Rab5 and Rab11 mediate transferrin and anti-variant surface glycoprotein antibody recycling in *Trypanosoma brucei*

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The mammalian-infective bloodstream form of *Trypanosoma brucei* possesses a highly active endocytotic system. Evasion of the host immune response by *T. brucei* is dependent on antigenic variation of VSG (variant surface glycoprotein), but additional mechanisms for removal of surface-bound antibody also operate. Four Rab proteins, Tb (trypanosomal) RAB4, 5A, 5B and 11 are located to the endosomal system; TbRAB5A and TbRAB11 co-localize with internalized anti-VSG antibody and transferrin. A live cell assay was used to record a single cycle of endocytosis of anti-VSG IgG and transferrin, their subsequent degradation within the endosomal system and exocytosis of the products. TbRAB5A and TbRAB11 were involved in the overall process of endocytosis, degradation and exocytosis, whereas TbRAB5B and TbRAB4 were not implicated. The kinetics of anti-VSG IgG and transferrin recycling depend on the nucleotide state of TbRAB5A and TbRAB11. These data, together with previous work, suggest that IgG and transferrin initially enter a TbRAB5A sorting endosome and are most probably recycled subsequently via a TbRAB11-dependent step. Analysis of the recycled IgG and transferrin demonstrated extensive degradation of these recycled proteins. Degradation of transferrin was enhanced in cells expressing increased amounts of TbRAB5A or TbRAB11 with a Ser → Asn mutation, but was decreased when active TbRAB11 was overexpressed. The extent of degradation of anti-VSG IgG was found to be unaffected by mutant Rab protein expression. The presence of an efficient mechanism for the removal of IgG bound to the external surface of *T. brucei* and its subsequent proteolysis within the recycling system suggests a role for this pathway in immune evasion.

Key words: antibody, endocytosis, Rab protein, small G protein, *Trypanosoma*, variant surface glycoprotein, vesicle trafficking.

INTRODUCTION

Evasion of the host immune response is a critical component of the survival strategy of any pathogen. Numerous mechanisms to achieve this evasion have been exploited by prokaryotic and eukaryotic pathogens and include sequestration, modulation of immune mechanisms and antigenic variation [1–5]. In the case of the protozoan parasite, *Trypanosoma brucei*, the predominant mechanism is antigenic variation. This organism expresses approx. 10^7 copies of the GPI (glycosylphosphatidylinositol)-anchored VSG (variant surface glycoprotein) on the external surface of the plasma membrane [6], which are proposed to act primarily as a steric barrier preventing antibody and complement binding to invariant surface determinants [5]. VSG is highly immunogenic, and a robust anti-VSG immune response can eliminate parasites from the bloodstream with high efficiency, but rapid switching of the expressed VSG gene to an immunologically non-cross reactive VSG results in chronic infection [4]. This process may be assisted by immunomodulatory mechanisms [7]. VSG is capable of rapid endocytosis and recycling; the protein has a long half-life and is neither shed nor subject to significant endogenous degradation [8]. Anti-VSG immunoglobulin bound to the surface of living parasites in *in vitro* cultures is rapidly internalized and degraded, whereas the stability of the VSG is unaltered [9]. Clearly, this process has the potential to prevent antibody-dependent destruction of trypanosomes during the immune response, and to be an important adjunct to antigenic variation.

The bloodstream form of *T. brucei* has an elevated level of endocytic activity compared with the insect form. This differential endocytic activity is unlikely to reflect an increased nutrient requirement in the bloodstream form because trypanosomes in both stages of the life cycle have similar replication times when measured during culture *in vitro*. Nevertheless, the bloodstream form does have an increased rate of accumulation of one essential nutrient, low density lipoprotein, compared with the procyclic form [10,11], as well as a specialized GPI-anchored receptor for endocytosis of host TF (transferrin) [12] that is not expressed in the procyclic form. TF is degraded, but the precise compartment where proteolysis takes place has not been defined [12,13]. Several distinct compartments within the endosomal system of trypanosomes have been described. These findings were based on the immunolocalization of several Rab GTPases, which are central components of vesicle transport systems. In particular, the bloodstream stage expresses two distinct TbRAB5 (trypanosomal RAB5) proteins that accompany fluid phase and receptor-mediated endocytic cargo, and are likely to be restricted in localization to early/sorting endosomes [11]. In addition, TbRAB11 and TbRAB4, both of which have homologues in higher eukaryotes, act within pathways that are responsible for recycling membrane proteins [14–17]. TbRAB5A and TbRAB5B proteins are constitutively expressed, but in the procyclic form they co-localize, whereas in the bloodstream form they display separate localizations and appear to mediate transport of distinct cargo sets. Specifically, TbRAB5A mediates trafficking of GPI-anchored VSG and the TF-receptor, whilst TbRAB5B mediates the

Abbreviations used: ORF, open reading frame; TbRAB4, 5 etc., trypanosomal RAB4, 5 etc.; $TbRAB4^{SN,QL,WT}$ etc, recombinant Rab with a Ser → Asn or Gln → Leu mutation, or the wild-type protein respectively; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid buffered saline; TF, transferrin; GPI, glycosylphosphatidylinositol; VSG, variant surface glycoprotein.

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trafficking of ISG (invariant surface glycoprotein)$_{100}$ [11,17]. Moreover, TbRAB11 is strongly upregulated in bloodstream stages, and partially co-localizes with TbRAB5A, VSG and the TF-receptor, suggesting that it may play a general role in recycling ligands. Using a novel live-cell assay to monitor endocytosis and recycling of anti-VSG IgG and TF, TbRAB5A and TbRAB11, but not TbRAB4 and TbRAB5B, are implicated as important components of the recycling system.

MATERIALS AND METHODS

Culture of bloodstream forms of *T. brucei*

Bloodstream forms of *T. brucei* MITat 1.2, derived from Lister strain 427 and expressing VSG 221, were cultured in HMI-9 medium, at 37 °C at 5 % CO_2 in medical flats (Corning) in a humid atmosphere. Continued expression of the 221 VSG was checked periodically by immunofluorescence, and always approached 100 %. Trypanosomes were maintained at densities between 10^5 and 5×10^6 cells/ml. Mutant trypanosomes were maintained in HMI-9 selection medium with 2.5 μg G418 or phleomycin (Gibco) as appropriate.

Generation of mutant trypanosomes

Cell lines were derived by transfection of various TbRAB ORFs (open reading frames) using the pXS519 plasmid (targeted to the tubulin locus on chromosome I), as described previously [11]. Following the transfection procedure, cultures were expanded in 24-well plates and cloned by limiting dilution as required. Integration and expression of the ectopic copy of the recombinant mutant Rab gene was verified by PCR [11] from genomic DNA using the primers 3pXS519 (5′-GGGAAAAGCCAACTAAATCGGC-3′) and 5pXS519 (5′-CGAAAGGGCACATCGGCACGC-3′) and by Western blot analysis. Silent restriction sites were introduced into the mutagenized TbRAB ORFs to facilitate verification that the correct construct had been integrated (see below and [11]).

Construction of mutant TbRAB isoforms and production of antibodies

Construction of the TbRAB5A and TbRAB5B mutants and production of rabbit polyclonal antibodies against these proteins have been described previously [11]. Rabbit anti-TbRAB11 antibody has also been described previously [16]. For TbRAB4, the complete ORF was amplified with the primers 5′-CGGAGGATCCCAACCATGTCAGAGAGATATC-3′ and 5′-GTGGAATTCAAATACCTAACAAGCACACG-3′. The product was digested with *Bam*HI and *Eco*RI and inserted into pGEX-3X (Amersham Biosciences). Polyclonal murine and rabbit antibodies were raised against affinity purified TbRAB4-GST (glutathione S-transferase) fusion protein expressed in *E. coli*, using RIBI (Sigma) as adjuvant and a minimum of four immunizations [15]. All antibodies were checked for cross-reactivity against a range of non-cognate TbRABs and by competition with the cognate TbRAB; in no case was cross-reactivity detected, and cognate competition was always achieved confirming specificity of the antibodies (results not shown). TbRAB4^{WT} (wild-type protein) was amplified from genomic DNA using the primers 5′-CGGAAAGCTTCAACCATGTCAGAGAG-3′ and 5′-TTTGGATCCAATACCTAACAAGC-3′. The TbRAB4^{SN} (Ser → Asn) mutation was introduced with the primers 5′-CGGAAAGCTTCAACCATGTCAGAGAGATATCAACAGTTAATGAAGTTAATTGTTGTTGGAGATAGCGGCACAGGTAAAAATTCTC-3′ and 5′-TTTGGATCCAATACCTAACAAGC-3′. The TbRAB4^{QL} (Gln → Leu) mutant was prepared using the primers 5′-CCGGTCTAGAAAGATACAAATCAG-3′ and CTGATTTGTATCTTTCTAGACCGGC-3′. PCR products were cut with *Bam*HI and *Hin*dIII and inserted into pXS219 vector and then subcloned into pUB39. The pUB39 construct was transfected into BF4 cells (trypanosome bloodstream-form cell line harbouring inducible T7) and selected with 2.5 μg/ml phleomycin in the presence of 1 μg/ml tetracycline [18].

The TbRAB11 ORF was amplified using the primers 5′-ATGAATTCCTTATTCTTTGATGAAGCCAAG-3′ and 5′-ATACCCGGGTTAACAGCACCCGCCACTCGC-3′, and then ligated into pBluescript. The SN and QL mutants were generated by megaprimer PCR. The primers 5′-CCAAAGCTTATGGAAGACATGAACCTTACG-3′ and 5′-GTAGCGAGTCATGAGGGAATTCTTCCCAACACCGCT-3′, were used to generate an S21N/N22S double point mutation PCR product within the portion of the recombinant gene encoding the N-terminal region of TbRAB11. This PCR product was then used as a megaprimer in a second PCR, employing 5′-TCTGTCATGAATTCGTTAACAGCACCCGCCACT-3′ to regenerate the entire ORF, which was then cloned into pXS519. Similarly, TbRAB11^{Q66L} was made by producing a mutagenic megaprimer with 5′-CCAAAGCTTATGGAAGACATGAACCTTACG-3′ and 5′-GCGGAAACGTTCTAGACCAGCGGTATCCCA-3′, followed by reconstruction of the ORF in a PCR using the megaprimer and 5′-TCTGTCATGAATTCGTTAACAGCACCCGCCACT-3′, and subcloning into pXS519. All mutant TbRABs were verified by DyeDeoxy sequencing using BigDye terminator chemistry on a 377 DNA sequencer (Applied Biosystems Inc.).

FITC-labelled probes

Polyclonal rabbit IgG anti-VSG 221 from *T. brucei* (MITat 1.2) was raised and purified as described previously [9]. IgG and bovine holotransferrin (Sigma) were covalently coupled to fluorescein isothiocyanate by standard methods and the labelled proteins separated from the reaction mixture by gel filtration on Sephadex G-25. The yield and fluorescein:protein molar ratio were determined by a standard method based on the absorbance of both the protein and the attached fluorescent probe, employing a correction for the contribution of the probe absorbance to that of the protein as described previously [19,20].

Continuous fluorescence single turnover assay

Full details of the derivation of the method can be obtained from H. P. Voorheis, Department of Biochemistry Trinity College, Dublin, Ireland. Unless otherwise mentioned, all manipulations were carried out on ice (< 4 °C). Cells were harvested from culture vessels, washed and resuspended in 5 ml of TES buffer [N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid buffered saline: 120 mM NaCl, 5 mM KCl, 3 mM $MgSO_4$, 16 mM Na_2HPO_4, 5 mM KH_2PO_4, 30 mM Tris, 10 mM glucose, 0.1 mM adenosine, pH 7.4] at 10^9 trypanosomes/ml at 0 °C. A preliminary incubation of typically 2×10^8 cells was then conducted in TES buffer at 30 °C for 15 min at a cell density of 10^7 cells/ml with stirring in order to allow any cycles of endocytosis–exocytosis of material derived from the culture medium to be completed. The incubation was terminated by diluting the suspension into a 4-fold excess (v/v) of ice-cold TES, centrifuging and resuspending at 2×10^8 cells/ml in ice-cold TES and then incubating with FITC–IgG or FITC–TF (final

concentrations of 20 μg/ml or 65 μg/ml respectively) for 30 min at 0 °C to allow the IgG or TF to bind to the cell surface. After two washes with TES buffer at 0 °C, the cells were resuspended in the same buffer at 0 °C to obtain a final stock cell suspension (2×10^9 cells/ml). An aliqout (60 μl) of this final cell suspension was added to a stirred fluorescence cuvette containing 3 ml of prewarmed isosmotic TES buffer. Fluorescence was recorded over a period of 1–2 h in an LS50B PerkinElmer fluorimeter (excitation wavelength, 476 nm; emission wavelength, 515 nm; both slit widths 5 nm). Data were sampled every 10 s in each of four cuvettes in parallel and recorded digitally with FL WinLab 5.0 (PerkinElmer). Correction for differences in cell concentration between cuvettes was accomplished by determining the concentration of cell protein in each cuvette at the termination of the assay.

Gel filtration chromatography

Following the completion of a single turnover experiment, using FITC-labelled anti-VSG IgG or FITC–TF, trypanosomes were centrifuged (20 min, 4000 ***g***) and the supernatant was filtered through a 0.2 μm filter and applied to a column (1 cm × 55 cm) of Bio-Gel P-2. The FITC-labelled material in the supernatant was eluted (20 °C, 0.3 ml/min) from the column with TES buffer and collected in 2.5 ml fractions. The fluorescence of each fraction was determined with a PerkinElmer LS50B fluorimeter. Data were normalized to the total fluorescence for that experiment. FITC–TF and FITC–IgG eluted at the void volume, whereas proteolysed FITC–TF and FITC–IgG (3 μg Pronase overnight at 37 °C) eluted with the total inclusion volume (determined with cysteine).

RESULTS

Generation of mutant trypanosomes

Many studies of Rab proteins in higher eukaryotes have demonstrated that a specific point mutation within the region of the GTP binding site (WDTAGQE: Q → L) results in a dominant active mutant protein which has dramatically reduced GTPase activity but retains affinity for GTP. Also, a specific point mutation in the first consensus GTP-binding region (SVGK: S → N) generates a dominant inactive protein with enhanced GDP-binding characteristics [21–23]. In the highly conserved GTP-binding regions of the TbRAB family, analogous mutations result in similar alterations to the GTP-TbRAB interaction, e.g. TbRAB5A, 5B [11], TbRAB18 [24], TbRAB31 and TbRAB2 (M. C. Field and H. I. Field, unpublished work); therefore this strategy is valid for trypanosomatid Rab proteins. The precise mechanism by which the mutations affect function *in vivo* is not completely clear, and is not confined to direct competition with the endogenous form. For example, some GTPase SN mutants are believed to sequester specific exchange factors, responsible for downstream function [25], and thereby effectively deplete the cell of wild-type Rab activity.

The intracellular localization and expression profiles of TbRAB4, 5A, 5B and 11 in bloodstream forms of *T. brucei*, as well as specific alterations to the endocytic behaviour of cells possessing either a TbRAB5A or a TbRAB5B mutant background, have been reported previously [11,15,16]. Parasites expressing SN or QL recombinant mutant Rab proteins or overexpressing the wild-type protein for each of the TbRAB4, 5A, 5B and 11 isoforms, from genomically integrated pXS519 or pUB39, were obtained by electroporation, selection and cloning (see the Materials and methods section) in all but one case. The TbRAB5A^{SN} form could not be generated despite repeated attempts [11]. All of the other TbRAB5 isoforms were ectopically expressed to a level 4–10-fold greater than the endogenous protein (Table 1). Genomic insertion of the plasmids encoding isoforms of TbRAB4 and TbRAB11 was verified by PCR (results not shown). Quantitative Western blot analysis (Table 1) confirmed that overexpression of the TbRAB11 isoforms was of the order of 4-fold compared with the endogenous protein, whereas expression of ectopic copies of TbRAB4 isoforms were either equal to the endogenous protein (TbRAB4^{SN} mutant) or more than 4-fold higher (all other mutant types of TbRAB4).

Table 1 Western blot analysis of TbRAB overexpression

Boiling-SDS lysates prepared from bloodstream-form cells expressing ectopic copies of various TbRAB proteins were fractionated by reducing SDS/PAGE, blotted on to nitrocellulose, and transferred protein was visualized by Ponceau S. TbRAB proteins were detected as described in the Materials and methods section by enhanced chemoluminescence. Data were quantified by densitometry using NIH Image, and loading normalized for total protein transferred by reference to Ponceau S stain. The specificity and derivation of the antibodies has been described previously [11,15,16]. Data are expressed as fold expression, with the endogenous level set at 1.0. *n* = number of independent determinations (derived from separately prepared lysates).

TbRAB protein	Isoform	Expression	*n*
4	WT	23.4 ± 2.0	2
	QL	24.2 ± 2.2	2
	SN	2.1 ± 0.4	2
5A	WT	4.1 ± 0.6	3
	QL	4.0 ± 0.5	3
	SN	1.0 ± 0.2*	3
5B	WT	11.1 ± 0.8	3
	QL	10.3 ± 1.1	3
	SN	10.2 + 1.2	3
11	WT	4.2 ± 0.8	2
	QL	3.7 ± 0.9	2
	SN	7.3 ± 0.7	2

* Undetectable expression of ectopic copy; not analysed further [11].

FITC-labelled anti-VSG IgG can be monitored as it progresses through the trypanosome endocytic pathway

The endocytic compartments of bloodstream-form trypanosomes are typical low pH internal compartments [26]; hence a pH-sensitive fluorophore will exhibit altered emission characteristics as a function of its location. For fluorescein, a decrease in emission can be correlated with endocytosis of the probe from the exterior of the cell into the endosomal system. A return to the initial fluorescence level will result if the probe is subsequently recycled to the exterior of the cell. Endocytosed antibody can be detected in defined endosomal compartments [11,15], confirming that immunoglobulin traverses the endosomal system of bloodstream form trypanosomes.

Trypanosomes were allowed to bind FITC-labelled anti-VSG antibody at 0 °C, washed to remove unbound antibody, and then allowed to endocytose in the absence of additional antibody in the supporting medium. Recorded fluorescence varied over time in a highly reproducible manner (Figure 1). The initial emission dropped rapidly to a minimum at approx. 300 s, and was then followed by a rapid return to the initial fluorescence at approx. 500 s (Figure 1A). The final fluorescence intensity was substantially greater than the initial value at the beginning of the experiment. The overshoot is at least partly explained by proteolysis,

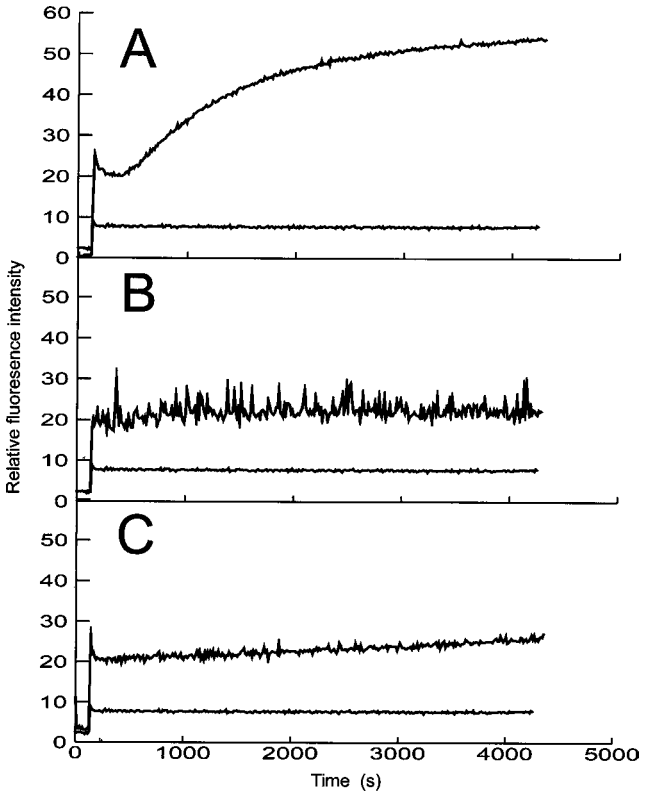

Figure 1 Change in fluorescence of FITC-labelled anti-VSG IgG following binding to the surface of MITat 1.2 bloodstream forms of *T. brucei*

Parental wild-type bloodstream form cells (MITat 1.2) were labelled with anti-MITat 1.2 VSG IgG, incubated in stirred fluorescence cuvettes and monitored for up to 5000 s. (**A**) Trypanosomes incubated in the presence of glucose. (**B**) Trypanosomes in the absence of glucose. (**C**) Trypanosomes in the presence of 1 mM chloroquine. In each panel, the lower trace is the autofluorescence from trypanosomes incubated in the absence of FITC-IgG. For cells incubated without glucose, cells were washed in glucose-free medium with an extra incubation for 5 min at 30 °C before rapidly lowering the temperature and washing prior to antibody labelling. For cells treated with chloroquine (1 mM), the inhibitor was added to the cuvette immediately before adding cells. Representative traces from at least three replicates are shown.

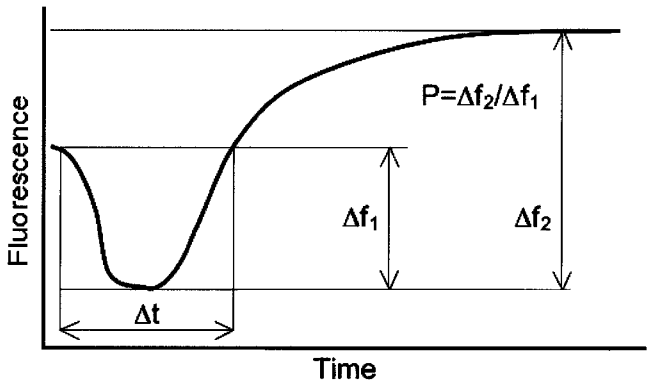

Figure 2 Schematic fluorescence time-course to illustrate derivation of parameters in Table 2

Following endocytosis of surface-bound fluorescent ligand, fluorescence decreases owing to the lower pH in endosomes/lysosomes. Fluorescence rises again as a combined result of the higher pH in recycling compartments plus a contribution from proteolysis of the protein probe. At the present time the relative contributions of these two factors are not known, and hence the parameters are simply comparative and do not make predictions concerning mechanism. Δt is the time from the beginning of endocytosis ($t = 0$) to a point when fluorescence reaches the initial value again; delayed recycling is accompanied by increased Δt. Δf_1 is the difference between the initial fluorescence and the minimum, Δf_2 is the difference between the final level and the minimum. P is the ratio $\Delta f_2/\Delta f_1$, and is a measure for how much the final fluorescence exceeds the initial value. For completely abrogated proteolysis, the fluorescence is determined by pH only, and P = 1.0.

as protease treatment of FITC–IgG results in increased fluorescence (results not shown). If cells are de-energized, by removal of glucose, or the internal pH of the endosomal compartments are raised by addition of chloroquine, these changes in fluorescence are lost. This is consistent with the model that the fluorophore traverses a low pH compartment during an energy-requiring transport process.

To facilitate comparison between cell lines, several parameters to describe the time-dependent changes in fluorescence were introduced. The time from the beginning of endocytosis to the return of the fluorescence to its initial emission, Δt, was found to be highly reproducible (Figure 2). This parameter can serve as a crude measure of how rapidly a probe traverses a portion of the recycling pathway. Δt for MITat 1.2 cells for FITC anti–VSG was 8.83 ± 1.25 min. Further, the extent to which the final emission exceeded the initial value can be expressed as P (Figure 2), the ratio of the fluorescence interval between the final emission level and the minimum (Δf_2) to the initial emission and the minimum (Δf_1) (Figure 2). The ratio P was also found to be highly reproducible. For MITat 1.2, ratio $P = 6.7 \pm 0.7$. The molecular basis for this parameter is somewhat complex and depends in part on the degree of degradation of the probe whereas traversing the endosomal system and most likely additional factors. Overall these data indicate that this approach is a valid method for investigation of recycling kinetics.

Effect of expression of TbRAB4, 5 and 11 mutant isoforms on recycling of FITC-labelled anti-VSG IgG

The 11 TbRAB mutant clones were analysed using continuous fluorescence assay under single-turnover conditions. Each group of mutants was analysed concurrently with the untransfected parental line. Analysis of the results obtained with the recombinant mutant isoforms of TbRAB11 were striking. First, there was no significant effect of the expression of any of the recombinant TbRAB11 isoforms on the rate of the endocytic portion of the recycling of FITC-labelled anti-VSG antibody present within the surface immune complexes of bloodstream forms of *T. brucei* (Figure 3A). However, marked effects were observed on the rate of exocytosis of the internalized antibodies from the endosomal compartment to the external supporting medium. The $TbRAB11^{WT}$ cells showed significant acceleration of the exocytic rate compared with the parent (Figure 3A), whereas dominant-active $TbRAB11^{QL}$ cells were even faster in exocytosis than the wild-type overexpressor (Figure 3A) and the dominant-inactive $Rab11^{SN}$ was markedly slowed in exocytosis compared with the parental cells. These data establish that the release of antibody from bloodstream form trypanosomes requires trafficking in a TbRAB11-dependent manner. The participation of TbRAB11 in vesicle trafficking from the endosomal compartment to the plasma membrane is consistent with previous observations on the trafficking of different cargo in other types of cells [27] and with observations on the location of TbRAB11 compared with internalized VSG antibody in trypanosomes [15].

None of the TbRAB4 mutant cells displayed any significant alteration at any stage in the recycling of FITC-labelled anti-VSG IgG (Figure 3B); the observed rates of initial fluorescence decrease and subsequent increase were identical to those of the

Table 2 Parameters for anti-VSG IgG and transferrin transport in TbRAB mutant trypanosome cells

An overview of characteristic parameters obtained in continuous uptake assays, as explained in the legend to Figure 4, is given. Δt and the ratio P are expressed as fold over parental for which Δt was 530 ± 75 s and P 6.7 ± 0.7 for FITC-anti VSG IgG transport and 345 ± 33 s and 6.8 ± 0.8 for FITC-transferrin transport. Par, parental bloodstream form cells (MITat 1.2); 4WT, TbRAB4 wild-type overexpressor; 4QL, TbRAB4 overexpressor; 4SN, TbRAB4 overexpressor; 5AWT, TbRAB5A wild-type overexpressor; 5AQL, TbRAB5A overexpressor; 5BWT, TbRAB5B wild-type overexpressor; 5BQL, TbRAB5B overexpressor; 5BSN, TbRAB5B overexpressor; 11WT, TbRAB11 wild-type overexpressor; 11QL, TbRAB11 overexpressor; 11SN, TbRAB11 overexpressor. Errors are the difference of independent experiments for $n = 2$ or the standard deviation for $n > 2$. The number of independent experiments (n) is indicated. *Significant difference ($P < 0.003$) to parental cells as indicated by Student's unpaired t-test, one-tailed distribution and assuming homoscedastic sample variance.

	IgG			Transferrin		
	Δt	Ratio P	n	Δt	Ratio P	n
Par	1.0 ± 0.14	1.0 ± 0.10	8	1.0 ± 0.10	1.0 ± 0.12	6
4WT	1.0 ± 0.08	1.0 ± 0.10	2	1.0 ± 0.07	1.0 ± 0.07	2
4QL	1.1 ± 0.03	1.0 ± 0.09	2	1.0 ± 0.07	1.0 ± 0.10	2
4SN	1.0 ± 0.05	1.0 ± 0.09	2	1.0 ± 0.06	1.0 ± 0.07	2
5AWT	1.8 ± 0.12*	0.7 ± 0.13*	3	1.7 ± 0.11*	0.7 ± 0.09*	3
5AQL	2.4 ± 0.10*	0.3 ± 0.12*	3	1.7 ± 0.11*	1.2 ± 0.07*	3
5BWT	1.0 ± 0.15	0.8 ± 0.07*	2	1.0 ± 0.14	1.0 ± 0.10	2
5BQL	1.1 ± 0.11	0.8 ± 0.09*	2	1.0 ± 0.09	1.0 ± 0.12	2
5BSN	1.0 ± 0.14	0.8 ± 0.12*	2	1.0 ± 0.10	1.0 ± 0.09	2
11WT	0.9 ± 0.04*	1.0 ± 0.12	3	0.9 ± 0.06*	1.0 ± 0.10	2
11QL	0.9 ± 0.04*	1.0 ± 0.10	3	0.8 ± 0.07*	1.0 ± 0.09	2
11SN	2.1 ± 0.06*	1.0 ± 0.12	3	2.2 ± 0.05*	1.0 ± 0.12	2

parental clone. This result is significant for two reasons: first, the data indicate that unlike many organisms where TbRAB4 is implicated in recycling of receptors, in bloodstream trypanosomes it does not play a significant role in this process ([28] and see below), and second, the data indicate that neither the genomic integration of a plasmid carrying a gene encoding a TbRAB isoform, nor its subsequent expression, interferes nonspecifically with endocytosis.

The effects on the recycling of FITC-labelled anti-VSG IgG in mutant trypanosomes carrying recombinant isoforms of TbRAB5A and TbRAB5B were restricted to the exocytic phase of the cycle (Figure 3C and 3D). However, significant effects on recycling were observed. For example, when any of the TbRAB5B isoforms were expressed, the exocytic rate was slowed to the same relatively small extent (Figure 3D), but when the TbRAB5A^{WT} was overexpressed the decreased rate was more marked than with any of the TbRAB5B isotypes. When the dominant-active TbRAB5A^{QL} isoform was expressed, the slowing of the exocytic phase was the greatest observed among all TbRAB5 isotypes and was comparable with that obtained with TbRAB11^{SN}. These data suggest that the rate of recycling is inversely proportional to the level of TbRAB5A/GTP.

Expression of either of the dominant-active isotypes, TbRAB5A^{QL} and TbRAB5B^{QL}, or expression of the dominant-inactive isotype, TbRAB5B^{SN} did not affect the entry rate. Consequently, either TbRAB5A and 5B are not significantly involved in the entry of this cargo in *T. brucei*, or the inward-directed vesicle trafficking system in *T. brucei* is already rate-limiting for an additional component, such that expression of the recombinant TbRAB5 isotypes is without observable impact. These data indicate that TbRAB11 and TbRAB5A are major factors in the recycling of anti-VSG IgG, and are fully consistent with our previous morphological work [11,15,16].

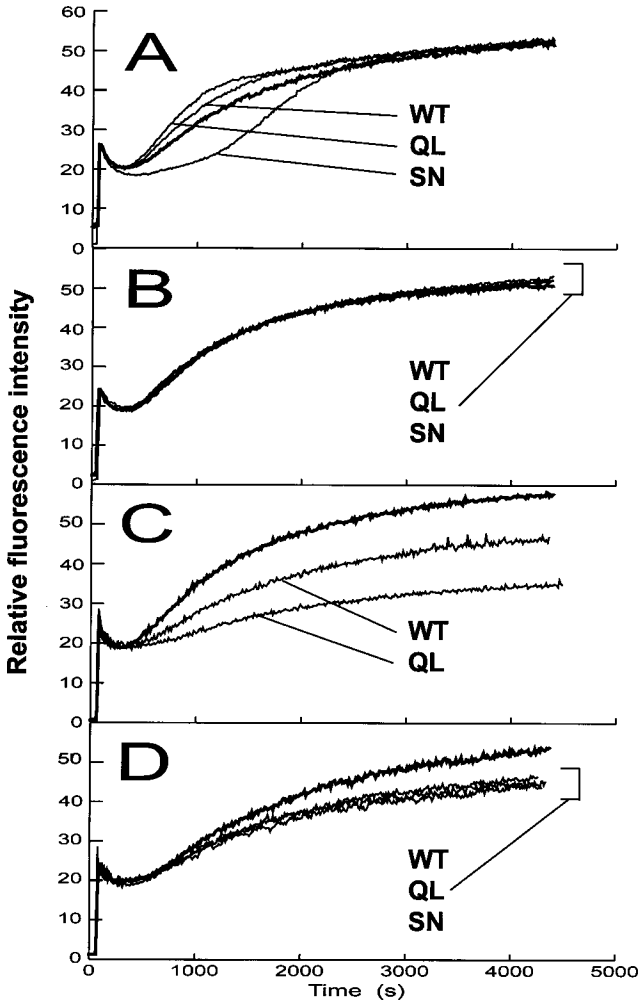

Figure 3 Effect of overexpression of TbRAB4, TbRAB5A, TbRAB5B and TbRAB11 isoforms on endocytosis and recycling of anti-VSG IgG

Bloodstream form trypanosomes were harvested from culture and prepared for the experiment, allowed to bind FITC-labelled anti-MITat 1.2 VSG IgG at 0 °C and then incubated at 30 °C in stirred fluorescence cuvettes and fluorescence changes recorded. Panels (**A–D**) show isoforms TbRAB11, TbRAB4, TbRAB5A and TbRAB5B respectively. In each case, wild-type parental cells are indicated with a thick line and other traces (WT, QL and SN) are as indicated. The experiments were performed twice (TbRAB4, TbRAB5B) or three times (TbRAB5A, TbRAB11) with essentially identical results.

Effect of expression of TbRAB4, 5 and 11 mutant isoforms on recycling of FITC-labelled TF

A similar experimental procedure was used to examine endocytosis and recycling of TF (see the Materials and methods section). Again, a rapid decrease in fluorescence was observed, followed by a return to the initial emission and an overshoot. Interestingly, the process was rather more rapid, with the fluorescence minimum being achieved at approx. 3.3 min, and the return to starting fluorescence at > 6.67 min, overall approx. 1.4-fold more rapid than IgG (Figure 4); a typical value of 5.75 ± 0.55 min for Δt for MITat 1.2 was found. These more rapid kinetics may simply reflect the size of the molecule or the efficiency of passage, and do not necessarily imply a distinct route. In addition, whereas the fluorescence in IgG experiments continued to rise, albeit slowly after 50 min (see Figure 3), for TF a clear plateau was obtained at 25 min, with a typical value for P of 6.8 for parental cells. Taken together, these data suggest that

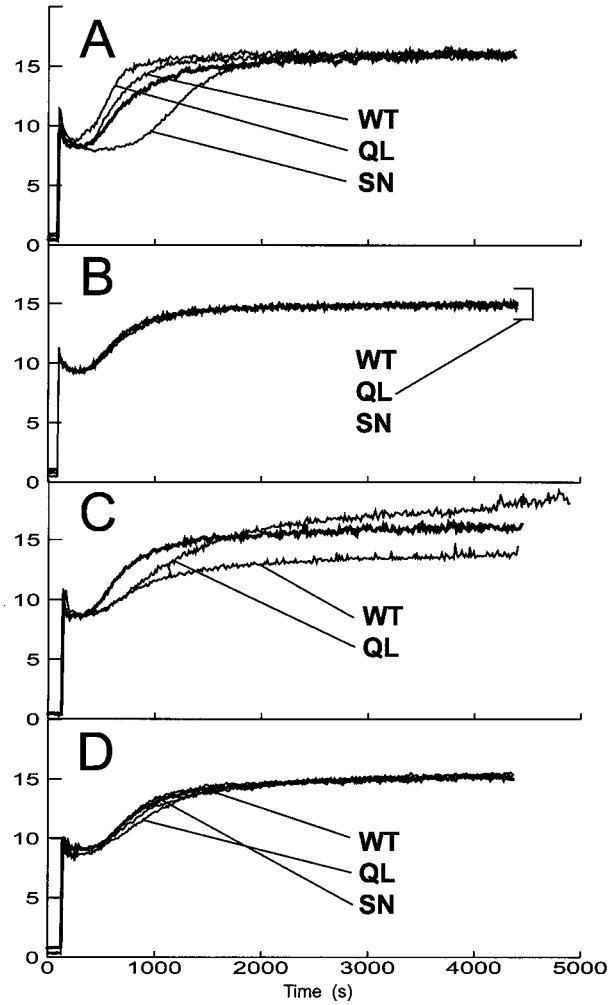

Figure 4 Effect of overexpression of TbRAB4, TbRAB5A, TbRAB5B and TbRAB11 isoforms on endocytosis and recycling of TF

Bloodstream form trypanosomes were prepared for the experiment, allowed to bind FITC-labelled TF at 0 °C and then incubated at 30 °C in stirred fluorescence cuvettes and fluorescence changes recorded. Panels (**A–D**) show isoforms TbRAB11, TbRAB4, TbRAB5A and TbRAB5B. In each case, wild-type parental cells are indicated with a thick line and other traces (WT, QL and SN) are as indicated. The experiments have been performed twice (TbRAB4, TbRAB5B, TbRAB11) or three times (TbRAB5A) with essentially identical results.

a rather more rapid system for the recycling of TF is present in *T. brucei* than for IgG. This may, in part, be a reflection of the heterogeneity of polypeptides in polyclonal IgG compared with the homogenous TF.

In the majority of cases, the data obtained for each of the mutant trypanosomes for FITC–TF were highly similar to those obtained with the respective cells for anti-VSG IgG transport. Overexpression of TbRAB11^{WT} and TbRAB11^{QL} selectively increased recycling of TF, whereas expression of TbRAB11^{SN} selectively decreased export of TF (Figure 4A). Expression of recombinant TbRAB4 mutant isoforms was without any detectable effects (Figure 4B). Trypanosomes expressing either the dominant-active TbRAB5A^{QL} and TbRAB5A^{WT} isoforms also led to a selective decrease of the export of internalized TF (Figure 4C).

Three significant differences between the effects of the TbRAB mutants on TF and anti-VSG IgG transport were detected. First, whereas ectopic expression of TbRAB5B isoforms was without a specific effect on recycling of TF (see Figure 4D where all TbRAB5B mutants show a similar decrease on recycling), a small but detectable influence was seen on anti-VSG IgG transport. Secondly, expression of TbRAB5A^{QL}, both slowed the export of internalized TF and led to an elevated final level of fluorescence (Figure 4C). This result was obtained in three replicates of the experiment, using separately prepared batches of cells, and did not result from a greater level of proteolysis (see below). Thirdly, the overall recycling rate for TF was approx. 1.4 times as fast as that observed for anti-VSG IgG; this increase was due almost entirely to the faster recycling step and not to the uptake phase (compare Figures 3 and 4).

IgG degradation is unaltered by TbRAB mutant expression

Altered endocytosis and recycling kinetics could be expected to affect proteolytic processing of immunoglobulin. Hence, IgG that had been recycled, i.e. released into the supernatant, was analysed after 4000 s. The contents of the assay vessel were centrifuged to remove cells and the supernatant (approx. 3 ml) was loaded directly onto a BioGel P2 column. Importantly, essentially no fluorescence was associated with the cells at this time (results not shown). Previous studies have shown that ^{125}I-labelled IgG is extensively degraded [9].

For MITat 1.2, gel permeation chromatograms revealed that the majority of fluorescence ($>65\%$) was associated with material eluting in the included volume, i.e. with a molecular mass of <1800 Da. The centroid of the major peak corresponded to the total volume (V_t, calibrated with cysteine), and represents remarkably efficient and essentially complete degradation of the antibody (Figure 5 and results not shown). Hence, these data fully confirm earlier observations that IgG is extensively degraded, and that the fragments are predominantly released from the cell. Significantly, none of the trypanosome cell lines analysed displayed altered gel permeation profiles (Table 3). Hence, despite significant effects on recycling rate, the TbRAB proteins did not affect the processing of endocytosed IgG.

TF degradation is altered in TbRAB5A and TbRAB11 mutants

When the recycled material from a TF–FITC experiment was subjected to gel permeation analysis, extensive degradation was also observed, in agreement with earlier work [13]. Further, this material also corresponded to $>90\%$ of fluorescence recovered at the end of the experiment, indicating a substantial recycling and release of degraded TF by the parasite. In this case, digestion was more complete than for IgG, with only 15% of fluorescence associated with material in the void volume. Again, the major included peak corresponded to V_t, suggesting essentially complete degradation (Figure 5).

As with the kinetic analysis, neither TbRAB4 not TbRAB5B mutant backgrounds affected the size distribution of the recovered material, suggesting little effect on proteolytic degradation of TF (Table 3 and Figure 5). In contrast, TbRAB5A and TbRAB11 mutants did significantly alter the state of recovered TF; the TbRAB5A^{WT}, 5A^{QL} and 11^{SN} mutants exhibited increased degradation, whereas the TbRAB11^{QL} mutant demonstrated decreased degradation. In all cases, only the proportion of material recovered in the included peak was altered (Figure 5 and Table 3), but in no case was the peak position altered, indicating no gross change to the process. These data are consistent with the recycling rate, and suggest that for TF, the extent of degradation is proportional to the time within the recycling system. For example the TbRAB11^{SN} background has a reduced recycling rate, and hence TF spends a longer period within the endocytic system; material recycled from these cells is more processed.

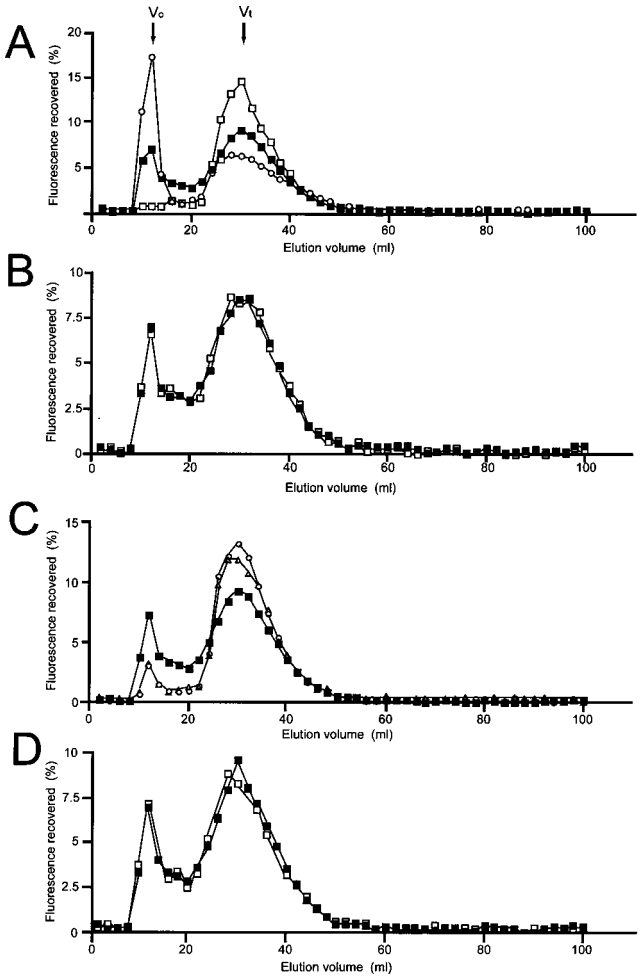

Figure 5 Effect of overexpression of TbRAB4, TbRAB5A, TbRAB5B and TbRAB11 isoforms on the degradation of TF

Following a round of endocytosis and recycling of TF, assays were terminated after 4000 s by centrifugation and the supernatants removed and chromatographed on a column of BioGel P2. (**A**) Wild-type parental cells (-■-), TbRAB11^{QL}-expressing cells (-□-), TbRAB11^{SN}-expressing cells (-○-). (**B**) Wild-type parental cells (-■-), TbRAB4^{SN}-expressing cells (-□-). (**C**) Wild-type parental cells (-■-), TbRAB5A wild-type overexpressing cells (-△-), TbRAB5AQ^{QL}-expressing cells (-○-). (**D**) Wild-type parental cells (-■-), TbRAB5B^{SN}-expressing cells (-□-). V_o and V_t indicate the positions of the column void volume and total included volume respectively.

Table 3 Influence of TbRAB proteins on degradation of IgG and transferrin in trypanosomes

Data are taken from gel filtration experiments on the supernatants from cells allowed to endocytose and recycle anti-MITat 1.2 VSG IgG (results not shown) and transferrin (data from Figure 5). Peak 1 corresponds to the fluorescence recovered in the void volume, whereas Peak 2 corresponds to the major peak within the included volume. Fluorescence recovered from the entire elution was normalized to 100 % to facilitate comparison between experiments. Par, parental bloodstream form cells (MITat 1.2); 4SN, TbRAB4 overexpressor; 5AWT, TbRAB5A wild-type overexpressor; 5AQL, TbRAB5A overexpressor; 5BSN, TbRAB5B overexpressor; 11QL, TbRAB11 overexpressor; 11SN, TbRAB11 overexpressor. Errors are the difference of independent experiments for $n = 2$ or the standard deviation for $n > 2$. The number of independent experiments (n) is indicated. *Significant difference ($P < 0.003$) to parental cells as indicated by Student's unpaired t-test, one-tailed distribution and assuming homoscedastic sample variance.

	IgG			Transferrin		
	% Peak 1	% Peak 2	n	% Peak 1	% Peak 2	n
Par	23.5 ± 0.5	66.8 ± 0.7	10	15.4 ± 1.0	68.0 ± 1.2	12
4SN	23.1 ± 1.0	66.6 ± 0.4	2	14.4 ± 1.3	68.3 ± 1.9	2
5AWT	23.8 ± 0.6	66.5 ± 0.5	2	5.2 ± 0.1	84.7 ± 2.6	2*
5AQL	23.3 ± 0.1	66.6 ± 0.9	2	5.2 ± 0.6	83.2 ± 1.4	3*
5BSN	23.0 ± 0.7	66.7 ± 1.3	2	14.5 ± 0.9	66.9 ± 0.6	2
11QL	24.2 ± 1.2	67.5 ± 2.0	2	32.7 ± 1.1	53.2 ± 1.8	3*
11SN	23.6 ± 1.5	66.8 ± 1.5	2	2.0 ± 0.2	89.9 ± 0.6	2*

DISCUSSION

Our data provide new evidence for the receptor-mediated endocytosis of surface immune complexes in trypanosomes, as well as providing confirmatory evidence for receptor-mediated endocytosis of TF [12]. These ligands have been demonstrated to enter acidic compartments that quench the fluorescence of an attached fluorescein probe. In addition, transit through the cell is energy dependent and leads to extensive internal proteolysis of both ligands. Proteolysis of IgG and TF was shown by increased fluorescence of the proteolysed ligands and their altered molecular mass following release from the cell.

The use of mutant Rab proteins has been of major value in determining the physiological role of Rabs in other cell systems. The approach is based upon the ability to make GTP-and GDP-locked forms of the proteins, with dominant effects over the endogenous Rab protein. Effects of the mutant TbRAB11 isoforms on ligand recycling are clear: both the wild-type over-expresser TbRAB11^{WT}, and the dominant positive, TbRAB11^{QL} have no effect on endocytosis but do accelerate exocytosis, i.e. the recycling phase. In addition, the dominant negative, TbRAB11^{SN} mutant had no effect on endocytosis but did slow the recycling portion of the cycle. These results agree with previous data, where TbRAB11 co-localized with both internalized anti-VSG IgG and with TF [11,15,29], and also with similar findings in mammalian cells where Rab11 acts on receptor-mediated endocytosis and recycling, including TF [27,30,31]. No influence was found for mutant TbRAB4 on ligand recycling, which is distinct from the mammalian system where Rab4 acts on receptor recycling and ligand recycling [32].

The effects of TbRAB5 on ligand cycling in bloodstream form *T. brucei* are more complex. Expression of TbRAB5B mutants slowed recycling but did not affect the endocytic part of anti-VSG IgG cycling, whereas their effects on TF recycling were much reduced or absent. The observation that TbRAB5A^{QL} decreases the recycling rate is consistent with observations that TbRAB5A^{QL} leads to moderately swollen endosomes [11]; an increased volume of these compartments would be expected to decrease the efficiency of exit of cargo from the early endosome, and hence slow the overall recycling rate. The mechanism for the small continuous increase in the final fluorescence of the material derived from FITC-labelled TF following transit through cells that express TbRAB5A^{QL}, is not yet clear, but is matched by an increase in the degradation of this material as assessed by gel filtration.

In mammalian cells Rab5 proteins are thought to be involved either close to or actually in the endocytic step and certainly act before Rab11 [22]. Overexpression of either the wild-type or QL isoform of TbRAB5A has no detectable effect on endocytosis of low density lipoprotein in the bloodstream form of the parasite, but does have a profound effect in the procyclic stage, where basal levels of endocytic activity are at least an order of magnitude lower [11]. It is possible that the level of endocytosis in the bloodstream form is so rapid, that a critical component is rate-limiting, with the consequence that it may be impossible to stimulate the process

further. In addition, the mechanism of endocytosis itself may differ substantially in the trypanosome from that described for higher eukaryotes, as the AP-2 (activator protein 2) complex appears absent from the genome, and GPI-anchored proteins are endocytosed via a clathrin-mediated pathway [14,33].

The fluorescein-tagged peptides derived from both TF and anti-VSG IgG were extensively degraded inside an endosomal compartment in the parent strain. The extent of proteolysis of IgG was unaffected by mutant TbRAB expression, but proteolysis of TF was increased by the expression of the mutant TbRABs ($TbRAB11^{SN}$, $TbRAB5A^{QL}$, $TbRAB5A^{WT}$) that slowed the overall recycling of TF. In addition, TF proteolysis was decreased by the expression of $TbRAB11^{QL}$, which accelerated recycling. The most probable reason for these effects is an increase or decrease in residence time of the ligand in the endosomal compartment, leading to an equivalent change in exposure time to the proteolytic activities responsible for degradation. The lack of a similar effect on IgG could be due to differences in the pathway taken by IgG and TF, or that TF and IgG significantly differ in protease sensitivities. The first possibility is supported by observations here that the transit time for TF through the endosome is significantly shorter than that for anti-VSG IgG.

A role for TF recycling via the endocytic system is well established as a mechanism for the nutritional uptake of iron [12], but the idea that recycling surface immune complexes play a role in the trypanosome life cycle is relatively new [9]. It is well established that antigenic variation is the major mechanism by which the African trypanosome avoids host immune defences. Endosomal recycling of surface immune complexes may contribute to the parasite defence repertoire against host antibody response, and is based on two recent findings. First, that bloodstream form trypanosomes can escape from antibody-mediated aggregates without injury and then remain competent to grow and divide at an undiminished rate [9], and second, the findings in the present study, which demonstrate that surface immune complexes can be endocytosed and the surface immunoglobulins degraded within the endosomal system. Many trypanosomes found in more primitive vertebrates than mammals, for example in fish, appear to lack a VSG and yet their infections in these hosts persist for many days, and even as long as several months [34]. The clearance mechanism for surface immune complexes may have arisen as an early evolutionary defence mechanism for the parasite, appearing before the advent of antigenic variation.

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