A developmentally regulated Rab11 homologue in *Trypanosoma brucei* is involved in recycling processes

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SUMMARY

Endocytosis in the parasitic protozoan *Trypanosoma brucei*, a deeply divergent eukaryote, is implicated as important in both general cellular function and virulence, and is strongly developmentally regulated. We report the characterisation of a previously undefined endosomal compartment in *T. brucei* based on identification of a new trypanosome gene (*TbRAB11)* homologous to Rab11/Ypt31. Northern and western analyses indicated that *TbRAB11* expression was significantly upregulated in the bloodstream stage of the parasite, the first trypanosome Rab to be identified with a developmentally regulated expression profile. In procyclic form parasites TbRAB11 localised to a compartment positioned close to the basal body, similar to mammalian Rab11. By contrast, in bloodstream form parasites, TbRAB11-containing structures were more extensive and the TbRAB11 compartment extended towards the posterior face of the nucleus, was more elaborate and was not always adjacent to the basal body. Colocalisation studies by light and confocal microscopy demonstrated that TbRAB11 was located on a compartment that did not correspond to other established trypanosomal organelles or markers. Using concanavalin A internalisation and temperature block procedures, TbRAB11 was observed on endomembranes anterior to the flagellar pocket that are juxtaposed to the collecting tubules. TbRAB11 colocalised with the trypanosomal transferrin receptor and internalised anti-variant surface glycoprotein. Further, we show that the collecting tubules contain TbRAB5A, suggesting that they are the trypanosomatid early endosome. Hence, TbRAB11 is present on endosomal structures that contain recycling cargo molecules and is under developmental regulation, suggesting a role in stage-dependent endocytic processes.

Key words: Rab11, Trypanosome, Recycling, Endosome, Flagellar pocket

INTRODUCTION

Rab proteins are a subgroup of the Ras superfamily of small GTPases involved in regulation of intracellular vesicle trafficking (Nuoffer and Balch, 1994). These proteins localise to specific membrane structures, for example, Rab5 is found uniquely on early endosomes (Bucci et al., 1992) and this localisation suggests that Rabs act as specificity determinants in vesicular trafficking processes (Ferro-Novick and Novick, 1993). Accurate vesicle targeting involves multiple levels of regulation and fidelity mechanisms operate at three or more distinct stages: vesicle budding, trafficking of vesicles through the cytoplasm, and fusion of the vesicle with the target membrane (Chavrier and Goud, 1999). In support of a regulatory role in vesicular trafficking Rabs have been implicated at each of these steps (Jedd et al., 1997; Echard et al., 1998; Ungermann et al., 1998) and are considered to be in an activated state when bound to GTP. Formation of trans-SNARE complexes involves regulatory factors including the Sec1-like proteins; correct functioning of these factors is regulated by Rab proteins such as Sly1p (a Sec1-like protein involved in ER to Golgi traffic in *Saccharomyces cerevisiae*) which, subsequent to recruitment to the t-SNARE Sed5p, is displaced from Sed5p by Ypt1p (Lupashin and Waters, 1997) permitting trans-SNARE complex assembly. Rab proteins themselves are insufficient to ensure complete targeting fidelity as a Ypt1p/Sec4p chimera can act at two transport steps without compromising cargo sorting (Brennwald and Novick, 1993). Rab proteins are also implicated in interactions with the cytoskeleton (Echard et al., 1998).

Endosomal endomembrane compartments can be viewed as being at the crossroads of flux through the endocytic and secretory pathways (Lemmon and Traub, 2000). For example, in *S. cerevisiae*, endosomal elements receive secretory traffic from Golgi elements and endocytosed material from the cell surface (Vida et al., 1993; Mulholland et al., 1999). Cargo is sorted in endosomes such that vacuolar destined proteins are routed to the late endosomal prevacuolar compartment while internalised resident surface membrane proteins may be recycled to the surface along with nascent plasma membrane proteins delivered from the Golgi (Gerrard et al., 2000; Wiederkehr et al., 2000). Mammalian cells appear to possess a more complex endosomal network. Endocytosed material initially traffics to early endosomes, regions characterised by the presence of Rab5 (Bucci et al., 1992), and may be targeted to late endosomes and ultimately lysosomes, recycled through

Rab4-positive peripheral endosomal elements (Bottger et al., 1997) or recycled through deeper, centriole-proximal Rab11-containing recycling endosomes (Ullrich et al., 1996).

Trypanosoma brucei is a parasitic protozoan with a digenetic life cycle. The procyclic form (PCF) resides within the tsetse fly whereas the bloodstream form (BSF) infects mammals and is exposed to immunological recognition. BSF parasites have a surface coat comprised predominately of the GPI-anchored variant surface glycoprotein (VSG; Ferguson, 1997). Through antigenic variation, antigenically distinct VSG isoforms are expressed throughout an infection (Pays et al., 1994), ensuring that the host is unable to accumulate levels of anti-VSG antibody sufficient to clear the infection. Large quantities of VSG are constitutively internalised at a specialised region of the plasma membrane known as the flagellar pocket (Duszenko and Seyfang, 1993), the sole site of endocytic membrane transport. The vast majority of internalised VSG is recycled to the plasma membrane (Seyfang et al., 1990). IgM and IgG anti-VSG antibodies may be generated faster than the VSG switch, but addition of anti-VSG antibody in vitro results in internalisation and recycling of the anti-VSG/VSG complexes; during this process the antibody is proteolytically degraded, but the VSG is returned intact to the plasma membrane. Hence, trafficking of VSG through the trypanosome endosomal system is likely to be important in immune evasion (O'Beirne et al., 1998).

The ultrastructure of the endocytic network of *T. brucei* has been described (Webster, 1989; Brickman et al., 1995). Following internalisation via coated pits at the flagellar pocket, endocytic cargo is delivered to tubular structures termed collecting tubules, where sorting occurs. Material destined for degradation (e.g. transferrin) is targeted to lysosomes whereas recycling cargo (e.g. VSG) is returned to the flagellar pocket. Whether these collecting tubules represent a homogenous membrane population has not been determined. To gain insight into collecting tubule organisation, VSG turnover and other endocytic mechanisms we are characterising the trypanosome endocytic system at the molecular level. Several constitutively expressed endosomal Rabs have been characterised, including TbRAB5A and TbRAB5B, implicated as early endosomal, and TbRAB4, potentially involved in a recycling pathway (Field et al., 1998). More recently we reported on TbRAB31, localised to the trans *Golgi* complex, and which may also have a role in recycling processes (Field et al., 2000). Here we describe a developmentally regulated Rab (TbRAB11), which is implicated in the recycling of two GPI-anchored proteins: VSG and the trypanosomal transferrin receptor.

MATERIALS AND METHODS

Chemicals, buffer components and media were from Sigma and of the highest grade available, unless otherwise indicated. Culture adapted BSF and PCF *T. brucei brucei* parasites, strain 427, were grown as previously described (Field et al., 1998). Standard molecular biology methods were carried out as described (Ausubel et al., 1994), unless indicated. Nitrocellulose and nylon membranes for western and northern blotting were from Amersham. Plasmids were grown in *Escherichia coli* XL1-Blue (Stratagene), unless otherwise described, following electroporation with a BTX600 ECM electroporator. PCR was performed using a 480 Thermal Cycler (Perkin Elmer) with *Pfu* polymerase (Stratagene) and gel embedded DNA purified using GeneClean (BIO101). Mini-preps were conducted using Spin kits from Qiagen.

Northern analysis

Total RNA was isolated from 10^8 PCF and BSF parasites (Ausubel et al., 1994). RNA was resolved through a denaturing formaldehyde gel (Parry and Alphey, 1994), blotted onto nylon membranes (Sambrook et al., 1989) and crosslinked using a UV Stratalinker (Stratagene) and hybridised overnight with a probe generated by the random priming method (Parry and Alphey, 1994) using the full *TbRAB11* open reading frame (ORF) as a template. Membranes were washed three times for 20 minutes in 0.2× SSC/0.1% SDS before exposure against X-Ray film (Kodak).

Cloning and expression of TbRAB11

5′ and 3′ regions of a *Rab11* homologue (*TbRAB11*) were identified on sheared DNA clones in the TIGR *T. brucei* database (www.tigr.org/tdb/mdb/tbdb/index.html). Primers to the ends of the *Rab11* homologue were used to amplify a product of approximately 650 bp that was cloned into pCR-Script (Stratagene). Sequencing of the product followed by BLAST analysis confirmed this gene as being highly homologous to *Rab11*. The nucleotide sequence has been deposited at GenBank, accession number AF152531. The *TbRAB11* ORF was cloned into the expression vector pGEX-2TK (Pharmacia) using the 5′ primer Tb11XF, which contained a *Bam*HI site (GACGTGGGATCCATGGAAGACCTGAACCTT) and the 3′ primer Tb11XB which contained an *Eco*RI site (TCTGTCATGAATTCGT-TAACAGCACCCGCCACT). The amplified product was cloned into the expression vector. All constructs were verified by DNA sequencing with a 377 DNA sequencer (Perkin Elmer) using Dye Terminator chemistry.

Recombinant protein expression and production of antibodies

TbRAB11 was expressed as a GST-fusion protein from pGEX-2TK in *E. coli* BL21 (Stratagene). Protein was purified on glutathione-sepharose 4B (Pharmacia) and cleaved to release TbRAB11 by incubation at room temperature with thrombin (New England Biolabs) for 30 minutes. Cleaved protein was quantitated and verified by SDS-PAGE followed by Coomassie staining. The released Rab protein was used to raise polyclonal antibodies in mice and rabbits using the MPL+TDM+CWS adjuvant system. 100 μg protein was administered to the rabbit and 20 μg protein to each mouse every injection. This regime was repeated six times in each case. Affinity purified antibodies were isolated using recombinant protein coupled to CNBr-activiated-sepharose 4B (Pharmacia). Recombinant protein was bound to the beads according to the manufacturers instructions and remaining reactive groups blocked with 1 M ethanolamine, pH 8.0 for 2 hours at room temperature. The resin was resuspended in 5 bed volumes of 10 mM Tris (pH 7.5) and transferred to a Poly-Prep column (BioRad). The resin was successively washed with 10 bed-volumes 10 mM Tris (pH 7.5), 10 bed-volumes 100 mM triethylamine (pH 11.5) and finally with 10 mM Tris (pH 7.5). Subsequent to loading, the column was washed with 20 bed-volumes 10 mM Tris (pH 7.5) and 20 bed-volumes 500 mM NaCl, 10 mM Tris (pH 7.5). Acid-sensitive antibodies were eluted with 10 bed-volumes of 100 mM glycine (pH 2.5). 1 ml fractions were collected directly onto 100 μl 1 M Tris (pH 8.0).

GTP hydrolysis assay

GTPase activity was determined as described (Foster et al., 1996) with modifications (Field et al., 2000). GST-fusion protein was purified as above, omitting the thrombin cleavage and verified by SDS-PAGE. Approximately 20 μg of protein was loaded with [α-^{32}P]GTP (10 μCi; 400 Ci/mmol; Amersham) in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM EDTA, 0.1 mM ethylene glycol-bis(β-aminoethyl)*n,n,n,n*-tetraacetic acid (EGTA), 0.1 mM dithiothreitol (DTT), 10 mM ATP

at 37°C for 10 minutes in a total volume of 100 µl. Beads were washed three times at 4°C in buffer (50 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 1 mM DTT and 1 mg/ml bovine serum albumin (BSA)). Samples were resuspended in wash buffer prewarmed to 37°C. Samples were taken and added to an equal volume of ice cold quench buffer (5 mM EDTA, 50 mM GTP, 50 mM GDP) and bound nucleotide eluted by incubation at 65°C for 5 minutes in elution buffer (1% SDS, 20 mM EDTA). Elutes were spotted onto polyethyleneimine-cellulose plates (Merck) and resolved by thin layer chromatography (TLC) in 0.75 M KH_2PO_4 (pH 3.4) and visualised on a phosphorimager screen (Molecular Dynamics). Following conversion of the data to TIFF, GDP/GTP ratios were calculated using NIH Image v.1.59.

Immunochemistry

Trypanosome cell pellets (10^7 cells) were resuspended in 100 µl boiling sample buffer and resolved by SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes by semi-dry electrophoresis under standard conditions for 40 minutes. Equivalence of loading was verified by Ponceau-S (Sigma) staining. Nonspecific binding sites were blocked in BLOTTO (0.2% Tween-20, 5% freeze dried milk, phosphate buffered saline pH7.6 (PBS)). Antibodies were also diluted in BLOTTO. Rabbit or mouse anti-TbRAB11 were used at 1:1000 and 1:100, respectively, and detected with mouse anti-rabbit horseradish peroxidase conjugate (Sigma) or goat anti-mouse horseradish peroxidase conjugate (Sigma). Bound conjugate was detected with ECL reagent.

Immunofluorescence

Indirect immunofluorescence microscopy was performed as previously described (Field et al., 1998). Antibodies were used at the following dilutions: rabbit anti-TbRAB11 1:500; mouse anti-TbRAB11 1:50; rabbit anti-TbRAB5A 1:200; rabbit anti-TbCLH 1:1000; mouse monoclonal anti-p67 1:1000 (gift of J. Bangs, Madison); mouse monoclonal BB4 1:1 (gift of K. Gull, Manchester), rabbit anti-trypanosome transferrin receptor 1:1000 (gift of P. Borst, Amsterdam). Secondary antibodies, anti-rabbit Cy3 (Sigma) and anti-mouse FITC (Sigma), were used according to the manufacturers instructions. The trypanosomal Golgi complex was stained using BODIPY-TR ceramide (Molecular probes) as previously described (Denny et al., 2000; Field et al., 2000). Cells were observed either on a Nikon Microphot-FX epifluorescent microscope attached to a Photometrics CH350-CCD camera or with a Laser Scanning Microscope 510 (Zeiss). Images were false-coloured and assembled using Adobe PhotoShop.

FITC-concanavalin A uptake

Concanavalin A (ConA) uptake was monitored as described, with modifications (Brickman et al., 1995). Uptake was followed by incubating 10^7 BSF parasites in 1 ml of media containing 100 µg FITC-ConA (Vector Labs). For pulse-chase experiments, parasites were harvested at mid-log phase, washed once and resuspended at a density of 10^8 parasites/ml in serum free media pre-equilibrated at 4°C. 100 µg of ConA was added per 10^7 cells and parasites incubated for 10 minutes. Subsequently, parasites were washed in serum-free media, resuspended in media pre-equilibrated at the desired temperature and incubated for 30 minutes. Biotinylated-ConA (Vector Labs) was used for lysosomal visualisation and FITC-ConA for all other assays.

Anti-VSG uptake

All manipulations were conducted using HMI9/1% BSA/protease inhibitors (Mini Complete, Sigma). BSF parasites expressing VSG 221 were cultured in media for 1 hour at 10^7parasites/ml. Rabbit anti-VSG 221 (gift of A. Pal) was added at 10 µg/ml and cells incubated at 37°C for 1 hour. After incubation, cells were washed twice in PBS, fixed for microscopy and co-stained using mouse anti-TbRAB11 antibodies. Rabbit and mouse antibodies were visualised as described above.

Bioinformatics

BLAST searches were conducted at NCBI (www.ncbi.nlm.nih.gov/blast) and the *T. brucei* database at TIGR (www.tigr.org/tdb/mdb/tbdb/index.html). Sequence alignments were performed using ClustalX and presented as SeqVu 1.1 documents. Phylogenetic reconstruction was done using PAUP release 4.0.

RESULTS

Identification of a Rab11 homologue in *T. brucei*

We have previously described three trypanosomal Rabs, 4, 5A and 5B, which are involved in endosomal endomembrane dynamics. To further extend understanding of the *T. brucei* endosomal system we searched the database for additional endosomal Rab sequences. tBLASTn analysis of the TIGR *T. brucei* database identified two shotgun sequences (TIGR clones: 8B12, 4O3) that encoded 5′ and 3′ regions of a gene with homology to mammalian Rab11. PCR allowed amplification of a full-length ORF 648 bp in length that codes for a protein with a predicted molecular mass of 23.5 kDa. Southern analysis indicates this gene is present as a single copy (data not shown). The translated ORF is 85% similar and has 81% identity with a recently identified Rab gene in *T. cruzi*,

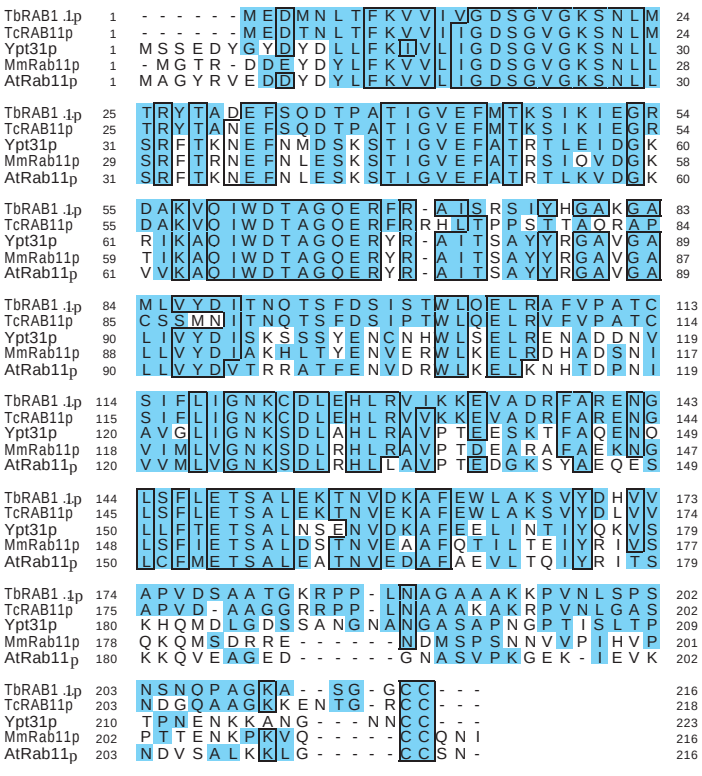

Fig. 1. TbRAB11 is a Rab11 / Ypt31 homolog. ClustalX alignment of the predicted translation of the ORF of TbRAB11 with its closest kinetoplastid, mammalian, yeast and plant orthologues identified by BLASTp analysis at GenBank. *Tc, T. cruzi; At, A. thaliana; Mm, M. musculus; Ypt, S. cerevisiae*. Boxes indicate identity and shading similarity.

TcRAB11. The TcRAB11 protein has been suggested to localise to lysosomal regions (Mauricio de Mendonca et al., 2000). The closest *S. cerevisiae* homologue to *TbRAB11*, *YPT31*, a gene whose product is implicated in traffic exiting the trans-Golgi network (TGN) (Jedd et al., 1997), exhibits 48% identity and 68% similarity. The nearest mammalian homologue, *Mus musculus Rab11* (*MmRab11*), shares 50% identity and 73% similarity. MmRab11 is involved in trafficking from the TGN in addition to regulating traffic flux through pericentriolar recycling endosomes (Chen et al., 1998; Ullrich et al., 1996). TbRAB31 is a Golgi-localised Rab that is also closely homologous to mammalian Rab11 (Field et al., 2000). *TbRAB31* shares 32% identity and 53% homology with *TbRAB11* but is distinct by phylogenetic analysis, forming an outgroup with *TbRAB2*, whereas *TbRAB11* groups with *TbRAB4* (data not shown). Finally, the closest plant homologue is *Arabidopsis thaliana Rab11* (*AtRab11*). This gene product possesses 54% identity and 74% similarity. A full alignment of protein sequences (except for TbRAB31) is shown in Fig. 1. As anticipated for a fully functional Rab the four nucleotide binding regions are conserved, as is the C-terminal prenylation motif.

TbRAB11 is developmentally regulated

We have information of the expression profile of six GTPases in *T. brucei*, all of which are constitutively expressed (Field et al., 1995; Field and Field 1997; Field et al., 1998; Field et al., 1999). By contrast, northern blot analysis using full length *TbRAB11* as a probe revealed a moderately abundant transcript at approximately 3 kb in the BSF stage, which was barely detectable in the PCF stage (Fig. 2A), suggesting that *TbRAB11* expression is developmentally regulated at the mRNA level. As trypanosome gene expression is predominantly controlled at the post-transcriptional level, we extended analysis to the protein level. Rabbit polyclonal antibodies raised against recombinant TbRAB11 recognised a single band at approximately 24 kDa that was significantly more abundant in BSF lysates compared with PCF (Fig. 2B). Equivalent loading of PCF and BSF lysates was verified by Ponceau S staining of the blot (data not shown). Reactivity was completely abolished by preincubation of the antibody with recombinant TbRAB11 confirming specificity (data not shown).

Several GTPase-deficient Rab proteins have been identified in *T. brucei* (Field et al., 2000; T.R.J. and M.C.F., unpublished), hence we wished to determine directly if TbRAB11 was an authentic GTPase. We compared the GTPase activity of recombinant GST-TbRAB11 fusion protein expressed in *E. coli* to that of GST-TbRAB2, a well characterised trypanosome GTPase (Field et al., 1999). GST-TbRAB11 bound levels of GTP similar to GST-TbRAB2, whereas the GTP hydrolytic rate of GST-TbRAB11 was approximately twofold lower than GST-TbRAB2 (Fig. 2C,D) and are in agreement with the conserved nucleotide binding motifs in the TbRAB11 sequence.

Subcellular localisation of TbRAB11

Developmentally regulated TbRAB11 expression suggests that the TbRAB11 pathway is more active in BSF, whereas all sequence data place TbRAB11 as being involved in endosomal processes, these are confined to the region between the nucleus and kinetoplast (Brickman et al., 1995; Field et al., 1998). By immunofluorescent microscopy, TbRAB11 localised to one or two distinct spots closely adjacent to the kinetoplast in PCFs, with extremely well conserved morphology in all cells analysed (Fig. 3A,B,C; Fig. 4). By contrast, in BSF parasites, the TbRAB11 staining was significantly more extensive (Fig. 3D,E,F), and although some of the staining retained a position adjacent to the kinetoplast in BSFs the stain was far more variable between cells, suggesting a more dynamic TbRAB11 structure, and spread towards the posterior face of the nucleus.

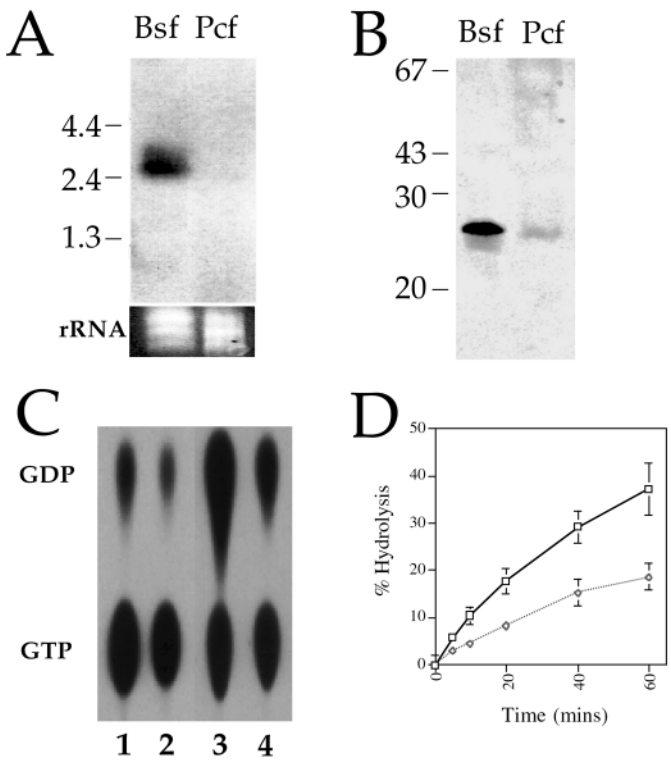

Fig. 2. TbRAB11 is a developmentally regulated GTPase. (A) Northern blot analysis demonstrating that BSF parasites express *TbRAB11* mRNA at a higher level than PCF. Total RNA was isolated from 10^7 cells and resolved on a formaldehyde gel. After transfer to a nylon membrane the blot was probed with the full length *TbRAB11* ORF. Positions of RNA standards in kb are shown to the left and ethidium bromide stain of the rRNA bands shown as a control for loading equivalence at the base of the figure. (B) Western blot analysis with affinity purified anti-TbRAB11 generated in rabbits using full length recombinant protein as antigen. Fresh parasite cultures were resuspended in protein sample buffer, 10^7 parasite equivalents loaded in each lane and resolved on a 12% SDS-polyacrylamide gel. After electrophoretic transfer to nitrocellulose, equivalence of loading was checked by Ponceau S staining. Molecular weight standards are indicated to the left in kDa. (C) GTPase assay using recombinant GST-TbRAB2 (lanes 1,3) and GST-TbRAB11 (lanes 2,4). Recombinant protein loaded with [α-^{32}P]GTP was incubated at 37°C for 0 minutes (lanes 1,2) or for 60 minutes (lanes 3,4). After incubation [α-^{32}P]GDP was separated from [α-^{32}P]GTP by thin layer chromatography. (D) Graphical representation of GTPase activity of recombinant GST-TbRAB2 (□) and GST-TbRAB11 (◇). Recombinant protein loaded with [α-^{32}P]GTP was incubated at 37°C for 0, 5, 10, 20, 40 or 60 minutes. After incubation [α-^{32}P]GDP was separated from [α-^{32}P]GTP by thin layer chromatography and the percent hydrolysis at each time point determined using phosphorimager analysis.

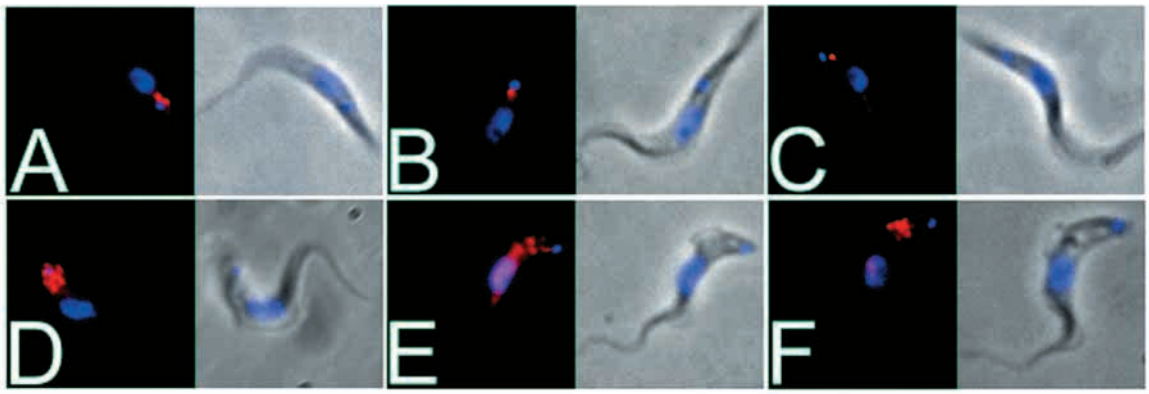

Fig. 3. TbRAB11 localisation in 427 BSF and PCF *T. brucei*. Immunofluorescence analysis of PCF (A-C) and BSF parasites (D-F). Parasites were stained with rabbit anti-TbRAB11 followed by an anti-rabbit Cy3 conjugate (red) and with DAPI for DNA (blue). Phase-contrast images merged with DNA stain are shown to the right of each fluorescence image.

TbRAB11 is positioned close to the basal body, a microtubule organising centre in trypanosomatids (Gull, 1999), as revealed by co-staining with basal body-specific monoclonal antibody BB4. The association was maintained throughout the cell cycle (Fig. 4) and at all stages of the mitotic process the TbRAB11 loci were closely adjacent to the basal body and kinetoplast (Fig. 4G-I). Coordinate regulation of endomembrane structures and the basal body/kinetoplast is a common feature in trypanosomes, and is clearly seen for early endosomes and the Golgi stack (Field et al., 1998; Field et al., 2000) and is potentially a mechanism for faithful organelle segregation. Significantly, juxtaposition of TbRAB11 with the basal body is reminiscent of the situation observed in mammalian cells for Rab11, which localises predominately to the pericentriolar recycling endosome in nonpolarised cells (Ullrich et al., 1996) and to the apical recycling endosome in polarised MDCK cells (Casanova et al., 1999), structures that are located close to the centriole.

TbRAB11 defines a new compartment of the trypanosome endosomal system

The position of TbRAB11 between the kinetoplast and nucleus is consistent with an endosomal function. To determine whether TbRAB11 localised to a previously characterised structure we compared TbRAB11 to a variety of established trypanosomal organellar markers. First, we looked at lysosomes, using antibodies to p67, a membrane protein predominately localised to lysosomes (Kelley et al., 1999). TbRAB11 was close to, but distinct from, the bulk of p67-positive membranes, with a limited colocalisation to fainter p67 staining membranes (Fig. 5A-D). This latter location probably results from p67 being present throughout the trypanosomal secretory and endosomal network while being concentrated in the lysosomes (Kelley et al., 1999). A second test for lysosomal location was conducted using biotinylated ConA, which binds to flagellar pocket glycoproteins and is subsequently trafficked through the cell to lysosomes. ConA chased at 37°C for 30 minutes is entirely lysosomal (Brickman et al., 1995). Using this procedure, TbRAB11 and lysosomal membranes are clearly distinct, despite the two organelles being closely associated (Fig. 5E,H). To determine whether TbRAB11 associated with Golgi membranes, BSF cells were co-stained for TbRAB11 and BODIPY-TR ceramide (Field et al., 2000); TbRAB11 was distinct from the Golgi stack, with TbRAB11 more posterior, extending towards the kinetoplast (Fig. 5I-L).

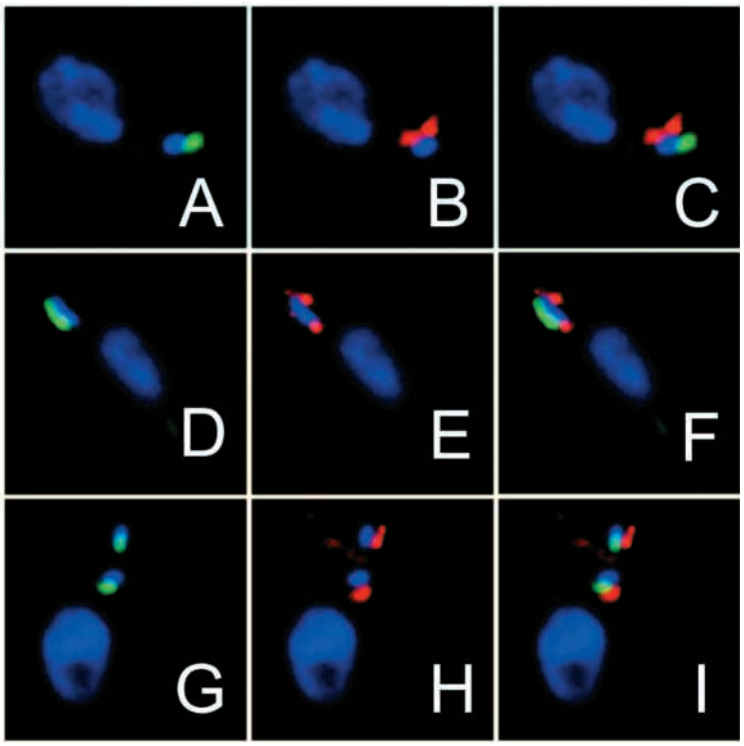

Fig. 4. TbRAB11 migrates coordinately with the basal body during mitosis. PCF parasites were treated for microscopy and stained with mouse anti-basal body (BB4) followed by an anti-mouse FITC conjugate (green), rabbit anti-TbRAB11 followed by an anti-rabbit Cy3 conjugate (red) and with DAPI for DNA (blue). For each image the basal body stain is shown to the left, the central figure corresponds to TbRAB11 and the right image is the merge. (A-C) Cells prior to entering mitosis with one kinetoplast and one nucleus. (D-F) Cells with one nucleus and an elongate duplicating kinetoplast and basal body. (G-I) Cells in which the kinetoplast and basal body have both duplicated while there is still a single nucleus.

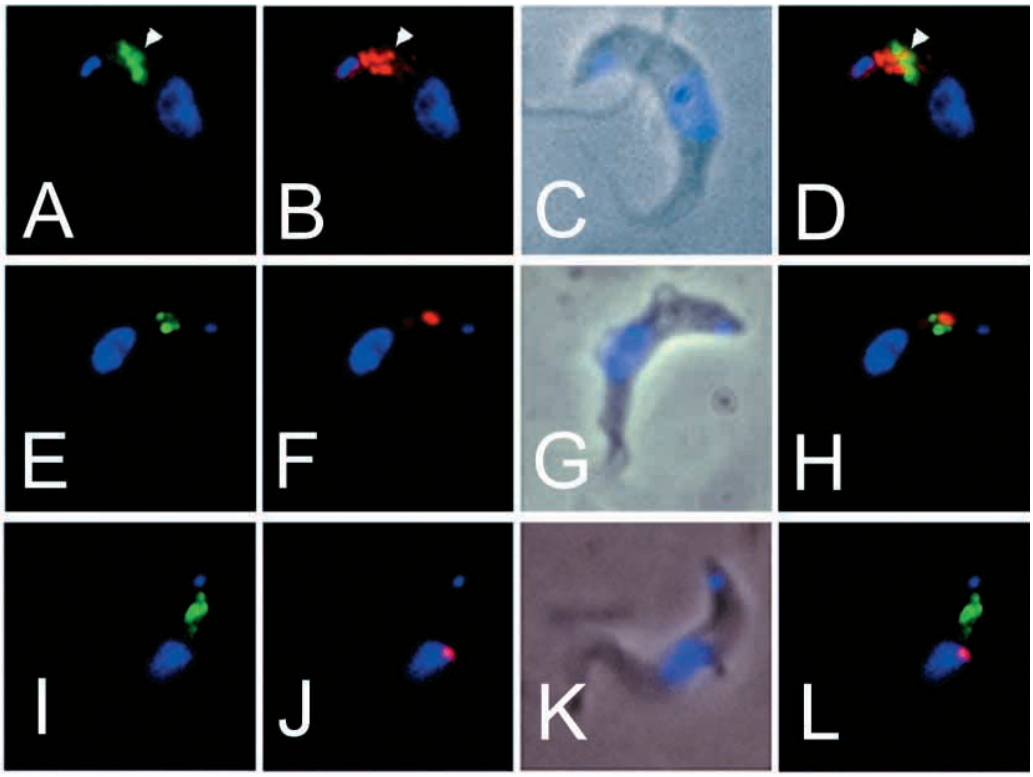

Fig. 5. TbRAB11 is absent from lysosomal and Golgi elements. **(**A-D) BSF parasites were treated for fluorescent microscopy and stained with mouse anti-p67 followed by an anti-mouse FITC conjugate (green), rabbit anti-TbRAB11 followed by an anti-rabbit Cy3 conjugate (red) and DAPI for DNA (blue). (E-H) BSF parasites were exposed to biotinylated ConA at 4°C for 10 minutes prior to chase for 30 minutes at 37°C to permit lysosomal ConA accumulation. Cells were then treated for microscopy and stained with FITC-avidin (green), rabbit anti-TbRAB11 followed by an anti-rabbit Cy3 conjugate (red) and DAPI for DNA (blue). (I-L) BSF parasites were treated with BODIPY-TR ceramide (red) to stain the Golgi (Field et al., 2000). Cells were subsequently processed for microscopy and counterstained with rabbit anti-TbRAB11 followed by an anti-rabbit Oregon Green conjugate (green) and DAPI for DNA (blue).

We next compared TbRAB11 with early endosomes (TbRAB5A) and clathrin (TbCLH). Co-staining BSF parasites for TbRAB5A and TbRAB11 followed by confocal microscopy revealed two endomembrane organelles that were predominately distinct but tightly juxtaposed, with restricted overlap at the margins of each compartment (Fig. 6A-C). Interestingly, in mammalian cells, Rab11 and Rab5A compartments rarely juxtapose because Rab5A is found at the periphery and Rab11 is situated more deeply, closer to the centrosome (Sonnichsen et al., 2000). Hence in *T. brucei* the relationship of Rab11 and Rab5 is more intimate. Recently we cloned the *T. brucei* homologue of the clathrin heavy chain gene (*TbCLH*; Morgan et al., 2001). Trypanosome clathrin is located to regions abutting the flagellar pocket and to predicted endosomal regions extending between the nucleus and kinetoplast. The majority of the TbRAB11 structures also contained clathrin, which may reflect an involvement in early endocytic events occurring at the flagellar pocket (Fig. 6D-F) or clathrin being situated on endosomal membranes involved in trafficking events to lysosomal regions and/or recycling events.

To discriminate between early and late endosomal compartments, FITC-ConA was internalised by BSF parasites for 15 seconds or 2 minutes and parasites stained for TbRAB11 or TbCLH. After 15 seconds FITC-ConA localised to a punctate spot marking the flagellar pocket with clathrin in close contact (Fig. 7G-I). By contrast, the majority of TbRAB11 was more distal from the ConA staining. Following two minutes of ConA internalisation the ConA has migrated to a more anterior position with substantial colocalisation with clathrin in both the flagellar pocket and more anterior regions (Fig. 7J-L). These structures correspond, at least in part, to previously described collecting

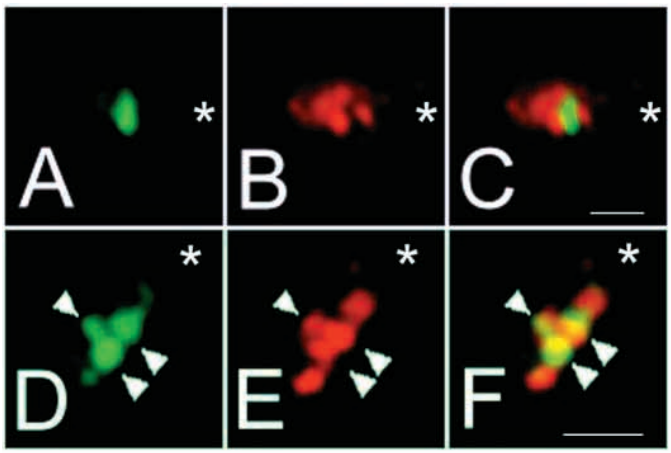

Fig. 6. The TbRAB11 compartment is juxtaposed to, but distinct from, early endosomes and partially colocalises with clathrin-coated structures. (A-C), Confocal microscopy was used to examine the relationship of TbRAB11 with TbRAB5A. Cells were stained with mouse anti-TbRAB11 followed by an anti-mouse FITC conjugate (green) and with rabbit anti-TbRAB5A followed by an anti-rabbit Cy3 conjugate (red). (D-F) Confocal fluorescent microscopy was used to examine the relationship of TbRAB11 with trypanosomal clathrin heavy chain. Cells were stained with mouse anti-TbRAB11 followed by an anti-mouse FITC conjugate (green) and with rabbit anti-TbCLH followed by an anti-rabbit Cy3 conjugate (red). C and F represent the merge in each case. Bars, 0.5 μm. * indicates the location of the nucleus; arrowheads indicate regions of colocalisation.

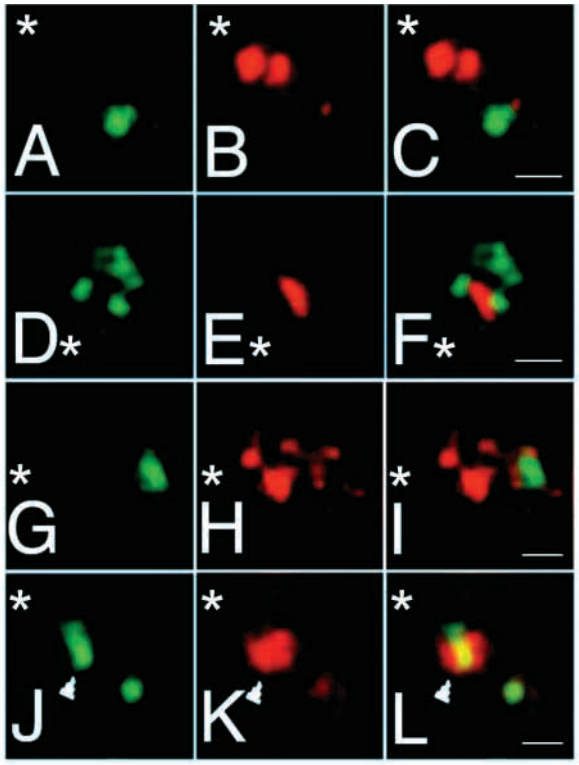

Fig. 7. TbRAB11 is not associated with endocytic events. BSF parasites were exposed to FITC-ConA (green) for 15 seconds (A-C,G-I) or two minutes (D-F,J-L) prior to treatment for microscopy. Cells were counterstained with either rabbit anti-TbRAB11 (red in A-F) or rabbit anti-TbCLH (red in G-L) followed by an anti-rabbit Cy3 conjugate. Arrowheads in panel J-L represent regions of colocalisation. Bars, 0.5 μm. * indicates the location of the nucleus.

tubules (Brickman et al., 1995). However, TbRAB11 still did not colocalise with FITC-ConA after 2 minutes of lectin uptake (Fig. 7D-F). Therefore, although TbRAB11 did not associate directly with lectin-containing endosomal compartments, the close juxtaposition is suggestive of interaction with these membrane systems.

TbRAB11 is associated with collecting tubules, TbRAB5A-positive structures

To determine more clearly the nature of the TbRAB11 compartment, we used defined temperature blockade to specifically label regions of the trypanosome endosomal system (Brickman et al., 1995). BSF cells were loaded with ConA on ice and transferred to media equilibrated at 4°C or 12°C. The low temperature prevents ConA from exiting the flagellar pocket, but at 12°C ConA migrates into, and becomes trapped within, the collecting tubules (Brickman et al., 1995). Staining with anti-TbRAB11 after 4°C chase (Fig. 8A-C) indicated that TbRAB11 was situated away from the flagellar pocket, but after a 12°C chase, the ConA had migrated to endosomal regions juxtaposed to TbRAB11 (Fig. 8D-F). By contrast, after a 12°C chase the vast majority of the anti-TbRAB5A and ConA overlapped (Fig. 8G-L), which was not observed at 4°C. Hence, TbRAB5A localises to the collecting tubules and TbRAB11 is present on an endomembrane compartment abutting these structures.

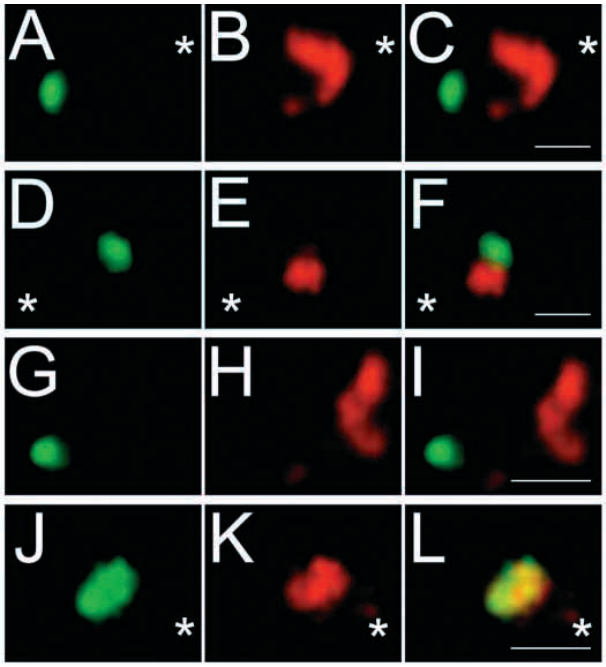

Fig. 8. The TbRAB11 compartment is juxtaposed to the collecting tubules. BSF parasites were exposed to FITC-ConA for 10 minutes at 4°C and chased in FITC-ConA free media equilibrated either at 4°C (A-C,G-I) or 12°C (D-F,J-L) for 30 minutes. Cells were subsequently processed for microscopy and stained for either TbRAB11 (red in A-F) or TbRAB5A (red in G-L). C,F,I,L are merged images. * indicates the location of the nucleus, except in G-I where the nucleus is just to the right-hand side of the field of view. Bars, 0.5 μm.

TbRAB11 membranes contain recycling cargo

The above data indicate that TbRAB11 is localised to the trypanosome endosomal system, intimately related with the collecting tubules and potentially has a role in recycling. To extend this hypothesis, we chose to determine whether the TbRAB11 compartment contained recycling cargo, specifically the trypanosomal transferrin receptor (ESAG6/7; Salmon et al., 1994; Kabiri and Steverding, 2000) and internalised anti-VSG (O'Beirne et al., 1998).

By confocal and epifluorescence, ESAG6/7 and TbRAB11 partially colocalise, indicating that this recycling receptor passes through the TbRAB11 compartment (Fig. 9). As TbRAB11 does not appear to be located on a compartment *en route* to the lysosome, we considered that it may also be involved in recycling of VSG. Internalisation of rabbit anti-VSG for 1 hour clearly demonstrated that the anti-VSG was partially localised within the TbRAB11 compartment, in addition to other regions of the cell (Fig. 10A-F). Hence, the presence of two known recycling markers, absence of lysosomal targeted cargo, close juxtaposition with the TbRAB5A collecting tubules and a high degree of sequence conservation with mammalian Rab11, all suggest that the TbRAB11 compartment is involved in recycling of trypanosomal surface proteins, and that the Rab protein has a role in regulating this process.

DISCUSSION

T. brucei has at least two early endosomal Rab proteins,

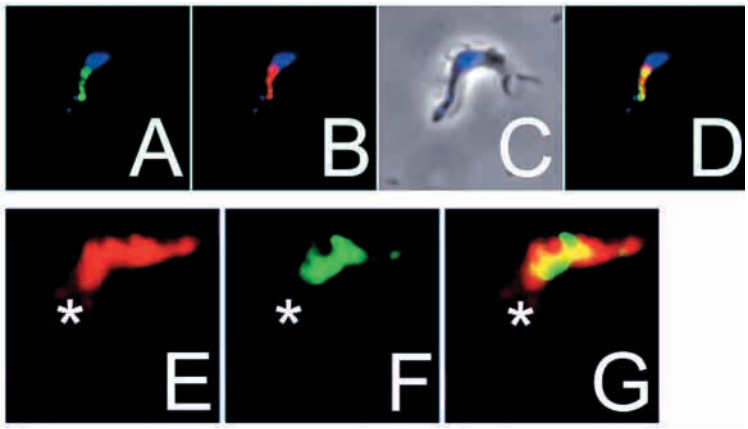

Fig. 9. The TbRAB11 compartment contains the trypanosomal transferrin receptor (ESAG6/7). BSF parasites were stained with mouse anti-TbRAB11 followed by an anti-mouse FITC conjugate (green), rabbit-ESAG6/7 followed by an anti-rabbit Cy3 conjugate (red) and DAPI for DNA (blue). A-D represent standard epifluorescent images, whereas E-G are confocal images; * represents the nucleus. D,G are merged images.

TbRAB5A and TbRAB5B, as well as TbRAB4, which is probably involved in recycling (Field et al., 1998). These features of the endosomal system are broadly similar with higher eukaryotes, although in humans and *S. cerevisiae* three Rab5 isoforms are known. In addition, we have described TbRAB31, a trans-Golgi Rab protein; the trans-Golgi, and TbRAB31-related proteins play important roles in recycling pathways in higher eukaryotes, but our data at present do not allow us to determine if TbRAB31 is involved in trypanosomal recycling (Field et al., 2000). We have now identified a further endosomal Rab in *T. brucei*, TbRAB11, which is homologous to higher eukaryotic Rab proteins that mediate deep recycling pathways. TbRAB11 has significant similarity to TbRAB31 at the primary structural level. By immunolocalisation and other studies we suggest that TbRAB11 is localised to developmentally regulated recycling endosomal membranes. The function of mammalian Rab11 has recently been explored in some detail using site-specific mutants, and is implicated as being involved in transport from the endosomal regions to the trans-Golgi network (Wilcke et al., 2000).

TbRAB11 localises to a compartment between the kinetoplast and posterior face of the nucleus. To define this compartment in more detail, BSF parasites were co-stained for TbRAB11 and a panel of established organellar markers. TbRAB11 was distanced from the Golgi stack, a location distinct to TbRAB31, a close homologue. Hence, although these two proteins may mediate closely integrated transport events, they clearly have disparate functions. Additionally TbRAB11 was posterior to p67-positive lysosomal regions, tightly juxtaposed to TbRAB5A collecting tubule elements and overlapped with the extensive clathrin membrane networks. Further, fluorescent ConA did not colocalise with TbRAB11, suggesting that the Rab is not associated directly with early endocytic membranes, despite colocalisation with TbCLH. Under conditions that accumulate ConA in the collecting tubules (Brickman et al., 1995) a close juxtaposition of part of the ConA-containing compartments with TbRAB11 membranes was observed, indicating that the TbRAB11 compartment is directly apposed with collecting tubule elements. Colocalisation with TbCLH suggests TbRAB11 may be involved with a clathrin dependent process (e.g. early endocytic cargo processing or transport following a trans-Golgi sorting step) but further work will be required to determine if these two proteins collaborate in the same process. These data indicate that TbRAB11 is associated with the endosomal system, but does not appear to be involved with early endocytic events or transport towards the lysosome. Based on the sequence, we considered that TbRAB11 was most likely to mediate a recycling process.

The relationship of the TbRAB11 compartment with recycling pathways was examined. Internalisation of anti-VSG antibody by BSF parasites revealed a tubular network of structures, which partially represents early endosomal regions, defined by TbRAB5A (B. Hall and M.C.F., unpublished), and presumably additional compartments involved in post-endocytic processing of internalised antibody. Significantly, we found TbRAB11 localised to a subdomain of the VSG-containing region. As TbRAB11 is not involved in early endocytic processes this subdomain most likely represents a compartment involved in returning the antibody to the flagellar

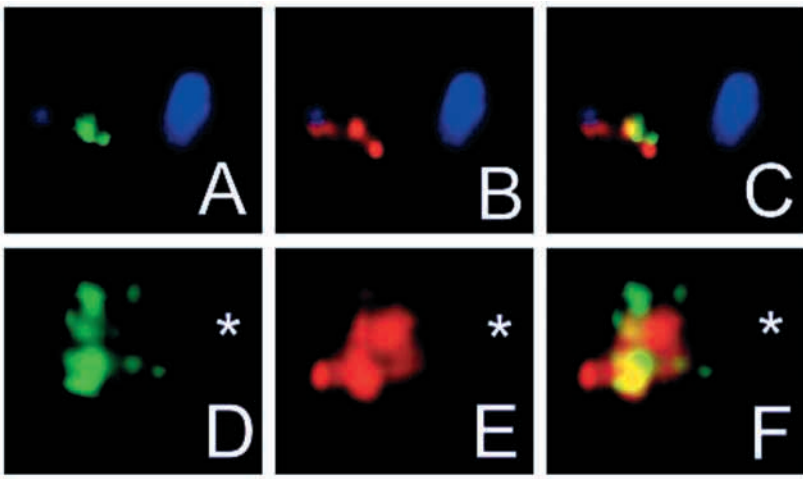

Fig. 10. Anti-VSG antibody traffics through the TbRAB11 compartment subsequent to internalisation. BSF parasites were incubated with anti-VSG antibody for 1 hour and subsequently processed for fluorescent microscopy. Rabbit anti-VSG was detected using an anti-rabbit Cy3 conjugate (red). TbRAB11 was detected using mouse anti-TbRAB11 followed by an anti-mouse FITC conjugate (green). (A-C) Epifluorescent images; blue, DAPI for DNA. (D-F) Confocal images; * represents the position of the nucleus. C,F are merged images.

pocket. ESAG6/7 (Salmon et al., 1994) is the trypanosome transferrin receptor, and recycles through the endosomal system (Kabiri and Steverding, 2000). By confocal microscopy ESAG6/7 localised to tubular-like structures situated between the flagellar pocket and nucleus, part of which colocalised with TbRAB11. This subpopulation of ESAG6/7 cannot be in the early endosome and hence likely represents recycling cargo.

VSG is the major surface coat protein in BSF *T. brucei* (Ferguson, 1997) and a vital component of the trypanosome immune evasion system. Additional virulence mechanisms also operate; most relevant here is trafficking of VSG and bound antibody through the trypanosomal endocytic/recycling system such that VSG is returned intact to the cell surface while antibodies are proteolysed and released into the medium (O'Beirne et al., 1998), resulting in cleaning of surface immunoglobulin from the parasite. In addition, many observations have shown that the BSF form of the parasite has a far greater level of endocytosis than the PCF (Overath et al., 1997), but this is unlikely to be simply based on nutrient requirement, as the generation times of the insect and mammalian forms of *T. brucei* are not vastly different. Although PCF parasites endocytose material (Overath et al., 1997) and can recycle cell surface receptors such as CRAM (Liu et al., 2000), these processes occur at a much reduced rate compared with the BSF stage. However, a need to rapidly clear immune complexes or antibody from the surface is clearly restricted to BSFs, and may represent a primary reason for the rapid endocytosis in this form.

Our evidence indicates that TbRAB11 defines a compartment associated with the early endosomes, and which contains two known recycling proteins. Based on sequence homology and other data, we suggest that TbRAB11 is most likely involved in recycling of VSG and ESAG6/7, and possibly other trypanosomal proteins. Importantly, both VSG and ESAG6/7 are anchored to the membrane via a GPI lipid, and hence we have no information at present on transmembrane proteins. An important feature of TbRAB11 is that it is strongly developmentally regulated. So far, this is a unique feature for a trypanosomal Rab protein, and is good evidence that TbRAB11 plays a more pronounced role in mediating BSF transport pathways than in the PCF, processes that are likely to be BSF specific. Several proteins involved in protein transport are upregulated in the BSF stage compared with the PCF; for example BiP is upregulated threefold, suggested to reflect secretory pathway requirements (Bangs et al., 1993), and the clathrin heavy chain, proposed to be important for control of major endocytic processes, shows a strong degree of developmental control (G.W.M. et al., unpublished). Increased expression of TbRAB11 may facilitate the antibody induced aggregation/disaggregation by assisting efficient trafficking of VSG/anti-VSG complexes through the processing endosomal compartments. The TbRAB11 compartment therefore potentially represents a developmentally regulated organelle dedicated principally to BSF recycling processes, and as such may contribute importantly to the survival of *T. brucei* within the mammalian host.

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