise a discrete structure located between the kinetoplast and nucleus in interphase procyclic cells; by contrast the structure is morphologically more complex in bloodstream form (BSF) parasites, consisting of at least two foci. TbRAB31 behaviour was also studied during the cell cycle; TbRAB31 always localised to a discrete structure that duplicated very early in mitosis and relocated to daughter cells in a coordinate manner with the basal body and kinetoplast, suggesting the involvement of microtubules. Additional evidence suggests that TbRAB31 localises to the trypanosome Golgi complex. Firstly, the interphase position of TbRAB31 is consistent with a Golgi location. Secondly, the TbRAB31 structure is also recognised by cross-reacting antibodies to mammalian

Abbreviations: β-COP, β-coatomer protein; BSF, bloodstream form; IFA, immunofluorescence assay; LY, Lucifer yellow; *TbRABn*, *Trypanosoma brucei* Rab*n* gene; TbRAB*n*, *T. brucei* Rab*n* protein.

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β-coatomer protein (β-COP), which localises to the Golgi in mammalian cells. Thirdly, the fluorescent ceramide analogue, BODIPY-TR-ceramide, a reliable marker of the mammalian Golgi apparatus, exhibited overlapping distribution with TbRAB31. The location of BODIPY-TR-ceramide was confirmed at the trypanosome Golgi by histochemistry with diaminobenzidine and electron microscopy.

Keywords: Cell cycle; Golgi apparatus; Kinetoplast; Small GTPase; Ypt31p; *Trypanosoma brucei*

1. Introduction

Trypanosoma brucei is a digenetic pathogenic protozoan flagellate and the causative agent of African sleeping sickness in humans and ngana in cattle. *T. brucei* is a useful model for study of secretion of glycosylphosphatidylinositol-anchored surface coat proteins, variant surface glycoprotein (VSG) in the mammalian bloodstream form (BSF) and procyclin in the procyclic tsetse fly dwelling stage [1]. The heavy commitment of the trypanosome to glycosylphosphatidylinositol-biosynthesis for the surface coat proteins has suggested this and other secretory processes as potential chemotherapeutic targets [2,3]. Vesicle trafficking in trypanosomes is a highly polarised process since all exo- and endocytosis takes place through the flagellar pocket, occupying some 2% of total plasma membrane [4]. Despite the potential importance of these mechanisms to drug metabolism and basic cell biology [3,5], few components of the trypanosome trafficking pathways have been identified, prompting our exploitation of the family of trypanosome Rab proteins as novel, functional markers [6,7].

The Rab family of small GTPases, part of the Ras superfamily, are essential components of vesicle trafficking and required for vesicle docking and fusion. The low intrinsic enzymatic activity of small GTPases is normally modulated by interaction with a number of proteins affecting the rate of nucleotide hydrolysis or exchange [8]. Modification of the Rab C-terminus by an isoprene moiety permits the localisation of Rabs on the cytoplasmic face of intracellular membrane structures [9]. Whilst the precise mechanisms by which Rabs operate is not completely understood, they most probably prime v- and t-SNARE proteins prior to fusion through interaction with Sec1p proteins, but may also play a more direct role by interaction with motor proteins, e.g. kinesins [10,11]. Fusion is only promoted by Rab•GTP and not Rab•GDP [12], so that the rate of GTP hydrolysis acts as an efficient flux control mechanism for individual vesicle transport steps. Rabs are inactivated and recycled back to their donor membranes in the GDP bound form. An attractive feature of the Rab protein family is that individual members are associated with a restricted number of transport steps and therefore potentially provide markers for specific organelles [11].

We have collected expressed sequence tags (ESTs) encoding *T. brucei* Rab proteins involved in exocytotic and endocytotic processes [6,13,7,14,15]. Trab7 was isolated together with *TbRAB2* from a single genomic clone: the two genes were oriented head to tail with only the 3′ untranslated region separating the former from the latter open reading frames [7]. TbRAB2 has been demonstrated to be present on the ER of the trypanosome [16]. Here we report on the biochemistry and subcellular localisation of the protein encoded by the Trab7 locus.

2. Materials and methods

2.1. Materials and molecular biology

Molecular biology materials and manipulations were performed as described [7]. Antibodies to TbRAB31 were previously described [7]. Monoclonal BB4 was used as a tissue culture supernatant [18]. Monoclonal 3A5 was from Sigma, polyclonal antibodies to β-COP were from Kreis [19]. Secondary antibodies were supplied by Sigma, Jackson Laboratories or Molecular

Probes. BODIPY-TR-ceramide was supplied by Molecular Probes. Purified recombinant human Rac protein was a gift from A. Ridley (Ludwig Institute, London, UK). Plasmid DNA was prepared using a Qiagen column, following the manufacturer s instructions. [^{32}P]-GTP was purchased from ICN. Lucifer yellow (LY), defatted bovine serum albumin solution and PBS tablets were from Sigma.

2.2. Cell culture and cell treatments

Procyclic *T. brucei brucei* strain 427 were grown in SDM79 and BSF cells strain 427 were grown in HMI-18 as described [7]. Cell numbers were determined with a Coulter Z1 Counter (Coulter Electronics). For ceramide labelling, washed trypanosomes were fed with ≈ 5 μM BODIPY-TR-ceramide conjugated with defatted bovine serum albumin, at 4°C for 1 h, washed and incubated for 30 min at growth temperature. Fluorescent ceramide:bovine serum albumin conjugates were prepared as follows: fluorescent ceramide was dissolved in ethanol at 500 μM, and diluted to 5 μM in a 1.8% solution (v/v) of defatted bovine serum albumin (Sigma) in serum free medium (Iscove Modified Dulbecco's Medium, BioWhittaker, for BSF), then incubated at 4°C for 1 h. This solution was used to resuspend trypanosomes after washing them three times in serum free medium.

2.3. Immunofluorescence (IFA)

Cells were washed twice in serum free medium (BSF) or phosphate buffered saline (procyclic forms), applied to polylysine coated slides (Sigma) and processed for IFA as described [20,16]. Cytoskeletons were prepared as described [21]. Antibodies were applied at dilutions of 1:200 (*anti*-TbRAB31), 1:500 (*anti*-BiP), neat (BB4), 1:50 (β-COP), 1:100 (3A5). Cells were examined on a Leica DMRXA epifluorescence microscope, or a Nikon Microphot II microscope, fitted with a Photometrics CH250 slow scan charge-coupled device camera. Digital images were captured using IP LAB SPECTRUM 3.1 software, then overlaid and assembled into figures using ADOBE PHOTOSHOP 5.0 (Adobe Systems).

2.4. 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, thereafter as described [22]. GTP hydrolysis assays were performed on recombinant TbRAB31 made as described (r-Trab7p [7]). GTP pull-down [28] was performed on 5 μg of purified fusion protein bound to glutathione beads, loaded with 10 μCi [α^{32}P]-GTP for 10 min at 37°C in Buffer C (50 mM Tris pH 7.5, 50 mM NaCl, 5 mM EDTA, 0.1 mM EGTA, 0.1 mM dithiothreitol, 10 μM ATP) in 100 μl total volume. A time = 0 sample was taken (10 μl) then beads were washed three times with ice cold Buffer D (50 mM Tris pH 7.5, 20 mM $MgCl_2$, 1 mM dithiothreitol, 1 mg ml^{-1} bovine serum albumin), resuspended in 100 μl Buffer D and then incubated at 37°C and a 10 μl aliquot taken after 15 min. The GTP hydrolysis assay using soluble TbRAB31 protein was loaded with GTP as above, and hydrolysis commenced after taking the time = 0 sample by adding 10 mM $MgCl_2$. Further aliquots were taken after 15, 30, 60 and 120 min. All samples were stopped by mixing with 10 μl Buffer E on ice (5 mM EDTA, 50 mM GTP, 50 mM GDP). Nucleotides were eluted by heating at 70°C for 2 min. One microliter was spotted onto a polyethyleneimine-cellulose thin layer chromatography plate (Sigma) and developed for 25 min in fresh Buffer F (0.6 M Na phosphate pH 3.4), air dried and radioactivity detected by autoradiography [17].

2.5. LY uptake assays

Trypanosomes were washed and resuspended in 100 μl serum-free medium containing 4 mg ml^{-1} LY. Replicate samples, each containing 10^7 cells, were incubated on ice or at 27°C for 2 h. Cells were washed five times in ice cold phosphate buffered saline (Sigma), lysed with 1% Triton-X100/10 mM Tris pH 7.5 and fluorescence measured on a Perkin Elmer LS50B fluorescence spectrophotometer as $[LY]_{27°C} - [LY]_{0°C}$ for each sample.

2.6. Electron microscopy

Cells were fed ceramide analogues as above, fixed 1 h in 3% glutaraldehyde in serum-free medium, pH 7.4 (Buffer A), washed in Buffer A + 0.2 M sucrose (Buffer B), incubated with 2 mM DAB in Buffer B for 10 min in the dark. Photoconversion was for 30 min using a mercury lamp at $\lambda_{ex} \sim 488$ nm. After washing in Buffer B and agar embedding, samples were dehydrated in a methanol series, treated with propylene oxide and embedded in TAAB resin (TAAB Laboratories, Reading, UK). Sections were cut and examined on a JEOL 1200EX MkII transmission electron microscope.

3. Results

3.1. Rab homology of Trab7p

Our previous analysis of the sequence of Trab7p was performed before completion of the yeast genome or the huge expansion in genome sequence data [7]. A tblastp search of the complete set of open reading frames of the *Saccharomyces cerevisiae* genome identified Ypt1p, Ypt31p and Sec4p as the closest homologues of Trab7p. Ypt1p is involved in ER to Golgi transport, Sec4p in a late step in exocytosis, and Ypt31p is required for exit from the Golgi [23,24]. Tblastp searches of the entire nonredundant NCBI database identified numerous Rab8, 11 and 13 homologues as well as *Leishmania major* Ypt1. All of these Rabs contain an effector domain that is partially conserved with the PTIGVD sequence in Trab7p [25–27]. In order to better discriminate the closest homologue of Trab7p we performed a phylogenetic reconstruction using the program PAUP (phylogenetic analysis using parsimony [7]). By this analysis Ypt31p and *Lotus japonicus* Rab11 were closest to Trab7p (data not shown). Two clear Rab11 homologues have recently been identified in *T. brucei*, distinct from Trab7p (T. Jeffries and M.C.F., unpublished data), excluding Rab11 as a functional assignment for Trab7. Based on the above and the apparent association of the protein with the Golgi complex (see below) we chose to rename Trab7p as TbRAB31.

3.2. Nonconserved GTPase motifs result in impaired GTPase activity

The sequence of TbRAB31 shows near-complete conservation of all major features of Rab proteins with two notable exceptions. Firstly, there is a single cysteine at the C-terminus (sequence-KWRC). Most Rab proteins contain two C-terminal Cys and are doubly geranylgeranylated, and this alteration in prenylation may significantly affect the stability of membrane association but, as TbRAB31 is mainly membrane associated, this was not pursued further. Secondly, two point mutations, V14 and S66, which in many other GTPases are G and A, respectively, are predicted to result in deficient GTPase activity based on similarity with human RhoE. In combination, but not separately, analogous substitutions abrogate enzymatic activity in RhoE [28]. As GTP hydrolysis is a central aspect of Rab protein function, we chose to investigate the activity of TbRAB31 further.

Recombinant TbRAB proteins were obtained by thrombin cleavage of glutathione-*S*-transferase (GST)-TbRAB fusion proteins expressed in *Escherichia coli*. Recombinant TbRAB31 showed essentially no GTPase activity in our assay (Fig. 1A and B) in contrast to recombinant human Rac and recombinant TbRAB2 (not shown) prepared at the same time as TbRAB31. Identical data were obtained with two separate preparations of TbRAB31. To ensure that the absence of GTPase activity in TbRAB31 was not due to degradation during purification we analysed the purified material by MALDI–TOF mass spectrometry. Three distinct species were found, consistent with complete protein, full length plus four residues from the GST linker, and a truncated form missing eight residues from the N-terminus (data not shown); none of these alterations were expected to substantially affect catalytic activity, since all the GTPase motifs remain intact. Taken together, these data indicate that TbRAB31 lacks significant GTPase activity in in vitro assays.

3.3. TbRAB31 is a functional GTP-binding protein

We next tested TbRAB31 to ensure that it was capable of binding GTP; recombinant GST-TbRAB31 (immobilised on glutathione-sepharose beads) bound GTP in a pull-down assay (Fig. 1C). This ruled out the possibility that failure to hydrolyse GTP was due to inability to bind the nucleotide and confirms that TbRAB31 is likely to be found in the GTP-locked activated configuration.

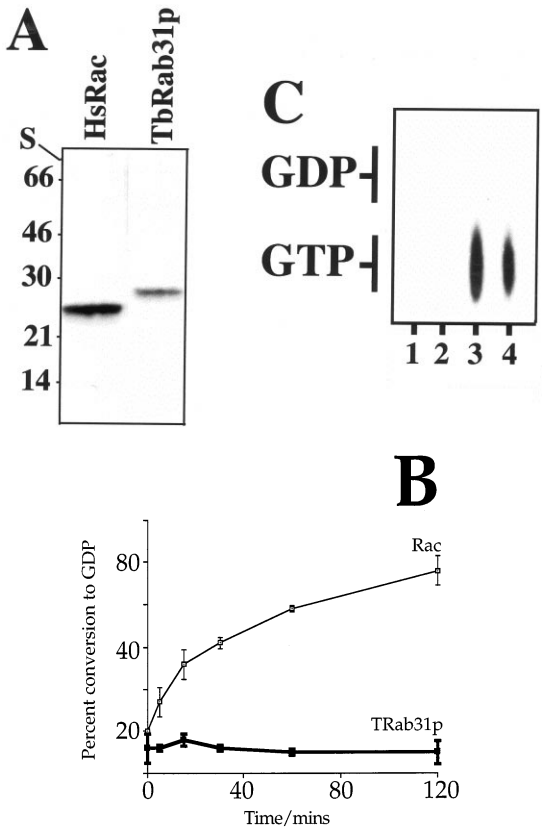

Fig. 1. TbRAB31 is GTP-locked. (A) Coomassie-stained SDS–PA gel of recombinant TbRAB31 (generated by thrombin-cleavage of N-terminal GST fusion proteins) and recombinant Rac; the same amounts of protein seen on this gel, ~1.2 μg, were used in the GTP hydrolysis assay. S marks the loading slot. (B) Time course for hydrolysis of GTP by TbRAB31 and Rac as detected by thin layer chromatographic analysis following the conversion of [α^{32}P]-GTP to [α^{32}P]-GDP. Data are representative of four experiments performed with two batches of protein. (C) Thin-layer chromatography separation of GTP and hydrolysed GDP from a GTP pull-down assay. Glutathione-sepharose beads were incubated with [α^{32}P]-GTP for 10 min, then washed and analysed by TLC: no radiolabel was detected (lanes 1, 2). In contrast, GST-TbRAB31 bound to glutathione-sepharose beads, when incubated with [α^{32}P]-GTP and washed, remained associated with GTP, demonstrating that TbRAB3 1 binds GTP. Migration positions of GTP and GDP are indicated. No Mg^{2+}, lanes 1, 3; with Mg^{2+} for 15 min, lanes 2,4.

3.4. Stable overexpression of TbRAB31

TbRAB31 is constitutively expressed at low levels in *T. brucei* and the protein is at the limit of detection [6,7,13]. We chose to stably overexpress native TbRAB31 to gain some insight into function and generated single-cell clones of procyclic *T. brucei* overexpressing TbRAB31 protein from a procyclin promoter [29]. Southern analysis demonstrated correct insertion into the tubulin locus in clone 427P31.1 (Fig. 2A) and, by Western blotting, this clone expressed 20-fold more TbRAB31 than wild type cells (Fig. 2B). Overexpressed TbRAB protein remained membrane-associated (not shown), and staining became more extensive (Fig. 2D, E). Ultrastructural examination of the cells showed clearly that there were no alterations in the membrane organisation of the ER, the Golgi stacks or other structures (S. Croft, M.C.F. and H.F., unpublished data) suggesting that the increased staining is a threshold effect, i.e. structures with little TbRAB31 in wild type cells can now be seen. In addition, by IFA, the position of TbRAB31 and its behaviour during the cell cycle were unchanged (see below).

Overexpression of TbRAB31 had a small effect on the overall GTP-binding profile of procyclic trypanosomes in a [^{32}P]-GTP overlay within the 21 kDa region (Fig. 2C), suggesting an alteration in GTP homeostasis. We would not expect to detect the excess TbRAB31 since even large amounts of recombinant TbRAB protein cannot always be detected by GTP overlay due to inefficient refolding of some GTPases. We also examined the overexpressing cells for defects in the exocytotic pathway [16]; no alteration in procyclin secretion or in glycosylphosphatidylinositol anchor precursor (PP1) biosynthesis was observed, suggesting that ER processes are unaffected by excess TbRAB31. The secretion of two other markers,

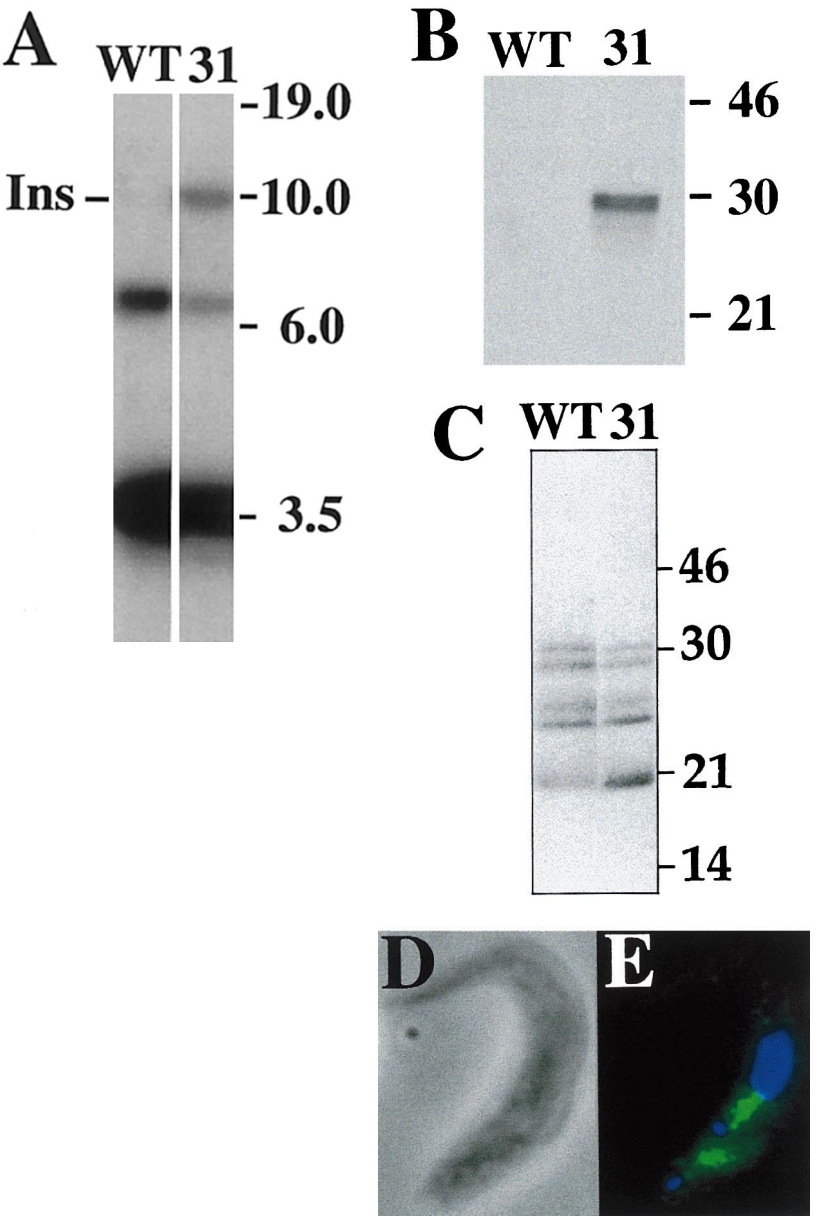

Fig. 2.

variant surface glycoprotein and soluble BiP, doubly transfected into the TbRAB31 overexpressing background, were likewise unaffected ([30], J. Bangs and M.C.F., unpublished data). Therefore, overexpression of TbRAB31 has no detectable effect on exocytotic processes.

Localisation data (see Section 3.7) suggest that TbRAB31 is present on the trypanosome Golgi complex. As membrane components are known to recycle through this compartment we considered the possibility that endocytotic events are affected by overexpression of TbRAB31 and tested fluid

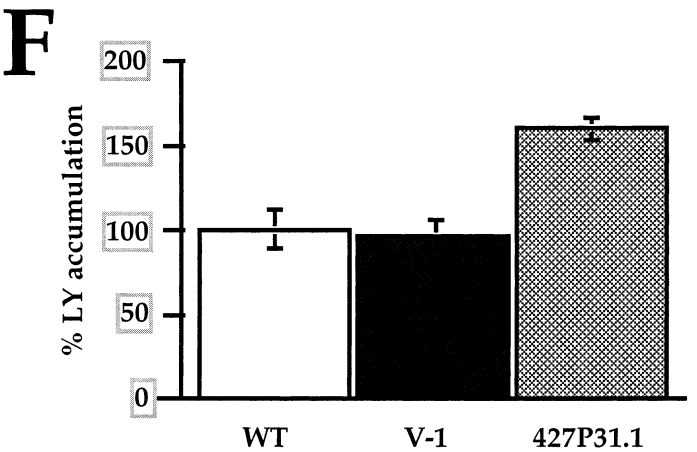

Fig. 2. TbRAB31 overexpression in 427P31.1 cells. (A) Southern blot of genomic DNA from wild type cells (WT), or a cloned cell line, 427P31.1 (31) transformed with pXS219myc.*TbRAB31*. Genomic DNA from each cell type was digested with BamHI and probed for insertion into the tubulin locus with a tubulin DNA probe detecting last relative to the β gene 3′ end. Molecular weight markers are in kilobase pairs (right). The βα. . . βαβ′ tubulin array was divided into 3.5 kb fragments by BamHI cutting the β gene [44]. The 6 kb band represents the tubulin gene from the 3′ end of the cluster (the last β gene is truncated before the BamHI site thus increasing the size of this fragment [45]). Insertion of the *TbRAB31*-containing plasmid into the allelic 6 kb fragment results in production of a ~10 kb fragment, and concomitantly reduces the intensity of that band (lane 31). (B) Western blot of 10^7 wild type (WT) or 427P31.1 (31) procyclic trypanosomes lysed in boiling SDS–PAGE sample buffer and electrophoresed on 15% reducing SDS–PAGE gels, blotted and probed with affinity purified antibodies to TbRAB31. (C) The GTP binding profile of trypanosome proteins is not significantly altered in 427P31.1 cells compared to wild type. 10^7 trypanosomes were lysed in boiling SDS–PAGE sample buffer, fractionated on reducing SDS–PAGE and renatured before blotting onto nitrocellulose and incubating with [^{32}P]-GTP and unlabelled ATP. Proteins binding GTP were detected by autoradiography. Excess unlabelled GTP eliminated all the signal (not shown). (D, E) IFA of TbRAB31 in a 427P31.1 cell: phase contrast (D); merge of TbRAB31 (green) and DNA (blue) stains (E). This cell is at an equivalent stage in the cell cycle to the cell shown in Fig. 4O and P. TbRAB31 stain appears larger and less discrete in cells overexpressing TbRAB31 but division and movement during the cell cycle are the same as in wild type cells, and movements remain distinct from and coordinated with the basal body complex (not shown). (F) LY uptake of procyclic trypanosomes overexpressing TbRAB31. LY uptake assays were performed as described (Section 2). Quadruplet samples were taken for the assay presented. The experiment was done at least three times in triplicate and the increase in LY uptake with overexpression of TbRAB31 ranged between 1.6- and 2.1-fold (1.6-fold increase shown). WT, wild type 427 strain procyclic trypanosomes; V-1, a clone of the same strain transfected with empty vector pXS219myc.

phase endocytosis using the fluorescent dye LY. Uptake of LY is very low in wild-type procyclic trypanosomes [31]. By immunofluorescence, LY uptake was significantly increased in 427P31.1 cells compared to wild type (data not shown). We quantified this effect by fluorescence spectrophotometry, measured as the accumulation of LY over 2 h as LY uptake reached a plateau at this time. Intracellular [LY] increased in 427P31.1 cells compared to wild type by at least 1.6-fold (Fig. 2F). This effect was not seen in procyclic cells transfected with the expression vector without an insert, demonstrating that the effect was due to the transgene. Therefore overexpression of TbRAB31 results in increased fluid-phase endocytosis in procyclic cells.

The increase in LY uptake was compared with that in BSF cells in our LY uptake assay: the $[LY]_{4\ h}/[LY]_{t=0}$ was 3.4-fold compared to 1.7-fold for 427P31.1 cells and 1.04-fold in wild type procyclic cells (data not shown). TbRAB31 is constitutively expressed at both the mRNA and protein levels in BSF and wild type procyclic cells, consistent with a role for TbRAB31 in some function other than direct control of the rate of endocytosis [6,7].

3.5. Localisation of TbRAB31

Previously, we showed by indirect immunofluorescence that TbRAB31 localised to a single, discrete compartment between the nucleus and the kinetoplast in the procyclic trypanosome [7]. This region of the cell contains many organelles including the Golgi apparatus, the bulk of the endocytotic vesicles [14] and cytoskeletal elements such as the basal bodies at the base of the flagellum and the flagellar pocket. It was therefore necessary to define precisely the location of TbRAB31.

Indirect immunofluorescence of 427P31.1 cells revealed markedly increased staining of the region between the kinetoplast and nucleus, confirming specificity of the antisera (Fig. 2). TbRAB31 was not associated with endocytic vesicles because IFA of BSF cells which had previously been fed Lucifer yellow, a fluid phase endocytic marker, showed no colocalisation (Fig. 3A and B). We compared the localisation of the basal body, another discrete element in the inter-nucleo-kinetoplast region. The TbRAB31 compartment occupied a cytoplasmic location distinct from the basal body (Fig. 3C–I), located between the nucleus and the basal body in interphase procyclic cells (Fig. 3C and D). Rab proteins are frequently associated with membraneous organelles. TbRAB31 is associated with the Triton X-100 soluble, non-cytoplasmic (membrane) fraction of cells as assessed by Western blotting, and is not associated with the extracted cytoskeleton of trypanosomes, by IFA or Western (data not shown).

3.6. Developmental regulation and cell cycle-dependent positioning of the TbRAB31 organelle

In contrast to procyclic cells, where there is a single TbRAB31 structure in interphase cells, the TbRAB31 compartment was seen as two or sometimes more discrete structures in interphase BSF (Fig. 3B and M, Fig. 5E) and as four (occasionally six or more) spots in mitotic BSF (Fig. 5C and F). Therefore the morphology of this structure is under developmental regulation, being more complex in the BSF.

The procyclic interphase trypanosome has a single TbRAB31 organelle (Fig. 3D, Fig. 4B). This compartment clearly divided just prior to the kinetoplast by elongation followed by fission (Fig. 4D, F and H). Procyclic cells with two kinetoplasts, i.e. entered into the nuclear S phase, contained two TbRAB31 compartments (Fig. 4J–T). The earliest documented event visible in the trypanosome cell cycle is the division of the basal body. By costaining for TbRAB31 and the basal body we obtained evidence that the TbRAB31 compartment may divide at the same time or slightly prior to the basal body as some cells had two TbRAB31 foci with a single basal body (Fig. 3F). In cells containing two TbRAB31 organelles and one elongated or recently divided kinetoplast, the foci change position, from close to the nucleus to adjacent to the two daughter kinetoplasts (Fig. 4H, J). During nuclear division the kinetoplasts and the basal bodies associate with the microtubular ar-

Fig. 3. TbRAB31 localises to the trypanosome Golgi apparatus. IFA of trypanosomes. Procyclic cells (C–L) or BSF cells (A, B, M–O) are shown in phase contrast (grey) overlaid with DNA (blue) and next to them the same cell stained for other markers as described. (A, B) Cell fed with LY (green) before fixing and staining for TbRAB31 (red). (C, D) Cell in interphase showing a single basal body (red) associated with the kinetoplast and a TbRAB31 compartment (green). (E, F) Cell entering cell cycle with kinetoplast in V-shape, costained for basal body (red) and TbRAB31 (green, arrowheads). (G, H) Cell at slightly later stage than E, F costained as E, F. (I) Cell in late stage of mitosis/cytokinesis with costaining as for E, F overlaid onto the phase contrast image. (J) Nomarski optics of cell in interphase costained for DNA (blue) and β-COP using monoclonal 3A5 (green) showing β-COP staining in same region as TbRAB31 (compare D). (K, L) Cell in late mitosis/cytokinesis stained as J shows staining of β-COP in same two regions as TbRAB31 (compare I). (M–O) Cell stained for TbRAB31 (M) and β-COP (N) using polyclonal antibodies shows colocalisation (yellow, O).

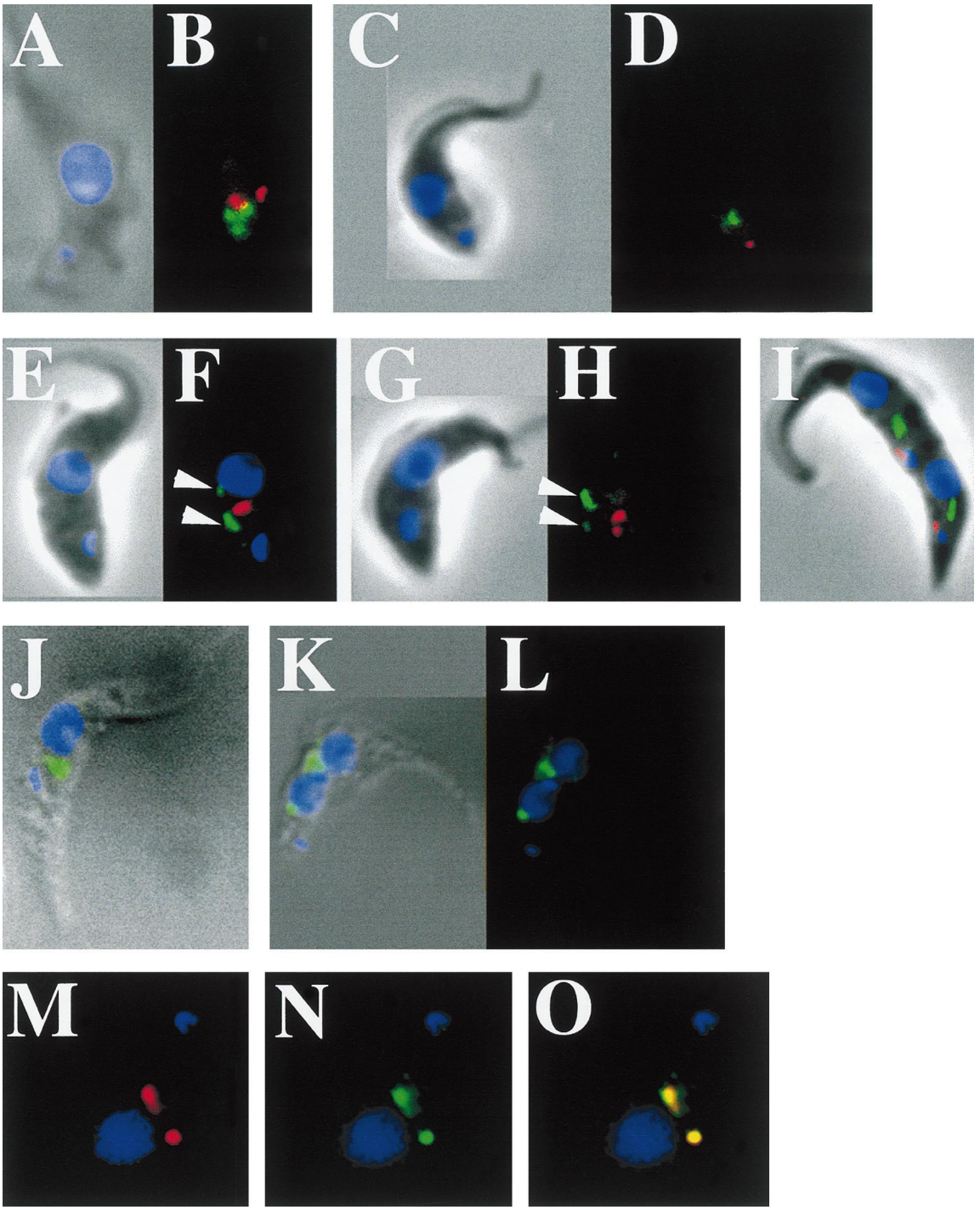

Fig. 3.

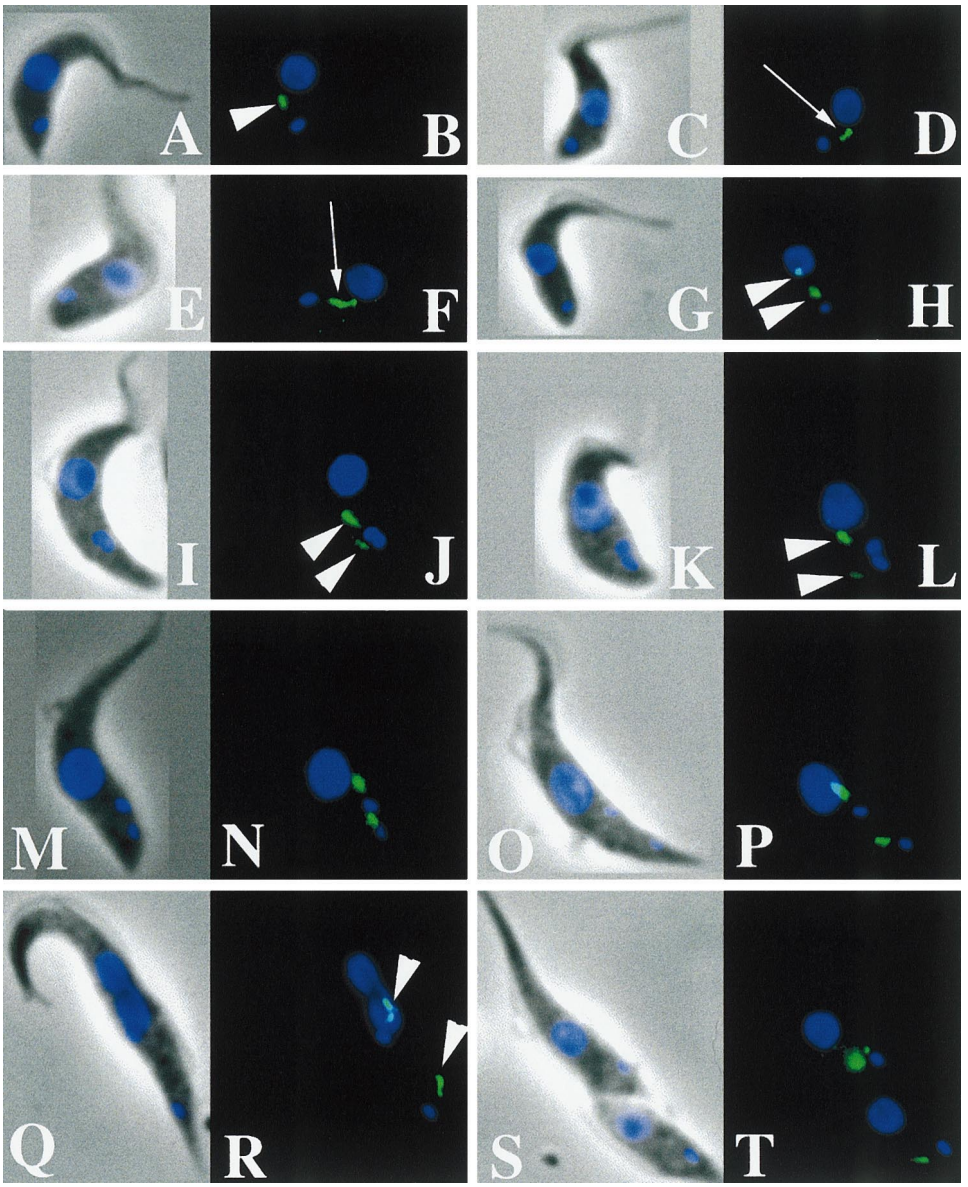

Fig. 4. Behaviour of the TbRAB31 compartment during the cell cycle. IFA of procyclic cells stained for TbRAB31 (green) and DNA (blue) showing DNA overlaid onto phase contrast images (grey). (A, B) Interphase cell showing normal resting TbRAB31 entity. (C, D) Late G1 cell with elongating TbRAB31 structure. (E, F) Slightly later stage than C, D with TbRAB31 aligned between the nucleus and extending to the kinetoplast. (G–T) Cells containing two TbRAB31 entities. (E–J) Cells where the kinetoplast has begun to divide; in H the TbRAB31 entity has just separated, in J the daughter TbRAB31 compartments are realigning and approaching the daughter kinetoplasts; in L, the TbRAB31 compartments move away from the kinetoplasts (in N the basal bodies are aligning with TbRAB31 and kinetoplasts, compare Fig. 3C–I). (M, N) A premitotic cell with closely aligned TbRAB31 and kinetoplasts. (M–R) The kinetoplasts continue to separate, preceded in their motion to the anterior of the cell by the Golgi. (Q, R) A mitotic cell with dividing nucleus and spindle: TbRAB31 compartments are still closely associated with kinetoplasts. (S, T) A cell late in cytokinesis showing the forming cell wall. At this stage the TbRAB31 compartments are resuming their normal position with respect to the kinetoplasts in the interphase cell (compare B).

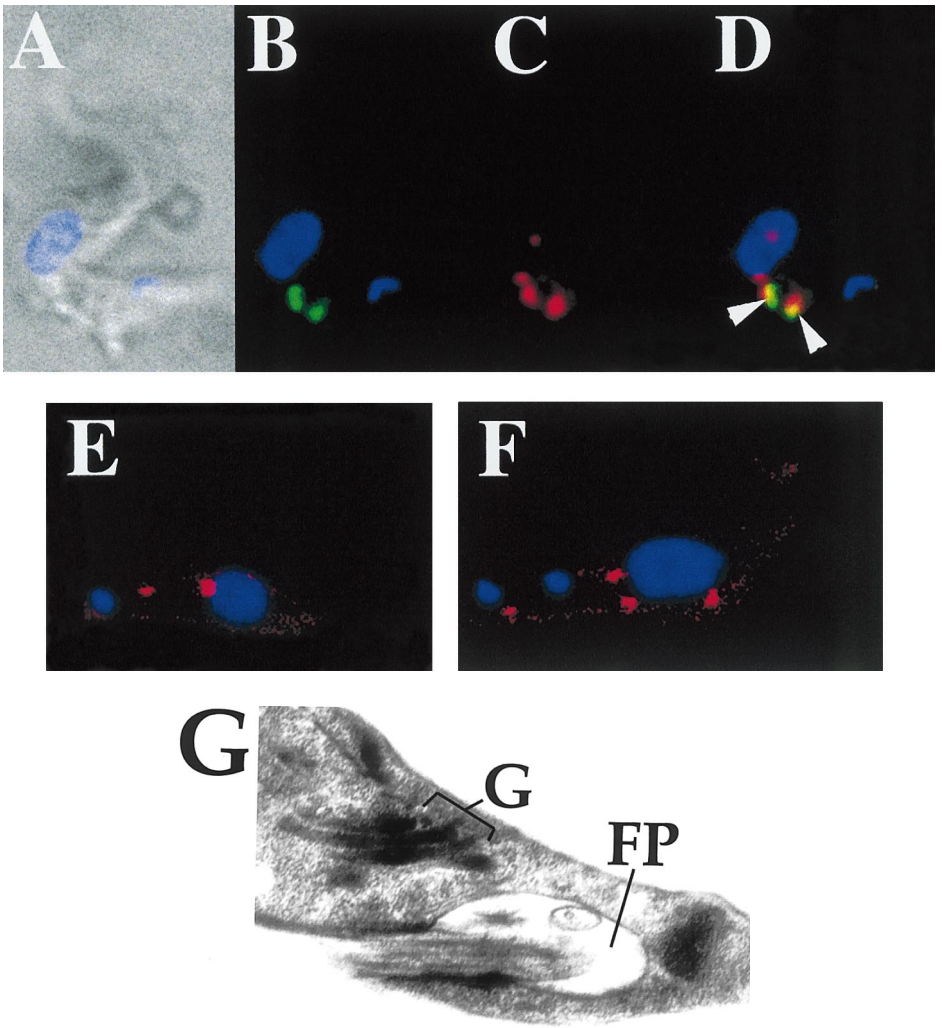

Fig. 5. TbRAB31 partially colocalises with the Golgi lipid marker, BODIPY-ceramide. Nomarski optics are shown in grey with DNA staining (Hoescht, blue) overlaid. (A–D) BSF cell fed with BODIPY-ceramide at 4°C for 1 h, then incubated at 37°C for 30 min for the metabolised lipid to concentrate in the Golgi apparatus, then fixed and stained for TbRAB31. Ceramide stain (red), TbRAB31 stain (green) and overlaid images which show partial overlap (D, arrowheads show costaining, yellow). This cell has entered into the cell cycle, and has a dividing kinetoplast. (E, F) Merged confocal images of all planes through BSF cells stained for TbRAB31 (red). Cell are in interphase (E) or entering mitosis (F). (G) BODIPY-ceramide fed to BSF trypanosomes labels the Golgi stacks. Electron micrograph of BSF cell fed with BODIPY-ceramide followed by photoconversion of DAB resulting in electron dense labelling of the Golgi apparatus.

ray as they migrate into the daughter cell bodies [32,33]. We observed that the daughter TbRAB31 organelles also position themselves alongside the kinetoplasts and basal bodies in a kinetoplast-basal body-TbRAB31 array, and migrate with them (Fig. 3I, Fig. 4N, P and R). This suggests association of the TbRAB31 organelle with the specialised microtubule array close to the flagellum during the mitotic cell cycle of the trypanosome.

3.7. Evidence for a Golgi association for TbRAB31

From the position of the TbRAB31 compartment, and its behaviour during the cell cycle, we considered that TbRAB31 may be Golgi associated. We sought to confirm this by further analysis. Repeated attempts at immuno-electron microscopy (EM) of trypanosomes to localise TbRAB31 have been unsuccessful, including immunocryo-EM. Significant labelling was not obtained even in clone 427P31.1 procyclic cells overexpressing TbRAB31 and is likely due to the low abundance of the TbRAB31 protein, and possibly also the nature of the epitope under EM fixation.

To circumvent the lack of EM data we used two established Golgi markers and attempted to colocalise these with with TbRAB31. 3A5, a monoclonal antibody recognising mammalian β-COP [34] cross-reacted with a structure in procyclic trypanosomes which mimicked the position and behaviour of TbRAB31 during the cell cycle (Fig. 3J–L). As monoclonal antibodies raised against higher eukaryotic antigens often fail to cross-react with trypanosome proteins we sought to confirm this reactivity with a second antibody and tested for colocalisation of TbRAB31 with polyclonal antibodies, specific for β-COP, which localise to the TGN of unperturbed Vero cells [19]; these β-COP antibodies also costained the TbRAB31 structure in BSF cells (Fig. 3M–O), consistent with TbRAB31 being Golgi associated.

We next used a nonimmunological method to label the trypanosome Golgi complex. BODIPY-ceramides localise to the Golgi apparatus in numerous cell types and specifically accumulate to this structure after feeding to live cells. In the related kinetoplastid *L. major*, BODIPY–TR-ceramide has been localised to the Golgi by colocalisation with a mutant of the HASPB (previously Gene B) protein that accumulates in the Golgi complex, demonstrated by cryoelectron microscopy [35]. BSF trypanosomes fed BODIPY–TR-ceramide exhibited similar levels of complexity, as well as partial colocalisation with TbRAB31 (Fig. 5A–D). We verified that the BODIPY-TR-ceramide stain was present in the Golgi apparatus, since the ceramide analogue can be used to photo convert diaminobenzidine to an electron dense stain [36]. BODIPY-TR-ceramide–bovine serum albumen conjugates were fed to BSF trypanosomes and taken up by endocytosis. Diaminobenzidine was photo converted and the strongest stain obtained in the Golgi stacks. Additional staining was also seen at the junction of the flagellar pocket with the cell body, consistent with these junctions being S the site of entry into the flagellar pocket and the route for endocytosis of the lipid label (Fig. 5G). The partial overlap of TbRAB31 staining with ceramide by IFA is therefore consistent with TbRAB31 labelling the trypanosome Golgi.

4. Discussion

Recent advances in the molecular cell biology and genomic analysis of *T. brucei* suggest that this organism has a highly developed secretory system. Based on the number of distinct TbRAB sequences identified, a total of 16 in mid 1999, it is probable that *T. brucei* is more complex in protein transport processes than the model eukaryote *S. cerevisiae*. We previously reported the location of TbRAB31, formerly Trab7p, in procyclic cells; here we have extended our analysis to demonstrate developmental and cell cycle-related aspects of the behaviour of this GTPase, as well as providing data suggesting that TbRAB31 is a Golgi-associated protein.

The function of TbRAB31 is probably influenced by its unusually low intrinsic GTPase activity. Since GTPase activity is a function that facilitates recycling of the Rab from the target membrane back to the donor membrane, the question is raised as to how TbRAB31 is cycled between organelles, and whether alternative associating factors are implicated in its function [37]. For enzymatically active Rab proteins, extensive work has shown that interaction with the GTPase Activating Protein leads to insertion of a critical arginine residue into the G protein active site, facilitating rapid hydrolysis. It is a possibility that an unusual GTPase activating protein is required to stimulate TbRAB31. Rab•GTP is competent for promotion of vesicle fusion, and hence

TbRAB31 is likely a potent stimulator of vesicle transport in the absence of a potent GTPase activating protein activity when compared with TbRAB2, which in vitro has a much shorter half life for the GTP-bound form. In this context, Rab13 which like TbRAB31 has a single Cys at the C-terminus, does not bind GDP dissociation inhibitor, but instead binds a phosphodiesterase δ-subunit which dissociates it from membranes [38]. Overexpression of TbRAB31 has little apparent effect in the physiology of the procyclic trypanosome and does not alter the ultrastructure of the cell. Of the biochemical assays available to us, only a small change in fluid-phase endocytosis was detectable.

The TbRAB31 compartment is clearly more complex in the BSF; most interphase BSF cells contain two or more foci, whereas equivalent procyclic cells have just one focus. This increased complexity presumably reflects increased activity in the TbRAB31 organelle in the BSF compared with the insect form (see below). Also, the structure is duplicated by a binary fission mechanism, with a clear duplication event occurring very early in the cell cycle. Thereafter, the TbRAB31 organelle relocates with the kinetoplast/basal body system. These latter two organelles have previously been shown to migrate based on a specialised microtubule-dependent system [33]; the inclusion of the TbRAB31 into this system suggests that a strongly polarised and coordinated mechanism for partitioning of organelles is present in *T. brucei*.

Several pieces of evidence suggest that TbRAB31 is Golgi associated. Firstly, the position of the TbRAB31 compartment is consistent with the position of the Golgi as observed by EM. Secondly, TbRAB31 colocalises in trypanosomes with two established markers of the Golgi apparatus, β-COP, a component of the COP-I coat, and BODIPY-TR-ceramide. The latter marker concentrates in the Golgi after feeding to trypanosomes, as confirmed by EM. Thirdly, division and duplication of the TbRAB31 organelle, apparently by binary fission, has been observed by EM for the trypanosome Golgi complex and is also typical of the behaviour of Golgi during the plant cell cycle (K. Gull, unpublished), [39,46]. We also note that the morphology of TbRAB31 in procyclic *T. brucei* is almost identical to that for the lipophosphoglycan (LPG) 1p-green fluorescent protein, an established Golgi protein in *L. major* promastigotes [40]. A recent elegant study using lipid dyes in *L. mexicana* identified a unique tubular structure, suggested to be part of the secretory system of this parasite, and potentially involved in glycoinositol phospholipid (GIPL) metabolism together with a similar morphology for the Golgi complex as suggested here [41]. Based on the data in this report we cannot assign TbRAB31 to a subcompartment of the Golgi complex. Increased fluid-phase endocytosis in the 427P31.1 cells is suggestive of a role in *trans*-Golgi network activity as this part of the Golgi complex has a function in recycling and hence endocytic processes. Creation of mutant forms of TbRAB31 will allow us to directly test this possibility.

Assignment of the TbRAB31 compartment as the *T. brucei* Golgi complex, together with its developmental stage and cell cycle behaviour highlights some important aspects of organellar biology. The mammalian Golgi is dispersed into over 100 vesicles during cell division, a mechanism to ensure accurate partitioning of Golgi contents to each daughter cell following cytokinesis [42]. Similar arguments have been made for the dispersed multiple mitochondria of eukaryotes. Kinetoplastids contain only a single mitochondrion and it appears that an alternative strategy is exploited to ensure daughter cells receive the correct organelles during cytokinesis. Coordination of the movements of the basal body, the kinetoplast and the flagellar pocket is dependent on specialised microtubules [33,32]. In trypanosomes, duplicative events and a strong association with the microtubular array results in a non random partitioning of the TbRAB31 compartment, basal body and kinetoplasts and hence may provide an alternate mechanism for Golgi inheritance. Also, the TbRAB31 structure exhibits a striking level of developmental variation; a single discrete spot in procyclics is replaced by at least two spots in the BSF, and occasionally even more complex structures, despite apparently constitutive expression levels as judged by both message and protein analysis [6,7]. Importantly, BODIPY-TR-ceramide staining is also more extensive in BSF cells than in procyclics. The

physiological significance of this developmental regulation is unknown, but is probably related to differences in the secretory pathway of the two stages of the trypanosome and, in particular, glycosylation requirements. Procyclic trypanosomes process their *N*-glycans very little, with surface proteins containing essentially only oligomannose type structures. By contrast, N-links on VSG molecules are highly processed, being predominantly complex class and frequently containing polylactosamine chains [43].

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