Available online at www.sciencedirect.com
SCIENCE DIRECT®
Experimental Parasitology 111 (2005) 160–171
Experimental Parasitology
www.elsevier.com/locate/yexpr

Trypanosoma brucei: TbRAB4 regulates membrane recycling and expression of surface proteins in procyclic forms

Belinda S. Hall [a], Arun Pal [a], David Goulding [a], Alvaro Acosta-Serrano [b], Mark C. Field [c,*]

[a] *Department of Biological Sciences, Imperial College, London SW7 2AY, UK*
[b] *Wellcome Centre for Molecular Parasitology, The Anderson College, University of Glasgow, Glasgow G11 6NU, UK*
[c] *The Molteno Building, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK*

Received 13 May 2005; received in revised form 4 July 2005; accepted 15 July 2005
Available online 13 September 2005

Abstract

TbRAB4 is the *Trypanosoma brucei* orthologue of the small GTPase Rab4, which is implicated in the control of early endocytosis and recycling processes. TbRAB4 is expressed constitutively in the procyclic and bloodstream stages suggesting an important function throughout the trypanosome life-cycle. Previous work from our laboratory has shown TbRAB4 to be essential in the bloodstream form. Induction of double-stranded TbRAB4 RNA expression leads to a specific reduction in TbRAB4 protein levels and inhibition of growth in procyclic form *T. brucei*, with alterations in uptake and recycling as measured with the fluorophore FM4-64. Trypanosomes overexpressing GTP-locked TbRAB4^{QL} mutants exhibit significant perturbations of endocytic and recycling pathways as well as disruption of surface expression of GPI-anchored proteins. Most significantly, both the endogenous GPI-anchored procyclins and an ectopically expressed GPI-anchored protein, the variant surface glycoprotein, are relocated from the surface to internal sites in TbRAB4 mutant cells. These data indicate that TbRAB4 is important in maintenance of normal surface expression of lipid-anchored proteins, and implicate recycling pathways as factors for modulation of surface protein expression in the procyclic trypanosome. The conservation of function of Rab4 throughout eukaryotic evolution demonstrated here indicates that the Rab4-mediated trafficking pathway is an extremely ancient component of the endocytic system.

Keywords: Trypanosoma; Protein transport; Vesicle trafficking; Endocytosis; Procyclin; GTPase; Trypanosomiasis; Membrane trafficking; Recycling; rab4; Developmental regulation; GPI

1. Introduction

The Rab family of small GTPases is a key regulator of the protein trafficking system (Zerial and McBride, 2001), and its role in regulation of the early endocytic system of crown eukaryotes has been well studied. Rab5 isoforms control entry into the early endosome, while Rab4 and Rab11 have been implicated in transport out of early endocytic compartments, with Rab4 involved mainly in rapid recycling and Rab11 in a slow recycling process that intersects with *trans*-Golgi derived pathways (Ullrich et al., 1996; Van der Sluijs et al., 1992). These three GTPases localise to overlapping subdomains of the early endosome (Sonnischen et al., 2000) whilst effectors shared between Rab5 and Rab4 (Rabaptin-5 and Rabenosyn-5, De Renzis et al., 2002), and between Rab4 and Rab11 (RCP, Lindsay et al., 2002) coordinate the sequential flow of endocytosed material through this system. Expression of constitutively active Rab4 in mammalian cells increases both the rate of recycling and lysosomal degradation of endocytosed proteins, whilst the rate of endocytosis is unaffected (McCaffrey et al., 2001). In certain specialised cells, Rab4

* Corresponding author.
E-mail address: mcf34@cam.ac.uk (M.C. Field).

0014-4894/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2005.07.005

has additional functions, including regulation of the formation of synaptic vesicles in neurons (De Wit et al., 2001), calcium-dependent exocytosis in platelets (Shirakawa et al., 2000), and translocation of the glucose transporter GLUT4 from internal vesicles to the plasma membrane in response to insulin in adipose and muscle tissue (Cormont et al., 2001).

TbRAB4, the *Trypanosoma brucei* orthologue to mammalian Rab4, localises to an early endocytic compartment and is closely associated with TbRAB5A, itself a key mediator of early endocytosis in this organism (Field et al., 1998; Hall et al., 2004a; Pal et al., 2002). TbRAB4 is expressed constitutively in both the insect and mammalian life stages, suggesting an important role throughout the life-cycle. Recycling of surface proteins has been reported for both bloodstream and procyclic forms, although the process is rather better understood in the former (Kabiri and Steverding, 2000; Liu et al., 2000; Seyfang et al., 1990). The recycling endosome-specific Rab protein, TbRAB11, is expressed at high levels in bloodstream forms and co-localises with recycling surface proteins such as the variant surface glycoprotein (VSG) and the transferrin receptor complex ESAG6/7 (Grunfelder et al., 2003; Jeffries et al., 2001), both of which are anchored to the membrane via a glycolipid. Whilst both TbRAB11 and TbRAB4 are essential to survival in bloodstream form parasites, recycling of surface proteins appears to be mediated exclusively by TbRAB11 in this life stage. RNAi of TbRAB11 results in an almost complete block to recycling of transferrin but loss of TbRAB4 has no effect (Hall et al., 2005). By contrast, accumulation of dextran is inhibited by TbRAB4 RNAi and enhanced by expression of a constitutively active TbRAB4 protein, indicating that TbRAB4 regulates the pathway directing fluid phase traffic to the lysosome (Hall et al., 2004b). However, whilst the routes followed by internalised surface protein, receptor-bound ligands, and fluid phase cargo are distinct in BSF, procyclics show less cargo segregation. In addition, procyclics express much lower levels of TbRAB11 than BSF and hence may be more dependent on TbRAB4-mediated recycling (Jeffries et al., 2001).

The major surface glycoproteins of the insect stage, procyclins, consist of a globular N-terminus followed by multiple repeats of the amino acids Glu-Pro (EP form) or Gly-Pro-Glu-Glu-Thr (GPEET)[1] and are attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor (Acosta-Serrano et al., 1999). Procyclic cells can express three isoforms of EP procyclin, EP1, EP2, and EP3, differing in N-terminal sequence and in the presence or absence of an N-glycosylation site, and two GPEET isoforms with different numbers of GPEET repeats. Expression of the various procyclins is co-ordinately controlled, suggesting that each may have distinct and vital functions (Vassella et al., 2001). Both EP and GPEET proteins are detected early during differentiation from the bloodstream form, with GPEET becoming predominant after 24 h (Acosta-Serrano et al., 2000). GPEET expression is transient and repressed from day seven both in the insect vector and in vitro, replaced by EP isoforms EP1 and EP3 (Acosta-Serrano et al., 2000; Vassella et al., 2000). Many culture-adapted procyclic lines express both EP and GPEET, with GPEET being the predominant surface protein (Butikofer et al., 1997). Procyclins appear to undergo little turnover and the importance of the trafficking system in maintaining the procyclin coat has not been addressed. By use of inducible RNAi and expression of a constitutively active mutant Rab4 isoform, we show that TbRAB4 is essential in the procyclic form and that TbRAB4-mediated trafficking participates in regulation of procyclin location and surface expression.

2. Materials and methods

2.1. Parasites

Laboratory adapted *T. brucei* Lister 427 strain procyclic trypomastigotes were cultured at 27 °C in SDM79 medium supplemented with 10% fetal bovine serum (Gibco) and 7.5 μg ml^{-1} haemin. The 29-13 procyclic line was cultured in the continuous presence of 25 μg ml^{-1} geneticin and 25 μg ml^{-1} hygromycin B (both from Sigma). For growth curves, 100 μl aliquots from 10 ml cultures were taken daily and counted in 10 ml Isoton II with a Z1 Coulter Counter (Beckman Coulter).

2.2. Immunochemistry

Wildtype TbRAB4 was amplified with the primers CGGAGGATCCCAACCATGTCAGAGAGATATC and GTGGAATTCAAATACCTAACAAGCACACG. The product was digested with *Bam*HI and *Eco*RI, and inserted into pGEX-3X (Amersham Pharmacia). Polyclonal murine and rabbit antibodies were raised against affinity purified TbRab4-GST fusion protein using RIBI (Sigma) as adjuvant (at least four immunisations spaced over a period of 4 months). Antibodies were affinity purified on CNBr-Sephadex-immobilised TbRAB4-GST. Monoclonal 5H3 and 247 antibodies, and polyclonal rabbit K1 antibody were kind gifts of Terry Pearson (University of Victoria, Canada), rabbit anti-117 VSG (MiTat1.4)

[1] *Abbreviations used:* BSA, bovine serum albumin; DAPI, 4′-6-diamidino-2-phenylindole; EP, Glu-Pro form of procyclin; FM4-64, *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide; GPEET, Gly-Pro-Glu-Glu-Thr form of procyclin; GPI, glyosylphosphatidylinositol; HF, hydrogen fluoride; IFA, immunofluorescence analysis; MALDI-TOF, matrix-assisted laser-desorption ionisation-time-of-flight; ORF, open reading frame; PBS, phosphate-buffered saline; RNAi, RNA interference; TFA, trifluoroacetic acid; VSG, variant surface glycoprotein.

antibody was a gift from Mark Carrington (Cambridge, UK). Polyclonal rabbit anti-TbRAB5A were used as described (Pal et al., 2002). For Western blotting, log phase procyclic parasites were harvested, washed once in PBS, and resuspended in Laemmli loading buffer. SDS–PAGE and blotting were performed as described (Pal et al., 2002). For immunofluorescence, cells were fixed in 4% paraformaldehyde and adhered to poly-L-lysine slides (Sigma). For staining of intact cells slides were washed in PBS. Alternatively cells were permeabilised with 0.1% Triton X-100. In both cases slides were blocked with 10% goat serum in PBS. Antibodies were diluted in blocking buffer. All incubations with primary and secondary antibodies were for 1 h at room temperature with the exception of anti-TbRAB4 antibodies, which were incubated on slides at 4 °C overnight. Cells were counterstained with DAPI before mounting with Vectashield (Vector Laboratories). Cells were examined under a Nikon Eclipse E600 microscope and images were captured using a Photometrix Coolsnap FX camera.

2.3. DNA manipulations

The TbRAB4^{QL} mutant was prepared using the primers CCGGTCTAGAAAGATACAAATCAG and CTGATTTGTATCTTTCTAGACCGGC. PCR products were cut with *Bam*HI and *Hin*dIII, and inserted into pXS219 vector. Log phase procyclic trypomastigotes were transfected with 10 μg plasmid DNA by electroporation and drug resistant cells were selected in the presence of 60 μg ml^{-1} geneticin for 2–3 weeks. Clones were isolated by limiting dilution and tested for genomic integration of plasmid DNA by PCR using primers against the pXS219 plasmid GGGTGGGCGTGCATTGAAA ATAGGG and GGAGGCGATATAAATGAAAGG AACC. Insertion of the mutant TbRAB4 isoform was determined by specific restriction digestion with *Xba*I for TbRAB4^{QL}. For VSG expression, parental procyclic trypomastigotes and TbRAB4 transfectants were transfected with the plasmid pXS2·117WT·Hyg (Bangs et al., 1997), kindly donated by Jay Bangs (University of Wisconsin, Madison), and selected for up to 5 weeks in the presence of 25 μg ml^{-1} hygromycin B. For RNAi analysis of TbRAB4, the entire TbRAB4^{WT} open reading frame was excised from pXS219 and inserted into p2T7 (La-Count et al., 2002) (kind gift from Doug LaCount, University of Iowa). The p2T7·TbRAB4 construct was digested with *Not*I and 5 μg purified DNA was transfected by electroporation into the tetracyclin responsive procyclic line PF 29-13 (Wirtz et al., 1999). Transfectants were selected for 4 weeks in the presence of 2.5 μg ml^{-1} phleomycin. The selected cells were maintained in the continuous presence of 2.5 μg ml^{-1} phleomycin, 25 μg ml^{-1} geneticin, and 25 μg ml^{-1} hygromycin B. For induction of double-stranded RNA, tetracycline was added to cultures at 1 μg ml^{-1}.

2.4. Uptake and recycling of FM4-64

Microscopic analysis of FM4-64 uptake and recycling was carried out by a modification of the assay described (Wiederkehr et al., 2000). Mid-log phase cells were harvested, washed twice in TES buffer (120 mM NaCl, 5 mM KCl, 3 mM $MgSO_4$, 16 mM Na_2HPO_4, 5 mM KH_2PO_4, 30 mM TES, 10 mM glucose, and 0.1 mM adenosine), then resuspended at a concentration of 1×10^7 ml^{-1} in TES. Cells were pre-equilibrated for 20 min at 4 or 27 °C, then 20 μM FM4-64 (Molecular Probes) was added. Cells were incubated for 15 min in the presence of FM4-64, then placed on ice, washed once in TES buffer, and fixed for 1 h at 4 °C with 4% paraformaldehyde. For assay of recycling, cells were allowed to take up the fluorophore as described, washed once, and then resuspended in TES, buffer at 1×10^7 ml^{-1}. Cells were incubated at 27 °C for 15 min, washed once in ice cold TES, and fixed as described. Fixed cells were adhered to poly-L-lysine slides for 30 min at room temperature, then mounted for analysis in Vectorshield. Images were captured as described for immunofluorescence. All images were accumulated under non-saturating conditions and exposure times were identical for each image unless indicated otherwise. Fluorescence intensity was quantified using Metamorph software (Universal Imaging Inc.). Values were obtained as the integrated grey-scale intensity within an area of fixed size minus the background intensity for an identically sized area within each cell. Background at 4 °C was subtracted to obtain uptake, and recycling values and means were calculated for 40–60 cells per sample. Significance was determined by student's *t* test.

2.5. Uptake of fluorescent dextran

Mutant and parental cells were harvested directly from mid-log phase cultures and resuspended at a concentration of 5×10^8 ml^{-1} in 50 μl aliquots of fresh complete medium. Alexa Fluor 488-labelled dextran 10,000 (Molecular Probes) was added to a concentration of 5 mg ml^{-1}. Cells were incubated at 27 °C for 30 min to 1 h and accumulation was stopped by the addition of 1 ml cold medium. Cells were washed, then fixed in 4% paraformaldehyde for 1 h before mounting onto poly-L-lysine slides (Sigma–Aldrich). Images were captured as described above. Fluorescence was quantified from images taken under identical conditions as described, using Metamorph Imaging software.

2.6. Biotinylation and detection of surface proteins

For analysis of total surface protein levels, log phase wildtype and mutant trypomastigotes (10^8) were harvested, and washed twice in ice cold PBS, pH 8.0, including 10 mM glucose, and then resuspended in PBS/glucose containing 1 mg ml^{-1} NHS-SS-biotin (Pierce).

Cells were incubated on ice for 15 min at 4 °C, and then 10 volumes of cold complete medium was added. Cells were centrifuged and washed twice in medium, then once in TBS. For procyclin analysis, pellets were delipidated with chloroform/methanol/water (10:10:3 v/v/v) and extracted with butanol (9%). Butanol extracts were dried and resuspended in non-reducing sample buffer. Samples (10^8 cell equivalents per lane) were separated on 12% SDS–PAGE gels and blotted onto nitrocellulose. EP and GPEET procyclin were detected with 247 and 5H3 antibodies, respectively, while biotin labelling was determined by incubating with streptavidin peroxidase (Sigma) in PBS/3% BSA for 1 h, then detecting with ECL. VSG was labelled in the same manner, but instead of butanol extraction, cells were lysed in 1 ml RIPA buffer (25 mM Tris–Cl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, and complete protease inhibitor cocktail (Roche)). Lysates were precleared by centrifugation at 10,000*g* for 10 min at 4 °C. A small aliquot was taken for determination of total VSG117 levels, then 1 μl rabbit anti-VSG117 serum was added, and the samples were incubated for 1 h with rocking at 4 °C. Protein A–Sepharose (Amersham Pharmacia Biotech) (25 μl) was added and the lysates were incubated for a further 1 h. The beads were washed three times in RIPA buffer and once in TBS, then resuspended in 50 μl non-reducing sample buffer. SDS–PAGE, blotting, and development were carried out as described above.

2.7. MALDI-TOF mass spectrometry

Mass spectrometric analysis was carried out as described (Acosta-Serrano et al., 1999). Briefly, butanol extracts of procyclics (10^8 cells) were dephosphorylated with 48% aqueous hydrofluoric acid (HF) for 18 h at 0 °C, dried, washed with water, and resuspended in 10 μl 0.1% TFA. An aliquot (5 μl) was mixed with sinnapinic acid matrix for analysis by negative-ion MALDI-TOF-MS using a Perceptive Biosystems Voyager Elite DE-STR mass spectrometer calibrated with insulin, thioredoxin, and apomyoglobin. Samples were tested at various dilutions from 1:10 to 1:50. To remove contaminants and confirm assignments, some samples were further treated with 40 mM TFA to cleave EP species at Asp-Pro bonds, and GPEET between Asp-13 and Gly-14 prior to analysis (Acosta-Serrano et al., 1999).

3. Results

3.1. TbRAB4 is required for growth of procyclic T. brucei trypomastigotes

Expression of TbRAB4 in two major life stages suggests an important function throughout the trypanosome life-cycle. To establish the importance of TbRAB4 expression in the insect form, the procyclic line 29-13 was transfected with p2T7·TbRAB4, containing the complete open reading frame (ORF) of TbRAB4 (La-Count et al., 2000, 2002; Ngo et al., 1998; Wang et al., 2000) and transformed cells were selected with phleomycin. Growth, morphology, and behaviour of uninduced cells were identical to untransfected cells. Induction of TbRAB4 RNAi had no initial effect but inhibition of growth was detectable after 7 days induction (Fig. 1A). By Western blot, a reduction in TbRAB4 protein levels was seen 48 h after initiation of RNAi, and no protein was detectable by 96 h (Fig. 1B). After incubation with tetracycline for 264 h (11 days), TbRAB4 protein reappeared and this was accompanied by a recovery of growth (data not shown). Residual TbRAB4 remained detectable by IFA for several days after initiation of induction but by day eight no protein was apparent (Fig. 1C). The effect on TbRAB4 was specific since no alteration to expression of TbRAB5A or TbRAB11 was detectable by Western analysis (data not shown). The RNAi-induced growth defect was comparatively slow in onset, suggesting that TbRAB4 function is important, but that cells can survive with a partially functional endocytic system for some time.

A range of morphological alterations was also observed in induced cells depleted of TbRAB4 (Fig. 1D); at least 50% of cells in induced cultures exhibited abnormality in shape, size or nucleus/kinetoplast positioning, and approximately 15% possessed abnormal copy numbers of nuclei and kinetoplasts ($n = 200$), but no specific block in the cell cycle was apparent. The pleomorphic appearance of these defects suggests that downstream of a defect in membrane traffic, multiple cellular functions are affected by TbRAB4 suppression.

3.2. Accumulation of endocytic markers in cells under TbRAB4 RNAi

The effect of TbRAB4 RNAi on general membrane uptake and recycling was assessed using the lipophilic fluorophore FM4-64, which is used similarly in yeast and mammalian cells. FM4-64 is fluorescent when associated with membranes and is taken up into cells via the endocytic pathway, and removed by recycling (Field et al., 2004; Nagamatsu et al., 2000; Wiederkehr et al., 2000). Cells were incubated with FM4-64 for 15 min to specifically label the early endocytic compartment; longer incubation with FM4-64 labels membrane compartments throughout the cell (V. Dhir and MCF, unpublished data). Cells were assayed following 8 days induction, a time chosen because at this point cells exhibit significant growth and phenotypic defects plus TbRAB4 protein was undetectable (Fig. 1).

In uninduced cells at 4 °C, low level labelling is seen and accumulation of FM4-64 at 27 °C is also compara-

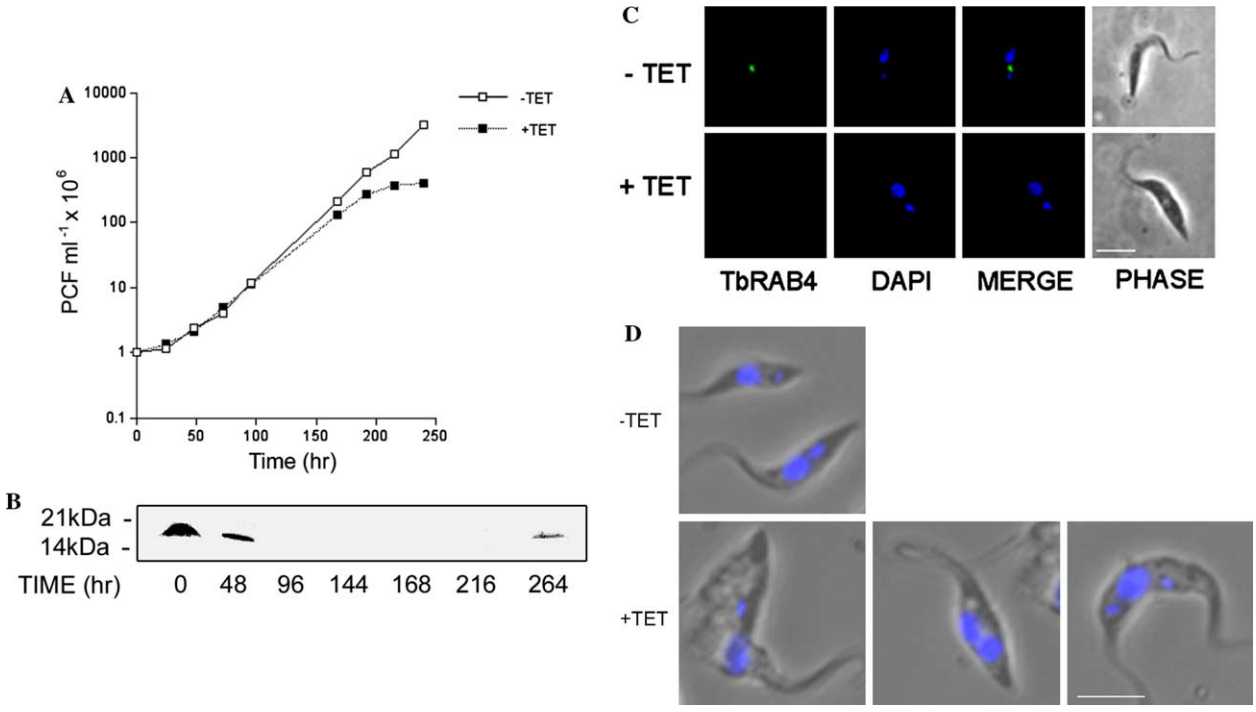

Fig. 1. RNAi indicates TbRAB4 is required for growth in *T.brucei* procyclic trypomastigotes. (A) Cumulative growth curve for 29-13 procyclic forms transfected with p2T7-TbRAB4 grown in the presence (closed) or absence (open) of 1 μg ml^{-1} tetracyline. Cultures were maintained between 0.5×10^6 and 2×10^7 cells ml^{-1} at all times. Cells were diluted at 96 h and 168 h. Fresh tetracycline was added at dilution. Each point represents the mean of tripicate samples. There is a significant decrease in growth after 168 h ($p < 0.01$). (B) Western blot of lysates of p2T7TbRAB4 cells cultured in the presence or absence of 1 μg ml^{-1} tetracycline for various times. Cells were washed in PBS, lysed directly in sample buffer and loaded onto 12% SDS–polyacrylamide gels at 2×10^7 cell equivalents per lane. TbRAB4 was detected with affinity-purified rabbit anti-TbRAB4 antibody. Equality of loading was confirmed by Ponceau S staining (data not shown). (C) Immunofluorescent localisation of TbRAB4 in cells incubated for 8 days in the absence or presence of 1 μg ml^{-1} tetracycline. Cells were fixed, blocked, and incubated overnight at 4 °C with 0.5 μg ml^{-1} affinity-purified polyclonal mouse antibody against TbRAB4-GST in PBS/10% goat serum/0.02% sodium azide, then for 1 h at room temperature with Oregon green 488 conjugated goat anti-mouse IgG. Nuclei and kinetoplasts were localised with DAPI. Merge shows localisation of TbRAB4 positive vesicles in the region involved in endocytosis, between the nucleus and kinetoplast. White bar represents 5 μm. (D) Effect of TbRAB4 RNAi on cell morphology: p2T7-TbRAB4 cells incubated with and without tetracycline for 8 days were fixed for 30 min in 3% paraformaldehyde, and adhered to slides. Cells were stained with DAPI. White bar represents 5 μm. Multiple changes in morphology are present in induced cells. Note, for example, swollen cell body (left panel), absence of a kinetoplast (middle panel), and abnormal kinetoplast location (right panel).

tively weak, consistent with previous reports of close to undetectable procyclic endocytic activity (Fig. 2A) (Pal et al., 2002). By contrast, induced RNAi cells exhibit increased FM4-64 accumulation in multiple vesicles between the nucleus and flagellar pocket. Following washing and reincubation for 15 min at 27 °C to allow recycling, very little FM4-64 was left in the uninduced cells, a result similar to *Saccharomyces cerevisiae* where 50% of endocytosed FM4-64 is recycled after 10 min. In induced cells, FM4-64 fluorescence after a recycling chase period is less intense and more localised than in the cells incubated at 27 °C alone but higher than after recycling in uninduced cells. Since cells in unsynchronised cultures can show a significant degree of heterogeneity in uptake and recycling, the levels of mean fluorescence were quantified (Fig. 2B). This analysis confirmed a significantly increased level of accumulation within the cells suppressed for TbRAB4 ($p < 0.001$). Recycling, expressed as the percent decrease in fluorescence on reincubation at 27 °C, is partially blocked in the RNAi cells ($p < 0.01$). Since the decrease in recycling is relatively minor compared to increased accumulation, the influence of TbRAB4 RNAi on recycling is likely less prominent than on endocytosis. Significantly, these data are in contrast with bloodstream forms where TbRAB4 RNAi has essentially no impact on recycling (Hall et al., 2004b), a process that appears to be entirely dependent on TbRAB11 (Hall et al., 2005; Pal et al., 2003).

The role of TbRAB4 on fluid phase endocytosis was examined using Fluor 488-labelled dextran 10,000. Induced and uninduced cells were incubated with dextran for 30 min at 27 °C. As with FM4-64, enhanced accumulation was observed in the TbRAB4 RNAi cells (Fig. 2C). Quantitation revealed that dextran uptake was significantly greater in induced cells ($p < 0.05$). Again these data contrast with fluid phase uptake in bloodstream forms, which is strongly inhibited by TbRAB4 RNAi (Hall et al., 2004b). These effects of TbRAB4 RNAi in procyclic form cells were unexpected. The differences between life stages may be due to the lower levels of TbRAB11 expression in procyclic cells or to

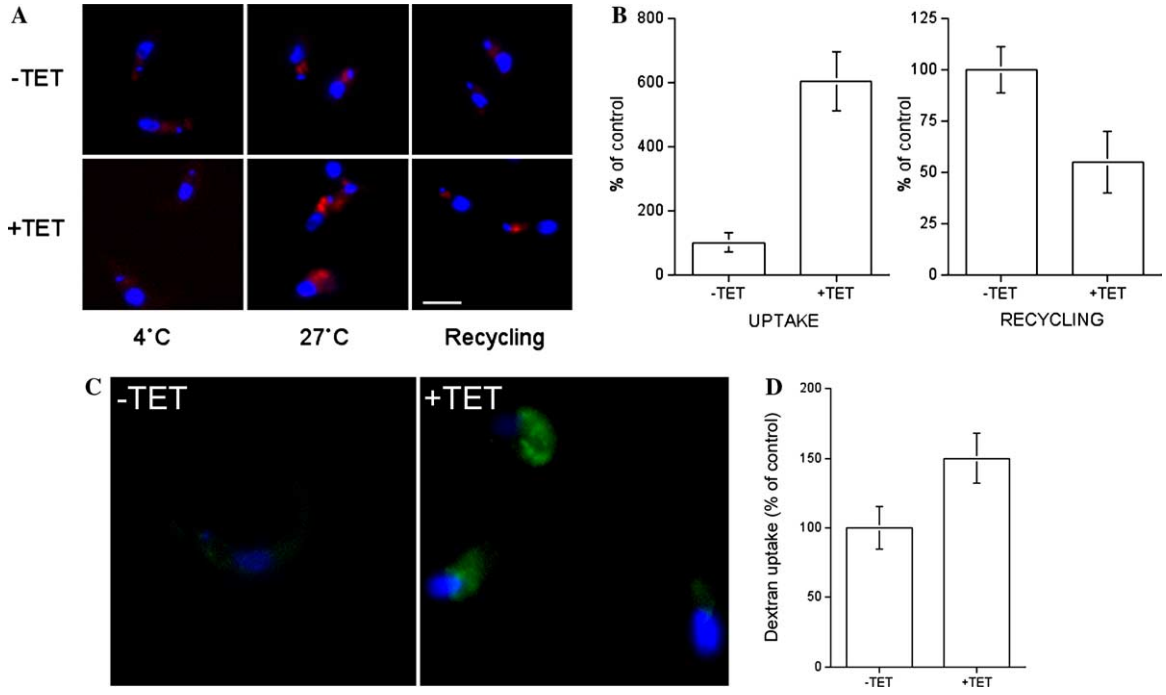

Fig. 2. Uptake and recycling in p2T7·TbRAB4 cells. (A) Cells incubated in the presence or absence of tetracycline for 8 days were incubated for 15 min at 4 or 27 °C in the presence of 20 μm FM4-64 (red). Increased internalisation is seen at the higher temperature for both induced and uninduced cells, but labelling is more intense in the induced cells. For recycling, FM4-64 labelled cells were washed and chased for 15 min. Label is barely detectable in uninduced cells, but still present in induced cells. Slides were counterstained with DAPI. Images were captured as described in Section 2. White bar represents 5 μm. Results are typical of multiple (>3) repeated experiments. (B) Quantitation confirms the effects of TbRAB4 RNAi on uptake and recycling of FM4-64. Fluorescence above background was quantified using Metamorph software for at least 40 cells from images taken under identical, non-saturating conditions, as described in Section 2. Recycling is calculated as the percent decrease in fluorescence following reincubation. Both uptake and recycling results are presented as percent fluorescence in uninduced cells. Bars represent mean values ± SEM. (C) Fluid phase uptake is also enhanced by TbRAB4 RNAi. Cells incubated in the presence or absence of tetracycline for eight days were incubated for 30 min at 28 °C with 5 mg ml^{-1} Fluor 488 Dextran 10,000, washed, fixed and adhered to slides. Slides are counterstained with DAPI. (D) Quantitation of Fluor 488 dextran uptake. Fluorescence intensity was determined for images captured under identical, non-saturating exposure conditions as described in Section 2. Bars represent the mean value for at least 50 cells ± SEM. Similar results were obtained in duplicate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

more fundamental differences in organisation of the endocytic system in the two cell types. It also cannot be excluded that the RNAi cells have accommodated suppression of TbRAB4 by alterations in other pathways.

3.3. Expression of dominant active TbRAB4

The constitutively active GTPase-locked TbRAB4 mutant (TbRAB4^{QL}) was expressed in procyclic cells (Hall et al., 2004b; McCaffrey et al., 2001; Van der Sluijs et al., 1992), and overexpression demonstrated by both Western blot (Fig. 3A) and immunofluoresence (Fig. 3B). Cloned cells showed a significantly increased lag period following dilution compared to the parental line, but exhibited similar growth rates once entering log phase (data not shown). In parental cells, IFA revealed vesicles positive for TbRAB4 between the flagellar pocket and nucleus (Fig. 3B), corresponding to the region of the cell where endocytic activity is concentrated. In cells expressing TbRAB4^{QL}, intense staining is seen in the same region, and hence the mutant protein is faithfully targeted. The relative increase in TbRAB4 staining in the mutant line is not as great as might be predicted from the Western data, but IFA is intrinsically less quantitative than immunoblotting plus IFA preferentially detects membrane associated Rab proteins whilst cytoplasmic (soluble) pools are substantially lost during preparation for microscopy. The localisation of TbRAB4 in the parental and mutant cells is consistent with that seen in bloodstream cells (Hall et al., 2004b). The majority of the cells in the TbRAB^{QL} lines have an overall shape that is normal (see above) at the light and electron microscopic level, but at the ultrastructural level most TbRAB4^{QL} cells have an increased number of enlarged vesicles close to the flagellar pocket compared to parental cells (see arrowheads, Fig. 3C). We have been unable to confirm the identity of these vesicles although their location is suggestive of endocytic origin and they are similar to enlarged vesicles observed in procyclic cells expressing TbRAB5A^{QL} (Pal et al., 2002).

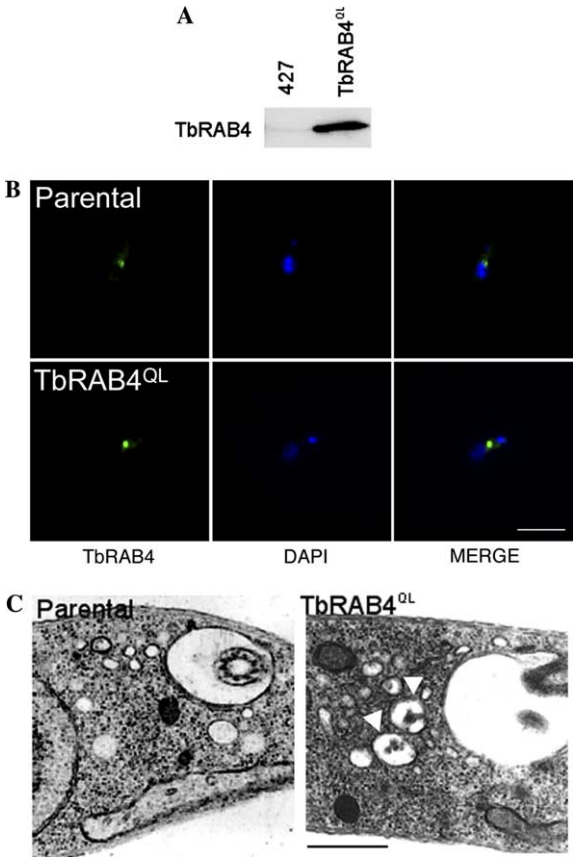

Fig. 3. Expression of mutant TbRAB4 in 427 strain procyclic trypomastigotes. (A) Western blot of lysates from wildtype and TbRAB4^{QL} 427 cells. Each lane contains 10^7 cell equivalents. Blots were incubated overnight at 4 °C with affinity purified rabbit anti-TbRAB4, then for 1 h at room temperature with HRP-conjugated anti-rabbit antibody prior to visualisation with luminol. Equality of loading was confirmed by Ponceau S staining (not shown). Note the exposure time used here is less than that for the blot shown in Fig. 1. (B) Immunofluorescent localisation of TbRAB4 in 427 cells and cells expressing TbRAB4^{QL}. Slides were incubated overnight with 0.5 μg ml^{-1} affinity purified polyclonal mouse antibody against TbRAB4-GST in PBS/10% goat serum/0.02% sodium azide, then for 1 h at room temperature with Oregon green 488 conjugated goat anti-mouse IgG. Nuclei and kinetoplasts were localised with DAPI. White bar represents 5 μm. (C) Electron micrographs of parental and transgenic cells. At least 10 images were analysed for each cell type and the images presented are representative. Black bar represents 1 μm.

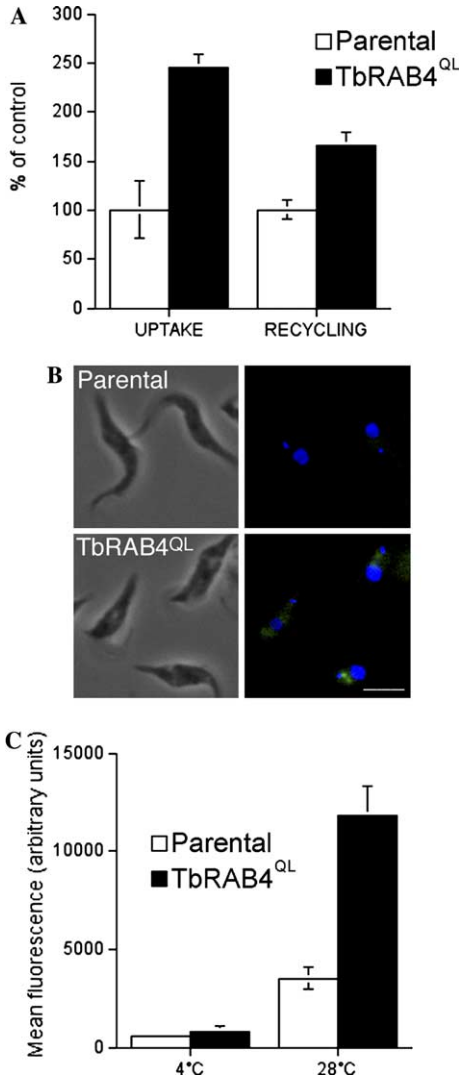

Fig. 4. Altered uptake and recycling in cells expressing TbRAB4^{QL}. (A) Mid-log phase cells were harvested and labelled with FM4-64 as described. Fluorophore uptake and recycling was quantified as described. Bars represent the mean values for at least 50 cells ± SEM. (B) Uptake of Fluor 488 Dextran 10,000 in parental and mutant procyclic cells. Cells were incubated for 1 h with the label, then fixed, and observed immediately. Similar results were obtained in duplicate experiments. (C) Quantitation of fluorescence in Fluor 488 dextran labelled cells. Mean fluorescence was measured as described for FM4-64. Each point represents the mean value for at least 40 cells ± SEM.

3.4. Uptake and recycling of FM4-64 in TbRAB4^{QL} procyclics

The effect of expression of TbRAB4^{QL} on FM4-64 uptake and recycling was assessed microscopically and quantified as described in Section 2 (Fig. 4A). Parental cells showed uptake and partial recycling of FM4-64. Somewhat surprisingly, similarly to cells lacking TbRAB4 expression, TbRAB4^{QL} cells show significantly higher levels of FM4-64 uptake at 27 °C than the parental line ($p < 0.001$). However, recycling levels were also increased in cells expressing TbRAB4^{QL} ($p < 0.05$). Enhancement of recycling in cells expressing TbRAB4^{QL} confirms that TbRAB4 contributes to regulation of membrane recycling in procyclic form *T. brucei*.

In bloodstream form cells, TbRAB4 is a specific regulator of fluid phase transport and expression of TbRAB4^{QL} enhances accumulation of fluid phase cargo (Hall et al., 2004b). To assess this role in procyclic cells, parental and TbRAB4^{QL} lines were incubated with fluorescent dextran (Figs. 4B and C). Dextran uptake was barely detectable in parental cells but was markedly increased in the cells expressing TbRAB4^{QL}, suggesting that this particular role of TbRAB4^{QL} is similar in both life stages. However, as both suppression and

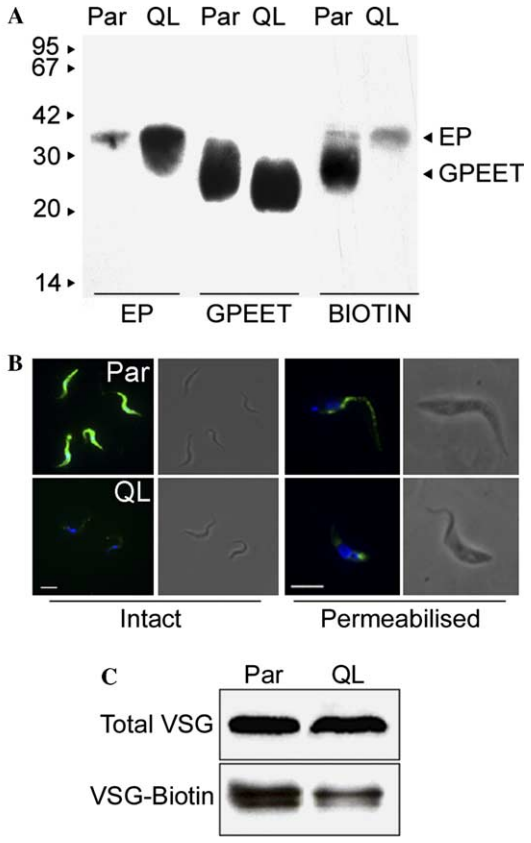

Fig. 5. Localisation of procyclin is affected by TbRAB4. (A) Butanol extracts of biotin-labelled parental (Par.) and TbRAB4^{QL} (QL) procyclic trypomastigotes separated by SDS–PAGE, blotted, and probed for EP, with MAb 247, for GPEET, with MAb 5H3, and for biotin with streptavidin-peroxidase. Arrows indicate migration of molecular weight markers in kilodaltons. (B) Fixed, unpermeabilised cells and cells permeabilised with 0.1% Triton x-100 stained with 5H3 antibody against phospho-GPEET procyclin (green). Slides were counterstained with DAPI and images are presented merged. White bar 5 μm. Permeabilisation results in reduced detection of surface proteins, a common phenomenon in most cell systems, due to selective solubilisation of the plasma membrane. (C) VSG was expressed ectopically in both parental and TbRAB4QL cells. Total VSG was detected by blotting of unfractionated lysates with rabbit anti-VSG. Biotin-labelled VSG was immunoprecipitated with anti-VSG 117 (MiTat1.4) antibodies and detected with streptavidin peroxidase as described. VSG is seen in these blots as a doublet, the lower band representing a proteolysed form that is retained by the cell (Bangs et al., 1997). Note greater level of biotinylated, surface VSG in parental cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

overexpression of the TbRAB4^{QL} mutant have similar effects on endocytosis, regulation of these pathways is clearly highly complex and more detailed analysis of interactions with additional regulatory components will be necessary for a full understanding of the role of TbRAB4.

3.5. Altered procyclin localisation in cells expressing TbRAB4^{QL}

To determine whether TbRAB4 has any function in trafficking of surface proteins, the role of TbRAB4 in regulation of procyclins was examined. Expression and localisation of procyclins was assessed in biotin-labelled wild type 427 and TbRAB4^{QL} procyclics. Immediately after labelling the cell surface with biotin at 4 °C, procyclins were purified by butanol extraction. Recovered proteins were separated by gel electrophoresis, transferred to nitrocellulose, and visualised with the anti-EP antibody MAb 247, the anti-GPEET antibody MAb 5H3 or streptavidin (Butikofer et al., 1997). Total EP was clearly increased in TbRAB4^{QL} cells (Fig. 5A). Biotin-labelled protein corresponding to surface EP was also increased, but quantitation revealed that the ratio of surface to total EP was higher in the parental line than in the TbRAB4^{QL} cells (see Table 1). Expression of mutant TbRAB4 had an even greater effect on GPEET location. Total GPEET levels were constant in both lines, but there was an apparent shift in GPEET mobility in the TbRAB4^{QL} line. Whilst GPEET represented the bulk of the biotin-labelled protein in parental 427 cells, GPEET protein was barely detectable at the surface in TbRAB4^{QL} cells. These data suggest that GPEET is mainly on the surface of wild type 427 procyclics, but is localised internally in the TbRAB^{QL} cells. Thus, an increase in TbRAB4 activity alters the distribution of procyclins, particularly GPEET, from external to internal sites. While it is possible that these changes are due to long term adaptation during selection and culture, we have not observed such effects in procyclic cells expressing other mutant Rab proteins that have been similarly cultured for extensive periods (Pal et al., 2002), indicating that the effect is specific for TbRAB4.

Attempts to analyse the effect of TbRAB4 RNAi on procyclin expression were compromised by lack of expression of GPEET in parental 29-13 cells. Incubation in low glucose is reported to induce re-expression of GPEET (Morris et al., 2002), but we found that these conditions were toxic to p2T7-TbRAB4 cells. Examination of EP levels and location showed no detectable change after induction of TbRAB4 RNAi (data not shown).

To confirm the relocalisation of GPEET detected by blot analysis, parental 427 and TbRAB4^{QL} cells were stained with MAb 5H3 (Fig. 5B). Fixed, unpermeabilised parental 427 procyclic forms show strong GPEET staining over the whole surface but very little surface GPEET is present on TbRAB4^{QL} cells. A distinct pattern of procyclin expression is seen in permeabilised cells. In parental cells, MAb 5H3 reacted only with surface proteins, with strongest staining along the flagellum. Again, TbRAB4^{QL} cells exhibited reduced surface GPEET expression, with no flagellar staining, but GPEET staining was seen at a single site close to the flagellar pocket. GPEET is a phospho-protein which is phosphorylated en-route to the surface (Butikofer et al., 1999, 2002). Since MAb 5H3 specifically recognises phosphorylated GPEET, this suggests that the procyclin is exported

Table 1
Relocalisation of GPI-anchored proteins in 427 procyclic cells overexpressing TbRAB4^{QL}

Protein	Total[a]		Biotin (surface)[a]		Ratio surface:total[b]	
	Parental	TbRAB4^{QL}	Parental	TbRAB4^{QL}	Parental	TbRAB4^{QL}
EP procyclin	100	560	100	170	1.00	0.23
GPEET procyclin	100	110	100	5	1.00	0.04
VSG (ectopic)[c]	100	100	100	56	1.00	0.54

Abundance of proteins was determined by Western blot analysis of butanol extracts (for procyclins) or immunoprecipitates (for VSG) (see Fig. 5A) and quantitated by densitometry. Note that both total and biotinylated EP levels are increased in the TbRAB4^{QL} line, but the increase in surface (biotin-labelled) protein is considerably less than the total, indicating that a smaller proportion of the EP procyclin is at the surface in these cells. However, expression of TbRAB4^{QL} has the greatest impact on surface expression of GPEET, which is reduced to 5% of control levels whilst the abundance of total protein is unchanged. Therefore, for both procyclin isoforms and VSG there is an increase in the proportion of the internal population. Similar results were obtained in duplicate experiments ($n = 2$).

[a] Data are expressed as percent, normalised to 100% for 427 procyclic trypanosomes.

[b] Represents the ratio of intensities of the protein and biotin-labelled bands. Due to differences between the detection methods, densitometry cannot be used to calculate the exact proportion of protein at the surface of the cell but can be used to infer relative levels of surface expression between the two cell lines.

[c] For analysis of ectopic VSG, procyclic cells were transfected with a 117 VSG expression construct (see Section 2).

normally and accumulates within the cell by internalisation from the surface. Co-staining of IFAs showed a partial co-localisation between GPEET and TbRAB4 (data not shown), suggesting that at least some of the internalised GPEET is present within the enlarged TbRAB4 compartment. By IFA, EP expression showed a high level of cell-to-cell variation, making interpretation of data difficult, but generally showed high levels of surface protein in both cell lines, supporting the biotinylation results (data not shown).

To determine the specificity of the effects of mutant TbRAB4 protein expression on procyclins, we transfected VSG 117 (MiTat1.4) into the parental and TbRAB4^{QL} cell lines. Both cell lines expressed similar levels of total VSG (Fig. 5C) but, as seen for procyclin, the parental line showed a higher level of surface expressed biotin-labelled VSG than the TbRAB4^{QL} cells. In procyclics surface VSG is gradually released by proteolysis into the medium (Bangs et al., 1997). The proportion of biotinylated VSG released by TbRAB4^{QL} cells is slightly lower than that found in parental cells (data not shown), so the difference in surface levels cannot be attributed to increased shedding of the protein from the surface of TbRAB4^{QL} cells. IFA staining of VSG showed a similar pattern to GPEET (data not shown). Hence, disruption of TbRAB4 function leads to a general relocalisation of surface GPI-anchored proteins.

3.6. Altered procyclin isoform expression in TbRAB4^{QL} cells

To investigate further the influence of TbRAB4 on procyclin, butanol extracts of parental and TbRAB4^{QL} cells were analysed by negative-ion MALDI-TOF mass spectrometry (Fig. 6). Samples were treated with hydrofluoric acid (HF), to remove the GPI anchor, and trifluoroacetic acid, which cleaves the protein at specific residues (Asp-Pro in EP and Asp-Gly in GPEET). The spectra obtained for the parental line, treated with HF alone (data not shown) or HF/TFA were similar to previous reports for 427 strain parasites, with relatively high levels of GPEET compared to the EP isoforms (Acosta-Serrano et al., 1999, 2000).

TbRAB4^{QL} mutants exhibited markedly altered spectra compared to the parental line (Fig. 6B). GPEET phosphorylation and glycosylation of EP were unchanged but the spectra showed an increase in all EP forms relative to GPEET. These findings fully confirm our Western blot and immunofluorescence data, and demonstrate categorically an upregulation in EP relative to GPEET in these mutants. The TbRAB4^{QL} line also appears to express a novel form of EP3, designated EP3-

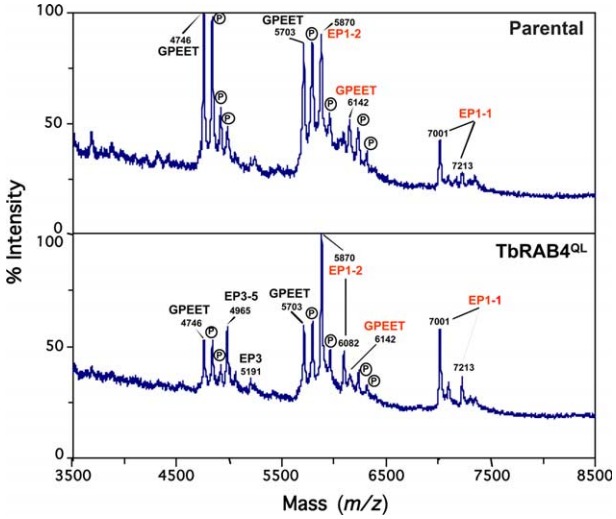

Fig. 6. Negative-ion MALDI-TOF mass spectrometric analysis of procyclin. Mass spectrum (m/z 3500–8500 region) of HF/TFA treated parental 427 butanol extracts (A) and TbRAB4^{QL} cells (B). Full length form of GPEET is highlighted in red and C-terminal fragments of EP-3 and GPEET are in black. The positions of some phosphorylated fragments are indicated by "P." (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

5, with 21 instead of the more usual 22 EP repeats (Fig. 6B). However, it is possible that this isoform is also present in the parental line, but overshadowed by a phospho-GPEET peak of similar size. Hence, TbRAB4-controlled pathways affect both EP and GPEET; the proportions of internal EP and GPEET procyclin are augmented and also total levels of EP are increased in TbRAB4^{QL} lines.

4. Discussion

In higher eukaryotes Rab4 is located on early endocytic compartments, closely associated with Rab5 and Rab11, and implicated in multiple functions, including rapid recycling of surface receptors, transport to the lysosome, exocytosis, cell adhesion, and response to insulin (Cormont et al., 2001; De Renzis et al., 2002; De Wit et al., 2001; Lindsay et al., 2002; McCaffrey et al., 2001; Nagamatsu et al., 2000; Roberts et al., 2001; Van der Sluijs et al., 1992). In *T. brucei* TbRAB4 shows a similar endosomal localisation to mammalian rab4 (Field et al., 1998). In the bloodstream form trypanosome the major recycling pathway responsible for recycling of GPI-anchored proteins involves TbRAB5A and TbRAB11, both of which are developmentally regulated (Pal et al., 2002; Hall et al., 2004a; Jeffries et al., 2001; Grunfelder et al., 2003; Pal et al., 2003). The importance of this pathway to the bloodstream stage is further underscored by evidence for a role in removal of surface-bound immunoglobulins.

In bloodstream form cells TbRAB4 is essential and is associated with control of fluid phase transport with no apparent role in recycling (Hall et al., 2004b). Here, we have addressed the function of TbRAB4 in the procyclic form, a life stage where endocytosis is down-regulated by at least 10-fold compared to the bloodstream form. The major findings of the present work are first that TbRAB4 is involved in multiple aspects of endocytic function in the procyclic stage parasite. Second, changes in surface protein expression induced by overexpression of a dominantly active mutant support a role for TbRAB4 in control of procyclin surface display, most likely due to the GTPase functioning in the endocytic and recycling pathways.

The data described here support a rather different role for TbRAB4 in the procyclic compared to bloodstream form. This was unexpected, but is likely due to differential features of the endosomal systems between these two stages. Significantly, TbRAB5A, TbRAB5B, and TbRAB11 are developmentally regulated, with expression of TbRAB11 being reduced in the insect stage by approximately 10-fold compared to the bloodstream form and a differential localisation for the TbRAB5 isoforms (Field et al., 1998; Jeffries et al., 2001; Pal et al., 2002). Since TbRAB4 is expressed constitutively (as judged by protein level and location) these observations raise the possibility of increased burden on TbRAB4-mediated recycling in the procyclic form. Impaired growth of TbRAB4 RNAi cells suggests a vital function in trafficking in the procyclic, whilst the profound effects of TbRAB4 RNAi on uptake and recycling of FM4-64 confirm the importance of the protein in early endocytosis. Despite the inhibition of FM4-64 recycling by TbRAB4 RNAi being relatively minor compared to the increase in uptake, the case for a specific role in recycling for TbRAB4 is further supported by increased recycling in TbRAB4^{QL}-expressing cells. However, the failure of TbRAB4 RNAi to completely block FM4-64 recycling shows that TbRAB4-independent recycling pathways may also be present. In contrast to bloodstream cells expressing TbRAB4^{QL}, which only show increased uptake of fluid phase markers (Hall et al., 2004b), TbRAB4^{QL} procyclic cells exhibit a generalised increase in endocytosis, with increased internalisation of FM4-64 as well as fluid phase cargo. Other experiments have indicated that uptake of LDL and biotinylated surface proteins is also enhanced in these cells (BSH and MCF, unpublished data).

The observation that TbRAB4 RNAi and TbRAB4^{QL} expression both cause increased uptake of endocytic markers, but have opposing effects on recycling, is surprising but not unprecedented. Simplistic models are not sufficient to describe the complex interactions between different Rab proteins and their binding partners with multiple endocytic and recycling pathways; for example, Rab22a, a mammalian Rab family protein associated with recycling. Disruption of expression by RNAi and expression of either a dominant negative or constitutively active Rab22a mutant each induces differential changes in recycling tubule structure but all lead to an inhibition of MHC recycling (Weigart et al., 2004). We have previously shown that bloodstream form cells respond to the block in transferrin receptor recycling by increasing receptor expression levels (Hall et al., 2005). It is possible that *T. brucei* procyclic cells are able to adjust to loss of TbRAB4 expression by upregulating other components of the trafficking system. The results obtained here suggest interactions of TbRAB4 with both uptake and recycling arms of the endocytic system and indicate a more general role for TbRAB4 in trafficking in procyclic forms than in bloodstream form cells.

Significantly, modulation of TbRAB4 expression also had effects on the GPI-anchored surface coat of the procyclic parasite, particularly GPEET procyclin. In general a more internal location for surface antigens was observed in TbRAB4^{QL}-expressing cells, suggesting that procyclins are recycled via a TbRAB4-dependent pathway. As well as altering location, the relative levels of EP and GPEET procyclin were affected in TbRAB4^{QL} cells. The influence of TbRAB4 on surface protein location

was also seen for ectopic VSG where a similar redistribution was observed. Hence the shift in location is most likely a general phenomenon due to a shift in the balance of endocytic flux. However, the greater impact of TbRAB4^{QL} expression on GPEET location may be an indication of the presence of specific mechanisms for the regulation of this procyclin.

Whilst procyclin expressed at the surface appears to be stable, internal protein pools are normally subjected to turnover and hence a shift in localisation may affect overall protein stability and hence abundance. It is possible that the apparent shift in molecular weight of GPEET in the TbRAB4^{QL} line is due to increased proteolysis of the internalised protein (Liniger et al., 2003). TbRAB4 may, therefore, play a role in the switch from GPEET to EP expression during procyclic development, controlling removal of the protein from the surface and promoting degradation. It is also possible that the change in location alters phosphorylation. However, we have been unable to confirm either of these explanations from the mass spectrometry analysis.

Alterations in procyclin localisation may have a functional significance: cold shock of bloodstream form parasites is sufficient to induce expression of EP procyclin in both slender and stumpy form parasites, but in slender form cells the protein is restricted to an endosomal location, while EP is present on the surface stumpy forms (Engstler and Boshart, 2004). Thus changes in the endocytic system are likely to be linked to developmental regulation of procyclin expression and parasite differentiation. Although transcriptional and post-transcriptional regulation of procyclins are well documented, little is known of post-translational methods of regulation (Engstler and Boshart, 2004; Hotz et al., 1998; Vassella et al., 1997; Vassella et al., 2000; Vassella et al., 2004). This work highlights the potential importance of endosomal trafficking in the control of parasite development.

Acknowledgments

We thank Clare Allen for advice, Mike Ferguson for MALDI-TOF access, Isabella Vlisidou for constructing p27T·TbRAB4, Mark Carrington for MiTat1.4 VSG antibodies, Terry Pearson for procyclin antibodies, and Doug La Count and James Bangs for p2T7 and pXS219·VSG117 plasmids, respectively. This work was supported by a programme grant (to M.C.F.) and a travelling research fellowship (to A.A.S.) from the Wellcome Trust.

References

Acosta-Serrano, A., Cole, R.N., Mehlert, A., Lee, M.G.-S., Ferguson, M.A.J., Englund, P.T., 1999. The procyclin repertoire of *Trypanosoma brucei*. Identification and structural characterization of the Glu-Pro-rich polypeptides. Journal of Biological Chemistry 274, 29763–29771.

Acosta-Serrano, A., Vassella, E., Liniger, M., Renggli, C.K., Brun, R., Roditi, I., 2000. The surface coat of procyclic *Trypanosoma brucei*: programmed expression and proteolytic cleavage of procyclin in the tsetse fly. Proceedings of the National Academy of Sciences (USA) 98, 1513–1518.

Bangs, J.D., Ransom, D.M., McDowell, M.A., Brouch, E.M., 1997. Expression of bloodstream variant surface glycoproteins in procyclic stage *Trypanosoma brucei*: role of GPI anchors in secretion. European Molecular Biology Organisation Journal 16, 4285–4294.

Butikofer, P., Ruepp, S., Bosching, M., Roditi, I., 1997. 'GPEET' procyclin is the major surface protein of procyclic culture forms of *Trypanosoma brucei brucei* strain 427. Biochemical Journal 326, 415–423.

Butikofer, P., Vassella, E., Ruepp, S., Boschung, M., Civenni, G., Seebeck, T., Hemphill, A., Mookherjee, N., Pearson, T.W., Roditi, I., 1999. Phosphorylation of a major GPI-anchored surface protein of *Trypanosoma brucei* during transport to the plasma membrane. Journal of Cell Science 112, 1785–1795.

Butikofer, P., Vassella, E., Mehlert, A., Ferguson, M.A.J., Roditi, I., 2002. Characterisation and cellular localisation of a GPEET procyclin precursor in *Trypanosoma brucei* insect forms. Molecular and Biochemical Parasitology 119, 87–95.

Cormont, M., Gautier, N., Ilc, K., Le Marchand-Brustel, Y., 2001. Expression of a prenylation-deficient Rab4 inhibits the GLUT4 translocation induced by active phosphatidylinositol 3-kinase and protein kinase B. Biochemical Journal 356, 143–149.

De Renzis, S., Sonnichsen, B., Zerial, M., 2002. Divalent Rab effectors regulate the sub-compartmental organization and sorting of early endosomes. Nature Cell Biology 4, 124–133.

De Wit, H., Lichtenstein, Y., Kelly, R.B., Geuze, H.J., Klumperman, J., van der Sluijs, P., 2001. Rab4 regulates formation of synaptic-like microvesicles from early endosomes in PC12 cells. Molecular Biology of the Cell 12, 3703–3715.

Engstler, M., Boshart, M., 2004. Cold shock and regulation of surface trafficking convey sensitization to inducers of stage differentiation in *Trypanosoma brucei*. Genes and Development 18, 2798–2811.

Field, M.C., Allen, C.L., Dhir, V., Goulding, D., Hall, B.S., Morgan, G., Veazey, P., Engstler, M.E., 2004. Morphological analysis of *Trypanosoma brucei* by light and electron microscopy. Microscopy and Microanalysis 10, 621–636.

Field, H., Farjah, M., Pal, A., Gull, K., Croft, S.L., Field, M.C., 1998. Complexity of trypanosomatid endocytosis pathways revealed by Rab4 and Rab5 isoforms in *Trypanosoma brucei*. Journal of Biological Chemistry 273, 32102–32110.

Grunfelder, C.G., Engstler, M., Weise, F., Schwarz, H., Steirhof, Y.-D., Morgan, G.W., Field, M.C., Overath, P., 2003. Endocytosis of a GPI-anchored protein via clathrin-coated vesicles, sorting by default in endosomes and exocytosis via TbRAB11-positive carriers. Molecular Biology of the Cell 14, 2029–2040.

Hall, B.S., Allen, C.L., Goulding, D., Field, M.C., 2004a. The small GTPases TbRAB5A and TbRAB5B are both essential and required for endocytosis in bloodstream form *Trypanosoma brucei*. Molecular and Biochemical Parasitology 138, 67–77.

Hall, B.S., Pal, A., Goulding, D., Field, M.C., 2004b. Rab4 is an essential regulator of lysosomal trafficking in trypanosomes. Journal of Biological Chemistry 279, 45047–45056.

Hall, B.S., Smith, E., Langer, W., Taoufiq, Z., Jacobs, L., Goulding, D., Field, M., 2005. TbRAB11-dependent recycling is essential in both bloodstream and procyclic form *Trypanosoma brucei*. Eukaryotic Cell. 4, 971–980.

Hotz, H.R., Biebinger, S., Flaspahler, J., Clayton, C., 1998. PARP gene expression: control at many levels. Molecular and Biochemical Parasitology 91, 131–132.

Jeffries, T., Morgan, G.W., Field, M.C., 2001. A Rab11 homologue in *Trypanosoma brucei* is involved in developmentally-regulated recycling processes. Journal of Cell Science 114, 2617–2626.

Kabiri, M., Steverding, D., 2000. Studies on the recycling of the transferrin receptor in *Trypanosoma brucei* using an inducible gene expression system. European Journal of Biochemistry 267, 3309–3314.

La-Count, D.J., Bruse, S., Hill, K.L., Donelson, J.E., 2000. Double-stranded RNA interference in *Trypanosoma brucei* using head-to-head promoters. Molecular and Biochemical Parasitology 111, 67–76.

La-Count, D.J., Barrett, B., Donelson, J.E., 2002. *Trypanosoma brucei* FLA1 is required for flagellum attachment and cytokinesis. Journal of Biological Chemistry 275, 15657–17588.

Lindsay, A.J., Hendrick, A.G., Cantalupo, G., Senic-Matuglia, F., Goud, B., Bucci, C., McCaffrey, M.W., 2002. Rab coupling protein (RCP), a novel Rab4 and Rab11 effector protein. Journal of Biological Chemistry 277, 12190–12199.

Liniger, M., Acosta-Serrano, A., Van Den Abbeele, J., Kunz Renggli, C., Brun, R., England, P.T., Roditi, I., 2003. Cleavage of trypanosome surface glycoproteins by alkaline trypsin-like enzyme(s) in the midgut of *Glossina Morsitans*. International Journal for Parasitology 33, 1319–1328.

Liu, J., Qiao, X., Du, D., Lee, M.G., 2000. Receptor-mediated endocytosis in the procyclic form of *Trypanosoma brucei*. Journal of Biological Chemistry 275, 12032–12040.

McCaffrey, M.W., Bielli, A., Cantalupo, G., Mora, S., Roberti, V., Santillo, M., Drummond, F., Bucci, C., 2001. Rab4 affects both recycling and degradative endosomal trafficking. Federation of European Biochemical Societies Letters 495, 21–30.

Morris, J.C., Wang, Z., Drew, M.E., Englund, P.T., 2002. Glycolysis modulates trypanosome glycoprotein expression as revealed by an RNAi library. European Molecular Biology Organisation Journal 21, 4429–4438.

Nagamatsu, S., Nakamighi, Y., Watanabe, T., Matsushima, S., Yamaguchi, S., Ni, J., Itagaki, E., Ishida, H., 2000. Localization of cellubrevin-related peptide, endobrevin, in the early endosome in pancreatic β-cells and its physiological function in exo-endocytosis of secretory granules. Journal of Cell Science 114, 219–227.

Ngo, H., Tschudi, C., Gull, K., Ullu, E., 1998. Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. Proceedings of the National Academy of Sciences (USA) 95, 14687–14692.

Pal, A., Hall, B.S., Nesbeth, D.N., Field, H.I., Field, M.C., 2002. Differential endocytic functions of *Trypanosoma brucei* Rab5 isoforms reveal a glycosyl-phosphatidylinositol-specific endosomal pathway. Journal of Biological Chemistry 277, 9529–9539.

Pal, A., Hall, B.S., Jeffries, T.R., Field, M.C., 2003. Rab5 and Rab11 mediate transferrin and anti-variant surface glycoprotein antibody recycling in *Trypanosoma brucei*. Biochemical Journal 374, 443–451.

Roberts, M., Barry, S., Woods, A., van der Sluijs, P., Norman, J., 2001. PDGF-regulated rab4-dependent recycling of $\alpha_v\beta_3$ integrin from early endosomes is necessary for cell adhesion and spreading. Current Biology 11, 1392–1402.

Seyfang, A., Mecke, D., Duszenko, M., 1990. Degradation, recycling, and shedding of *Trypanosoma brucei* variant surface glycoprotein. Journal of Protozoology 37, 546–552.

Shirakawa, R., Yoshioko, A., Horiuchi, H., Nishioka, H., Tabuchi, A., Kita, T., 2000. Small GTPase Rab4 regulates Ca^{2+}-induced alpha-granule secretion in platelets. Journal of Biological Chemistry 275, 33844–33849.

Sonnischen, B., De Renzis, S., Nielsen, E., Rietdorf, J., Zerial, M., 2000. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. Journal of Cell Biology 149, 901–914.

Ullrich, O., Reinsch, S., Urbe, S., Zerial, M., Parton, R.G., 1996. Rab11 regulates recycling through the pericentriolar recycling endosome. Journal of Cell Biology 135, 913–924.

Van der Sluijs, P., Hull, M., Webster, P., Male, P., Goud, B., Mellman, I., 1992. The small GTP-binding protein rab4 controls an early sorting event on the endocytic pathway. Cell 70, 729–740.

Vassella, E., Reuner, B., Yutzy, B., Boshart, M., 1997. Differentiation of African trypanosomes is controlled by a density sensing mechanism which signals cell cycle arrest via the cAMP pathway. Journal of Cell Science 110, 2661–2671.

Vassella, E., Van den Abeele, J., Butikofer, P., Renggli, C.K., Furger, A., Brun, R., Roditi, I., 2000. A major surface glycoprotein of *Trypanosoma brucei* is expressed transiently during development and can be regulated post-transcriptionally by glycerol or hypoxia. Genes and Development 14, 615–626.

Vassella, E., Acosta-Serrano, A., Studer, E., Lee, S.H., Englund, P.T., Roditi, I., 2001. Multiple procyclin isoforms are expressed differentially during the development of insect forms of *Trypanosoma brucei*. Journal of Molecular Biology 312, 597–607.

Vassella, E., Probst, M., Schneider, A., Studer, E., Renggli, C.K., Roditi, I., 2004. Expression of a major surface protein of *Trypanosoma brucei* insect forms is controlled by the activity of Mitochondrial enzymes. Molecular cell Biology 15, 3986–3993.

Wang, Z., Morris, J.C., Drew, M.E., Englund, P.T., 2000. Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters. Journal of Biological Chemistry 275, 40174–40179.

Weigart, R., Yeung, A.C., Li, J., Donaldson, J.G., 2004. Rab22a regulates the recycling of membrane proteins internalised independently of clathrin. Molecular and Cellular Biology 15, 3758–3770.

Wiederkehr, A., Avaro, S., Prescianotto-Baschong, C., Haguenauer-Tsapis, R., Riezman, H., 2000. The F-Box protein Rcy1p is involved in endocytic membrane traffic and recycling out of an early endosome in *Saccharomyces cerevisiae*. Journal of Cell Biology 149, 397–410.

Wirtz, E., Leal, S., Ochatt, C., Cross, G.A., 1999. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Molecular and Biochemical Parasitology 99, 89–101.

Zerial, M., McBride, H., 2001. Rab proteins as membrane organizers. Nature Reviews Molecular Cell Biololgy 2, 107–117.