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Variant Surface Glycoprotein, Transferrin Receptor, and ERAD in *Trypanosoma brucei*

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SUMMARY

Variant surface glycoprotein (VSG) is central to antigenic variation in African trypanosomes. VSG is highly abundant and stable. Although much prior work documents that VSG is efficiently synthesized and exported to the cell surface, it was recently claimed that 2–3x more is synthesized than required, the excess being eliminated by ER Associated Degradation (ERAD) (Field et. al, *PLoS One* 5: e8468, 2010). We reinvestigate turnover of multiple VSGs by steady state and biosynthetic protocols and find no evidence for rapid degradation, consistent with a model whereby VSG synthesis is precisely regulated to match requirements for a functional surface coat on each daughter cell. However, using a mutated version of the ESAG7 subunit of the transferrin receptor (E7:Ty) we confirm the presence of functional ERAD in trypanosomes. E7:Ty fails to assemble into functional receptors and accumulates in the ER, consistent with retention of misfolded protein, and its turnover can be rescued by the proteasomal inhibitor MG132. These data, along with the presence of ERAD orthologues in the *T. brucei* genome, confirm ERAD in trypanosomes. We discuss scenarios in which ERAD could be critical to bloodstream parasites, and how these may have contributed to the evolution of antigenic variation in trypanosomes.

Keywords

variant surface glycoprotein; transferrin receptor; trypanosome; antigenic variation; ERAD

INTRODUCTION

African trypanosomes (*Trypanosoma brucei* spp.) are anciently derived eukaryotic parasites that cause human African trypanosomiasis (sleeping sickness). They are transmitted by tsetse flies (genus *Glossina*) and replicate by binary fission in the bloodstream and extracellular tissues of the mammalian host. Bloodstream form (BSF) trypanosomes survive by a process of antigenic variation in which serologically distinct populations of parasites arise, only to be eliminated by corresponding acquired host immune responses (Schwede et al., 2010, Rudenko, 2011). Following each wave of parasitemia new variant populations emerge necessitating a new immune response, a process that continues until the eventual demise of the host. The linchpin of this process is the variant surface glycoprotein (VSG)

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(Cross, 1975) [extensively reviewed in (Cross, 1996, Morrison *et al.*, 2009, Schwede *et al.*, 2010, Manna *et al.*, 2014a) to cite just a few]. VSG is a homodimeric (Auffret *et al.*, 1981), N-glycosylated (Holder, 1985), glycosylphosphatidylinositol (GPI) anchored (Ferguson *et al.*, 1988) surface protein. Extraordinarily abundant, $\sim 5 \times 10^6$ VSG dimers ($\sim 10\%$ of total cell protein) make a monolayer that completely covers the external cell surface protecting the BSF parasite from host-derived antibodies targeting underlying invariant antigens (Vickerman, 1969). It is the VSG-specific adaptive immune response that limits each wave of parasitemia. The *T. brucei* genome has >1000 *VSG* genes and pseudogenes, but only one is transcribed at a time from a single active telomeric Expression Site (ES, ~ 15 per genome) (Hertz-Fowler C, 2008). Antigenic variation by combined mechanisms of sequential monoallelic expression and segmental gene conversion insures that each parasite has an essentially unlimited repertoire of serotypes (Hall *et al.*, 2013, Mugnier *et al.*, 2015).

VSG protein is synthesized on membrane bound ribosomes (Lheureux *et al.*, 1979) and imported into the ER where it is subject to rapid addition of the GPI anchor and N-glycans (Bangs *et al.*, 1985, Bangs *et al.*, 1986, Ferguson *et al.*, 1986). C-terminal GPI addition, which requires completed translation and translocation, is so rapid that in some instances it can occur before addition of upstream N-glycans is finished (Bangs *et al.*, 1986). During this early phase of post-translational processing VSG is in transient physical association with the ER molecular chaperone, BiP (Bangs *et al.*, 1996). Other typical eukaryotic ER quality control machinery such as calreticulin, glucosidase II, UDP-glucose:glycoprotein glucosyltransferase and protein disulfide isomerase (Conte *et al.*, 2003, Jones *et al.*, 2005, Schwartz *et al.*, 2005, Izquierdo *et al.*, 2009) have been characterized in trypanosomes, and additional putative chaperones have been identified in the genome by homology (Engstler *et al.*, 2006, Field *et al.*, 2010). The extent to which any of these contribute to VSG folding is not clear, but it is likely that the resident ER quality control machinery beyond BiP does participate in this process. However, even in the absence of chaperones purified VSG has intrinsically reversible denaturation kinetics, albeit with intact internal disulfide bonds, suggesting that VSG folding is inherently favorable in energetic terms (Rehaber *et al.*, 1990).

Once folding and dimerization of newly synthesized VSG is complete, transport via the Golgi to the cell surface is very rapid ($t_{1/2} \sim 15$ min) (Bangs *et al.*, 1986, Duszenko *et al.*, 1988). Pulse-chase analyses have consistently demonstrated that all VSG is delivered to the cell surface within one hour of synthesis, indicating that the combined processes of assembly and transport are exceptionally efficient (Bangs *et al.*, 1986, Duszenko *et al.*, 1988, Hall *et al.*, 2005, Hall *et al.*, 2006, Allen *et al.*, 2007, Sutterwala *et al.*, 2008, Sevova *et al.*, 2009). ER exit is dependent on the GPI-anchor (Böhme *et al.*, 2002, Triggs *et al.*, 2003), and selection into transport vesicles at ER exit sites is mediated by a specific subset of COPII coat proteins (Sevova *et al.*, 2009). Once incorporated into the surface coat VSG is a dynamically stable protein. It is constantly endocytosed, but is efficiently recycled to the plasma membrane (Engstler *et al.*, 2004) such that it is lost from cells with a half time of more than five cell cycles ($t_{1/2} > 30$ hr) (Bulow *et al.*, 1989, Seyfang *et al.*, 1990).

The transferrin receptor (TfR) of BSF trypanosomes, which is structurally related to VSG, is a heterodimer of ESAG6 (E6) and ESAG7 (E7) subunits that form ~ 3000 functional

receptors per cell (Steverding *et al.*, 1995). They are almost identical from N-termini to the C-terminus of E7, but E6 is longer and has a C-terminal GPI attachment peptide (Ligtenberg *et al.*, 1994, Salmon *et al.*, 1994). *E6* and *E7* genes are encoded at the 5' end of all *VSG* expression sites (Hertz-Fowler C, 2008) (Fig. 1) and are transcribed coordinately from the active ES promoter, although ~20% of all steady state TfR mRNA comes from low-level transcription of the other 'inactive' ESs (Ansorge *et al.*, 1999). Unlike *VSG*, which has two GPI anchors and localizes to the cell surface, TfR has a single GPI and localizes to the flagellar pocket and endosomal compartments. It has been proposed that these differences in GPI valence are the primary determinant for post-Golgi trafficking of these two essential BSF membrane proteins (Schwartz *et al.*, 2005). Alternatively, it has been argued that TfR is prevented from accessing the external plasma membrane by a saturable retention mechanism in the FP (Mussman *et al.*, 2003, Mussman *et al.*, 2004).

That *VSG* is the critical virulence factor contributing to the success of the infectious bloodstream stage of the life cycle is indicated by RNAi knockdown experiments (Sheader *et al.*, 2005, Smith *et al.*, 2009). Specific silencing of *VSG* expression leads to a rapid pre-cytokinesis cell cycle arrest and a global shutdown of protein translation. Co-expression of a different *VSG* rescues these phenotypes. Interestingly, expression of the native *VSG* in these dual expressing cells is reduced in compensation for the presence of the new coat protein. These findings suggest that trypanosomes have an innate bookkeeping system for maintaining a fixed steady state level of total *VSG*. This is intuitively logical since trypanosomes devote ~10% of total protein biosynthetic capacity to the production of this single molecule - there must be strong selection pressure to produce only what is needed to generate a complete surface coat for each daughter cell.

However, it has recently been claimed that trypanosomes actually synthesize up to 3-fold more *VSG* than needed to form a functional surface coat (Field *et al.*, 2010, Manna *et al.*, 2014b). The basis for this claim is that short-term pharmacological inhibition (MG132) of proteasomal activity resulted in an apparent and dramatic increase in steady state *VSG* levels. These authors concluded that *VSG* folding is an inherently inefficient process commonly leading to misfolded polypeptides that are eliminated by the eukaryotic ER-Associated Degradation (ERAD) pathway (Brodsky, 2012, Hampton *et al.*, 2012), which involves recognition and retro-translocation from the ER, ubiquitinylation, and proteasomal degradation in the cytosol. This is certainly possible as the core ERAD machinery is present in the *T. brucei* genome (Engstler *et al.*, 2006, Field *et al.*, 2010). However, if this were the case one would expect rapid loss of a large portion of all newly synthesized *VSG*; a prediction that is not supported by any of the aforementioned pulse-chase analyses of *VSG* synthesis. Our original investigation of *VSG* synthesis did indicate a slow loss of *VSG* (~25%) in the hour immediately following initial biosynthesis (Bangs *et al.*, 1986) (see Fig. 1 therein), but this is insufficient to account for the rapid turnover of up to 2/3rd of all newly synthesized *VSG* as claimed by (Field *et al.*, 2010). Likewise no evidence for significant turnover of newly synthesized *VSG* was apparent in the contemporaneous studies of other investigators (Ferguson *et al.*, 1986). Coupled with the well-established efficiency of *VSG* transport to the plasma membrane, these data are incompatible with significant retro-translocation and cytoplasmic degradation of newly synthesized *VSG*.

In this work we reevaluate the issue of VSG folding and turnover in the early secretory pathway with the purpose of resolving these conflicting data sets. In full agreement with the overwhelming amount of published data on VSG biosynthesis, we find that endogenous native VSGs are not subject to ERAD – rather newly synthesized VSG is rapidly and quantitatively folded and exported from the ER. These results are consistent with the notion that VSG has evolved to be expressed and incorporated into the surface coats of daughter cells with maximum efficiency. However, in the process of ongoing unrelated studies investigating intracellular trafficking of TfR we have generated a ‘failed’ epitope tagged E7 subunit that display all the hallmarks of ERAD substrates. We use this fortuitous reporter to confirm for the first time that the ERAD process is functional in trypanosomes. Finally, we propose circumstances under which ERAD could be critical to survival of bloodstream form parasites, and how this may have contributed to the evolution of antigenic variation as a means to avoid host acquired immunity.

RESULTS

Native VSG is not subject to ERAD

We have reinvestigated synthesis and short-term turnover of VSG by pulse-chase metabolic radiolabeling using the same basic protocol as in our prior work (Bangs *et al.*, 1986). BSF parasites expressing VSG221 [the same variant antigenic type as used by (Field *et al.*, 2010)] were pulse radiolabeled for 2 minutes and the fate of radiolabeled VSG during the chase was assessed by immunoprecipitation, SDS/PAGE and phosphorimaging (Fig. 2A). No loss of newly synthesized VSG was observed during the 1 hour chase period, which is equivalent to ~4 half-lives for VSG transport to the cell surface (Bangs *et al.*, 1986, Duszenko *et al.*, 1988). At this point essentially no VSG synthesized during the pulse should remain in the ER. As retrotranslocation out of the ER to the cytosol would be expected to be a relatively early event, these data confirm that assembly and forward trafficking of VSG221 from the ER is an extremely efficient process. These pulse-chase data are generally consistent with our original observations (Bangs *et al.*, 1986), although the slow loss of VSG in that study was not evident here. At least three factors likely contribute to this minor discrepancy: 1) use of culture-adapted trypanosomes with more appropriate media (Hirumi *et al.*, 1994); 2) use of more sensitive quantification technology (phosphorimaging vs. fluorography/densitometry); and 3) minor variation between different VSGs (VSG221 herein vs. ILTat1.3 in our prior work). In any case, neither data set is compatible with 3-fold excess synthesis of VSG followed by ERAD-mediated turnover as proposed by (Field *et al.*, 2010).

We next attempted to verify the effect of selective inhibitors of the proteasome (MG132 and lactacystin) on steady state levels of cell associated VSG. In our hands the concentration of MG132 (20 µg/ml, 42 µM) used in the Field *et al.* (2010) study was lethal in short-term (4 hr) BSF cultures. Therefore, we used an empirically determined short-term maximum non-trypanocidal concentration (25 µM). Lactacystin was used at a concentration (5 µM) previously validated in trypanosomes (Van Hellemond *et al.*, 2000). As a control the thiol protease inhibitor FMK024 was used at a concentration (20 µM) sufficient to prevent degradation of lysosomally targeted cargo (Silverman *et al.*, 2013). Log phase BSF trypanosomes expressing VSGs 221, 117 or ILTat1.3 were cultured in the presence of

vehicle or inhibitors, and changes in steady state levels were assessed by western blotting with specific anti-VSG antibodies (Fig. 2B). Simultaneous blotting with antibody to cytoplasmic Hsp70 was used as a loading control. None of these treatments affected steady state levels of either endogenous VSG221 or VSG117 (Fig. 2B & C). There was a small but reproducible increase in ILTat1.3 VSG with FMK024 treatment, which may account for the modest short term turnover seen in our original pulse-chase analyses (Bangs *et al.*, 1986). However, this would be consistent with degradation in the lysosome, likely due to endocytosis of surface VSG. Collectively these results confirm that native wild type VSG is not degraded in any detectable amount by the proteasome, and consequently that it is not normally subject to turnover via the ERAD pathway. Nor is there significant lysosomal turnover of VSGs in the time frame of these experiments, consistent with our previous results (Triggs *et al.*, 2003).

TfR RNAi and expression of epitope tagged ESAG7

The presence of genes for the ERAD machinery in the *T. brucei* genome begs the question of whether this process does function in trypanosomes. Such studies require a bona fide ERAD substrate, which has fortuitously emerged from ongoing studies on TfR trafficking. We developed a system for exclusive expression of an epitope tagged E7 subunit (Fig. 1). First, a pan-specific RNAi cell line targeting all TfR transcripts (both E6 and E7), whether from the active ES (~80%) or the ~15 silent ESs (~20%) was created. An RNAi^R Ty-tagged E7 gene (E7:Ty) was then used to replace the endogenous wild type E7 orf in the active BES1 expression site (Fig. 1). Induction of dsRNA was increasingly inhibitory for growth over a 3-day period (Fig. 3A). The lag in onset of growth inhibition is likely due to a slow depletion of excess intracellular iron stores (Steverding, 1998, Taylor *et al.*, 2010). Expression of E7:Ty did not impact normal growth of the TfR RNAi cell line, but as expected, it was unable to rescue growth when wild type TfR was silenced (Fig. 3A) as there is no partner E6 subunit to form a functional heterodimer. Consistent with this result, TfR silencing specifically abolished endocytosis of Tf in both cell lines (Fig. 3B). No negative effect was observed on uptake of tomato lectin (TL), a surrogate for receptor-mediated endocytosis that binds poly-N-acetyllactosamine glycoconjugates in the flagellar pocket (Nolan *et al.*, 1999, Alexander *et al.*, 2002). There was actually a modest elevation in TL uptake suggesting an increase in, or accessibility to, available TL ligands within the flagellar pocket in the absence of TfR. qRT-PCR analyses confirmed that all wild type E6 and E7 transcripts were effectively knocked down (~80%) in both cell lines when silencing was induced (Fig. 3C). Interestingly, silencing of native E6/E7 in the E7:Ty cell line led to a marked ~10-fold increase in the steady state level of the RNAi^R E7 transcript. Immunoblotting with anti-TfR (Fig. 3D) confirmed the overwhelming depletion of wild type E6 and E7 proteins in the parental RNAi cell line (lane 1 vs. 2). Wild type E6 was also depleted in the E7:Ty cell line (lane 3 vs. 4), and presumably wild type E7 as well, although this was obscured by a dramatic up-regulation of RNAi^R E7:Ty protein. Up-regulation of RNAi^R E7:Ty protein in the absence wild type TfR subunits was confirmed by the matched anti-Ty blot (lanes 7 vs. 8), and mirrors the observed increase in specific message. This phenomenon, which mimics changes in TfR expression seen when cells are shifted from bovine to canine sera (Mussman *et al.*, 2003, Mussman *et al.*, 2004), is discussed below.

Neither the cytoplasmic marker Hsp70 nor the ER marker BiP were affected by TfR silencing, confirming the specificity of TfR loss and E7:Ty up-regulation.

Epitope tagged ESAG7 is non-functional

The functionality of E7:Ty was assessed by a sequential pull down protocol looking for association with native wild type E6, and if so, whether such complexes were capable of binding to transferrin (Fig. 4). Lysates were subjected to primary (1^o) and secondary (2^o) pull down with either anti-Ty or transferrin-conjugated beads (Tf-beads), and then to a final tertiary (3^o) pull-down with anti-TfR, anti-Ty or Tf-beads. All pull-downs were then immuno-blotted with anti-TfR (Panel A) and anti-Ty (Panel B). 1^o and 2^o pull-down with anti-Ty removed essentially all E7:Ty from lysates, with no apparent co-pull down of E6 (Panel A; lanes 1 & 2; 4 & 5), suggesting that E7:Ty is largely unable to heterodimerize with native E6. Subsequent 3^o pulldown with anti-TfR detected stoichiometric amounts of native E6 and E7 (Panel A; lane 6), and minor amounts of E7:Ty (Panel B; lane 6). Similar patterns were seen in 3^o pull-downs with Tf-beads (Panels A & B; lane 3), confirming this as functional E6/E7 TfR heterodimers remaining after removal of E7:Ty. The small level of anti-Ty reactive species present in each 3^o pull-down suggests that a low level of E7:Ty is incorporated into functional heterodimers. This interpretation is supported by the reciprocal pull-downs. 1^o and 2^o selection with Tf-beads removed essentially all functional E6/E7 heterodimers from lysates (Panel A; lanes 7 & 8; 10 & 11), leaving behind large amounts of E7:Ty detected in subsequent 3^o pull downs with anti-Ty or anti-TfR (Panels A & B; lanes 9 & 12, respectively). However, in this case a small level of anti-Ty reactive species was seen in 1^o pull downs with Tf-beads (Panel B; lanes 7 & 10) consistent with inclusion of some E7:Ty in functional heterodimers. Tagged E7:Ty can be discriminated from native E7 by its slower electrophoretic mobility. This is evident when the E7 region of the 1^o Tf-bead pull downs (lanes 7 & 10) of the original two-channel image are digitally segregated into anti-Ty (green) and anti-TfR (red) images representing E7:Ty and total TfR respectively (Panel C). The anti-Ty signal confirms the presence of E7:Ty in functional TfR heterodimers, but the anti-TfR signal indicates that this is a minor fraction of the total E7 in Tf-bead pull-downs. Collectively these data suggest that E7:Ty is largely unable to associate with native E6. Most of the functional TfR is composed of heterodimers of native E6 and E7, but a small portion of E7:Ty does form functional heterodimers. The source of native E7 is likely the 'silent' expression sites (discussed below).

Localization of E7:Ty

Immunofluorescent staining was performed to determine the localization of E7:Ty in the presence and absence of wild type TfR subunits. Without TfR silencing the pattern of staining was variable, but the most common localization of E7:Ty was discrete foci in the post nuclear region, including the flagellar pocket (Fig. 5A, arrowheads). Costaining with the ER marker BiP was never observed. Such localization is typical for native functional TfR, and is consistent with the presence of a low level of functional E6/E7:Ty heterodimers, as observed in the sequential pull-down experiments. In contrast, when native TfR was silenced E7:Ty staining markedly overlapped with BiP. Occasionally discrete foci of non-ER staining in the post-nuclear region was observed (Fig. 5A, star), the significance of which is not clear. These results indicate that when expressed without native E6, E7:Ty localizes at

steady state to the ER. This apparent 'relocalization' is due in large part to the ~10-fold increase in RNAi^R E7:Ty under TfR silencing, but aggregation of misfolded E7:Ty and/or retention by the ER quality control machinery may also contribute. Alternatively, E7 can form non-GPI anchored homodimers (Salmon *et al.*, 1994) and, by analogy to GPI-minus VSG (Schwartz *et al.*, 2005), these might be expected to have delayed forward trafficking with consequent ER accumulation.

Turnover of E7:Ty

The facts that E7:Ty largely does not assemble into functional TfR heterodimers and localizes prominently to the ER in the absence of E6 are consistent with retention of misfolded polypeptides by the ER quality control machinery. This begs the question of how trypanosomes dispose of this unwanted protein, by transport to the lysosome or by ERAD? We first investigated this issue using a cycloheximide (CHX) 'chase' protocol in the RNAi^R E7:Ty cell line. Cells were incubated with CHX to block protein synthesis, and turnover of steady state TfR subunits was then followed over time by immunoblotting. FMK024 or MG132 were included to block lysosomal or proteasomal degradation respectively. We have previously shown that native TfR is completely rescued from lysosomal degradation by FMK024 (Schwartz *et al.*, 2005). This protocol was executed without (Fig. 6A) and with RNAi silencing of native TfR (Fig. 6B); the results were essentially identical except that the elevated expression of E7:Ty in silenced cells provided a clearer picture of E7:Ty turnover. Without any treatment the steady state level of E7:Ty increased dramatically during the 4 hr incubation (lanes 1–3). This is because the high short-term culture density (5×10^6 /ml) results in localized hypoxia leading to up-regulation of TfR expression (Mussman *et al.*, 2004). Conversely, when new synthesis was blocked with CHX E7:Ty was substantially lost from cells within 4 hours (Fig. 6A, lanes 4–6). FMK024 treatment was ineffective in rescuing E7:Ty (lanes 7–9), while turnover was partially blocked/delayed in the presence of MG132 (lanes 10–12). An ~30 kDa fragment is an apparent partial proteolytic product derived from the E7 subunit (lanes 11 & 12, star).

In a second approach, E7:Ty turnover was followed by quantitative pulse-chase metabolic radiolabeling, again with and without silencing of endogenous TfR (Fig. 7). The results are entirely consistent with those of the CHX chase protocol. FMK024 was completely unable to rescue E7:Ty turnover, whereas MG132 dramatically reduced the rate of degradation. Collectively these results indicate that the E7:Ty is not turned over in the lysosome as is native TfR, but rather is degraded in the proteasome consistent with the ERAD process. Furthermore, this turnover occurs in both normal and silenced cells indicating that E7:Ty degradation is largely independent of the presence of native TfR subunits in keeping with exclusion of the tagged subunit from functional TfR heterodimers.

DISCUSSION

Native VSG and ERAD

We have carefully re-examined the evidence that VSGs are synthesized in excess with up to 2/3rd of all nascent polypeptides being consigned via ERAD to proteasomal degradation. In doing so we repeated both our original pulse-chase analysis (Bangs *et al.*, 1986) and the

protease inhibitor treatments of (Field *et al.*, 2010). In the first instance we confirmed the finding that there is no turnover of VSG synthesized in a short pulse radiolabel (2 min) during the ensuing chase period (1 hr). In the second, using three different VSGs from two different *T. brucei* serodemes we were unable to detect any effect of proteasomal inhibitors on steady state protein levels. We conclude that VSG is not normally subject to ERAD. We can offer no explanation for the singular results of (Field *et al.*, 2010), but our findings are consistent with many generally accepted properties of VSG including: i) trypanosomes already devote 10% of total protein synthesis to VSG (Cross, 1975); ii) newly synthesized VSG is quantitatively transported to the cell surface (Bangs *et al.*, 1986, Duszynski *et al.*, 1988, Hall *et al.*, 2005, Hall *et al.*, 2006, Allen *et al.*, 2007, Sutterwala *et al.*, 2008, Sevova *et al.*, 2009); iii) VSG folding is intrinsically favorable (Rehaber *et al.*, 1990); and iv) trypanosomes have an innate bookkeeping system that functions at the level of mRNA abundance to precisely regulate total VSG synthesis (Smith *et al.*, 2009). Collectively these data indicate that trypanosomes have evolved to coordinate VSG synthesis and folding with maximum efficiency and parsimony, generating just enough VSG in each cell cycle to provide a full complement to each daughter cell. In keeping with this fundamental property, trypanosomes have a streamlined secretory pathway for rapid VSG transport to the cell surface (Sevova *et al.*, 2009), and an efficient recycling machinery for maintenance of an intact surface coat (Engstler *et al.*, 2004).

Evidence for functional ERAD

Despite the fact that VSGs are not subject to ERAD, our results with the E7:Ty reporter indicate that trypanosomes do use this pathway to cope with misfolded proteins in the early secretory pathway. E7:Ty was originally engineered for use in separate ongoing studies of TfR trafficking, but failed in this regard, notably in its inability to assemble with native E6 into functional TfR heterodimers. The likely reason for this is the presence of non-native sequences on both the N- and C-termini. E7:Ty was created by contingency based on preexisting reporters and vectors in our laboratory. Consequently, following removal of the signal sequence it has ten residues deriving from the N-terminus of mature EP1 procyclin and the ten residues of the C-terminal Ty epitope tag. One or both of these segments must account for the aberrant behavior of this reporter, which raises the issue of why replacing the functional native BES1 *E7* gene with non-functional *E7:Ty* does not impact growth. Presumably native E7 subunits provided by the silent expression sites are enough to maintain functional TfR for viability (Ansorge *et al.*, 1999). Alternatively, the few E7:Ty subunits that do assemble may be sufficient for transferrin acquisition.

E7:Ty is expressed from the active BES1 expression site under control of the native E7 3' UTR. Thus it is subject to external stimuli such as transferrin starvation and hypoxia that are known to up regulate TfR expression (Mussman *et al.*, 2003, Mussman *et al.*, 2004). This phenomenon is recapitulated when native E6 and E7 are eliminated by RNAi silencing. Without E6 subunits no functional receptors can be made, which is equivalent to transferrin starvation, leading to an ~10-fold increase in RNAi^R E7:Ty mRNA and protein. Regulation of expression in trypanosomes is post-transcriptional, with modulation of mRNA stability by cis-acting elements in 3' UTRs being the major mechanism (Clayton, 2014), and it is likely that a similar phenomenon is at work with TfR. Under these conditions E7:Ty

overwhelmingly localizes to the ER. This relocalization may be apparent due in part to upregulation in the absence of functional TfR, but it is also likely that misfolding and aggregation prevent ER exit.

The failure of E7:Ty to assemble into functional receptors, and its dramatic accumulation in the ER under TfR silencing raises the issue of how this protein is dealt with and whether ERAD may be at play. We assessed the effect of proteasomal or lysosomal protease inhibitors on both steady state levels of E7:Ty when protein synthesis was blocked, and on active turnover in biosynthesis experiments. In each instance FMK024 failed to rescue E7:Ty, while MG132 treatment greatly retarded turnover leading to substantial accumulation of intact and partially degraded protein. These findings were the same regardless of whether native TfR was present or absent, indicating that mode of turnover is an innate property of the E7:Ty reporter. Collectively these findings – failure to assemble, accumulation in the ER, and rescue by proteasomal inhibitor – provide the first functional evidence for the ERAD process in trypanosomes. Consistent with this evidence orthologues of all the critical ERAD components are present in the *T. brucei* genome including EDEM (Tb927.8.2910-2940), OS-9 (Tb927.11.10700), Hrd1 (Tb927.9.5260) and p97 (TbVCP, Tb927.10.5770) (Roggy *et al.*, 1999, Lamb *et al.*, 2001, Engstler *et al.*, 2006, Field *et al.*, 2010). Further work will be required to confirm the functionality of these proteins in relation to trypanosomal ERAD.

ERAD, Misfolded VSG & Antigenic Variation

The question remains as to how ERAD would be of benefit to trypanosomes – when would the ability to dispose of misfolded proteins be critical for survival? It is easy to envision stress induced misfolding in the procyclic insect stage, which exists in the harsh environment of the tsetse midgut and may be exposed to temperature extremes due to natural vector biology. Non-dividing transmissive forms, short stumppies (mammal-to-tsetse) and metacyclics (tsetse-to-mammal), which are preadapted for passage between different thermal environments, may also have need of ERAD capability for pre- and post-transmission cellular remodelling. In contrast, it has been argued that bloodstream stage trypanosomes exist in such a well controlled environment that severe stress events are unlikely (Koumandou *et al.*, 2008). However, there is one core aspect of bloodstream infection where ERAD could provide a critical edge for parasite survival – antigenic variation. VSGs are expressed from telomeric expression sites (ES), of which there are ~15 per genome (Barry *et al.*, 2005, Marcello *et al.*, 2007, Rudenko, 2011) (Fig. 1A). Only one is active at a time, and the main mode of antigenic variation is gene conversion (duplication activation) whereby the resident ES *VSG* gene is over-written with a distinct gene from silent subtelomeric arrays (Robinson *et al.*, 1999). There are ~1500 *VSG* genes in the repertoire, most of which are incomplete. Initially in infection antigenic variation favors switching of intact donor genes, but later increasingly utilizes partial *VSG* sequences (segmental gene conversion) leading to production of mosaic genes. This has the benefit of increasing the available repertoire of antigenically distinct VSGs, but has one inherent disadvantage. Inevitably during antigenic variation, which occurs at rates up to 10^{-2} in authentic wild isolates (Turner, 1997), portions of incompatible donor genes will be fused with portions of the resident ES gene, creating VSGs that are incapable of folding. The result will be a catastrophic switch from a functional to a ‘failed’ VSG that cannot assemble into dimers, leave the ER, and/or form a

viable surface coat. Such cells would have a fixed surface coat composed of the original VSG while continuing to synthesize and accumulate the failed VSG. Ultimately this would be fatal, either from internal intoxication and/or by onset of immune responses to the surface VSG - unless two things occur. First, there must be sufficient 'disposal capacity' to temporarily cope with the immediate stress of excessive misfolded VSG. Next, a second round of antigenic variation must occur to restore expression of a functional VSG gene. Antigenic variation is a stochastic process that occurs continuously in the background of active infection. Given a window of time individual cells might survive a catastrophic switch, and ERAD may be the process that provides that opportunity.

Finally, the likely production of ‘failed’ VSGs may provide a rationale for one of the longstanding puzzles of antigenic variation, the existence of multiple expression sites. Antigenic variation by gene conversion is a very efficient process, and a priori there is no obvious need for more than one ES, yet trypanosomes have evolved and maintain ~15. This allows a second mode of switching; coordinate repression of the active ES and activation of a second ES bearing a different VSG. The evolutionary transition from the ancestral state of a single ES to multiple ESs may have been driven in part by the selective advantage of having a rapid and fool-proof alternative mechanism for switching in the event of a failed VSG arising by gene conversion. It has been alternatively proposed that the selection pressure leading to expansion of the ES repertoire was the ability to make TfRs adapted to the sera of different mammalian hosts (Bitter *et al.*, 1998, Gerrits *et al.*, 2002). There are considerable sequence polymorphisms in native ESAG6 and ESAG7 encoded by different ESs, and the TfRs generated by each have variable affinities for transferrins from different host species. As the trypanosome never ‘knows’ what host it may be transmitted to, it would be advantageous to have the ability to rapidly switch to an ES encoding a more favorable TfR. These two mechanisms are not at all exclusive, and it may be that both synergistically provided the selection pressure leading to ES amplification.

EXPERIMENTAL PROCEDURES

Cell lines and culture

All work was carried out with bloodstream form of the commonly used Lister 427 strain of *T. brucei brucei* (variant antigenic type MITat1.2 expressing VSG221, VAT MITat1.4 expressing VSG117), or with the ILTat1.3 VAT of the ILTar1 serodeme (Rice-Ficht *et al.*, 1981), grown in HMI-9 (Hirumi *et al.*, 1994). The tetracycline-responsive single-marker (SM) MITat1.2 BSF cell line (Wirtz *et al.*, 1999) was used for all experiments involving inducible RNAi silencing of endogenous TfR expression.

TfR RNAi cell line

Using SM cells as the parental, a TfR RNAi cell line was generated. First, the active BES1 expression site (Berriman *et al.*, 2002, Hertz-Fowler C, 2008) was ‘locked’ by inserting a blasticidin cassette downstream of the promoter (see Fig. 2) (pHNES221B1.6 construct from Gloria Rudenko, Imperial College). Next a TfR RNAi construct was generated in the pLEW100v5X:Pex11 stem loop vector (Silverman *et al.*, 2011). Using bacterial artificial chromosome (BAC) DNA containing the BES1 expression site [clone H25N7, (Berriman *et*

al., 2002), gift of Gloria Rudenko] as template, a 651 bp fragment (nts -50 to 601, targeting shared codons 1-200 and 5' UTR sequences of both the ESAG6 and ESAG7 orfs) was PCR amplified with nested 5' XhoI/BamHI and 3' AscI/NdeI sites. The PCR product was sequentially inserted upstream of the Pex11 stuffer using BamHI/AscI, and downstream in the opposite orientation using XhoI/NdeI. All cloning steps were confirmed by sequencing and the resultant plasmid was linearized with NotI for transfection of the 'locked' SM cell line. Transfection was by electroporation as described previously (Burkard *et al.*, 2007, Sevova *et al.*, 2009). Clonal cell lines were derived by limiting dilution and selection with phleomycin. Induction of anti-TfR double-stranded RNA was achieved by addition of 1 µg/ml of tetracycline.

Epitope Tagged RNAi Resistant ESAG7

An RNAi resistant (RNAi^R) C-terminally Ty epitope tagged ESAG7 (E7:Ty) orf was generated for unrelated studies on trafficking of TfR. For reasons presented herein this failed as a trafficking reporter, but fortuitously was found to act as an ERAD substrate. This reporter was generated by contingency based on other constructs available in the laboratory and precise details will not be described here, but see Fig. 1. Briefly, using our pXS6 vector (Silverman *et al.*, 2011) as the backbone the RNAi^R E7:Ty genomic replacement construct was assembled as follows (5'-3'): 5' UTR targeting regions (nts -489 to 1; relative to the E7 orf); puromycin resistance cassette; βα-tubulin intergenic region; EP1 procyclin signal sequence (codons 1-38, cleavage site at 27/28) (Clayton *et al.*, 1989, Roditi *et al.*, 1999); the E7 orf minus the native signal sequences (nts 58-1023, codons 20-341) with an in frame C-terminal Ty epitope (EVHTNQDPLD); 3' UTR targeting region (nts 1-524; relative to E7 stop codon). E7 segments were PCR amplified from H25N7 BAC DNA. All segments were confirmed by sequencing. To recode the E7 reporter for RNAi resistance the N-terminal region from the EP1 signal sequence (XhoI) to the an internal BamHI site (nt 739) was chemically synthesized (Integrated DNA Technologies, Coralville, IO), synonymously altering codons to the next most frequently used codon in *T. brucei* housekeeping genes (Parsons *et al.*, 1991, Horn, 2008). Alignment of wild type and RNAi^R sequences are presented in Fig. S1. This synthetic DNA was placed in the E7:Ty construct using XhoI/BamHI. The resultant RNAi^R reporter was linearized with ClaI/FseI for homologous replacement of the endogenous E7 gene in the active BES1 expression site of the TfR RNAi cell line (Fig. 1). Transfection and clonal selection was as described above.

Immunological Reagents

The following antibodies have been described in our prior publications (Bangs *et al.*, 1986, Silverman *et al.*, 2011, Tiengwe *et al.*, 2015): rabbit anti-VSG221, anti-VSG117, anti-ILTat1.3 VSG, anti-BiP, and anti-Hsp70. Mouse anti-Ty was from the UAB Hybridoma Facility (Birmingham, AL). Rabbit anti-TfR (BES1 specific) was a generous gift of Dr. Piet Borst (Netherlands Cancer Institute, Amsterdam). Secondary reagents for western blotting were IRDye680- and IRDye800-conjugated goat anti-rabbit and anti-mouse IgG (*Li-Cor*, Lincoln NB). Secondary reagents for immunofluorescent imaging were species-specific Alexa-conjugated goat anti-IgG as appropriate (Molecular Probes, Eugene, OR).

Treatment with protease inhibitors

Log phase BSF trypanosomes were adjusted to 2.5×10^5 /ml in HMI-9 growth media and cultured with DMSO alone, or with final concentrations (in DMSO) of lactacystin or MG132 (Sigma-Aldrich, St. Louis, MO, 5 μ M and 25 μ M, respectively), or FMK024 (morpholinourea-phenylalanine-homophenylalanine-fluoromethyl ketone; 20 μ M; MP Biomedicals, Aurora, OH) for 4 hours. Cell extracts were prepared and subjected to immunoblotting with anti-VSG. Alternatively, BSF cells were pretreated (15 min) with inhibitor and then pulse-chase radiolabeled as described below in the continued presence of inhibitor.

Metabolic labeling and immunoprecipitation

Pulse-chase radiolabeling of log-phase cultured BSF trypanosomes with [35 S]methionine/cysteine, and subsequent immunoprecipitation of labeled polypeptides were performed as described previously (Tazeh *et al.*, 2007, Peck *et al.*, 2008). Pulse and chase times are indicated in the figure legends. All immunoprecipitates were fractionated by 12% SDS-PAGE, and gels were analyzed by phosphorimaging using a Molecular Dynamics Typhoon FLA 9000 system with native ImageQuant Software (GE Healthcare, Piscataway, NJ).

Endocytosis Assay

Endocytosis was assayed by flow cytometry as described in (Silverman *et al.*, 2011). Ligands were Alexa488 conjugated bovine transferrin and tomato lectin (Molecular Probes)

Immunoblotting

Gels were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a Trans-Blot Turbo apparatus (BioRad, Hercules, California). Membranes were blocked and probed with appropriate dilutions of primary and secondary antibodies in Odyssey Blocking Buffer (*Li-Cor*). All washes were with PBS, 0.5% Tween20. Quantitative fluorescent signals were scanned on an Odyssey CLx Imager (*Li-Cor*).

Data analyses

Phosphorimages and fluorescent blot scans were quantified with ImageJ software (<http://imagej.nih.gov/ij/>). For analysis of specific band intensities, signals were corrected by subtraction of the signal from equivalent unlabeled areas of each lane. All subsequent data management was performed with Prism4 software (GraphPad Software, Inc., San Diego CA).

qRT-PCR

Specific transcript levels were determined using quantitative RT-PCR (qPCR). Total RNA was isolated from log phase cultures using RNeasy Mini kit (Qiagen, Valencia, CA). RNA was treated with DNaseI on-column using RNase-Free DNase Set (Qiagen) and cDNA synthesized using iScript cDNA synthesis kit (BioRad). qPCR was performed using diluted cDNAs and Power SYBR green PCR Master Mix (Life Technologies, Carlsbad, CA) with oligonucleotide pairs specifically targeting transcripts for wild type E6 and E7, and E7Ty. The positions of these primers are indicated in the sequence alignment presented in Fig. S1.

TbZFP3 (Tb927.3.720, nts 241–301) was used as the control amplicon. Amplification was performed using an Applied Biosystems StepOne Real-Time PCR System (Life Technologies, Carlsbad, CA). For each transcript post-amplification melting curves indicated a single dominant product. Transcript levels were normalized to TbZFP3 as endogenous control. All calculations and normalizations were done using StepOne software, version 2.2.2. Reactions were performed in triplicate, and means $\pm$ standard errors of the means (sem) for three biological replicates are presented.

Epifluorescence microscopy

Immunofluorescence (IFA) microscopy was performed with formaldehyde fixed/detergent permeabilized cells as described in (Tazeh *et al.*, 2007). Cells were also stained with DAPI ($0.5 \mu\text{g ml}^{-1}$) to reveal nuclei and kinetoplasts. Serial image stacks (0.2 micron Z-increment) were collected with capture times from 100–500 msec (100 $\times$ PlanApo, oil immersion, 1.46 na) on a motorized Zeiss Axioimager M2 stand equipped with a rear-mounted excitation filter wheel, a triple pass (DAPI/FITC/Texas Red) emission cube, differential interference contrast (DIC) optics, and an Orca ER CCD camera (Hamamatsu, Bridgewater, NJ). Images were collected with Volocity 6.1 Acquisition Module (Improvision Inc., Lexington, MA) and individual channel stacks were deconvolved by a constrained iterative algorithm, pseudocolored, and merged using Volocity 6.1 Restoration Module. Unless otherwise stated all images presented are summed stack projections of merged channels. The xyz pixel precision of this arrangement has been validated in (Sevova *et al.*, 2009) (see Figure S1 therein).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Modification of the Tfr locus

A. Map of the BES1 telomere showing the promoter proximal positions of ESAG6 and ESAG7 orfs (Berriman *et al.*, 2002). ESAG orfs are numbered. Flag indicates the ES promoter. Under-bar indicates the region of E7 modification. B. Expanded diagram of the promoter proximal region and integration constructs. (1) Blasticidin insertion cassette (Bsd). (2) RNAi^R E7:Ty replacement construct. The ESAG tagging constructs contain (5'-3'): 5' UTR targeting sequences; puromycin resistance cassette; tubulin β α -intergenic region (Tub Int); EP1 procyclin signal sequence (SS, not to scale); the E7 orf minus the native signal sequence (ss); the Ty epitope tag (not to scale); 3' UTR targeting sequences. Overbar indicates region of E7 RNAi resistance. Internal BamHI sites (B) are indicated.

Figure 2. Turnover of endogenous VSG

A. Log phase BSF MITat1.2 trypanosomes were pulse radiolabeled with [35 S]Met/Cys (2 min) and then chased with complete medium. At the indicated times samples were collected and specifically immunoprecipitated with anti-VSG221. (Top) Immunoprecipitates were fractionated by SDS/PAGE and visualized by phosphorimaging. A representative gel is presented with the mobility of VSG (V) and molecular mass markers (kDa) indicated. (Bottom) Phosphorimages were quantified and VSG recovery is presented as the fraction of initial VSG at time zero (mean $\pm$ sem, $n=5$ biological replicates). B. Log phase BSF trypanosomes expressing the indicated VSGs (221, 117, ILTat1.3) were cultured (4 hr, 2.5×10^5 cells per ml) in the presence of DMSO (D), 5 μ M lactacystin (L), 25 μ M MG132 (M), or 20 μ M FMK024 (F). Cell viability and morphology remained normal throughout the incubation period (not shown). Washed cells from equal culture volumes (corresponding to 10^6 initial cells) were fractionated by SDS/PAGE, and simultaneous immunoblotting was performed with specific rabbit anti-VSG (V) and anti-Hsp70 (H) sera. Fluorescent blots were digitally imaged on an Odyssey CLx Imager (*Li-Cor*). Images for each VSG blot are representative of multiple biological replicates ($n=3$). C. The ratio of corrected VSG signal to corrected Hsp70 signal (V:H), normalized to the DMSO controls, is presented for each VSG (means $\pm$ sem, $n=3$ biological replicates).

Figure 3. RNAi Silencing of TfR

The parental TfR RNAi cell line alone (Par), or containing the RNAi^R E7:Ty construct (E7:Ty), were cultured without or with tetracycline. A. Cell density was enumerated by hemocytometer and cultures were adjusted to starting density daily. All subsequent analyses were performed at 24 hours of silencing. B. Receptor mediated uptake of fluorescent transferrin (Tf) and tomato lectin (TL) was measured by flow cytometry. Data are presented as mean fluorescent intensity (MFI $\pm$ s.e.m.) for three biological replicates and are normalized to un-silenced control cells. C. Wild type RNAi^S E6 and E7 (E6^{WT}, E7^{WT}) and RNAi^R E7:Ty (E7^{Ty}) transcript levels were assessed by qRT-PCR. Specific primers are indicated in Fig. S1. Results are normalized to uninduced controls and are presented as fold-change for five (Par) or three (E7:Ty) biological replicates (mean $\pm$ s.e.m.). D. Cell extracts were subjected to simultaneous immunoblotting with rabbit anti-TfR and mAb anti-Ty. The anti-TfR (left) and anti-Ty (right) channels were digitally segregated for presentation. Separate blots were probed independently with rabbit anti-Hsp70 or anti-BiP as loading controls (below). All lanes have 5×10^6 cell equivalents. Mobilities of molecular mass markers are indicated. Asterisks indicate non-specific background polypeptides consistently observed with anti-TfR.

Figure 4. E7:Ty Sequential Pull-Down

Cell extracts were prepared from the E7:Ty cell line without silencing of native E6/E7. Lysates were affinity precipitated sequentially with anti-Ty (1° & 2° , lanes 1 & 2; 4 & 5) and then once more with Tf-beads (3° , Tfb, lane 3) or anti-TfR (3° , lane 6). Alternatively, lysates were affinity precipitated sequentially with Tf-beads (1° & 2° , lanes 7 & 8; 10 & 11) and then once more with anti-Ty (lane 9) or anti-TfR (3° , lane 12). A. & B. Pull downs (5×10^6 cell equivalents per lane) were subjected to simultaneous immunoblotting with rabbit anti-TfR and mAb anti-Ty. The anti-TfR (A) and anti-Ty (B) channels were digitally segregated for presentation. Vertical white lines were placed after digital contrasting to demark the four independent data sets. Arrowheads indicate mobility of E7:Ty in the anti-TfR blot. C. The E7 region of the 1° Tfb pull downs (lanes 7 & 10) of the original two-channel image were digitally excised, and then each channel was subtracted leaving the anti-Ty signal (green) or anti-TfR signal (red) representing E7:Ty and total E7 respectively. The mobilities of E7:Ty and native E7 are indicated.

Figure 5. Location of E7:Ty under TfR silencing

IFA was performed with the RNAi^R E7:Ty cell line without (A, Tet-) or with (B, Tet+, 24 hr) prior TfR silencing. Staining of fixed permeabilized cells was with rabbit anti-BiP (left, green) and mAb anti-Ty (center, red). Three channel merged images with DAPI staining are presented (left). In each case, deconvolved summed stacked projections for two individual cells are presented. Flagellar pocket localizations of E7:Ty in control cells (arrowhead) and discrete post-nuclear foci (star) of E7:Ty in silenced cells are indicated.

Figure 6. Turnover of E7:Ty (I)

E7:Ty cells without (A. tet-) or with (B. tet+) TfR RNAi induction (24 hr), were cultured (5×10^6 /ml) with or without cycloheximide (CHX⁻, CHX⁺, 100 μ g/ml) to stop protein synthesis. Cells were also treated with the FMK024 (FMK, 20 μ M) or MG132 (MG, 25 μ M) as indicated. Samples were collected at 0, 2, or 4 hours and immunoblotted with anti-Ty or anti-Hsp70 (5×10^6 cells/lane). Mobilities of E6 and E7 species are indicated on the left; molecular mass markers on the right. Arrowheads (right) indicate non-specific bands that can be used to compare exposure/expression levels between the anti-Ty blots in Panels A & B. The mobility of the MG132-protected E7:Ty fragment is indicated by a star.

Figure 7. Turnover of E7:Ty II

Turnover of E7:Ty was assayed by pulse/chase (15 min/4 hr) radiolabeling in control (A. Tet-) and TfR silenced (B. Tet+, 24 hr) E7:Ty cells. As indicated FMK024 (Panel A, 20 μ M) or MG132 (Panel B, 25 μ M) were included. Control was DMSO vehicle. In each case the top panels are typical phosphorimages of anti-Ty immunoprecipitates with and without inhibitors. Graphs represent quantification of the loss of the full length E7:Ty reporter during the chase period for three biological replicates (mean $\pm$ s.e.m, n = 3).