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Expression of bloodstream variant surface glycoproteins in procyclic stage *Trypanosoma brucei*: role of GPI anchors in secretion

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Using transformed procyclic trypanosomes, the synthesis, intracellular transport and secretion of wild-type and mutant variant surface glycoprotein (VSG) is characterized. We find no impediment to the expression of this bloodstream stage protein in insect stage cells. VSG receives a procyclic-type phosphatidylinositol-specific phospholipase C-resistant glycosyl phosphatidylinositol (GPI) anchor, dimerizes and is *N*-glycosylated. It is transported to the plasma membrane with rapid kinetics ($t_{1/2}$ ~1 h) and then released by a cell surface zinc-dependent metalloendoprotease activity, a possible homolog of leishmanial gp63. Deletion of the C-terminal GPI addition signal generates a soluble form of VSG that is exported with greatly reduced kinetics ($t_{1/2}$ ~5 h). Fusion of the procyclic acidic repetitive protein (PARP) GPI anchor signal to the C-terminus of the truncated VSG reporter restores both GPI addition and transport competence, suggesting that GPI anchors play a critical role in the folding and/or forward transport of newly synthesized VSG. The VSG–PARP fusion is also processed near the C-terminus by events that do not involve *N*-linked oligosaccharides and which are consistent with GPI side chain modification. This unexpected result suggests that GPI processing may be influenced by adjacent peptide sequence or conformation.

Keywords: glycosyl phosphatidylinositol anchor/gp63 metalloendoprotease/secretion/trypanosomes/variant surface glycoprotein

Introduction

African trypanosomes are pathogenic kinetoplastid protozoa responsible for human and veterinary trypanosomiasis in sub-Saharan Africa. They are digenetic parasites with a life cycle that alternates between the insect vector (tsetse flies) and the bloodstream of mammalian hosts. Both forms are commonly studied in the laboratory and each elaborates a distinct stage-specific glycosyl phosphatidylinositol (GPI)-anchored protein: variant surface glycoprotein (VSG) in bloodstream trypanosomes (Cross, 1975) and procyclic acidic repetitive protein (PARP, also called procyclin) in the procyclic insect stage (Mowatt and Clayton, 1987; Roditi *et al.*, 1987; Richardson *et al.*, 1988). These molecules comprise the major cell surface component, and consequently are the major secretory cargo, of their respective stages of the life cycle. Not surprisingly then, VSG and PARP have provided many insights into the secretory pathway of these ancient eukaryotes. The adaptation of VSG as a recombinant secretory reporter (Bangs *et al.*, 1996) promises even more insights to come.

VSG (10–20% of total bloodstream protein) constitutes a dense monolayer surface coat that completely envelopes the cell (Vickerman, 1969; Cross, 1975). It is by the sequential expression of distinct VSG genes, a process called antigenic variation, that the parasite avoids the host immune response (reviewed in Vickerman *et al.*, 1993). A homodimer (Auffret and Turner, 1981), VSG is synthesized in the endoplasmic reticulum (ER) where it is modified rapidly by core *N*-linked glycosylation and GPI addition (Bangs *et al.*, 1985, 1986; Ferguson *et al.*, 1986; Duszenko *et al.*, 1988). Newly synthesized VSG has been shown to interact physically with BiP (Bangs *et al.*, 1996), an ER molecular chaperone. It is presumably at this early stage that dimerization takes place, as is the case with secretory macromolecules in other eukaryotic cells (Hurtley and Helenius, 1989). Thereafter, VSG is transported through the cells ($t_{1/2}$ 15 min), with concomitant oligosaccharide processing, to the cell surface where it is incorporated into the surface coat.

In contrast to VSG, PARP (1–3% of total procyclic protein) has an essentially non-variant sequence (Mowatt and Clayton, 1987) consisting of an ~40 amino acid N-terminal domain containing a single *N*-linked glycan followed by a C-terminal domain composed of a variable number (~30) of Glu–Pro dipeptide repeats. The repeat domain is believed to have a rigid and extended conformation (Roditi *et al.*, 1989). The precise function of PARP is not known, but its relative abundance suggests that it forms a surface coat for protection in the hydrolytic environment of the tsetse fly midgut (Stebeck and Pearson, 1994); the dipeptide repeat domain has been demonstrated to be essentially protease resistant (Ferguson *et al.*, 1993). Nothing is known concerning the post-translational processing and intracellular transport of PARP.

The VSG GPI anchor and PARP GPI precursor (and presumably the mature PARP anchor) have identical Manα1–2Manα1–6Manα1–4GlcN glycan core structures linked α1–6 to phosphatidylinositol (reviewed in Englund, 1993). The VSG anchor contains exclusively dimyristoyl glycerol (Ferguson *et al.*, 1985) while the PARP anchor contains 1-stearoyl-2-*lyso* glycerol and is palmitylated on the inositol (Field *et al.*, 1991). A consequence of this lipid arrangement is that, unlike the diacylglycerol (DAG) VSG anchor, the PARP structure is resistant to phosphatidylinositol-specific phospholipase C (PI-PLC; Field *et al.*, 1991). Both structures bear distinct side chain modifications to the core glycan. VSG has a variable

number of α-linked galactose residues (Ferguson *et al.*, 1988) and, although its precise structure is not known, the PARP GPI anchor has a poly-*N*-acetyllactosamine side chain with terminal sialic acids (Ferguson *et al.*, 1993). Both of these side chains are synthesized after attachment of the core GPI anchor to protein (Bangs *et al.*, 1988; Ferguson *et al.*, 1993). Presumably, the *N*-acetyllactosamine structures of the PARP anchor are attached during intracellular transport, but sialylation occurs after export and is mediated by a cell surface trans-sialidase activity (Pontes de Carvalho *et al.*, 1993).

Expression of VSG and PARP are coordinately regulated; bloodstream trypanosomes that are induced *in vitro* to differentiate to procyclic forms simultaneously repress expression of VSG and induce the expression of PARP (Roditi *et al.*, 1989). During the differentiation process, the turnover of VSG increases dramatically (from $t_{1/2}$ ~32 h to ~13 h) as VSG is shed from the surface of differentiating cells (Bulow *et al.*, 1989). Release occurs by proteolytic cleavage of VSG near the C-terminus, generating a soluble truncated form (Ziegelbauer *et al.*, 1993). The protease(s) mediating release, which is apparently activated or expressed during differentiation, is not susceptible to many commonly used protease inhibitors.

Using a stable transformation system, we have shown previously that a recombinant form of VSG, truncated at the site of GPI addition, is exported in a soluble manner from procyclic cells (Bangs *et al.*, 1996). In this study, we extend these results to include both full-length wild-type and truncated forms of two distinct VSGs. We find that wild-type VSG is synthesized, dimerized and GPI anchored in procyclic trypanosomes and that it is exported to the cell surface efficiently ($t_{1/2}$ ~1 h). It is then shed into the medium by a proteolytic event that mimics the release of VSG from differentiating bloodstream cells. Proteolysis is sensitive to chelating agents, including 1,10-phenanthroline, suggesting involvement of a cell surface zinc metalloprotease. Export of the truncated form of VSG is greatly reduced ($t_{1/2}$ ~5 h) relative to the rate of GPI-anchored VSG transport, suggesting a role for GPI anchors in protein folding and/or forward transport from the ER. Fusion of the PARP GPI addition sequence to truncated VSG not only restores rapid transport, but also results in apparent modification of the GPI anchor during intracellular transport. This finding suggests that adjacent protein sequence may influence modification of the core GPI structure.

Results

Expression of VSG in procyclic trypanosomes

In the process of developing soluble secretory reporters for expression in transformed trypanosomes, we truncated a VSG gene (VSG 117) such that the C-terminal peptide sequence specifying GPI addition was deleted (Bangs *et al.*, 1996). This reporter, 117Δgpi, is secreted from procyclic trypanosomes, albeit inefficiently. To investigate this phenomenon further and to determine if full-length wild-type VSG can be synthesized, GPI anchored and transported with fidelity in procyclic insect stage trypanosomes, we generated a procyclic cell line expressing unmodified VSG 117 (117wt) (see Figure 1). In addition, an analogous set of reporters was generated using the

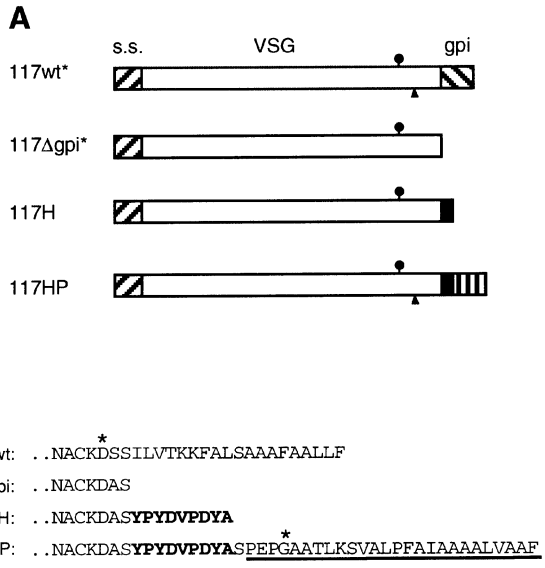

Fig. 1. VSG secretory reporters. (**A**) Diagrams (not to scale) of the various VSG 117-derived secretory reporters used. Domain regions are: N-terminal signal sequence (▨); mature VSG coding region (□); VSG GPI anchor sequence (▧); HA9 epitope (■); PARP GPI anchor sequence (▥). The approximate positions of the single *N*-linked glycosylation site at Asn453 (●) and the C-terminal proteolytic cleavage site (▲) are indicated. Asterisks; analogous VSG 221wt and 221Δgpi reporters were also prepared. (**B**) C-terminal amino acid sequences of the VSG 117 reporters. Sequences are aligned from the C-terminus of mature VSG 117. Sequences derived from the PARP C-terminus (underlined) and the HA9 epitope (bold) are indicated. Asterisks denote sites of GPI addition in the native VSG 117 and PARP proteins. Sequences are from Boothroyd *et al.* (1981) and Clayton and Mowatt (1989), and amino acid numbering, here and in the text, is relative to the initiation methionine residue of VSG 117.

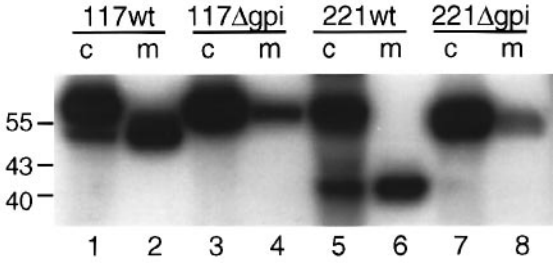

Fig. 2. Expression of recombinant VSG reporters. Transformed procyclic cell lines expressing different VSG 117 and VSG 221 reporters, as denoted, were radiolabeled with [^{35}S]methionine for 4 h. Cell (c) and media (m) fractions were prepared, and labeled polypeptides were immunoprecipitated with anti-VSG 117 (lanes 1–4) or anti-VSG 221 (lanes 5–8) antibody. Immunoprecipitates were analyzed by SDS–PAGE and fluorography. Lanes 5 and 6, 10^7 cell equivalents; all other lanes, 5×10^6 cell equivalents. A scan of a 36 h exposure is presented. Scale refers to relative molecular mass in kDa.

distinct VSG 221 gene (221wt and 221Δgpi). All cell lines were analyzed in parallel by metabolic radiolabeling and specific immunoprecipitation from cell and media fractions (Figure 2).

As previously reported, a polypeptide of the expected M_r (~59 kDa) is synthesized in, and secreted from, the 117Δgpi cell line (lanes 3 and 4). However, the ratio of exported to cell-associated protein is low, suggesting that this reporter is poorly secreted. Likewise, deletion of the GPI anchor addition sequence results in low level secretion of VSG 221 (lanes 7 and 8). In the 117wt cell line, an

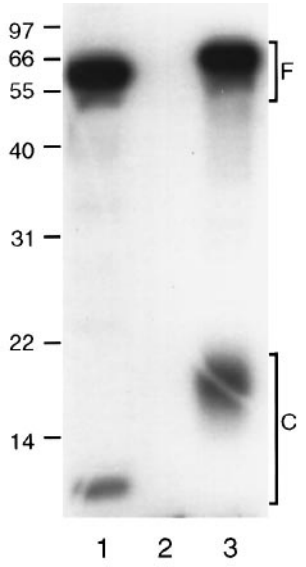

Fig. 3. [^{3}H]Palmitate labeling of 117 VSGs. 117wt (lane 1), 117Δgpi (lane 2) and 117HP (lane 3) cell lines were metabolically radiolabeled with [^{3}H]palmitate as described in Materials and methods. Radiolabeled polypeptides were immunoprecipitated with anti-VSG 117, fractionated on a 15% SDS–polyacrylamide gel and analyzed by fluorography. A scan of a 6 week exposure is shown. All lanes contain 4×10^7 cell equivalents. Full-length polypeptides (F) and C-terminal fragments (C) are denoted. Scale refers to relative molecular mass in kDa. (Note: discontinuities in the lower molecular mass region of the gel are cracks caused by drying.)

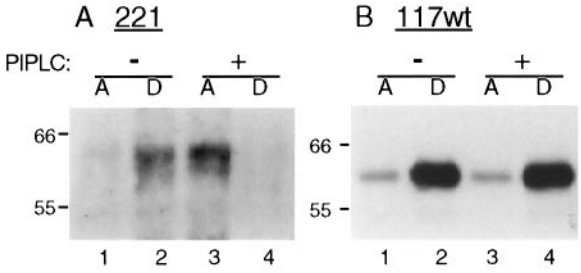

Fig. 4. GPI-PLC treatment of procyclic VSG. 117wt procylic cells were radiolabeled with [^{35}S]methionine for 2 h and extracted in 1% Triton X-114. Purified bloodstream membrane-form 221 VSG was added to the extract and samples were incubated for 2 h with (+) or without (–) GPI-PLC. Aqueous (A) and detergent (D) fractions were generated by phase separation and analyzed for 221 (**A**) and 117 (**B**) VSG by immunoblotting and immunoprecipitation, respectively. Scale refers to relative molecular mass in kDa. A scan of an overnight exposure is presented in (**B**). (Note: 117 tVSG is not detected in this exposure because the labeling period is shorter than in Figure 2.)

abundant cell-associated polypeptide of the predicted M_r (~59 kDa) is also detected (lane 1), but two unexpected results were obtained. First, a smaller minor form (~53 kDa) of cell-associated VSG is also apparent (lane 1) and, second, this 53 kDa form is abundant in culture medium derived from these cells (lane 2). A similar pattern of expression is seen in the 221wt cell line (lanes 5 and 6), although the soluble fragment is smaller, indicating that release of truncated VSG (tVSG) is not restricted to a single variant antigen. The magnitude of the truncations, especially that of 221 tVSG, suggests that release occurs by proteolytic cleavage upstream of the C-terminal membrane anchor.

Recombinant 117wt protein is GPI anchored

Before proceeding with an analysis of intracellular transport of recombinant VSGs, we wished to prove that the full-length reporters are indeed properly GPI anchored in procyclic cells. Therefore, both 117wt and 117Δgpi cells were metabolically radiolabeled with [^{3}H]palmitate (Figure 3). Palmitate is a component of the PARP GPI anchor (Field *et al.*, 1991) and would be expected to specifically label GPI structures on recombinant VSG synthesized in procyclic cells. Labeled full-length VSG was detected in the 117wt cell line (lane 1), suggesting that VSG is properly GPI anchored. The failure to detect labeled VSG in 117Δgpi cells (lane 2) indicates that labeling is dependent on the presence of the C-terminal GPI addition peptide. To confirm this, we restored palmitate labeling (lane 3) by placing the authentic PARP GPI addition peptide at the C-terminus of the 117Δgpi reporter (117HP, see Figure 1). Collectively, these data indicate that the label has been incorporated into a bona fide GPI structure. No labeled polypeptides corresponding to tVSG were observed in any cell line, but palmitate was incorporated into small polypeptides derived from both the 117wt (lane 1) and 117HP (lane 3) cell lines. In keeping with our conclusion that tVSG release results from cleavage proximal to the C-terminus, these species must be the residual C-terminal GPI-anchored fragment. The larger size of the 117HP fragment is due, in part, to the inclusion of extra amino acids during the construction of this reporter (see Figure 1).

To characterize the nature of the VSG GPI anchor further, we tested for sensitivity to GPI-specific phospholipase C (GPI-PLC). If a PARP-type inositol-acylated anchor is attached to VSG in procyclic cells, it should be resistant to the action of this enzyme (Field *et al.*, 1991). Radiolabeled 117wt cells were extracted by Triton X-114 phase separation and the hydrophobic proteins were subjected to digestion with trypanosomal GPI-PLC. Purified bloodstream membrane-form 221 VSG was included in the digests as a positive control. Reaction products were fractionated by phase separation and analyzed (Figure 4) by immunoblotting (221 VSG) or immunoprecipitation (117 VSG). Untreated 221 VSG partitions exclusively in the detergent phase (Figure 4A, lane 1 versus 2) and GPI-PLC treatment substantially reverses this pattern (Figure 4A, lane 3 versus 4), consistent with the removal of DAG from the GPI anchor. Untreated procyclic 117wt VSG is also found in the detergent phase (Figure 4B, lane 1 versus 2), but GPI-PLC treatment has no effect on this distribution. This result indicates that a PARP-type anchor is added to VSG when expressed in procyclic cells.

Kinetics of VSG transport

We next determined the precise kinetics for intracellular transport of VSG in procyclic cells. Both the 117wt and 117Δgpi cell lines were analyzed by pulse–chase radiolabeling followed by immunoprecipitation of VSG polypeptides from cell and media fractions. Transport and release of 117wt VSG was monitored over a 4 h chase (Figure 5A). Initially, all of this reporter is cell associated and is detected exclusively as full-length VSG (lane 1). During the chase, this form disappears from the cell (lanes 2–5), concomitant with the appearance of 117 tVSG in the medium (lanes 6–10). The kinetic half-time of 117wt

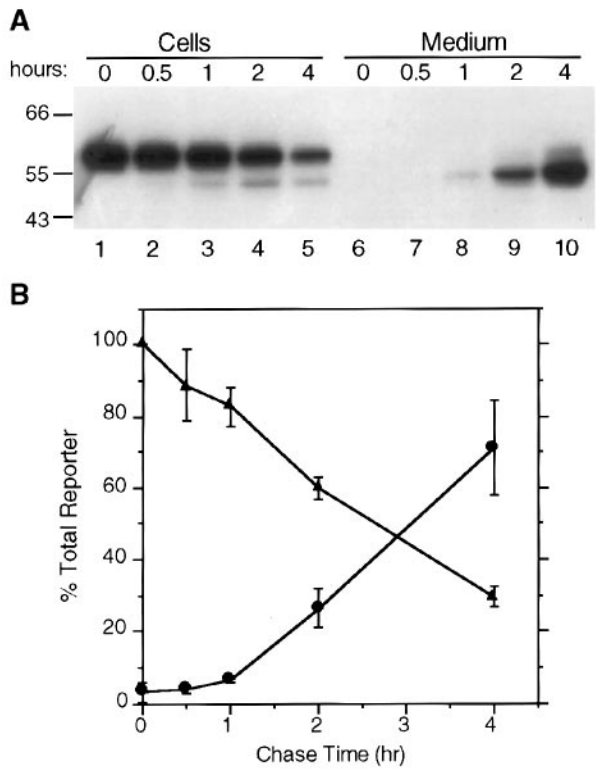

Fig. 5. Kinetics of 117wt VSG secretion. Transformed procyclic cells expressing recombinant 117wt VSG were pulse-labeled with [^{35}S]methionine for 15 min and then chased for 4 h. (**A**) At the indicated chase times, aliquots were separated into cell and medium fractions and radiolabeled VSG polypeptides were analyzed as in Figure 2. All lanes contain 5×10^6 cell equivalents. A scan of a 3 day exposure is presented. Scale refers to relative molecular mass in kDa. (**B**) Repetitions of this experiment (n = 3) were analyzed by densitometry, and the kinetics of transport were determined as described previously (Bangs *et al.*, 1996). Data are presented as percentage of total reporter at time zero (ordinate) versus chase time (abscissa). ▲, cell-associated reporter; ●, secreted reporter.

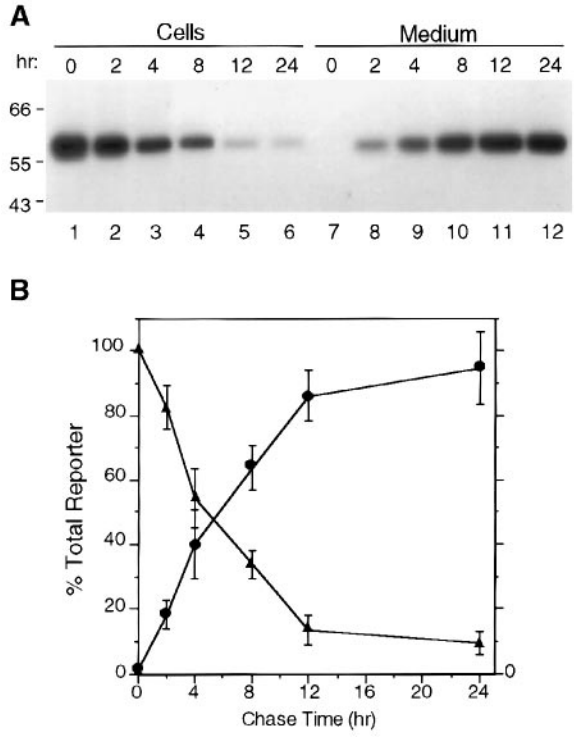

Fig. 6. Kinetics of 117Δgpi VSG secretion. Transformed procyclic cells expressing recombinant 117Δgpi VSG were pulse-labeled for 15 min and then chased for 24 h. At the indicated chase times, aliquots were separated into cell and medium fractions and analyzed as in Figure 5. A representative fluorogram is presented in (**A**). Densitometric analyses of repetitions (n = 3) are presented in (**B**). ▲, cell-associated reporter; ●, secreted reporter.

transport and release, as measured by the disappearance of cell-associated full-length VSG, is ~2.5 h (Figure 5B). However, there is a distinct lag in export, best visualized in the curve for appearance of tVSG in the medium. Identical results were obtained for the 221wt cell line (data not shown).

These results are consistent with a two-step process in which newly synthesized VSG is first transported to the cell surface where it transiently resides as a GPI-anchored homodimer. Subsequent proteolytic cleavage, proximal to the C-terminus of each monomer, results in quantitative release as dimeric tVSG. The smaller size of 221 tVSG (see Figure 2) can be accounted for, in part, by cleavage further from the C-terminus and in part by an *N*-linked oligosaccharide, immediately upstream of the GPI anchor (Holder, 1985; Carrington *et al.*, 1991), that remains with the C-terminal fragment. Inherent in the model is the presence at the cell surface of a heterodimeric VSG intermediate, composed of one full-length GPI-anchored monomer and one tVSG monomer. Consistent with this prediction is the presence of a small level of cell-associated 117 tVSG later in the chase period (Figure 5A, lanes 2–5). Another prediction is that the true rate of intracellular transport should be faster than the measured rate of release. Additional data to support this model are presented below.

Export of 117Δgpi was monitored over a 24 h chase period (Figure 6A) and a $t_{1/2}$ for export of ~5 h, with no apparent lag, was determined (Figure 6B). While this is slow relative to export of both 117wt VSG and another soluble recombinant reporter, BiPN ($t_{1/2}$ ~1 h) (Bangs *et al.*, 1996), it is nevertheless efficient since essentially all of the labeled 117Δgpi VSG is exported to the medium by the end of the chase period (compare lanes 1 and 12). Identical results were obtained for the 221Δgpi cell line (data not shown).

The PARP GPI sequence restores transport competence

The data presented above suggest that the absence of a GPI anchor reduces the efficiency of VSG transport. If so, the 117HP reporter, with the PARP GPI anchor sequence fused at the C-terminus, should display wild-type kinetics. To control for the presence of the HA9 epitope in 117HP, a matched GPI-minus reporter (117H) was also prepared (see Figure 1). Pulse–chase analyses (Figure 7) indicate that neither the manner nor rate of export of 117HP (Figure 7A) or 117H (Figure 7B) VSG differ significantly from that of the original 117 constructs. Thus, addition of a GPI signal is sufficient to restore transport competence, confirming that the transport defects in 117Δgpi and 117H are related to the absence of a GPI anchor. However, in contrast to 117wt (see Figure 5A), the size of 117HP VSG increases during intracellular transport. Initially detected as an immature form (~65 kDa; Figure 7A, lane 1), during the chase period it is converted

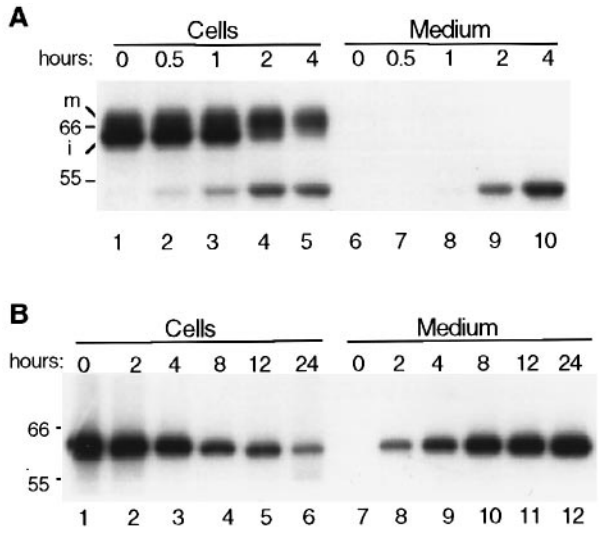

Fig. 7. Restoration of the GPI-plus phenotype. Transformed procyclic cell lines expressing recombinant VSG 117 reporters were pulse-labeled for 15 min and then chased for 4 h (**A**, 117HP) or 24 h (**B**, 117H). At the indicated chase times, aliquots were separated into cell and medium fractions and analyzed as in Figure 5, except that (A) is an 8–15% gradient gel. All lanes contain 5×10^6 cell equivalents. Scans of 2–3 day exposures are presented. Scale refers to relative molecular mass in kDa.

to a mature form (~68 kDa) and subsequently is released from cells (Figure 7A, lanes 2–5). These findings suggest that 117HP, but not 117wt, VSG is modified during intracellular transport.

Endoglycosidase treatment of procyclic VSGs

One possibility for post-translational processing of 117HP is the *N*-linked oligosaccharide at Asn453 of the 117 VSG sequence (see Figure 1). Although processing of *N*-linked oligosaccharides does occur in bloodstream forms, it has not been reported in procyclic trypanosomes. To address this question, we treated radiolabeled procyclic VSG with endo-β-*N*-acetylglucosaminidase H (Endo H), an enzyme that removes unprocessed *N*-linked oligosaccharides (Figure 8). Endo H treatment coordinately decreased the sizes of both full-length and truncated forms of 117wt (lanes 1 and 2) and 117HP (lanes 3 and 4) VSG by an amount consistent with the removal of a single high-mannose (i.e. unprocessed) oligosaccharide. It is also apparent that tVSG fragments derived from both reporters are essentially the same size (compare lanes 1 versus 3, and 2 versus 4). Since the 117HP and 117wt proteins (i) both have C-terminal GPI anchors, (ii) differ only in their C-terminal amino acid sequences and (iii) generate identical glycosylated tVSG fragments, a common cleavage site must exist downstream of Asn453 in the shared VSG 117 amino acid sequence, consistent with the generation of different sized C-terminal fragments (Figure 3). These results also demonstrate that the *N*-linked oligosaccharide is not involved in maturation of 117HP and that the site of processing is proximal to the C-terminus.

We have also found that the two *N*-linked oligosaccharides attached to 221wt VSG in procyclic trypanosomes are fully Endo H sensitive (unpublished observations). In bloodstream cells, this VSG bears one high-mannose (sensitive) and one complex (resistant)

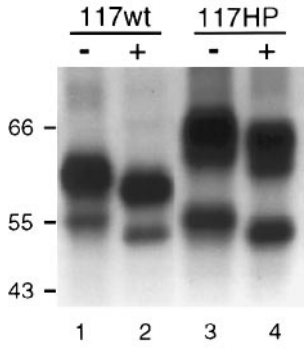

Fig. 8. Endoglycosidase treatment of 117wt and 117HP VSG. Cell lines were radiolabeled for 4 h, and 117wt (lanes 1 and 2) and 117HP (lanes 3 and 4) VSG polypeptides were immunoprecipitated from cell lysates. Immunoprecipitates were mock treated (–, lanes 1 and 3) or digested with Endo H (+, lanes 2 and 4). Reactions were fractionated on 8–15% SDS–polyacrylamide gels and visualized by fluorography. All lanes contain 10^7 cell equivalents. Scans of 36 h exposures are presented. Scales refer to relative molecular mass in kDa.

oligosaccharide (Zamze *et al.*, 1991). This result, together with similar findings for endogenous p67, a highly glycosylated lysosomal membrane protein (Brickman and Balber, 1994; Kelley *et al.*, 1996), suggests that procyclic cells are unable to process protein-linked high-mannose oligosaccharides to complex structures.

Release of VSG by a metalloprotease

The release of tVSG from procyclic cells provides a convenient assay that in reality measures a two-step process, transport and release. To study the release process alone, we surface-biotinylated the 117wt cell line and then followed the appearance of biotinylated 117 tVSG as a function of time (Figure 9A). Initially, all biotinylated VSG was present on the cell surface as both full-length and truncated VSG in approximately equal proportions. During the subsequent incubation, this doublet of surface VSG coordinately decreases concomitant with the exclusive appearance of tVSG in the medium. The presence of a large steady-state pool of cell surface tVSG, that has no membrane anchor of its own, is consistent with our interpretation that a heterodimer of full-length VSG and tVSG is an intermediate of the release process. Unlike the transport and release of biosynthetically labeled VSG, there is no lag when only the surface pool of VSG is assayed. These results indicate that the estimated $t_{1/2}$ for release of biosynthetically labeled VSG is an underestimate of the actual rate for intracellular transport.

The data presented thus far are consistent with the release of tVSG by a proteolytic event, but attempts to block release with a mixture of protease inhibitors (antipain, chymostatin, pepstatin and leupeptin) were unsuccessful. As none of these inhibitors would be expected to effect metalloproteases, we tested the ability of chelators of divalent cations to block release of tVSG (Figure 9B). Biotinylated cells were incubated in physiological saline in the presence of EDTA (lanes 5 and 6), EGTA (lanes 7 and 8) or 1,10-phenanthroline (lanes 9 and 10). Millimolar concentrations of each of these chelators substantially reversed the pattern of release seen in the untreated cells (lanes 1–4), indicating a divalent cation requirement. In an identical assay performed in complete TM-P medium,

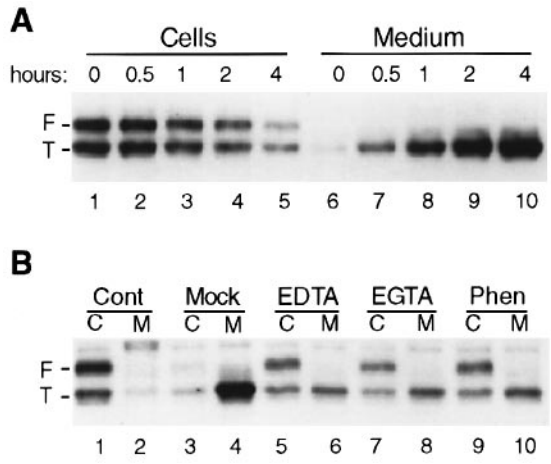

Fig. 9. Release of cell surface 117wt VSG from procyclic trypanosomes. 117wt cells were surface biotinylated as described in Materials and methods. (**A**) Kinetics of release of cell surface 117wt VSG. Biotinylated cells were cultured at 27°C in TM-P medium and, at the indicated times, aliquots were separated into cell and media fractions. (**B**) Inhibition of release by chelators of divalent cations. Biotinylated cells were cultured for 4 h at 27°C in supplemented PBS containing 3 mM EDTA, EGTA or 1,10-phenanthroline and then separated into cell and media fractions. Control biotinylated cells were analyzed with (Mock) or without (Cont) incubation. (A and B) 117 VSG polypeptides were specifically immunoprecipitated from all samples and fractionated by SDS–PAGE. Following electrophoretic transfer to nitrocellulose, biotinylated VSG was detected with streptavidin–HRP conjugate. All lanes contain 5×10^6 cell equivalents. Full-length (F) and tVSG (T) forms of 117wt VSG are indicated. Scale refers to relative molecular mass in kDa.

which is replete with millimolar levels of Ca^{2+} and Mg^{2+}, only phenanthroline inhibited release (unpublished observations). The ability of phenanthroline to inhibit in the presence of these cations strongly implicates some other metal ion, presumably Zn^{2+}, as the active cofactor.

Discussion

VSG is the major surface protein of the bloodstream stage of the trypanosome life cycle; its expression is tightly repressed in the insect stage. Using cell lines stably transformed with genes for two distinct VSGs (117 and 221), we find that there is no impediment to the 'inappropriate' expression of these proteins in procyclic cells. Collectively, our data argue for a general model whereby newly synthesized procyclic VSG is GPI anchored, dimerized and *N*-glycosylated. It is then transported to the plasma membrane where it enters a pool of cell surface full-length homodimeric VSG. Immunofluorescence assay indicates a uniform distribution over the entire cell (unpublished data). This pool is subject to proteolytic cleavage at a site proximal to the C-terminus, by a resident surface protease, generating first a membrane-bound heterodimer and then freely soluble homodimeric tVSG. In support of this model, velocity sedimentation analysis indicates that 117 tVSG exists in solution as a homodimer of the expected size (unpublished observations). Alternatively, it is possible that surface VSG is internalized, cleaved in an endosomal compartment and then released by recycling. However, the high steady-state level of tVSG on the cell surface and the fact that little detectable endocytosis occurs in procyclic trypanosomes (Langreth and Balber, 1975) argue against this interpretation. Three distinct issues arising from the experimental basis of this model merit discussion.

Release of cell surface VSG

Our results with transformed trypanosomes mimic those of Ziegelbauer *et al.* (1993) concerning the release of VSG from bloodstream cells during *in vitro* differentiation to procyclics. Using trypanosomes naturally expressing VSG 117, they found that a protease activity elaborated during differentiation was responsible for releasing the unneeded bloodstream surface coat. We have extended their findings in several important ways. First, the protease is present throughout the procyclic stage of the life cycle and is therefore likely to provide some essential function related to the survival of the parasite in the midgut of the tsetse fly vector.

Second, the protease is a metalloenzyme, with Zn^{2+} being the probable metal cofactor; the identification of discrete N- and C-terminal proteolytic fragments indicates it to be endoproteolytic. Precedent exists for cell surface zinc metalloendoprotease activities in the kinetoplastid protozoa, the gp63 gene family of the genera *Leishmania* and *Crithidia* (Medina-Acosta *et al.*, 1993). Recently a *T.brucei* gp63 homolog has been identified by sequencing of random genomic clones, and Northern analysis indicates that it is expressed in both bloodstream and procyclic cells (N.El-Sayed and J.E.Donelson, submitted). Thus, it is probable that we have identified a corresponding biochemical activity in the insect stage of the trypanosome life cycle.

Finally, 117wt and 117HP reporters produce identical tVSG fragments, suggesting that the site of cleavage is not determined by relative distance from the C-terminus (see Figure 1). Ziegelbauer *et al.* (1993) located cleavage to a sequence (amino acids 466–468) downstream of the glycosylation site at Asn453, which falls between two distinct subdomains of intrachain disulfide linkages (Allen *et al.*, 1982). VSG 221, which has a different C-terminal sequence and conformation, produces a smaller tVSG fragment (see Figure 2). Consequently, this protein must be cleaved further from the C-terminus, probably in the boundary region between the major N- and C-terminal domains (Carrington *et al.*, 1991). Thus, substrate conformation (availability of exposed polypeptide chain) and accessibility to the protease active site may control cleavage site selection. As noted by others (Ferguson *et al.*, 1993; Ziegelbauer *et al.*, 1993), the Glu–Pro repeat region of PARP is protease resistant. We suggest that this may be an essential adaptation for stability of the major procyclic surface molecule in a membrane containing a robust protease activity.

GPI anchor addition and processing

Our results demonstrate that natural GPI addition signals of VSGs function effectively in procyclic cells. Procyclic VSG is resistant to hydrolysis by recombinant *T.brucei* GPI-PLC, indicating that a PARP-type inositol-acylated GPI anchor is attached. Thus, it is the biosynthetic capability of procyclic cells (i.e. the steady-state level of GPI precursors), not the primary sequence of the addition signal, that determines the structure that is transferred to GPI acceptor proteins. Although our results do not estab-

lish that all VSG molecules expressed in procyclic cells receive a GPI anchor, it seems likely that most do since the export of 117wt VSG is so efficient. Failure to GPI-anchor significant amounts of VSG could have three predictable outcomes, all leading to intracellular retention: (i) retention of the hydrophobic GPI anchor signal peptide (discussed below); (ii) conversion to a partial reaction product covalently attached to the GPI transamidase (Udenfriend and Kodukula, 1995); and (iii) hydrolysis of the GPI transamidase adduct resulting in an enzymatically truncated equivalent of our VSGΔgpi constructs (Udenfriend and Kodukula, 1995).

Analysis of the 117HP reporter, which has the PARP GPI anchor signal fused to VSG 117 (see Figure 1), suggests that additional modifications of the GPI core can occur during intracellular transport. No such processing was found with the native 117wt reporter. The GPI anchor is the most likely site of processing since: (i) the single *N*-linked oligosaccharide at Asn453 is Endo H sensitive and therefore not processed; (ii) 117wt and 117HP produce identical tVSG fragments, indicating that processing must occur downstream of the cleavage site; and (iii) non-GPI-anchored reporters (117Δgpi and 117H) are not processed. The PARP GPI anchor acquires a sialylated poly-*N*-acetyllactosamine side chain (Ferguson *et al.*, 1993), and a similar structure may be assembled on 117HP during transport. If so, an unexpected implication of these findings is that adjacent primary amino acid sequence may influence GPI processing. A 15 residue sequence upstream of the PARP GPI addition site distinguishes 117HP from the unprocessed 117wt reporter (see Figure 1). This sequence may simply have a conformation that allows access of the appropriate glycosyltransferases to the GPI core. Alternatively, the primary sequence may provide a specific recognition signal needed for GPI processing. Any part of the extra sequence could provide such a signal, but the tripeptide, Pro–Glu–Pro, derived from the authentic PARP C-terminus, would be the most likely basis for specific PARP-type processing of the 117HP anchor. Studies are underway to address these possibilities, as well as the chemical nature of the modification. However, it should be noted that peptide-directed glycosylation is not without precedent: the GlcNac-1-phosphotransferase that catalyzes the first step in the synthesis of mannose-6-phosphate lysosomal targeting signals (Kornfeld and Mellman, 1989) and the UDP-Glc:glycoprotein glucosyltransferase that participates in the calnexin cycle (Sousa and Parodi, 1995) recognize folded and unfolded protein determinants, respectively.

The role of GPI anchors in transport

We have made careful estimations of the rate of transport for the various forms of 117 VSG expressed in procyclic cells. The half-time for export of full-length GPI-anchored VSG (~2.5 h), as measured by release of tVSG, is likely to be an overestimation since the end point is a measure of two processes, rate of transport and rate of proteolytic release. Consistent with this premise, release of biosynthetically labeled 117wt has a lag phase whereas release of surface biotinylated 117wt is immediate. Thus, a more realistic estimate may be made by considering the processing events involved in the maturation of 117HP, the bulk of which are likely to occur during intracellular transport. Collectively, these data suggest that the true rate of transport for GPI-anchored VSG in procyclic trypanosomes is closer to $t_{1/2}$ ~1 h, similar to the rate for a soluble secretory reporter, BiPN (Bangs *et al.*, 1996).

In contrast, the rate of transport of VSG in the absence of a GPI anchor can be assessed directly by release into the medium. Surprisingly, the rate of 117Δgpi transport ($t_{1/2}$ ~5 h) is greatly reduced relative to transport of 117wt VSG. This cannot be attributed to some deleterious mutation of the 117 coding region since wild-type kinetics are restored by replacement of a GPI anchor addition signal (117HP). The simplest conclusion is that the GPI anchor plays some essential role in intracellular transport. Disruption of GPI attachment has been shown to block transport of a number of GPI-anchored proteins in both yeast and animal cells (Delahunty *et al.*, 1993; Field *et al.*, 1994; Chen *et al.*, 1996; Doering and Schekman, 1996). However, in each of these cases, reporter proteins retained the C-terminal hydrophobic domain, making it impossible to separate a loss of GPI-mediated functionality from an adverse effect of the hydrophobic peptide. In our case, the defect in transport is related directly to the loss of the GPI anchor.

Two preliminary results from our laboratory are relevant to this issue (unpublished data). First, double immunofluorescence assays with the 117Δgpi cell line indicate that intracellular VSG is coincident with the ER molecular chaperone BiP, suggesting that the bottleneck is in exit from the ER. Second, secreted 117Δgpi VSG is dimerized as judged by velocity sedimentation analysis. Thus, it may be that in the absence of a GPI anchor the 117 protein fails to fold efficiently and is retained by ER quality control mechanisms (Hammond and Helenius, 1995) until folding is complete. Alternatively, GPI anchors may be required as a positive factor in mediating forward transport of VSG from the ER, perhaps by directing lateral movement of cargo proteins into budding ER transport vesicles via interaction with lipid bilayer microdomains. Evidence for interactions of GPI anchors with sphingolipid microdomains does exist (Hanada *et al.*, 1993, 1995). Furthermore, inhibition of ceramide synthesis has been shown to reduce transport of Gas1p, the major GPI-anchored protein in yeast (Horvath *et al.*, 1994). A 'lipid clustering' model was proposed for this finding and it may be that such a mechanism is operative in trypanosomes, which do have significant levels of sphingolipid (Patnaik *et al.*, 1993). Work is currently underway to resolve these alternative models, the results of which will have direct bearing on the transport of GPI-anchored proteins in all eukaryotic cells.

Materials and methods

Maintenance and manipulation of trypanosomes

The growth, maintenance, transformation and metabolic radiolabeling ([^{35}S]methionine/cysteine) of cultured strain 427 procyclic trypanosomes have been described (Bangs *et al.*, 1996). All experiments were performed with uncloned stably transformed procyclic cell lines. For labeling with [9,10-^{3}H]palmitic acid, cells were cultured (6 h) in TM-P media (Overath *et al.*, 1986) without fetal bovine serum and containing labeled fatty acid [200 μCi/ml as an equimolar complex with fatty acid-free bovine serum albumin (BSA)].

To biotinylate procyclic cell surfaces, cells were washed three times in ice-cold phosphate-buffered saline with 1 mg/ml glucose (PBSG) and resuspended at 10^8 cells/ml. Sulfo NHS-biotin (Pierce Chemical Co, Rockford, IL) was added to 200 μg/ml from a freshly made 10× stock

in PBSG. Cells were incubated on ice for 20 min and then diluted to 10^7/ml with PBSG supplemented with 5.5 mM proline, 10 mM glycerol. After the addition of 1/100 volume of 1 M NH_4Cl, cells were washed three times in ice-cold supplemented PBSG, resuspended at 10^7/ml in pre-warmed TM-P medium and cultured at 27°C. For chelator inhibition experiments, biotinylated cells were incubated in supplemented PBSG. In all metabolic radiolabeling and biotinylation procedures, cells were monitored throughout the experimental period by microscopy, and viability as judged by motility was excellent. Only incubation with phenanthroline resulted in reduced motility at the end of 4 h incubation, but cells remained intact.

Construction of reporter genes

Procedures were performed by protocols according to either Sambrook *et al.* (1989) or the manufacturer's instructions. All reporter genes were generated by PCR and the products were cloned into either pBluescript II SK– (pBSII, Stratagene Inc., San Diego, CA) or the HA9 epitope-tagging vector, pHA9 (Bangs *et al.*, 1996), using unique flanking restriction sites. The ends of all inserts were sequenced (Sequenase Kit, United States Biochemical Corp., Cleveland, OH) to confirm identity. Ultimately, all reporters were cloned into the procyclic expression vector, pXS2 (Bangs *et al.*, 1996), for transformation of procyclic trypanosomes. In some cases, products were cloned directly into pXS2 and the 3′ end of the inserts were sequenced using primer JB85.

VSG 117 reporters (Figure 1) were amplified using the plasmid pGB117.1 containing the *T.brucei* VSG 117 basic copy gene (Benards *et al.*, 1981) (a generous gift of Dr John Boothroyd, Stanford University) as template with the following synthetic deoxyoligonucleotide primer combinations: JB77/JB80, the entire 117 basic copy gene (117wt); and JB77/JB79, the 117 basic copy minus the C-terminal GPI addition signal (117Δgpi). 117H was constructed by cloning the *Hin*dIII–*Nhe*I fragment bearing the 117Δgpi coding region into *Hin*dIII–*Nhe*I-cut pHA9F, fusing the nine amino acid HA9 epitope to the C-terminus of 117Δgpi. 117H was modified further by placing the PARP B2α GPI anchor sequence at the extreme C-terminus. This was accomplished by amplifying from the cloned PARP B2 locus (a generous gift of Dr Keith Wilson, Stanford University) the portion of the PARP gene encoding the C-terminus using primers JB144/JB122. The PCR product was cut with *Mlu*I–*Eco*RI and inserted into the *Mlu*I–*Eco*RI sites of 117H, generating 117HP. The VSG 221 gene (Carrington *et al.*, 1991) was amplified from total MITat1.2 bloodstream trypanosome RNA by RT–PCR using primers JB146/JB147 for full-length VSG 221 (221wt) and JB146/148 for the GPI-deleted form (221Δgpi). The latter was cloned into *Cla*I–*Mlu*I-cut pHA9F to provide an in-frame stop codon.

Synthetic deoxyoligonucleotides

The following synthetic deoxyoligonucleotides were used for PCR. Sequences complementary to the target template are in upper case; added sequences are in lower case. Restriction sites are underlined; all sequences are 5′ to 3′: JB77, cagggatccaagcttATGGACTGCCATACA, 5′ sense primer for VSG 117 (codons 1–5) with *Bam*HI and *Hin*dIII sites; JB79, acggaattcttagctagcATCTTTGCAAGCATT, 3′ antisense primer for VSG 117 (codons 499–503) with *Eco*RI and *Nhe*I sites; JB80, acggaattcCTAAAAAAGCAAGGC, 3′ antisense primer for VSG 117 (codons 521–525) with an *Eco*RI site; JB85, GTTTTACAAGAAA-CAGCAGG, 3′ sequencing primer for pXS2 reporter inserts, complementary to PARPint sequence 100–120 bp downstream of the multicloning site; JB122, aaagaattcTTAGAATGCGGCAACGAG, 3′ antisense primer for PARP B2α (codons 141–146) with an *Eco*RI site; JB144, aaaaacgcgtcACCTGAACCTGGTGCTGC, 5′ sense primer for PARP B2α (codons 120–125) with a *Mlu*I site; JB146, aaaatcgatATGCCTTCCAATCAGGAG, 5′ sense primer for VSG 221 (codons 1–6) with a *Cla*I site; JB147, aaagaattcTTAAAAA-AGCAAAACTGC, 3′ antisense primer for VSG 221 (codons 472–477) with an *Eco*RI site; JB148, aaaaacgcgtaGCTGCTTCCTGTGGTG, 3′ antisense primer for VSG 221 (codons 455–459) with a *Mlu*I site.

Immunoprecipitation, immunoblotting and electrophoresis

Cell lysis conditions, specific immunoprecipitation with anti-VSG antibodies, electrophoresis conditions and fluorography have been described previously (Bangs *et al.*, 1996). All lysates and culture supernatants were supplemented with 1× protease inhibitor cocktail (PIC: 2 μg/ml each leupeptin, pepstatin, chymostatin and antipain) prior to immunoprecipitation. Unless otherwise stated in the text, all samples were fractionated by 12% SDS–PAGE. Rabbit anti-VSG 117 and anti-VSG 221 were negatively selected on VSG 221 and VSG 117 Sepharose columns, respectively, to remove antibodies to the cross-reacting determinant (Barbet and McQuire, 1978). Anti-VSG 117 was affinity purified further on a VSG 117 Sepharose column. For immuno and streptavidin blotting, gels were transferred electophoretically to 0.45 μm NitroPure membranes (MSI, Westboro MA) and molecular weight markers were visualized by Ponceau S staining. Membranes were blocked in immunobuffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Tween-20, 0.01% SDS) containing 5% non-fat dry milk. After rinsing, membranes were incubated for 1 h with primary antibody diluted in immunobuffer with 1% normal goat serum and then washed (4× 10 min) with immunobuffer. Blots were incubated (1 h) in secondary antibody [goat anti-rabbit IgG–horseradish peroxidase (HRP) conjugate, Kirkegaard and Perry Laboratories Inc. (KPL), Gaithersburg, MD], washed as before, and specific staining was detected by chemiluminescence (LumiGLO, KPL). For biotinylation experiments, blocked membranes were probed directly with streptavidin–HRP conjugate (KPL).

PI-PLC treatment of VSG

Triton X-114 extracts were prepared as follows. Radiolabeled transformed trypanosomes (2.5×10^7) expressing 117wt VSG were incubated (15 min, 0°C) in extraction buffer (EB: 125 μl; 50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 2× PIC) containing 1% pre-condensed Triton X-114 (Bordier, 1981). The lysate was cleared by microcentrifugation (10 min, 0°C) and phase separation was induced at elevated temperature (15 min, 37°C). Following microcentrifugation (10 min, room temperature), the detergent phase was resuspended to the original volume with ice-cold EB. Samples (2×50 μl, 10^7 cell equivalents) were supplemented with ~1 μg of purified bloodstream membrane-form 221 VSG (Mensa-Wilmot *et al.*, 1995) and then mock-treated or treated at 27°C with hourly additions of 4500 U of recombinant *T.brucei* GPI-PLC (a generous gift of Dr Kojo Mensa-Wilmot, Univeristy of Georgia). After 2 h, the samples were subjected to phase extraction and the aqueous and detergent phases were adjusted to 200 μl, 0.5% Triton X-114. Equal portions of each were analyzed for 117 VSG by immunoprecipitation or, following 10% trichloroacetic acid (TCA) precipitation, for 221 VSG by immunoblotting.

Endoglycosidase H treatment of VSG

Immunoprecipitated polypeptides (2×10^7 cell equivalents) were eluted from protein A–Sepharose beads by boiling in 1% SDS (200 μl) and, following addition of hemoglobin (20 μg) and NP-40 (to 1%) as carrier, were precipitated with 10% TCA. Pellets were washed three times in acetone, dried and dissolved in denaturation buffer (10 μl: 50 mM sodium citrate, pH 5.5, 1% 2-mercaptoethanol, 0.5% SDS). After boiling (5 min), samples were diluted with renaturation buffer (40 μl: 50 mM sodium citrate, 1.25% NP-40, 0.02% NaN_3, 2× PIC) and split into two 25 μl aliquots. Samples received either Endo H (New England Biolabs, 1 μl, 1000 U) or mock addition, and were incubated at 37°C for 20 h with fresh additions at 16 h. Reactions were terminated by TCA precipitation as described above and analyzed by SDS–PAGE/fluorography.

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