GPI valence and the fate of secretory membrane proteins in African trypanosomes

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Summary

Progression of GPI-anchored proteins in bloodstream African trypanosomes correlates with GPI-valence: homodimeric VSG (2 GPI) is a surface protein; heterodimeric transferrin receptor (1 GPI) localizes in the flagellar pocket; homodimeric GPI-minus VSG (0 GPI) is rapidly degraded in the lysosome. We test this relationship using three native secretory/endocytic proteins as monomeric GPI-plus and -minus reporters. GPI-minus procyclin trafficks to the lysosome and is degraded. GPI-plus procyclin trafficks to the flagellar pocket/cell surface and is released (~50%) with an intact anchor, the remainder (~50%) is degraded in the lysosome. GPI-plus BiPNHP, derived from the ER marker BiP, is released quantitatively (>80%), while GPI-plus p67HP, derived from the lysosomal marker p67, turns over by both release (~15%) and lysosomal degradation (>50%). Turnover of endogenous transferrin receptor occurs primarily by lysosomal degradation (>90%). Thus shedding of monovalent GPI reporters correlates inversely with lysosomal targeting. We propose that mono-GPI reporters cycle through the flagellar pocket and endosome until they are disposed of by either shedding or lysosomal targeting. Partitioning between these fates may be a function of individual physical properties. Release is likely due to the exclusive use of C-14:0 myristate in the bloodstream stage GPI anchor. Up-regulation of transferrin receptor by culture in dog serum resulted in prominent cell surface localization, but not in elevated release. Surface receptor was non-functional for ligand binding suggesting that it may be bivalent homodimers of the GPI-anchored ESAG6 receptor subunit.

Key words: Trypanosome, Glycosylphosphatidylinositol, Flagellar pocket, Transferrin receptor, Protein sorting

Introduction

African trypanosomes are phylogenetically ancient parasitic protozoa that cause human (sleeping sickness) and veterinary (Nagana) disease throughout the sub-Saharan continent. The trypanosome life cycle alternates between the bloodstream of the mammalian host and the midgut of the arthropod vector, tsetse flies of the genus *Glossina*. As an essential adaptation to their respective niches, each stage elaborates a unique surface coat that is composed of a single abundant protein, variant surface glycoprotein (VSG) in mammalian bloodstream forms, and procyclin in the procyclic insect forms. There are upwards of a 1000 *VSG* genes encoding antigenically distinct homodimeric proteins, and it is by varying the expression of these genes that the bloodstream parasite avoids the host immune response (Donelson, 2003). Monomeric procyclin has two isoforms defined by C-terminal repeat motifs (Roditi and Clayton, 1999); EP procyclin has ~30 repeats of Glu-Pro and GPEET procyclin has six repeats of Gly-Pro-Glu-Glu-Thr. Both forms are substantially protease resistant to afford protection in the hydrolytic milieu of the tsetse midgut (Acosta-Serrano et al., 2001), and perhaps also against an endogenous procyclic-specific cell surface metalloprotease (Bangs et al., 1997; LaCount et al., 2003). Both VSGs and procyclins are attached to membranes by glycosylphosphatidylinositol (GPI) membrane anchors.

Not surprisingly, given its abundance and its importance to pathogenesis, VSG is the most studied protein in African trypanosomes. Newly synthesized VSG is rapidly modified in the endoplasmic reticulum by *N*-glycosylation and by attachment of the C-terminal GPI-anchor (Bangs et al., 1985; Ferguson et al., 1986). Homodimerization occurs during this early stage of transport (McDowell et al., 1998; Triggs and Bangs, 2003). During subsequent transport from the ER and through the Golgi both GPI and *N*-glycans are modified, and thereafter VSG is incorporated via the flagellar pocket into the surface coat with an overall transport rate of $t_{1/2}$ ~15 minutes (Bangs et al., 1986; Bangs et al., 1988; Duszenko et al., 1988; Mayor et al., 1992). VSG recycles constantly between the plasma membrane and internal endosomal compartments (Overath et al., 1997), yet is remarkably stable in bloodstream trypanosomes ($t_{1/2}$ >30 hours) (Bulow et al., 1989; Seyfang et al., 1990). However, the rate of membrane uptake within the flagellar pocket is exceedingly rapid (an entire surface coat equivalent in <12 minutes) (Engstler et al., 2004; Grünfelder et al., 2003), thus sorting back to the surface must be efficient to avoid degradation in the lysosome. Recent studies have defined the intracellular pathway of VSG sorting (Engstler et al., 2004; Grünfelder et al., 2003). Uptake is via clathrin-coated vesicles (Class I) followed by delivery to a RAB5$^+$ early endosomal compartment. After maturation into a RAB11$^+$ recycling endosome, lysosomal cargo is distilled into small clathrin-coated vesicles (Class II), leaving behind VSG$^+$

exocytic carriers that fuse with the flagellar pocket. Within this pathway there is a steep gradient of lateral VSG density moving from internal compartments to the plasma membrane [ER (1×), Golgi (3×), flagellar pocket/plasma membrane (50×), endosomes (10×)] (Grünfelder et al., 2002). Unlike VSG, nothing is known about the transport and turnover of endogenous procyclins in insect stage trypanosomes. Nevertheless it is assumed that GPI anchors strongly influence membrane protein trafficking in both life cycle stages.

The only other GPI-anchored protein to be studied in trypanosomes is the bloodstream stage-specific transferrin receptor (TfR). TfR is a heterodimer of the *ESAG6* and *ESAG7* gene products (Ligtenberg et al., 1994; Salmon et al., 1994). These subunits have high sequence similarity with each other, as well as overall structural similarity to VSGs (Carrington and Boothroyd, 1996), but only ESAG6 is GPI-anchored. The steady state location of this single GPI heterodimer is in the flagellar pocket; it is not normally detectable on the external plasma membrane. However, modest overexpression induced by transferrin starvation leads to 'spill-over' onto the cell surface (Mussman et al., 2004; Mussman et al., 2003), TfR is continuously endocytosed bearing serum transferrin, which is delivered to the lysosome, and the receptor is recycled to the flagellar pocket. The half-life of TfR has been variously estimated to be 0.7-7.0 hours (Biebinger et al., 2003; Kabiri and Steverding, 2000; Mussman et al., 2004) and turnover apparently occurs in the lysosome (Steverding et al., 1995).

We have previously studied the role of GPI anchors in trypanosomes by expression of modified GPI-minus VSG reporters (Bangs et al., 1997; McDowell et al., 1998; Triggs and Bangs, 2003). In procyclic trypanosomes GPI-minus VSG is delayed in ER exit, but is ultimately secreted quantitatively. In bloodstream trypanosomes these reporters are also apparently delayed in ER exit, but once exit is achieved they are delivered to and degraded in the lysosome ($t_{1/2}$ ~1 hour depending on the VSG). Similar results were obtained with an analogous GPI-minus version of TfR (Biebinger et al., 2003) and with VSGs (Böhme and Cross, 2001). Collectively these findings suggest that GPI anchors function in the early secretory pathway, and that in bloodstream parasites GPIs also play a critical role in post-Golgi targeting to the cell surface.

Our findings, in conjunction with the known behavior of native VSG and TfR present an intriguing correlation between GPI valence and the ultimate fate of proteins within the secretory pathway of bloodstream trypanosomes: native VSG (GPI^2) has a steady state cell surface localization and is exceptionally stable; TfR (GPI^1) is located in the flagellar pocket and is turned over more rapidly; and GPI-minus VSG (GPI^0) is delivered to the lysosome and degraded most rapidly. We now present data in bloodstream trypanosomes with a series of proteins of the native secretory/endocytic pathways engineered to have a valence of GPI^1 and GP^0, and also with native TfR. These reporters conform generally to the 'valence correlation', but surprisingly we find that GPI^1 membrane proteins can also be released from viable bloodstream trypanosomes with intact GPI anchors. A simple model is proposed for parsing these reporters between the alternate fates of release versus lysosomal degradation, and the implications of our novel findings for trafficking of native GPI-anchored proteins in trypanosomes are discussed.

Materials and Methods

Maintenance and manipulation of trypanosomes

All stable transformants were generated in cultured monomorphic bloodstream forms of the Lister 427 strain of *Trypanosoma brucei brucei* (MITat 1.2, expressing VSG221, referred to herein as BS221). The maintenance, transformation and metabolic radiolabeling of cultured bloodstream cells have been described in previous publications (Bangs et al., 1997; McDowell et al., 1998; Triggs and Bangs, 2003). The *N*-glycan synthesis inhibitor tunicamycin (Sigma, St Louis MO) and the thiol protease inhibitor FMK024 (morpholinourea-phenylalanine - homophenylalanine-fluoromethyl ketone; Enzyme Systems Products, Livermore, CA) were dissolved in DMSO and added to cultures at dilutions of at least 1/100 to give final concentrations of 1 μM and 20 μM, respectively. For induction of native TfR expression cells were cultured in HMI9 media (Hirumi and Hirumi, 1994) containing either 10% fetal bovine serum (control) or 10% canine serum (Equitech-Bio, Kerrville, TX) ±200 μg/ml bovine holotransferrin (Sigma).

Immunoprotocols, antibodies and electrophoresis

Immunoprecipitation and electrophoresis have been described previously (Bangs et al., 1997; McDowell et al., 1998; Triggs and Bangs, 2003). The only modification was that for immunoprecipitation of EP procyclin reporters cells were solubilized in 1× RIPA salts (50 mM TrisHCl, pH 8.0, 100 mM NaCl) containing 0.5% SDS, denatured (5 minutes, 95°C), and then adjusted to 1× RIPA salts with 1% NP40, 0.5% deoxycholate, 0.1% SDS. All cell lysates and media fractions were supplemented with protease inhibitors (final concentrations 2 μg/ml each of leupeptin, antipain, chymostatin and pepstatin, and 0.1 mM tosyllysine chloromethyl ketone). Rabbit and mouse anti-BiP, rabbit anti-VSG221 and monoclonal anti-p67 are described in (Alexander et al., 2002; Bangs et al., 1996; McDowell et al., 1998). Monoclonal 247 anti-EP procyclin was purchased from Cederlane Laboratories Ltd. (Hornby, Ontario). Rabbit anti-HA and monoclonal 12CA5 anti-HA were purchased from Zymed (San Francisco, CA) and BabCO (Richmond, CA), respectively. Rabbit anti-EP procyclin was prepared by immunization with a synthetic $(EP)_9$ peptide coupled to keyhole limpet hemocyanin (anti-EP_9). Monoclonal anti-*Leishmania* paraflagellar rod and rabbit anti-transferrin receptor were kindly supplied by Diane McMahon-Pratt (Yale University) and Piet Borst (Netherlands Cancer Institute, Amsterdam), respectively. Biotinyl-tomato lectin was purchased from Vector Laboratories, Burlingame CA.

Gels were exposed on a Molecular Dynamics Typhoon system and phosphorimages were quantified in the native ImageQuant Software (Amersham Biosciences, Piscataway, NJ). For each lane, densities of identical regions of interest were measured and corrected by subtraction of the density for an equivalent unlabeled region.

Construction of reporter genes

All reporters are shown diagrammatically in Fig. 1 and were cloned into the constitutive expression vectors $pXS5^{neo}$ (Alexander et al., 2002) using flanking 5′ *Hin*dIII and 3′ *Eco*RI sites. Vector plasmids were linearized with *Xho*I and stably transformed bloodstream cell lines were generated as described in (Triggs and Bangs, 2003).

Lectin staining of live trypanosomes

The flagellar pocket of live bloodstream trypanososomes was stained with biotinyl-tomato lectin as described previously (Alexander et al., 2002). Immunostaining of fixed/permeabilized bloodstream (methanol/acetone) and procyclic (formaldehyde) stage parasites has been previously described (Alexander et al., 2002). Specific antibody staining was visualized with the appropriate Alexa 488- and Alexa 633-conjugated goat anti-IgG secondary reagents (Molecular Probes,

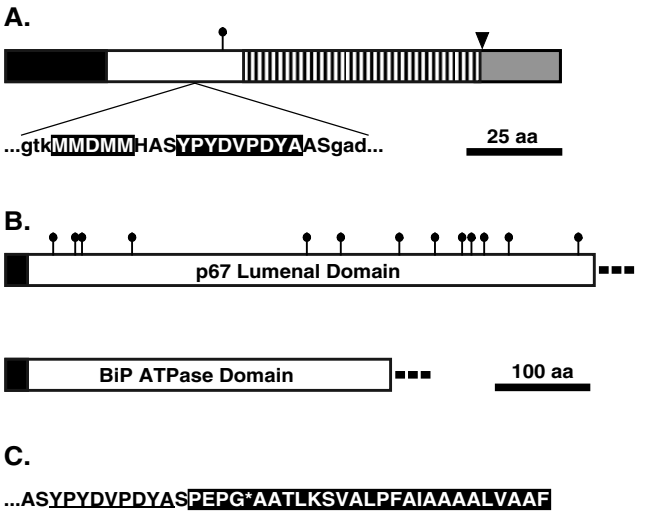

Fig. 1. Diagram of reporter constructs. (A) Diagram of the full length EPMH reporter (above). Native EP1 procyclin domains (from N to C terminus) are: signal sequence, black box; mature N-terminal domain, white box, EP repeat domain, stripped box; GPI attachment peptide, gray box. The single EP1 *N*-glycosylation site (lollipop) and the C-terminus of the EPMHΔgpi reporter (arrowhead) are indicated. The position (codon 50) and sequence of the inserted cassettes are indicated (below). The insert sequence is in upper case, the methionine and HA cassettes are highlighted in black, and flanking native sequences are in lower case. (B) Domains and *N*-glycosylation (lollipops) patterns of the p67HP and BiPNHP GPI[1] reporters: native signal sequences, black boxes; ectoplasmic domains, white boxes. The C-terminal HA and EP procyclin GPI-peptide sequences are indicated by dots. BiPNHP and p67HP contain codons 1-415 and 1-616 of the native sequences, respectively. (C) C-terminal amino acid sequence of the p67HP and BiPNHP GPI[1] reporters including the HA epitope (underlined) and the EP procyclin GPI attachment peptide (black box). * indicates GPI attachment site.

Seattle, WA). Biotinyl-tomato-lectin staining was visualized with Alexa633 streptavidin (Molecular Probes). Cells were counterstained with 500 ng/ml DAPI. Serial image stacks (0.2 micron Z-increment) were collected at 100× (PlanApo oil immersion 1.4 NA) on a motorized Zeiss Axioplan IIi equipped with a rear-mounted excitation filter wheel, a triple pass (DAPI/FITC/Texas Red) emission cube, differential interference contrast (DIC) optics, and a Zeiss AxioCam B&W CCD camera. Fluorescence images were deconvolved by a constrained iterative algorithm, pseudocolored, and merged using OpenLabs 4.0 software (Improvision, Lexington, MA). Unless stated otherwise, capture times for all images were 200-500 milliseconds.

Immunoelectron microscopy

Fresh samples were embedded in 2% agarose in 0.1 M phosphate buffer (PB), pH 7.4 at 50°C. After cooling, samples were fixed (30 minutes) in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M PB, sectioned into 100 μm slices, fixed again (2 hours), and then quenched with 0.1% sodium borohydride in 0.1 M PB (10 minutes). The slices were incubated in 0.1% Triton X-100 (30 minutes) and then blocked (1 hour, RT) with Aurion Goat Blocking Agent (Aurion, Wageningen, The Netherlands). After rinsing in incubation buffer [IB: PBS+0.1% BSA-c (Aurion)], slices were incubated in anti-EP (mAb247, 1:500 in IB, 2 hours at RT, then 16 hours at 4°C). Following extensive rinsing, slices were incubated with goat anti-mouse IgG F(ab′)$_2$ Ultra-Small gold conjugate (Aurion) (1:100 in IB, 2 hours at RT, then 16 hours at 4°C). Stained slices were rinsed sequentially in IB and PBS, and then postfixed in 2% glutaraldehyde in 0.1 M PB (30 minutes) and finally in PB. Postfixed slices were rinsed in Enhancing Conditioning Solution (ECS, Aurion) and then developed in Silver Enhancement Solution (Aurion) for 1.25 hours. Enhancement was terminated in 0.3 M sodium thiosulfate in ECS (5 minutes) followed by rinsing in ECS. Enhanced slices were further fixed with 0.5% osmium tetroxide in 0.1M PB (30 minutes) and then serially dehydrated in ethanol. Samples were transferred into propylene oxide and flat-embedded in Spurr's epoxy resin (Electron Microscopy Sciences, Hatfield, PA). Sections (60-90 nm) were cut on a Reichert-Jung Ultracut-E Ultramicrotome and contrasted with Reynolds' lead citrate and 8% uranyl acetate in 50% EtOH. Ultrathin sections were mounted and observed with a Philips CM120 electron microscope. All images were captured with a MegaView III (Soft Imaging System; Lakewood, CO) side-mounted digital camera.

Transferrin binding

BS221 cells (~5×10^7), grown in HMI9 media supplemented with either 10% canine or fetal bovine serum for 48 hours, were washed with HEPES buffered-saline (HBS) and resuspended in 0.8 ml HMI9 media lacking serum but supplemented with 10 mg/ml bovine serum albumin to reduce nonspecific binding. Tf-gold (0.2 ml bovine holo-transferrin conjugated to 5 nm gold suspended in PBS to an OD_{520} of 5.8, Aurion) was added and the cells were incubated at 8°C for 1 hour followed by washing in HBS to remove unbound Tf-gold. Cells were then fixed in 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for ~20 hours at 4°C. The cells were post-fixed in 1% osmium tetroxide in the same buffer for 1 hour at RT. Samples were dehydrated, embedded, sectioned, and observed as described above.

Results

Validation of procyclin reporters in procyclic trypanosomes

Before using engineered procyclin reporters for trafficking studies in bloodstream stage trypanosomes we first validated their behavior in procyclic cells where procyclin is normally expressed. These reporters, which are based on native EP1 procyclin, have an inserted methionine cassette to facilitate detection by metabolic radiolabeling and an HA epitope to allow discrimination from endogenous EP procyclins (see Fig. 1A). The reporters were prepared in a GPI[1] (EPMH) and a GPI[0] (EPMHΔgpi) format. We have previously demonstrated in procyclic trypanosomes that transgenic reporters (VSG and transialidase) are delivered to the cell surface in GPI-anchored form, and are quantitatively secreted in GPI-minus form, albeit slowly (Bangs et al., 1997; McDowell et al., 1998). The slow rate of export of the GPI-minus reporters is manifested by elevated steady state localization in the ER. Procyclic cell lines expressing both EP procyclin reporters were analyzed by metabolic radiolabeling and immunofluorescence (data not shown). EPMH was stably incorporated into the surface coat. EPMHΔgpi accumulated in the ER but was ultimately secreted. These results are consistent with the known behavior of native procyclin, as well as recombinant GPI-plus and GPI-minus reporters in transgenic procyclic cells.

Expression of procyclin reporters in bloodstream trypanosomes

Expression of the procyclin reporters in stable bloodstream cell

lines was investigated by pulse radiolabeling in the presence and absence of tunicamycin, an inhibitor of *N*-glycan synthesis (Fig. 2A, top). No procyclin polypeptides were detected under either condition in cell or media fractions from untransformed control cells (lanes 1 and 2, 7 and 8). Furthermore, in all cell lines tested endogenous VSG 221 behaved as expected, a single species of ~55 kDa that was reduced in size by tunicamycin treatment consistent with the loss of 2 *N*-glycans (lanes 1-6, bottom). No VSG was detected in the media fractions (Fig. 2A bottom, lanes 7-12).

In untreated cells the EPMH reporter was detected as a sharp ~40 kDa precursor band and a mature ~55 kDa smear (lane 3, P and M). The precursor/product relationship of these glycoforms is confirmed in the following experiments. The precursor size was reduced ~2 kDa by tunicamycin treatment consistent with utilization of the single *N*-glycosylation site (compare lanes 3 and 4). Tunicamycin treatment partially reduced the size of the mature form indicating that the *N*-glycan is modified, but that additional post-translational processing must also be occurring. This second mode of processing, the nature of which is unclear, is likely related to the GPI anchor, as a similar phenomenon was not observed with the otherwise identical GPI^0 EPMHΔgpi reporter (see below). Unexpectedly, the mature glycoform was also detected in the accompanying media fractions (lanes 9 and 10) suggesting that the reporter is exported from cells. Inhibition of *N*-glycosylation had no effect on release of mature EPMH (lane 10).

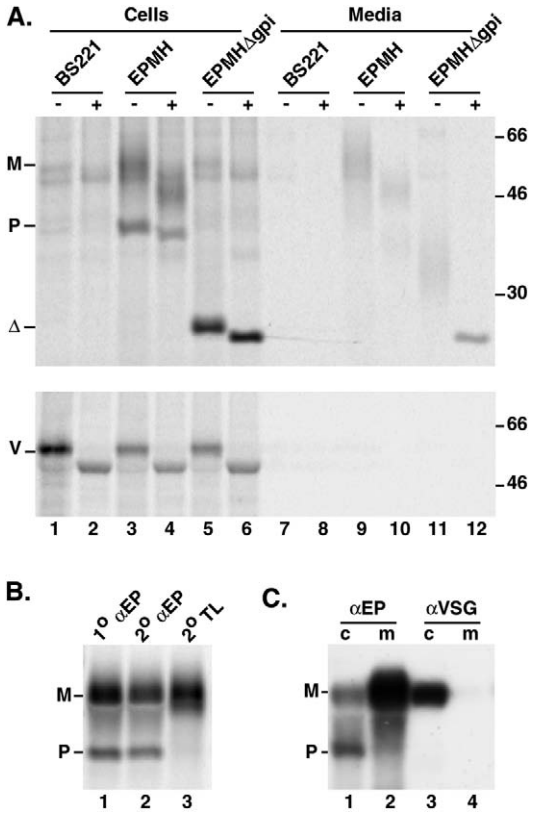

Fig. 2. Expression of EPMH reporters in bloodstream trypanosomes. (A) Control untransformed bloodstream cells (BS221) and cell lines expressing EPMH or EPMHΔgpi were preincubated (1 hour) with (+) or without (–) tunicamycin, and then metabolically radiolabeled with [^{35}S]Met/Cys (1 hour) in the continued absence or presence of inhibitor. Reporters and endogenous VSG were immunoprecipitated from cell and media fractions with anti-EP_9 (top, 2×10^7 cell equivalents/lane) and anti-VSG221 (bottom, 2×10^5 cell equivalents/lane) antibodies. Mobilities of the precursor (P), and mature (M) forms of EPMH, of EPHMΔgpi (Δ), and of VSG (V), are indicated on the left; mobilities of molecular mass markers on the right. (B) EPMH cells were radiolabeled with [^{35}S]Met/Cys for 2 hours and then immunoprecipitated with anti-EP_9 (lane 1). Identical anti-EP_9 immunoprecipitates were solubilized and reprecipitated with either anti-EP_9 (lane 2) or biotinyl-tomato lectin (lane 3). All lanes contain 2×10^7 cell equivalents/lane. (C) EPMH cells were radiolabeled with [^{3}H]Myristate for 4 hours and labeled polypeptides were immunoprecipitated from cell and media fractions with anti-EP_9 (5×10^7 cell equivalents/lane) or anti-VSG221 (1×10^6 cell equivalents/lane). All samples were analyzed by SDS-PAGE and phosphorimaging (panels A and B) or fluorography (panel C).

To determine the nature of the EPMH *N*-glycan modifications radiolabeled reporter was first immunoprecipitated with anti-EP_9, and then following solubilization reprecipitated with either anti-EP_9 or with tomato lectin (Fig. 2B). Tomato lectin specifically binds the unusual giant poly-*N*-acetyllactosamine (pNAL) chains that are attached to *N*-glycans of many proteins in the endomembrane system of bloodstream trypanosomes (Atrih et al., 2005; Nolan et al., 1999). Only the mature processed glycoform was detected by reprecipitation with tomato lectin (lane 3) indicating that the 40 kDa glycoform is an immature precursor to the larger fully modified glycoform. This experiment does not allow a definitive assignment of the site of addition of tomato-lectin-reactive pNAL, but this is likely to be the single *N*-glycan of EPMH given the precedent for this sort of modification of *N*-glycans in bloodstream trypanosomes, and the magnitude of the size shift seen in tunicamycin-treated cells, which is too large to be compatible with the loss of a more typical high mannose or complex oligosaccharide.

Finally, to establish that the EPMH reporter is actually GPI-anchored, cells were metabolically radiolabeled with [^{3}H]Myristate and procyclin polypeptides were immunoprecipitated from cell and media fractions (Fig. 2C). Both glycoforms were detected in the cell fraction (lane 1), but surprisingly, radiolabeled mature glycoform was also detected in the media indicating that release is not dependent on GPI hydrolysis (lane 2) by endogenous GPI-phospholipase C (GPI-PLC). As expected [^{3}H]Myristate-labeled VSG 221 was only detected in the cell-associated fraction (compare lanes 3 and 4). These results confirm that the procyclin GPI attachment sequence functions in bloodstream trypanosomes, and suggest that proteins with a GPI^1 valence can be released from bloodstream trypanosomes with an intact GPI anchor. No

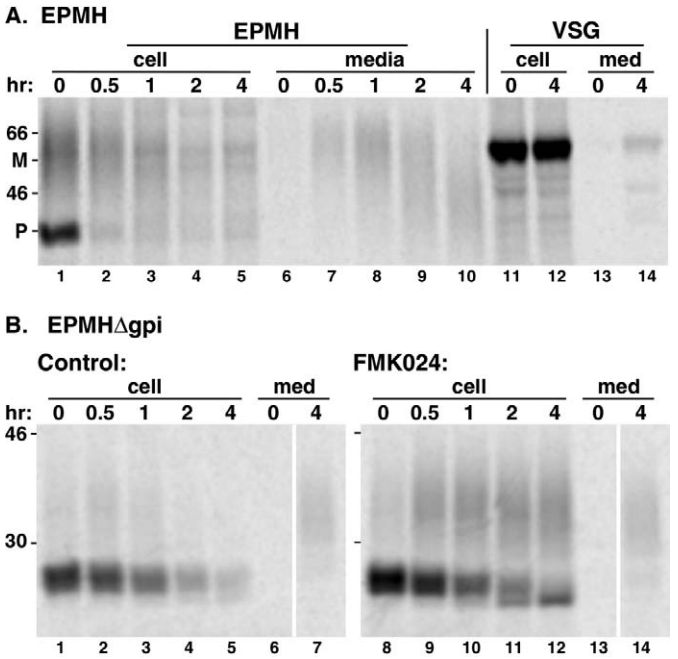

Fig. 3. Turnover of EPMH reporters in bloodstream trypanosomes Transgenic bloodstream cell lines expressing either EPMH (panel A) or EPMHΔgpi (panel B) were pulse radiolabeled with [^{35}S]Met/Cys for 15 minutes and then chased with complete media. At the indicated times polypeptides were immunoprecipitated from cell and media fractions with either anti-EP$_9$ (2×10^7 cell equivalents) or anti-VSG (2×10^5 cell equivalents). Samples were analyzed by SDS-PAGE and phosphorimaging. (A) 'M' and 'P' indicate the mobility of mature and precursor EPMH polypeptides. (B) As indicated, the EPHMΔgpi cells were either untreated or treated with FMK024 (20 μM) for 15 minutes prior to radiolabeling, and subsequently for the duration of the pulse/chase periods. Vertical white stripes indicate irrelevant regions of phosphoimages that were digitally removed to simplify presentation. The mobilities of molecular mass markers are indicated in kDa.

labeling of the EPMHΔgpi reporter was detected (data not shown) indicating that myristate incorporation was GPI-dependent.

Fate of procyclin reporters in bloodstream trypanosomes

Pulse/chase turnover analyses were performed to precisely determine the fate of the procyclin reporters in bloodstream cell lines (Fig. 3). At the end of the short pulse radiolabel the GPI-anchored EPMH reporter was detected as both the immature and mature glycoforms (Fig. 3A, lane 1). During the chase the precursor band was rapidly processed to the larger form, which subsequently disappeared from the cell fraction (lanes 2-5). Much of the processed reporter was exported to the external media by the end of the chase period (lanes 6-10). Again, release of the reporter was not due to GPI hydrolysis or membrane blebbing as no endogenous VSG was released from the cells (lanes 11-14), which remained fully viable during the chase period. Quantitative analyses revealed that 50% of EPMH reporter was exported to the media (Table 1). There was a modest increase in cell-associated recovery in the presence of FMK024, a selective inhibitor of thiol proteases,

Table 1. Recovery of procyclin reporters in bloodstream trypanosomes

Reporter (–/+ FMK024)	Cells*	Media*	Total†	Decay‡ $t_{1/2}$ (minutes)
EPMH (–)	18.4±3.6	49.8±6.2	68.2	67
EPMH (+)	27.5±3.8	44.6±5.4	72.1	NA§
EPMHΔgpi (–)	8.5±0.4	17.0±1.4	25.5	41
EPMHΔgpi (+)	51.9±7.9	12±1.4	64.0	NA§

*Recovery at T_4 as a percent of T_0 cell-associated reporter (mean ±s.d., n=3). Data were analyzed in Prism 4 (GraphPad Software Inc. San Diego, CA).
†Sum of cell+media recoveries.
‡Loss of cell-associated reporter from best fit equation.
§Not applicable.

but total recovery was unchanged (68% versus 72%). We have previously used protection by FMK024 as a benchmark for delivery to the lysosomal compartment (Alexander et al., 2002; Triggs and Bangs, 2003). These results suggest that the overall turnover of cell-associated EPMH ($t_{1/2}$ ~67 minutes) is due primarily to extracellular release. The basis for the gradual decrease in size of the released EPMH reporter (lanes 7-10) is not known, but it is consistently observed and could be due to proteolysis and/or glycan trimming.

Analyses of the EPMHΔgpi cell line indicates that this ~28 kDa reporter also disappears from cells during the chase (Fig. 3B, lanes 1-5), but very little of the reporter is actually secreted into the media fraction (lanes 6 and 7, 17%). Treatment with FMK024 leads to greatly elevated recovery of cell-associated reporter (8% versus 52%), much of which is detected as a higher molecular mass smear (lanes 8-12), indicating that *N*-glycan processing can occur with this soluble reporter. FMK024 had no effect on recovery in the media fraction (lanes 13 and 14; Table 1). These data suggest that the overwhelming fate the GPI-minus reporter is degradation in the lysosomal compartment.

Localization of procyclin reporters in bloodstream trypanosomes

To determine the localization of the procyclin reporters in bloodstream parasites we performed IFA analysis. In the EPMH cells both anti-EP$_9$ and anti-HA antibodies gave discrete and precisely overlapping signals in the flagellar pocket region just anterior of the kinetoplast (Fig. 4, panels A-D, arrowhead) validating both antibodies for detection of the reporter in bloodstream cells. The EPMH signal did not overlap to any degree with either the lumenal ER marker BiP (Fig. 4, panels E-H) or the lysosomal membrane marker p67 (Fig. 4, panels I-L). To confirm the site of EPMH localization as the flagellar pocket, live trypanosomes were exposed to biotinyl-tomato lectin at 5°C, conditions that allow external access to the lumen of the pocket, but which inhibit subsequent endocytosis (Langreth and Balber, 1975). Tomato lectin binds poly-*N*-acetyllactosamine-containing *N*-glycans on membrane glycoproteins in bloodstream trypanosomes, and hence is a specific marker for the flagellar pocket/endosomal system (Alexander et al., 2002; Nolan et al., 1999). After washing and fixation the cells were stained with anti-HA antibody to detect the reporter, and also with antibody to the paraflagellar rod, which extends the entire extracellular length of the flagellum

but does not enter the cell body per se (Gull, 1999). Anti-HA staining exactly overlaps the signal from extracellular tomato lectin at a site precisely between the end of the paraflagellar rod and the posterior kinetoplast (Fig. 4, panels M-P, arrowhead) unequivocally establishing the flagellar pocket as the steady state location for the GPI_1 EPMH reporter.

Cell surface localization of the EPMH reporter might be expected as an intermediate step in export and release, but the standard methanol/acetone fixation we use for IFA gave little indication of surface localization (panels A-P). However, low intensity surface staining was seen by an alternative formaldehyde fixation (panels Q-T), but required longer exposures (⩾1 seconds). Therefore, to investigate this issue more carefully immunoelectron microscopy was performed on fixed non-permeable cells (Fig. 5). No staining was observed with anti-EP antibody on untransfected control cells (panel A), but a clear signal of enhanced gold staining was routinely detected on the cell body and flagellum of EPMH expressing cells (panels B-D). Thus, at least a portion of the GPI^1 reporter is present on the cell surface before final release into the media.

In cells expressing EPMHΔgpi the GPI^0 reporter typically presented a reticular pattern of staining (Fig. 6; panels B,F,J) that co-localized predominantly with the ER marker BiP (panel D), but with some discrete staining in the lysosomal region (panel D, arrowhead), and modest co-localization with the lysosomal marker p67 (panel H, arrowhead). However, cells

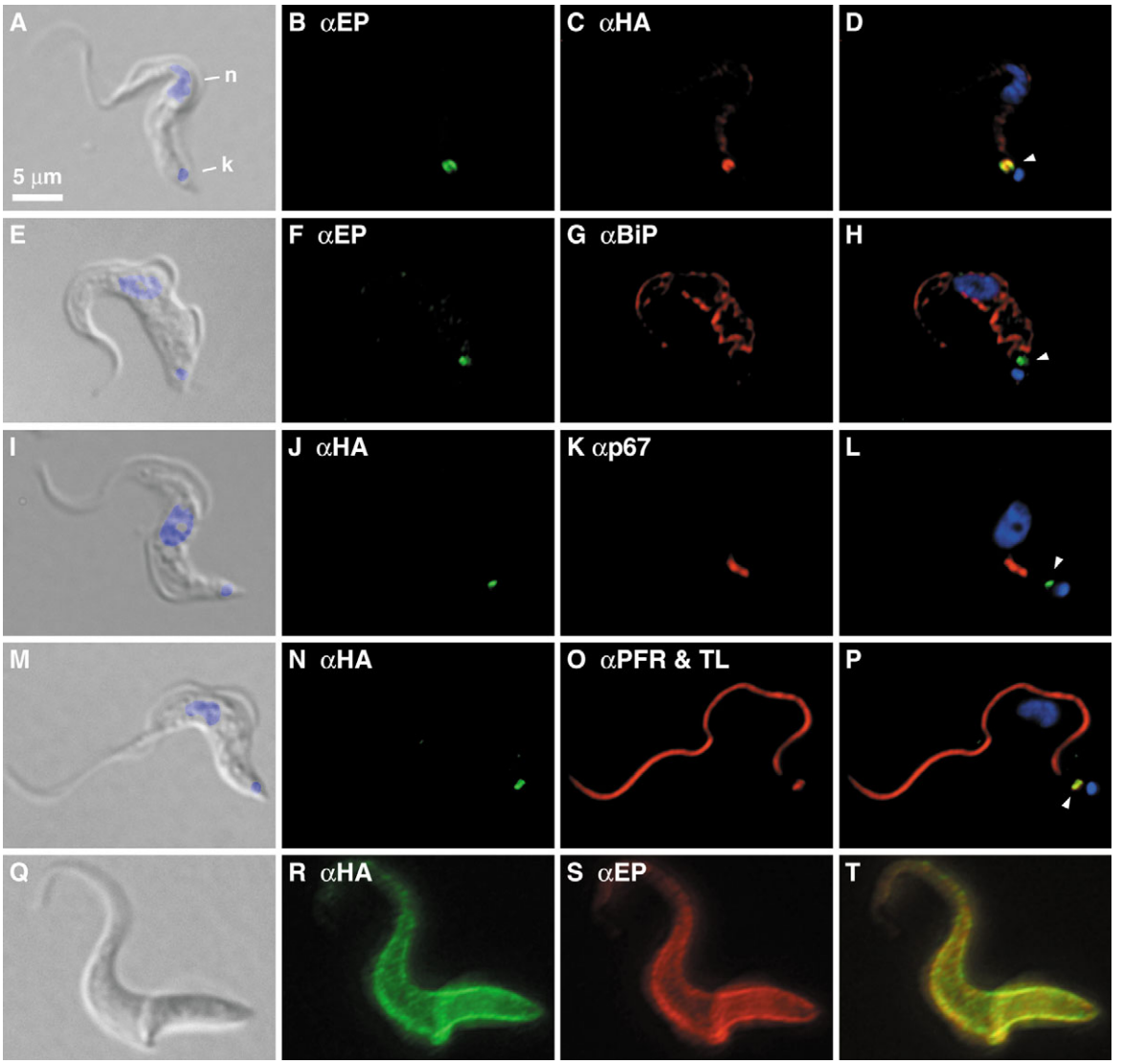

Fig. 4. Immunolocalization of EPMH. (A-P) Methanol/acetone fixed bloodstream cells expressing the GPI^1 EPMH reporter were stained with the indicated primary antibodies and visualized by epifluorescence microscopy. (A-D) Anti-EP_9 and anti-HA. (E-H) Anti-EP_9 and anti-BiP. (I-L) Anti-HA and anti-p67. (M-P) Live trypanosomes were exposed to biotinyl-tomato lectin (TL) at 5°C to allow flagellar pocket binding. After washing, cells were fixed and stained with anti-HA and anti-paraflagellar rod (PFR) antibodies. Paraflagellar rod and tomato-lectin staining were visualized simultaneously in the red channel with appropriate secondary reagents. Three consecutive Z-slices were merged to generate the red channel image. (Q-T) Cells were formaldehyde fixed without permeabilization and stained with anti-HA and anti-EP_9. All panels: left column, merged DIC/DAPI images; right column, merged three channel fluorescent images (arrowheads indicate flagellar pocket localization). DAPI staining (blue) reveals nucleus (n) and kinetoplast (k) localization (indicated in panel A only).

were occasionally observed in which more robust colocalization with p67 was evident (panel L, arrowhead). When cells were pretreated with FMK024 to block lysosomal degradation the reporter signal in the ER was diminished and elevated post-nuclear staining (Fig. 6, panels M-P) was seen in a region that also stained prominently for the lysosomal marker p67 (Fig. 6, panels Q-T). The co-localization of EPMHΔgpi and p67 is not precise, rather it interdigitates as if the enlargement of the lysosomal compartment induced by FMK024 separates the signals deriving from membrane-bound p67 and the soluble reporter. We have seen a similar phenomenon when comparing the localization of endocytosed transferrin and p67 in FMK024-treated cells (Alexander et al., 2002). It is not clear why the ER signal diminishes with FMK024 treatment (identical exposure times were used for all anti-EP_9 images), but we have also seen a similar, albeit less pronounced, effect with GPI-minus VSG (Triggs and Bangs, 2003). Overall these localizations are consistent with the pulse-chase data indicating that GPI^0 EPMHΔgpi is delivered to the lysosome where it is subject to degradation by endogenous protease activities

Behavior of other GPI^1 reporters in bloodstream trypanosomes

To confirm that the behavior of the EPMH reporters was not an artifact of the extreme polyanionic EP procyclin sequence we constructed additional GPI^1 reporters based on two endogenous proteins of the secretory pathway, the lumenal ER marker BiP and the lysosomal membrane protein p67 (see Fig. 1B). We have previously demonstrated that the soluble N-terminal ATPase domain of BiP (BiPN) is secreted from bloodstream trypanosomes with an ~50% recovery in the media fraction (Triggs and Bangs, 2003). Conversely we have shown that the soluble lumenal domain of p67 (p67ΔTM) fails to be secreted from bloodstream stage parasites, rather it is quantitatively delivered to the lysosome where it is degraded (Alexander et al., 2002). Both of these soluble reporters were fused to the HA-tag (H) and EP procyclin (P) GPI addition sequence (see Fig. 1C) to generate GPI^1-anchored BiPNHP and p67HP reporters.

In pulse chase analyses the BiPNHP reporter (Fig. 7A) was first detected as a discrete precursor (lane 1, ~55 kDa) that was converted to a larger mature form during subsequent intracellular transport (lanes 2-5). Ultimately the mature form was released into the media with high efficiency (>80%, lanes 6-10). Treatment with FMK024 to block lysosomal degradation did not alter the pattern of processing or recovery of the mature form (Fig. 7B). BiP has no *N*-glycosylation sites, therefore it is likely that conversion to the mature form is due to GPI modification as we suggested above for the EPMH reporter. As is the case for EPMH, the nature of this GPI modification is unknown. These results support the observation made with EPMH that a single GPI anchor cannot prevent release from cells. However, since soluble (GPI^0) BiPN is secreted from bloodstream trypanosomes, we wished to test this more rigorously with a reporter that is not normally secreted, p67. The p67HP reporter behaved in a manner qualitatively similar with that of the EPMH and BiPNHP reporters (Fig. 7C). It is initially detected as an ~100 kDa glycoform (lane 1) and is processed to a larger mature form (lanes 2-5) that is subsequently released from cells (~15%, lanes 6-10). Processing to the mature form is due to extensive addition of pNAL to *N*-linked glycans (Alexander et al., 2002).

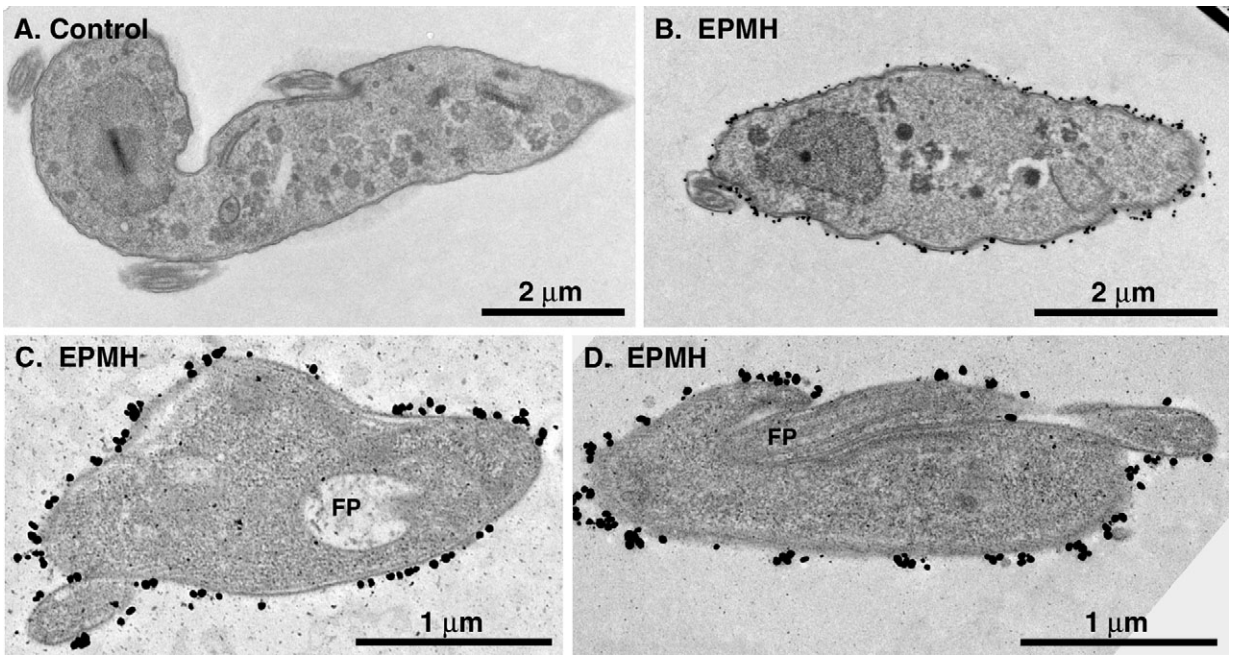

Fig. 5. Surface localization of EPMH by immuno-electron microscopy. Fixed cells were stained with monoclonal anti-EP antibody and colloidal gold-conjugated secondary antibodies prior to silver enhancement and preparation for electron microscopy. Untransformed BS221 cells (A) were used as a negative control for cell lines expressing EPMH (B-D), Note that this fixation technique does not allow detection of reporters in the lumen of the flagellar pocket (FP).

FMK024 treatment did not increase the fractional recovery of released p67HP, but the cell-associated fraction was increased indicating that most of the GPI-anchored reporter is still delivered to the lysosome (Fig. 7D). Collectively these results with exogenous reporters indicate that a single GPI anchor is sufficient to rescue secretory reporters, either in part or in full, from lysosomal targeting in bloodstream stage trypanosomes, but is not sufficient to prevent release from the cell surface.

Behavior of endogenous TfR

Finally we investigated the fate of TfR, an endogenous GPI[1] protein (Fig. 8A). Initially TfR is detected as a doublet of the ESAG7 and the immature ESAG6 subunits (lane 1). During the chase ESAG6 is converted to the mature form (lanes 2-5) by *N*-glycan processing (Steverding et al., 1994). Both forms coordinately disappear from the cell fraction during the chase period ($t_{1/2}$ ~1.5 hours), but very little (<5%) is recovered in the media. FMK024 treatment completely abolishes turnover of the TfR subunits without affecting the level of released receptor (Fig. 8B). Consistent with the findings of Steverding et al. (Steverding et al., 1995), these results indicate that the predominant fate of TfR is lysosomal degradation.

The level of endogenous TfR expression [2-3×10^3 molecules per cell (Steverding et al., 1995)] is relatively low compared with the expected levels of the various reporter constructs from our constitutive expression vector. To test if elevated expression of TfR would affect its relative distribution between lysosomal degradation and shedding we used transferrin starvation as a means to induce higher synthesis rates. Starvation can be achieved by culture in serum with low affinity transferrin ligand, e.g. canine serum,

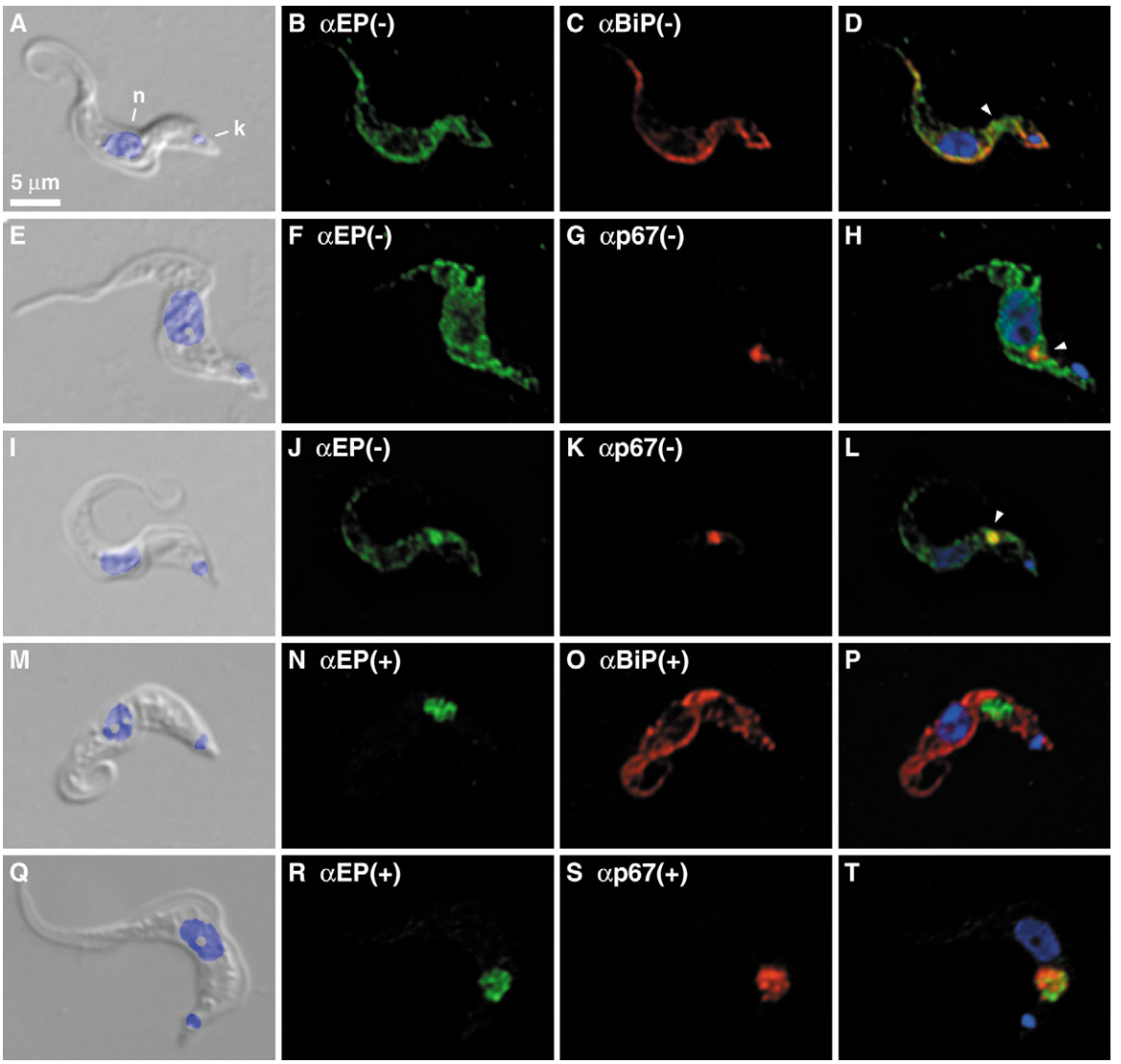

Fig. 6. Immunolocalization of EPMHΔgpi. Bloodstream cells expressing EPMHΔgpi were mock-treated (panels A-L; –) or cultured with FMK024 for 1 hour (panels M-T; +). Cells were fixed/permeabilized, stained with the indicated antibodies, and visualized by epifluorescence microscopy. (A-D,M-P) Stained with anti-EP_9 and anti-BiP antibodies. (E-L,Q-T) Stained with anti-EP_9 and anti-p67 antibodies. All panels: left column, merged DIC/DAPI images; right column, merged three channel fluorescent images (arrowheads indicate lysosomal localization). DAPI staining (blue) reveals nucleus (n) and kinetoplast (k) localization (indicated in A only).

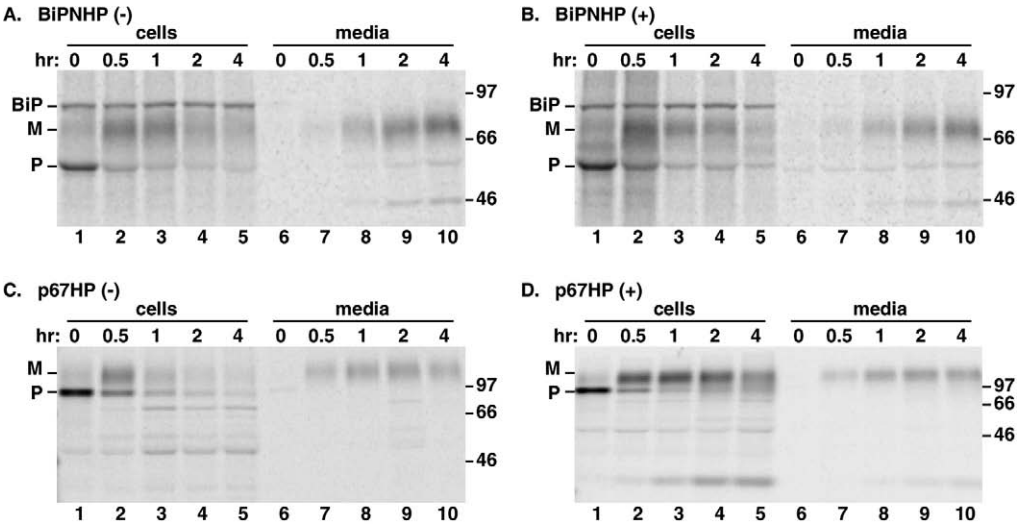

Fig. 7. Fate of other GPI_1 reporters. Bloodstream trypanosome cell lines expressing BiPNHP (A,B) or p67HP (C,D) in the absence (–) or presence (+) of FMK024, were pulse (15 minutes)-chase radiolabeled. Cell and media fractions were immunoprecipitated with specific anti-BiP (A,B) or anti-HA (C,D). All samples were analyzed by SDS-PAGE and phosphorimaging. Mobilities of precursor (P) and mature (M) forms of the BiPNHP and p67HP reporters, and of endogenous full-length BiP are indicated on the left of A-D.

resulting in a ~fivefold elevation in steady state TfR levels (Mussman et al., 2004; Mussman et al., 2003). We too find that culture (48 hours) in canine serum elicits an approximately sevenfold to tenfold increase in steady state TfR relative to cells cultured in bovine serum (estimated by immunoblotting of serially diluted cell extracts; data not shown). Supplementation with bovine holotransferrin greatly reduced this effect. Starved and control cells were subjected to pulse-chase analysis to determine the fate of up-regulated TfR (Fig. 8C). Transferrin starvation increased the de novo synthesis of both subunits 14-fold (compare lanes 1 and 5), and transferrin significantly reduced this effect (lane 9). However, the recovery of released TfR (T_4 media fraction) did not increase as a percentage of initial synthesis (T_0 cell fraction) in the induced cells (lanes 5 and 8, 2%) relative to control cells (lanes 1 and 4, 7%). Thus, increased synthesis does not result in more shedding, despite the fact that elevated TfR expression results in prominent surface localization (Fig. 9B,C) as was originally demonstrated by Mussman et al.

Given our findings with the recombinant GPI^1 reporters it seemed contradictory that increased surface expression would not also lead to increased release of the heterodimeric TfR. To test whether this surface receptor was actually functional, live cells were exposed to Tf:gold conjugates at 8°C to minimize endocytosis and binding was visualized by electron microscopy. Gold particles were found in the flagellar pocket and nearby endosomal elements in uninduced cells (Fig. 9D,G) and likewise to a much greater extent in transferrin-starved cells (Fig. 9E,H). However, under neither condition was binding seen on the external flagellum or cell surface. Competition with holotransferrin completely blocked Tf:gold binding (Fig. 9F,I). These results strongly suggest that surface TfR in starved cells is not functional heterodimer. A possible

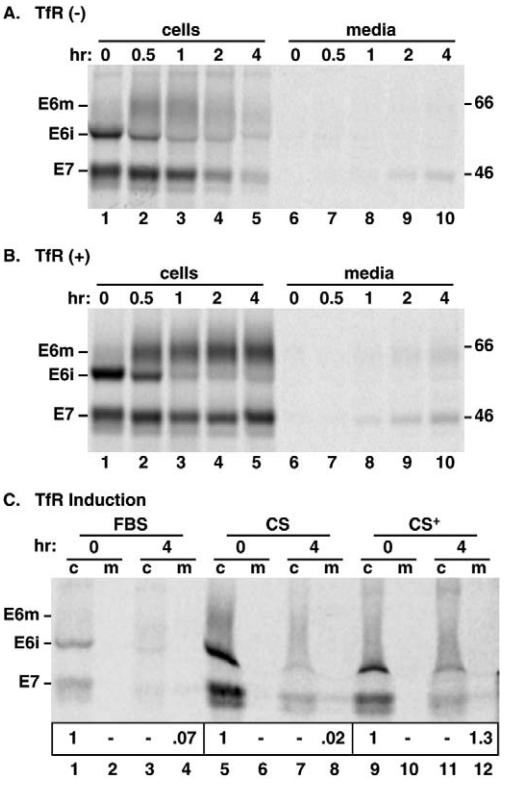

Fig. 8. Upregulation of TfR expression. Bloodstream 221 cells were pulse-chase radiolabeled as in Fig. 7 in the absence (A) or presence (B) of FMK024, and cell and media fractions were prepared at the indicated times. TfR polypeptides were immunoprecipitated with anti-TfR and analyzed by SDS-PAGE and phosphorimaging. (C) Bloodstream 221 cells were cultured 48 hours in HMI9 media containing 10% fetal bovine serum (FBS), 10% canine serum (CS), or 10% canine serum plus 200 μg/ml bovine transferrin (CS^+). Cells were then pulse labeled (15 minutes) and chased under the same conditions. Cell (c) and media (m) fractions were prepared at 0 and 4 hours and analyzed as above. Fractional recovery of released ESAG7 polypeptide for each set is presented below (boxed). The relative amounts of initial ESAG7 are 1.0:14.0:6.1 for FBS (lane 1):CS (lane 5):Cs^+ (lane 9). Distortions of lanes 5-12 are reproducible and probably result from non-specific precipitation of components of dog serum in lanes 6, 8, 10 and 12. (A-C) Mobilities of mature and immature forms of ESAG6 (E6m and E6i) and ESAG7 (E7) are indicated on the left. Mobilities of molecular mass standards are indicated on the right. All lanes contain 10^7 cell equivalents.

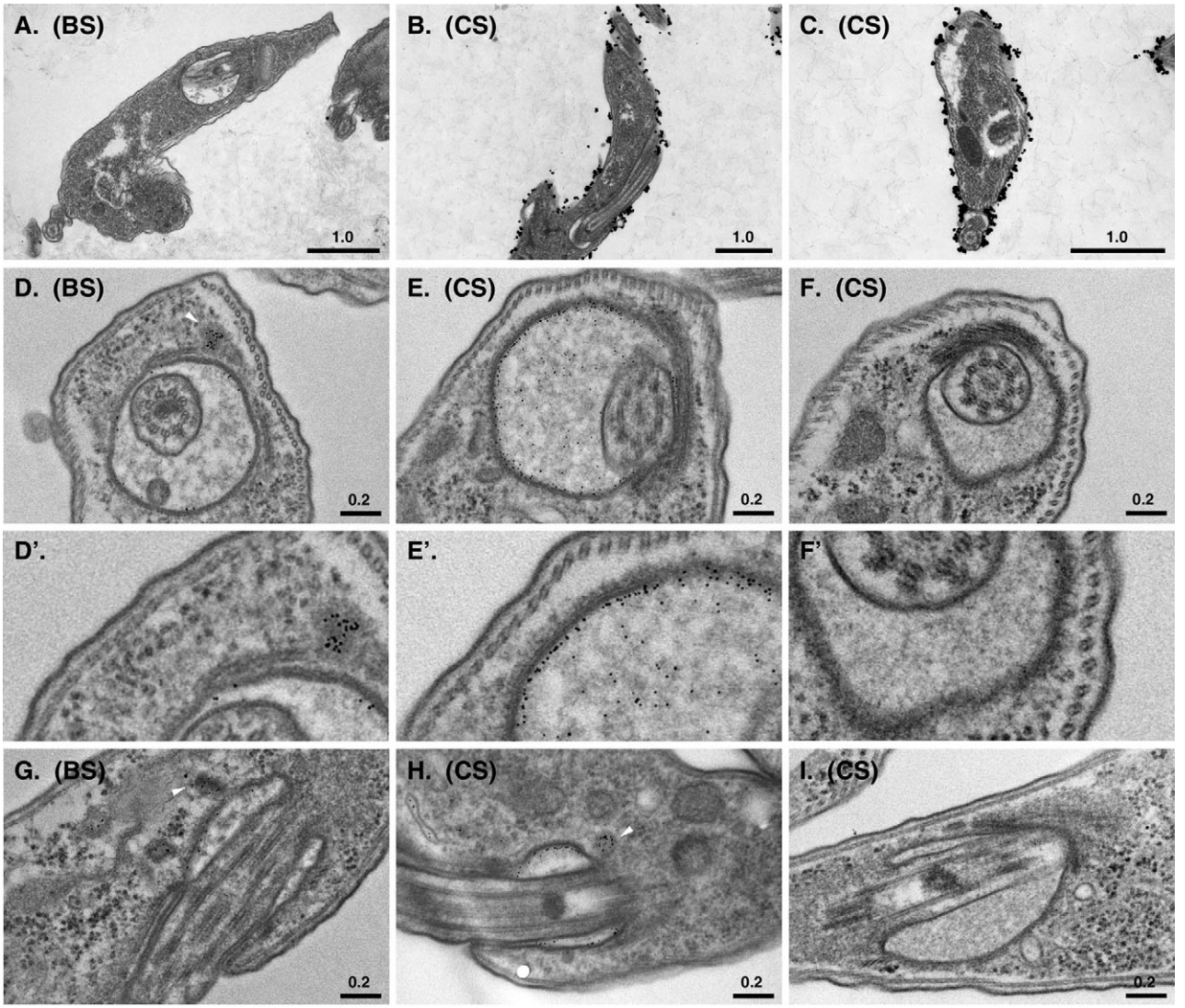

Fig. 9. Surface localization of TfR. Bloodstream 221 cells were cultured 48 hours in HMI9 media containing 10% fetal bovine serum (BS, panels A,D,G) or 10% canine serum (CS, panels B,C,E,F,H,I). (A-C) Fixed cells were stained with anti-TfR antibody and colloidal gold-conjugated secondary antibodies prior to silver enhancement and preparation for electron microscopy. Note that this fixation technique does not allow detection of TfR in the lumen of the flagellar pocket (FP). (D-I) Live cells were incubated with Tf:gold (5 nm) conjugate at 8°C and prepared for electron microscopy as described in Materials and Methods. 1 mg/ml bovine holotransferrin was included in F,I as a competition control for specific binding. Arrowheads indicate endosomal elements containing Tf:gold. Size scales are indicated in μm. Selected regions of panels D, E and F are magnified 2× in panels D′, E′ and F′, respectively.

explanation for these seemingly contradictory results is proposed below.

Discussion

To assess the apparent correlation between GPI valence and the progression of membrane proteins in bloodstream trypanosomes we engineered GPI^1 and GPI^0 reporters based on monomeric GPI-anchored EP procyclin. The behavior of these constructs in procyclic cell lines was consistent with both endogenous procyclins and other transgenic GPI-minus reporters (Bangs et al., 1997; McDowell et al., 1998) validating their use as secretory reporters. In bloodstream cells their behavior was generally in accordance with the 'valence correlation'. Similar to GPI-minus VSG (Triggs and Bangs, 2003), EPMHΔgpi is largely delivered to the lysosome. And similar to endogenous TfR, GPI-anchored EPMH has a steady state localization in the flagellar pocket and on the cell surface. To our surprise however, the EPMH reporter is released from cell membranes with an intact GPI anchor, a phenomenon that we have confirmed with the additional GPI^1 reporters, BiPNHP and p67HP. We have also investigated the fate of TfR in bloodstream trypanosomes and find little evidence for significant release of this endogenous GPI^1 protein, rather it is preferentially delivered to the lysosome and degraded, consistent with previous reports (Steverding et al., 1995). Collectively then, there is a decreasing order for release of GPI^1 reporters (BiPN>EPMH>p67HP>TfR) that correlates

inversely with delivery to the lysosome. What controls parsing between these alternative fates is not clear, but it is likely that structural differences strongly influence the process since these reporters all have distinct profiles: BiPNHP is a compact globular structure without *N*-glycosylation; EPMH is highly polyanionic with a single pNAL-modified *N*-glycan; p67HP has 14 *N*-glycans with extensive pNAL modification; and TfR is a bulky *N*-glycosylated heterodimer, also with pNAL modifications.

Release of EPMH with an intact GPI anchor was initially surprising, however, there is precedence for desorption of GPI[1] proteins from trypanosomal membranes. Ultrastructual studies show that TfR is dispersed within the lumen of the flagellar pocket, unlike VSG, which is intimately associated with the pocket membrane (Geuskens et al., 2000; Salmon et al., 1994; Steverding et al., 1994). The behaviors of both TfR and our GPI[1] reporters are likely related to the exclusive use of myristate (C-14:0) in the diacylglycerol portion of the bloodstream form GPI anchor (Buxbaum et al., 1994; Masterson et al., 1990). The biological effect of this eccentricity is readily extrapolated from the desorption of free and macromolecule-bound phospholipids from model bilayer membranes. Dimyristoyl phosphatidylethanolamine desorbs from membranes with a rate ($t_{1/2}$ ~14 minutes at 37°C) consistent with the extent of release observed for GPI[1] reporters in our system, and furthermore, attachment of bulky hydrophilic head groups, e.g. apotransferrin, significantly enhances the rate of desorption (Silvius and Leventis, 1993; Silvius and Zuckerman, 1993). Lengthening the acyl chains by two (C-16:0, palmitoyl) or four (C-18:0, stearoyl) methylene groups reduces the PE desorption rate ~60-fold and ~1500-fold, respectively. It is logical then that the EPMH reporter is not shed from procyclic trypanosomes because this stage of the lifecycle synthesizes a more hydrophobic lysoacylglycerol (C-18:0), inositol acylated (mixed C-16:0 and C-18:0), GPI anchor (Treumann et al., 1997), and also because the lower temperature (27°C) of procyclic cultures will decrease the desorption rate (Silvius and Leventis, 1993). A strong biological imperative for why native VSG is a dimer can also be inferred. If bloodstream form trypanosomes have a surface glycocalyx composed of a single major GPI-anchored protein, and if that anchor is composed uniquely of dimyristoylglycerol, then dimerization is essential to increase the avidity of membrane association. Indeed, VSG is an extremely stable protein, turning over with a halftime in excess of 30 hours (Bulow et al., 1989; Seyfang et al., 1990), and yet low-level transfer of intact VSG to neighboring erythrocyte membranes can still be detected (Rifkin and Landsberger, 1990). It is also worth noting that monovalent GPI-anchored lipophosphoglycan with a lysoacylglycerol chain length of C-24:0 to C-26:0 is also readily shed from the surface of the related kinetoplastid parasite *Leishmania* (Ilg et al., 1992).

We propose a simple model for the general behavior of GPI[1] proteins in bloodstream stage parasites based on the documented trafficking of VSG and endocytic cargo (Engstler et al., 2004; Grünfelder et al., 2003; Overath and Engstler, 2004). This model assumes that dissociation/reassociation from membranes can occur at any point during trafficking through the secretory/endocytic pathways, but dissociation at extracellular membranes is effectively irreversible because serum lipoproteins will act as a sink for monomer transfer (Silvius and Zuckerman, 1993). A GPI[1] reporter may arrive at the flagellar pocket in either a membrane-associated or soluble state. If membrane-associated it may diffuse laterally out onto the surface of the cell body, from which it will dissociate with a finite half-life. If already dissociated, it may then exit via the confined opening of the pocket. Alternatively, the GPI[1] reporters can be endocytosed in either free or membrane-bound states and delivered to endosomal compartments for subsequent sorting. Reporters in the free state when they arrive at the Rab11$^+$ recycling endosome are likely to be included in the Class II clathrin-coated vesicles that mediate trafficking to the late endosomal and lysosomal compartments as demonstrated for the fluid-phase markers, ferritin and horseradish peroxidase (Engstler et al., 2004). GPI[1] reporters that are membrane-bound at this point will be excluded from coated vesicles and returned by default to the flagellar pocket via exocytic carriers as shown for endogenous VSG (Overath and Engstler, 2004). It is also possible that membrane association of internal GPI[1] reporters is favored by the close proximity of enclosing membranes, and that GPI[1] proteins are just not as effectively excluded from coated vesicles as is GPI[2] VSG. In either case, recycling and sorting will continue until any single reporter molecule is either released into the media or delivered to the lysosome for degradation. An additional layer of cargo sorting may take place at the flagellar pocket, where soluble GPI[1] reporters may be at a disadvantage for exit. There is little evidence for release of soluble secretory proteins in bloodstream stage parasites. Indeed, the only soluble reporter we have found to be effectively secreted is the truncated BiPN reporter (Triggs and Bangs, 2003). Other soluble reporters, e.g. the lumenal domain of p67 or GPI-minus VSG, are primarily delivered to the lysosome (Alexander et al., 2002; Triggs and Bangs, 2003). Thus membrane attachment may facilitate lateral diffusion onto the cell surface, whereas soluble reporters are more prone to endocytic uptake. Another factor that may contribute to retention of soluble cargo is the poorly defined ground substance of the flagellar pocket lumen. The pocket is rich in extensive pNAL-bearing glycoconjugates (Nolan et al., 1999) and it has been suggested that these glycans may form a ‘gel-like’ matrix that could retard egress (Atrih et al., 2005).

Our results are directly relevant to the recent finding that transferrin starvation induces upregulation of native TfR (ESAG6/ESAG7 heterodimer) expression, leading to spill-over of receptor onto the surface of bloodstream stage parasites (Mussman et al., 2004; Mussman et al., 2003), a result that we have fully reproduced here. This was interpreted to indicate a saturable mechanism for TfR retention in the flagellar pocket, although no evidence for a ‘TfR receptor’ exists. Nevertheless, a retention mechanism could explain why, of all our GPI[1] reporters, native TfR is the least likely to be released from intact bloodstream trypanosomes. However, some explanation is also required for why spill-over does not lead to increased shedding of TfR from the cell surface, when our results so clearly indicate that a single GPI anchor will not maintain cell association. One possibility stems from the ability of both ESAG6 and ESAG7 to homodimerize when expressed independently (Salmon et al., 1994). Nothing is known about the relative affinities for homotypic versus heterotypic subunit association, but native homodimerization cannot be excluded given that the two ESAGs are nearly identical in sequence. If

so, then GPI^0 ESAG7 homodimers should be delivered to the lysosome and degraded, as was observed for heterodimers of ESAG7 and GPI-minus ESAG6 (Biebinger et al., 2003) and for GPI-minus VSG (Triggs and Bangs, 2003). And, like native VSG, GPI^2 ESAG6 homodimers should be stably cell-associated, which would account for surface localization without significant shedding into the media. Consistent with this explanation, and with the fact that ESAG6 homodimers are non-functional (Salmon et al., 1994), we are unable to detect direct binding of Tf:gold to the cell surface of transferrin-starved cells, despite the presence of abundant immuno-reactive material over the entire surface. Very similar results were presented by Mussman et al. (Mussman et al., 2003), although they did detect low-level binding of biotinyl-Tf to the surface of transferrin-starved cells. However, specific binding was restricted to the flagellar pocket opening and the proximal membrane of the flagellum, and was conspicuously absent from the larger cell body. Collectively then, the lack of transferrin binding and the lack of TfR shedding strongly argue against the presence of significant amounts of functional GPI^1 TfR heterodimers on the surface of transferrin-starved cells. Additional work will be required to confirm that ESAG6 homodimers are present on the cell surface.

In summary, our work confirms that GPI valence strongly influences post-Golgi trafficking of membrane proteins in bloodstream stage trypanosomes. Furthermore, these effects cannot be ignored in interpreting the behavior of native GPI^1 proteins, i.e. procyclin and TfR, when expression is induced in this stage of the lifecycle. A future area that requires attention is the fate of additional reporters with a GPI^2 valence and this work is already underway in our lab. Consequently our understanding of the role of GPI anchors in protein trafficking in these important pathogens should soon come into sharper focus.

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