Complexity of Trypanosomatid Endocytosis Pathways Revealed by Rab4 and Rab5 Isoforms in Trypanosoma brucei

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Small G proteins of the Rab family are responsible for vesicle fusion and control flux during intracellular transport. Rab5 is important in endosome maturation and Rab4 in recycling of endocytic material. Three Rab5 isoforms identified so far in mammals and three in the yeast genome suggest that conservation of multiple Rab5 isoforms is required for sophisticated regulation of endocytosis. Trypanosoma brucei homologues of Rab5 and Rab4 (TbRab5A and TbRab4) have been identified. Here we report cloning of a second Rab5 homologue, TbRab5Bp. The TbRab5A- and -5B genes are not linked in the genome, and phylogenetic reconstruction indicates that multiple Rab5 isoforms in yeast, mammals, and trypanosomes evolved independently. Northern blots demonstrate that TbRab5A, -5B, and TbRab4 messages are expressed in bloodstream form (BSF) and procyclic forms of the parasite even though endocytosis is not very active in the latter form. mRNA levels of TbRab5A and -4 are constitutive. Multiple-sized TbRab5B messages at very low abundance are detected, with greater expression in BSF. Also, the TbRab5B mRNA has a large 3′-untranslated region suggestive of potentially complex regulation, and therefore TbRab5Bp may be an important regulator of differential endocytosis levels between BSF and procyclic stage parasites. Affinity purified antibodies raised to C-terminal peptide sequences of all three TbRab proteins recognized small vesicular cytoplasmic structures, which for TbRab5A and -5Bp are predominantly near the flagellar pocket. TbRab5Bp colocalizes with invariant surface glycoprotein 100 (ISG100), a protein entering the endocytotic pathway through the ISG pocket. TbRab5 proteins are therefore components of the endocytic pathway. TbRab4p localizes to vesicular structures throughout the cytoplasm, with some overlap with TbRab5Bp, but the majority occupying a different compartment to the TbRab5s. Therefore the trypanosome endosomal system has been functionally dissected for the first time; these reagents provide a unique opportunity for manipulation of the protozoan endosomal system to further our understanding of drug uptake mechanisms and virulence.

Rabs are a family of small GTPases essential for membrane vesicle trafficking in eukaryotes (1). Each Rab localizes to a distinct subset of organelles; the C terminus is responsible for intracellular localization (2), while the N terminus also recognizes the target organelle and is required for vesicle fusion (3). The Rab system controls organelle-specific flux by regulating the ability of the t- and v-SNARE partners to interact, promoting docking and fusion (4, 5). The amount of time that Rab5 remains GTP-bound governs the lifetime of the fusion-competent state of the docking vesicle (6).

Humans and yeast each have three identified Rab5 isoforms in the endosome system which colocalize, suggesting complex regulation or redundancy (7–10). Dominant negative Rab5 decreases endosome size, whereas constitutively active Rab5 leads to endosomal swelling and fusion (11). Phospholipase A2 and phosphatidylinositol 3-kinase are implicated in Rab5 function (12, 13), suggesting close integration with signal transduction pathways. Rab4 is involved in recycling of membrane material back to the plasma membrane from endosomal compartments (14). Human Rab4a is subject to cell-cycle-dependent phosphorylation and is involved in insulin-mediated translocation of glucose transporters (15, 16). A second isoform, Rab4b, is not phosphorylated (17).

Trypanosoma brucei sp., the causative agent of Ngana in ungulates and sleeping sickness in humans, is a parasitic protozoan pathogen seriously affecting public health and the economy in endemic regions. Suramin, a drug currently used in management of African trypanosomiasis, is believed to enter the parasite via endocytosis (18). Resistance to lysis by human serum is mediated by inducible, selective control of endocytosis (19). We can genetically engineer the Rab system to manipulate endocytosis in T. brucei and study drug uptake. 1 T. brucei additionally provides a convenient model system for elucidation of cell biological processes in a divergent eukaryote with a strongly directional endocytotic/recycling system, where a very small portion of the total membrane (2–3%), Ref. 20 is the site of all endo- and exocytosis. Recycling is very active in T. brucei, with more than 95% of endocytosed variant surface glycoprotein (VSG) (21). Here we report on the subcellular location and evolution of three TbRab proteins involved in endocytosis in T. brucei.

EXPERIMENTAL PROCEDURES

Trypanosomes—Culture-adapted bloodstream form (BSF) 3 T. brucei, strain 427, were grown as described (22). Cells were quantitated with a

† H. Field and M. C. Field, unpublished data.
3 The abbreviations used are: BSF, bloodstream form; EST, expressed sequence tag; FP, flagellar pocket; GST, glutathione S-transferase; IFA, immunofluorescence analysis; ISG, invariant surface glycoprotein; kb, kilobase(s); nt, nucleotides; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; TLF, trypanosome lytic factor; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid.
Z2 Coulter Counter (Coulter Electronics, UK). For boiling SDS lysates, freshly harvested trypanosomes were added to SDS-PAGE sample buffer (23) at 95 °C, heated for 10 min, and reduced with dithiothreitol. Treatment with fluorescent endocytic markers was at 3–4 mg/ml added from 10× stock, in 100 μl of culture medium, with (for Texas red-dextran, Molecular Probes) or without (for Lucifer yellow, Sigma) serum.

**Nucleic Acids and Recombinant DNA Methods—**Nitrocellulose for Western and Southern blotting was from Schleicher and Schuell, and nylon for Northern blots was from Amersham Pharmacia Biotech. Radioisotopes were from Amersham International, ION, and NEN Life Science Products. Vector pGEX2tk and glutathione-Sepharose-4B beads were from Amersham Pharmacia Biotech, and the pQE30 vector and nickel-agarose from Qiagen Inc. Plasmids were cloned in *E. coli* coli XL1-Blue (Stratagene) following transformation by electroporation with a BTX 600 ECM electroperator. PCR products and gel-embedded DNA were purified using PCR cleanup kits (Promega), while plasmid and λ DNA were purified using QIagen kits following the manufacturer’s instructions. Small scale plasmid preparations were performed using the Promega Wizard system. Oligonucleotide primers were obtained from Genosys.

**Polymerase Chain Reaction—**PCR was performed in 50–μl reactions with 25 pmol of each primer in ammonium buffer (Bioline), 2.5 mM MgCl₂, 1 unit of Taq polymerase (Bioline) in a 480 thermal cycler (Perkin Elmer) typically as follows: 95 °C, 15 min (1 cycle); 5 cycles of 94 °C, 1 min; 56 °C, 1 min; 72 °C, 3 min (5 cycles). Primers for subcloning and assembling ORFs for expression were: 5BH3 (5'-TCCAGCTTATACAGGGCGCTGCG-3') and 5BKON (5'-CCTGACGCTGGGTACTGACGGAGC-3'). PCR products were ligated into pGEX-3X, yielding GST-TbRab5Ap fusion protein.

**Construction of a Full-length TbRAB5A ORF—**The TbRAB5A ORF (a gift from Dr. Nabib El-Sayed) was constructed from clone 22, encoding the N-terminal half of TbRAB5A, and a fragment from clone T378, which encodes the major ORF of TbRab5Ap in pBluescript (29). The ORF was verified by restriction analysis and sequencing. The plasmid was cleaved with XhoI (3' to the ORF) and blunted with Klenow, and the ORF was released with BamHI and ligated into BamHI/SmaI-cleaved pGEX-3X (Amersham Pharmacia Biotech) to create an in-frame fusion with glutathione S-transferase (GST; a synthetic 4-amino acid linker is used). The construct was verified by expression in *E. coli*, producing a 56-kDa protein, the predicted molecular mass for a GST-TbRab5Ap fusion.

**Construction of a Full-length TbRAB5B ORF—**The TbRAB5B ORF (a gift from Dr. Nabib El-Sayed) was constructed from clone 33, encoding the N-terminal half of TbRAB5B, and a fragment from clone 22, encoded the 3' half of TbRAB5B. The ORF was released with BamHI and ligated into BamHI/SmaI-cleaved pGEX-3X (Amersham Pharmacia Biotech) to create an in-frame fusion with glutathione S-transferase (GST; a synthetic 4-amino acid linker is used). The construct was verified by expression in *E. coli*, producing a 56-kDa protein, the predicted molecular mass for a GST-TbRab5Bp fusion.

**Protein Electrophoresis and Western Blotting—**Proteins were separated by reducing SDS-PAGE. For Western blots, 5 × 10⁶–2 × 10⁷ cells per lane were electrophoresed on 15% SDS-polyacrylamide minigels and blotted (Amersham Pharmacia Biotech) onto 0.45 μm nitrocellulose._authentication filters were prepared as follows: full-length ORFs from some rRNA species at 1600, 1900, and 2300 bases. Probes were prepared by high salt transfer Southern blotting (25) and probed at low stringency (2). Typical yields were 0.4–4 mg of recombinant TbRab protein/ligther E. coli. Antibodies to TbRab5Ap, -5Bp, and -5Bp were raised against synthetic peptides designed from the C termini and covalently bound to bovine serum albumin. Additional antibodies were generated using synthetic peptides, to facilitate detection and derivatization. Peptides WENG-HAEHTLDYGKPRKFS (−5A), WYGQAGRELPTQRKQKEGC (−5A), and ACKGVLQGPNTRSSCCG (−5B) were synthesized (Dr. R. Leatherbarrow, Department of Chemistry, Imperial College) and verified for purity by high performance liquid chromatography and for sequence by fast atom bombardment-mass spectrometry, following standard methods. A portion of the peptide was iodinated with iodobeads (Pierce) or Bolton-Hunter reagent (−5B) and used as a tracer. Peptides were coupled to bovine serum albumin with an efficiency of ~40%, using m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce) following standard procedures (24). The conjugate was used to immunize rabbits and rats using the MPL®+ TDM + CWS Adjuvant System (Sigma). Animals were exsanguinated, and serum was stored at −4 °C with azide. For affinity purification, peptide was coupled to either thiol-Sepharose 4B or Sepharose 4B as described following (22), the manufacturer’s instructions. Antibodies were affinity purified from serum after 50% ammonium sulfate precipitation (31).

**Computer Analysis—**Computer analysis was described as (22). Fig. 1 was generated using SEQVU (Garvan Institute of Medical Research, Sydney, Australia).

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3 N. El-Sayed, unpublished data.
RESULTS

Isolation of cDNA of a Second T. brucei Rab5 Homologue—A T. brucei homologue of mammalian Rab5 was previously isolated (T.b. Rab5, Ref. 29); we designate it TbRab5Ap. A second Rab5 homologue was identified in T. brucei by reverse transcriptase PCR using a degenerate primer to the WDTAGQE motif of Rab GTPases. An EST, Rtb4 (27), was used to probe a T. brucei BSF cDNA library. A full-length cDNA of 2.0 kb was cloned and sequenced. The ORF was hypothetically translated and identified as a Rab5 homologue (Fig. 1A) by BLAST search of GenBank™ and designated TbRab5Bp. Neither T. brucei Rab5 clearly corresponded to a particular Rab5 subclass.

Comparison of TbRab5Bp with -5Ap and other Rab5 protein sequences (Fig. 1A, and Table I) confirmed the conservation of canonical Rab protein features, i.e. GTP-binding regions, effector loops, and dicysteinyl C-terminal isoprenylation signal (34), but two differences were seen between TbRab5Ap and -5Bp. The first is an insertion in TbRab5Ap (residues 61–77) with a high Ala and Gly content; this region corresponds to a loop between \( \beta \)-sheets 2 and 3 in the Ras structure and is predicted to be on the protein surface and highly mobile. The second is a deletion in -5Bp within the hypervariable region (Fig. 1A); this is more common as similar but smaller indels are found in this region of mammalian Rab5 s and TbRab5Ap when compared with Ypt51p, -52p, and -53p (not shown).

Phylogenetic Reconstruction of the Rab5 Lineage—By BLAST

FIG. 1. T. brucei has at least two Rab5 homologues. A, clustal alignment of the hypothetical ORFs of TbRab5Ap and -5Bp from T. brucei, together with their closest orthologues from GenBank™. Boxes indicate identity and shading indicates homology. The indels in TbRab5Ap and -5Bp are underlined. Cf, C. familiaris. B, phylogenetic reconstruction using PAUP (43) suggests that Rab5 families have evolved several times. TbRab5Ap and -5Bp were examined in relation to protein homologues representative of sequences from all three mammalian Rab5 subclasses and the three yeast Ypt5s. Rab5 sequences from the same order emerge as monophyletic, suggesting that divergence of the mammalian, fungal, and trypanosomatid lineages predates emergence of multiple Rab5 isoforms. The tree was obtained from 1000 branched and bound bootstrap replicates after a branched and bound search. Cf, C. familiaris; Hs, H. sapiens; Ypt, S. cerevisiae; Tb, T. brucei. Ran/TC4 was chosen as an outgroup, and monophyletic TbRab2p/-8p were included as a control. Numbers are percent confidence for various branch points following bootstrapping. Horizontal distances represent relative genetic distance.
search, the nearest homologue of TbRab5Ap was Ypt51p and of -5Bp was Canis familiaris Rab5c (Table I). TbRab5Ap and -5Bp were as similar to each other as to their nearest homologues (Table I) so could not be assigned to specific Rab5 subgroups. A phylogeny for the Rab5 lineage was constructed, using Clustal (4.0) followed by PAUP, on sequences representative of the subclasses of Rab5 so far identified in mammals (Rab5a, -5b, and -5c) and the three Rab5 homologues from Saccharomyces cerevisiae (Ypt51p, -52p, and -53p). Rab5 proteins from any one of the three lineages were monophyletic (Fig. 1).

Genomic Organization—Because TbRab5Ap and -5Bp are closely related, we asked whether the genes encoding them (TbRAB5A and -5B) were linked in the genome. Trypanosome genomic DNA, digested with a panel of restriction enzymes, each lane of a Northern blot and probed with ORFs from and -5c) and the three Rab5 homologues from (Table I) so could not be assigned to specific Rab5 subgroups. A phylogeny for the Rab5 lineage was constructed, using Clustal (4.0) followed by PAUP, on sequences representative of the subclasses of Rab5 so far identified in mammals (Rab5a, -5b, and -5c) and the three Rab5 homologues from Saccharomyces cerevisiae (Ypt51p, -52p, and -53p). Rab5 proteins from any one of the three lineages were monophyletic (Fig. 1).

Genomic Organization—Because TbRab5Ap and -5Bp are closely related, we asked whether the genes encoding them (TbRAB5A and -5B) were linked in the genome. Trypanosome genomic DNA, digested with a panel of restriction enzymes, was Southern blotted and probed with the ORFs of TbRAB5A and -5B (Fig. 2A). No cross-hybridization between probes was detected using plasmids as targets at low stringency (not shown). There was no coincidence between the hybridization patterns obtained with TbRAB5A and -5B, reflected in the different maps obtained for the two genes (Fig. 2B).

The TbRAB5B probe revealed the presence of a restriction fragment-length polymorphism (Fig. 2B, asterisk) giving a doublet in SacII digests although previous Southern blot analysis of the trypanosome genome with the Rtb4 probe (the 5’ third of the -5B ORF) suggested that TbRAB5B was single copy (27). In our original EST study, we isolated a second partial cDNA (Rtb9) closely related to Rtb4 but with a small number of substitutions (27). These data suggest that Rtb4 and Rtb9 are derived from allelic copies of TbRAB5B.

There was no additional hybridization with the TbRAB5A or -5B probes. As these two probes do not cross-hybridize at low stringency, there are no sequences more closely related to TbRAB5A than -5B and vice versa in the trypanosome genome, but more distantly related TbRAB5B genes may have been undetectable and therefore cannot be formally excluded. Southern analysis with the TbRAB4 ORF revealed a single copy, with no evidence for linkage between TbRAB4 and any other TbRab gene so far studied (Fig. 2A, Ref. 27).

Expression of TbRAB Genes—Poly(A)-enriched RNA from 10⁶ trypomastigotes (3.4 µg from BSF and 10 µg from procyclics: the differing RNA yield is a known phenomenon⁴) was used in each lane of a Northern blot and probed with ORFs from TbRAB4, -5A, and -5B (Fig. 3). TbRAB4 detected a single major species migrating at ~0.7 kb, with the ORF of 718 nt suggesting minimal untranslated sequence (UTR), and TbRAB5A detected a single species of ~1.0 kb, with the ORF of 930 nt so there is again very little UTR. In contrast, TbRAB5B detected larger message species. As the 5’ UTR is 150 nt and the ORF 612 nt, the 3’ UTR is ~1.2 kb and could potentially include regulatory sequences. The message levels were at a ratio of ~10:20:1 for TbRab4:-5A:-5B, with -5B particularly difficult to detect because of low abundance and molecular weight heterogeneity, differing in BSF (3.8, 2.4, 2.1*, 1.8, 1.35 kb) and procyclics (3.8*, 2.3, 1.95* kb, where asterisks indicate the prominent message sizes). The 3.8-kb message probably represents an unprocessed nuclear transcript. The 2.0-kb cDNA clone containing TbRAB5B is consistent with a message size of 2.1–2.4 kb.

For quantitation, the blots were reprobed with tubulin as a constitutively expressed standard; the signal intensity was at ~1:4 for BSF:procyclics and ~1:1 with the mass of RNA loaded. When normalized to tubulin, both TbRab4 and -5A messages were constitutive. The 3.8-kb TbRab5B message is also constitutive; conversely the ~1.8–2.4-kb -5B transcripts normalized to tubulin at a ratio of 1:0.5, indicating more processed -5B message in BSF than in procyclics. In separate experiments loading equal masses of BSF and procyclic poly(A)-enriched RNA or poly(A)-selected RNA, hybridization signals for TbRAB5B were more intense in BSF, confirming that TbRab5Bp is developmentally regulated⁵ (Ref. 27).

GTPase activity was assessed in the two life stages by GTP overlay of lysates from 10⁷ BSF or procyclic cells (Fig. 4). Total protein levels are approximately equal for both BSF and procyclic cells (Fig. 4).¹ The pattern of GTP-bound bands was essentially identical, while significantly more GTP was bound

| Table I |
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| Homology between TbRab5Ap, -5Bp, and closest homologues in the data base |
| | C. familiaris | S. cerevisiae | TbRab5Ap | TbRab5Bp |
| Rab5c | 72.7 | 64.6 | 63.4 |
| Ypt51p | 52.5 | 65.5 | 61.6 |
| TbRab5Ap | 44.0 | 47.1 | 63.3 |
| TbRab5Bp | 47.0 | 41.9 | 45.2 |

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¹ J. Boothroyd, personal communication.

⁵ H. Field, A. Pal, and M. C. Field, unpublished data.
by BSF lysate than procyclic.

Production and Characterization of Specific Antiseria to TbRab4p, -5Ap, and -5Bp—Rat (anti-TbRab4p and -5Ap) and rabbit antisera (-5Bp) were raised against synthetic peptides corresponding to the C termini of the TbRab sequences and affinity purified, and the specificity of anti-TbRab5Ap and -5Bp sera were checked by Western blotting against lysates of E. coli expressing recombinant TbRab5Ap and (His)6-TbRab5Bp. No signal was observed using anti-TbRab4p or -5Ap antibodies in Western blotting on trypanosome lysates (Fig. 5B, lane 3, and data not shown). Extra residues added to the TbRab5Ap and -4p peptides may explain their relative lack of reactivity in Westerns, but inactivity in Western blotting is commonly observed for anti-Rab C-terminal peptide antibodies that work well in IFA and for antipeptide antibodies in general. Antibodies to TbRab5Bp reacted against two bands at 24 and 17 kDa. The TbRab5B protein is 21.8 kDa and is expected to be doubly geranylgeranylated so probably corresponds to the 24-kDa band. The 17-kDa band is most likely a degradation product because it was also detected by Western blotting in some E. coli lysates.

Immunolocalization of TbRab4p, -5Ap, and -5Bp—Based on sequence similarity, it is most probable that the TbRab4p, -5Ap, and -5Bp are trypanosome homologues of Rab4 and Rab5. To confirm that these proteins are components of the trypanosome endosomal system, we determined the location of the TbRab proteins. All three antibodies recognized a large number of vesicular structures in the trypanosome cytoplasm. Staining for both TbRab5Ap and -5Bp produced very similar patterns with small vesicular structures concentrated at high density between the nucleus and kinetoplast in interphase cells, suggesting that the majority of TbRab5 compartments were located closer to the FP which is consistent with an active role in endocytosis (Fig. 6A). Indeed, the similarity of the staining was highlighted by colabeling of cells for both TbRab5Ap and -5Bp; this demonstrated that the vast majority of the vesicles contained both proteins, with only a small subpopulation bearing only one of the TbRab5 s (Fig. 6A). No obvious difference was seen between procyclic and BSF (Fig. 6, A–C). Interestingly, in mitotic cells we found that the TbRab5Ap/5B compartment migrated from the posterior region of the cell to a position anterior to the nucleus (Fig. 6B). The repositioning occurred as the kinetoplast divided (not shown).

To confirm that TbRab5Ap and -5Bp were indeed located on compartments associated with the trypanosome endocytic system, we costained BSF cells with antibodies to TbRab5Ap and ISG100, a recently characterized cell surface protein that is also present throughout the endosomal system including the lysosomes (36). The majority of ISG100 colocalized with TbRab5Bp, particularly in a number of vesicles between the kinetoplast and the nucleus (Fig. 6C, arrowheads). These data, taken together with the high degree of sequence relatedness, confirm TbRab5Ap and TbRab5Bp as Rab5 homologues positioned at

**Fig. 3.** Developmentally regulated expression of TbRAB5B but not -5A or -4. Northern blot analysis of poly(A)-enriched RNA extracted from 10^6 BSF (B) or procyclic form (P) T. brucei. Identical blots were probed with the ORFs of TbRAB4, -5A, and -5B and washed at low and high stringency. Molecular weights are in kilobases. Left-most lanes are TbRab4 probed at low stringency, and the remainder are at high stringency. The same blots were reprobed with tubulin (not shown), and the ratio of message in BSF compared with procyclic cells was determined for each message (see text). TbRab5B mRNA is more shown), and the ratio of message in BSF compared with procyclic cells was determined for each message (see text). TbRab5B mRNA is more abundant in BSF than procyclic when normalized to tubulin, whereas -4 and -5A messages are found at approximately equal levels in both life stages.

**Fig. 4.** Total small G protein activity is increased in BSF compared with procyclic cells. A, ligand blot analysis of total GTP-binding proteins in BSF (B) and procyclic (P) T. brucei. Boiling SDS lysates were prepared (10^7 cells/lane), fractionated by reducing SDS-PAGE, renatured, transferred to nitrocellulose, and overlaid with [32P]GTP in a solution containing ATP. Control lanes were co-exposed to excess unlabeled GTP, GDP, or ATP. GTP-binding activity is recovered in the 17–30 kDa range and is ~10-fold greater in the BSF. Longer exposure confirms that the pattern of bands is similar in procyclic and BSF cells (not shown). The experiment has been repeated four times with essentially identical results. B, Coomassie-stained proteins from two lanes of the gel used for panel A showing equivalent loading in BSF (B) and procycles (P). Marker sizes are shown in kDa.

**Fig. 5.** Characterization of affinity-purified antisera to TbRab5Ap and -5Bp. A, antipeptide antibodies affinity purified from rat (anti-TbRab5A, at 1:100) or rabbit (anti-5Bp, at 1:300) sera were used to probe Western blots prepared from lysates of E. coli strains expressing recombinant GST-TbRab5Ap and (His)_6-TbRab5Bp. Detection was with ECL™ (Amersham Pharmacia Biotech) for 1–5 min. B, Western blots of 10^7 trypanosomes/lane, with rabbit anti-TbRab5Ap antibodies at 1:20 dilution, or anti-TbRab5Bp antibodies at 1:200 dilution. Wild type TbRab5Bp is detectable in trypanosomes at 24 kDa; a 17-kDa band (asterisk) is probably a degradation product (see “Results”). Detection was with ECL™.
**FIG. 6.** **TbRab5Ap and -5Bp are associated with endosomal compartments.** Immunofluorescence analysis of procyclic and BSF trypanosomes. 

**A**, procyclic trypanosome stained for DNA (blue), TbRab5Ap (green), and -5Bp (red). Phase contrast images of the cell (right) and merged fluorescence is shown (left, coincident staining is yellow). Note the significant, but not complete, coincidence of staining for the two Rab5s. 

**B**, reorganization of the TbRab5Ap (top) and -5Bp (lower) compartment during mitosis in procycles. Interphase (left) and corresponding mitotic cells (right) are shown. DNA (blue) and TbRab5 stain (red) are overlaid onto phase contrast images of the cell, with the kinetoplast (small) at the posterior end of the cell. In interphase cells, the majority of TbRab5p staining is between the kinetoplast and the nucleus whereas in the mitotic cell most TbRab5p is anterior of the respective nucleus. 

**C**, BSF trypanosome stained with antibodies to TbRab5Bp (red), ISG100 (green), and DNA (blue). Right-most panel shows merged fluorescence data with coincident staining in yellow. Arrowheads indicate some of the endosomal vesicle structures where there is coincident staining. ISG100 stain is mainly in lysosomal structures in this example, as previously reported (arrows).
Endocytotic structures. In addition, the similarity of the staining in panel B (procyclic cells) compared with panel C (BSF) confirms the presence of endocytic structures in both life stages; TbRab5Ap staining in BSF cells (not shown) is likewise similar to that of procycles (Fig. 6, A and B).

TbRab4p antibodies recognized numerous small compartments throughout the cell in both procyclic and BSF trypanosomes (Fig. 7). A small amount of colocalization with TbRab5Bp (Fig. 7A) and -5Ap (not shown) was detected, suggesting that TbRab4p is also a component of the endosomal system, but is distinct from the majority of the TbRab5p compartment. Similar to TbRab5p, we also observed a migration during mitosis to an anterior position (Fig. 7B). TbRab4p mostly did not colocalize with ISG100 (Fig. 7C), confirming the separate identity of the Rab4 compartment. These data are consistent with that expected for a Rab4 homologue involved in recycling (37) and, with the sequence similarity (29), show TbRab4p to be a functional trypanosome Rab4. Again, comparison of procyclic and BSF (Fig. 7) demonstrates the constitutive presence of the TbRab4p compartment. Therefore, in trypanosomes as in mammals, the endosomal system is divided between an endosomal compartment controlled by functionally distinct Rab proteins.

**Fig. 7.** *TbRab4p is associated with early endocytotic vesicles.* A, procyclic form trypanosome stained for DNA (blue), TbRab4p (green), and TbRab5Bp (red). Phase contrast image of the cell (right) and merged fluorescence is shown (left, coincident staining is yellow). Arrowheads indicate positions of coincident staining for TbRab4p and -5Bp. Most of the staining does not overlap. B, TbRab4p (green) location in interphase (left) and mitotic procyclic cells (right), TbRab4p stain (green), and DNA (blue) is overlaid onto phase contrast images of the cell, positioned with the kinetoplast (small blue structure) at the bottom. Note a similar migration to the anterior pole of the cell as for TbRab5A/-5Bp, with the exception that in interphase there are already greater numbers of TbRab4p vesicles in the cell anterior. C, BSF stained for DNA (blue), ISG100 (green), and TbRab4p (red). Phase contrast images of the cell (right) and merged fluorescence is shown (left, coincident staining is yellow). Essentially no colocalization is seen for the two proteins.
redundant Rab5 homologues and a separate recycling system communicating with the endosomal pathway and governed by a Rab4 homologue.

**DISCUSSION**

We show here that the endosome system of the African trypanosome has multiple Rab5 isoforms. The two TbRab5 protein sequences, TbRab5Ap (29) and TbRab5Bp (this report), are clearly distinct; divergence is most apparent in the occurrence of insertions in the center of the molecule and within the hypervariable C-terminal region. Southern analysis demonstrated that TbRAB5A and -5B genes are not closely linked in the trypanosome genome, so emergence of these two isoforms has been via duplication followed by genomic rearrangement. This is distinct from two other *T. brucei* Rab genes, TbRAB2 and TbRAB8, which arose by tandem duplication but have not separated (22). Southern blotting also revealed a restriction fragment length polymorphism in TbRAB5B, and the second allele probably encodes EST Rtb9 (27).

TbRab4p, 5Ap, and -5Bp are all associated with the endocytotic pathway as demonstrated by IFA, containing with specific anti-peptide antibodies and anti-ISG100 antibodies. The compartments recognized by these antibodies indicates that the endocytotic network is extensive and present in both life forms even though endocytosis is not an active process in procyclins. The inter-nucleo-kinetoplast region of the cell contains the FP and is the primary site of endocytotic activity, as visualized by Lucifer yellow (a fluid phase endocytosis marker1) or TR-dextran uptake (19). This is also the region where anti-TbRab5Ap, -5Bp, and ISG100 staining is most concentrated. TbRab4p-containing vesicles are also located anterior of the nucleus, in contrast to TbRab5p vesicles. There is also very little colocalization between TbRab4p and TbRab5p although the majority of the two TbRab5 proteins are present on the same vesicle population. We have shown that the Golgi apparatus is found in the inter-nucleo-kinetoplast region, posterior and adjacent to the nucleus (22), and would be expected to recycle components to and from the endocytotic pathway.

The different patterns for TbRab4 and TbRab5 proteins demonstrate specific subpopulations of vesicles consistent with a complex endosomal system similar to that of higher eukaryotes. The observation of identical staining patterns for TbRab5 homologues suggests that, as in mammalian and yeast systems, the Rab5 compartment is one entity. This division into Rab5 and Rab4 compartments suggests that the trypanosomal endocytotic system is more like that of mammals than that of yeast, where a Rab4 homologue has not been specifically designated (10). To confirm that TbRab5Ap and -5Bp are localized on completely overlapping populations of vesicles, and because of the difficulty of resolving small vesicles in trypanosomes at the light microscopy level, we are producing transgenic trypanosomes overexpressing wild-type and mutant forms of the TbRab proteins to alter endosome size and are addressing this question directly.

We speculate that the reorientation of the TbRab5p and TbRab4p endosome systems from posterior to anterior of the nucleus during cell division may play a role in cell-cycle-dependent plasma membrane events and suggest that the TbRab4p and TbRab5p compartments are under coordinate control, presumably by cytoskeletal elements. Movements of the kinetoplast and basal body during the cell cycle are microtubule-driven (38) and endosome maturation is also microtubule-dependent (39). These data suggest that during cytokinesis, movement of membrane-bound organelles, specifically endosomes, the kinetoplast, basal body, and Golgi apparatus,6 is coordinated by microtubules and possibly other cytoskeletal elements, ensuring accurate partitioning to the daughter cells.

Northern blots suggest developmental regulation of the TbRab5B message, which is more abundant in the BSF. In contrast, the TbRab5A and TbRab4 messages are constitutive compared with tubulin. As well as being stage specifically up-regulated, the TbRab5B message has an extended 3'UTR compared with the -5A and -4 messages, which have virtually no UTR sequence. The TbRab5B message is clearly expressed at much lower levels than the -5A message, indicating that TbRab5Ap is more abundant than -5Bp although this was not tested at the protein level. This suggests that the putatively more abundant TbRab5Ap may do the bulk of the work in promoting targeting and fusion in endocytosis, whereas TbRab5Bp, expressed at low levels, is implicated in the regulatory control of endocytotic levels between the two life stages. Procyclic cells do not endocytose markers such as TR-dextran which are taken into the BSF within minutes (40). Endocytotic activity therefore correlates with TbRab5B message levels and may be related to differential message processing observed between BSF and procyclic cells. This is the first observation of developmental regulation of TbRab protein expression and suggests that TbRab5Bp is an important component of parasite life-stage developmental response to different environments. The rate of endocytosis is also seen to be related to Rab4 and Rab5 expression in hepatocytes (41). This has general implications for these small G proteins as important regulators of the cell’s response to altered environment.

A model where one Rab5 does the work and a second Rab5 is responsible for stage-specific regulation is consistent with the situation in *S. cerevisiae*, where the more abundant Ypt51p is essential for transport of secreted proteins, whereas Ypt52p and Ypt53p appear redundant and the genes without phenotype when deleted (8, 10). Our analysis represents the first functional dissection of a multiprotein Rab5 family, because in the yeast and mammalian systems there is no proposed role for the apparent redundancy in the Rab5/Ypt5p systems. Interestingly, endocytosis may be stimulated in procyclins by overexpression of a secretory Rab protein causing an increase in fluid phase marker (Lucifer yellow) uptake, correlating with the appearance of clathrin coated pits1, suggesting that the components required for endocytosis are latent within the procyclic cell, consistent with our observations of TbRab4p, -5Ap, and -5Bp in both life stages.

Qualitative differences in endocytosis are particularly dramatic for *T. brucei* spp. *T. brucei brucei* is lysed by human serum because it actively endocytoses from human serum a lytic factor (TLF, a haptoglobin-related component of high density lipoprotein), whereas *T. brucei rhodesiense*, normally resistant to human serum can become sensitive to TLF during serial passage through mice, because specific endocytosis of this factor is no longer suppressed (19). Procyclic *T. brucei brucei* are resistant because of their complete inability to endocytose exogenous TLF. Lysis of BSF *T. brucei* by tumor necrosis factor α is likewise dependent on endocytosis of the cytokine (42), and there is increased sensitivity to lysis by tumor necrosis factor α in trypanosomes at high parasitemia compared with trypanosomes at lower cell densities. These observations clearly imply that manipulation of endocytosis by the trypanosome is stage-specific and is a potential virulence mechanism.

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