Streamlined Architecture and Glycosylphosphatidylinositol-dependent Trafficking in the Early Secretory Pathway of African Trypanosomes

Elitza S. Sevova and James D. Bangs

Department of Medical Microbiology and Immunology, University of Wisconsin School of Medicine and Public Health, Madison, WI 53706

Submitted July 2, 2009; Revised August 14, 2009; Accepted September 10, 2009

Monitoring Editor: Benjamin S. Glick

The variant surface glycoprotein (VSG) of bloodstream form Trypanosoma brucei (Tb) is a critical virulence factor. The VSG glycosylphosphatidylinositol (GPI)-anchor strongly influences passage through the early secretory pathway. Using a dominant-negative mutation of TbSar1, we show that endoplasmic reticulum (ER) exit of secretory cargo in trypanosomes is dependent on the coat protein complex II (COPII) machinery. Trypanosomes have two orthologues each of the Sec23 and Sec24 COPII subunits, which form specific heterodimeric pairs: TbSec23.1/TbSec24.2 and TbSec23.2/TbSec24.1. RNA interference silencing of each subunit is lethal but has minimal effects on trafficking of soluble and transmembrane proteins. However, silencing of the TbSec23.2/TbSec24.1 pair selectively impairs ER exit of GPI-anchored cargo. All four subunits colocalize to one or two ER exit sites (ERES), in close alignment with the postnuclear flagellar adherence zone (FAZ), and closely juxtaposed to corresponding Golgi clusters. These ERES are nucleated on the FAZ-associated ER. The Golgi matrix protein Tb Golgi reassembly stacking protein defines a region between the ERES and Golgi, suggesting a possible structural role in the ERES:Golgi junction. Our results confirm a selective mechanism for GPI-anchored cargo loading into COPII vesicles and a remarkable degree of streamlining in the early secretory pathway. This unusual architecture probably maximizes efficiency of VSG transport and fidelity in organellar segregation during cytokinesis.

INTRODUCTION

Trypanosoma brucei spp. are phylogenetically ancient parasitic protozoa, responsible for African trypanosomiasis (sleeping sickness) in humans and the veterinary disease Nagana in cattle. Transmitted by the tse-tse fly (Glossina spp.) vector, T. brucei have a digenetic life cycle alternating between the bloodstream form (BSF) in vertebrate hosts and the procyclic form in tsetse flies (Glossina spp.). Trypanosomes are phylogenically ancient parasitic protozoa, responsible for African trypanosomiasis (sleeping sickness) in humans and the veterinary disease Nagana in cattle. Transmitted by the tse-tse fly (Glossina spp.) vector, T. brucei have a digenetic life cycle alternating between the bloodstream form (BSF) in vertebrate hosts and the procyclic form in tsetse flies (Glossina spp.).

© 2009 by The American Society for Cell Biology
into the forming vesicle (Barlowe et al., 1994; Kuehn et al., 1998). Cargo capture is thought to involve interaction between specific motifs in the cytoplasmic domains of cargo molecules and the Sec24 subunit and to a lesser extent with Sec23 (Miller et al., 2003, 2005; Mossessova et al., 2003; Cai et al., 2007; Farhan et al., 2007; Mancias and Goldberg, 2007). Subsequently, the Sec13/Sec31 heterotetramer is recruited, concentrating the prebudding complexes, and stimulating membrane deformation and subsequent vesicle scission from the ER (Matsuoka et al., 1998).

In Saccharomyces cerevisiae, GPI anchors facilitate loading of cargo into COPII vesicles. This process is influenced by de novo synthesis of ceramide (Horvath et al., 2002). Disruption of p24 expression from the ER (Matsuoka et al., 1998). Cargo capture is thought to involve interaction between the Sec13/Sec31 heterotetramer and the 3′-untranslated region (UTR) sequence of each gene. The Sec13/Sec31 heterotetramer and the 3′-UTR were digested with KpnI/Sacl and stably transfected into BS221 cells. In total, four clonal cell lines were developed, each with a different combination of tagged Tbs23c and Tbs23c components: Tbs23c1:HA/Tbs23c1:Ty, Tbs23c2:HA/Tbs23c1:Ty, Tbs23c1:HA/Tbs23c2:Ty, and Tbs23c2:HA/Tbs23c2:Ty. In frame chromosomal integration of the cDNA from glycosyltransferase TbgT135Y has been described previously (Sutterwala et al., 2008).

**Northern Analysis**

Total RNA was extracted from mid-log phase parasites by using TRIzol reagent (Promega) according to the manufacturer’s instructions. PCR-generated probes (Tbs23c1, nt 939-1743; Tbs23c2, nt 1025-1857; Tbs24c1, nt 1028-1900; and Tbs24c2, nt 1025-1900) were labeled with [32P]dCTP with a random priming kit (Stratagene, CA) and desalted on ProbeQuant 96 G-50 columns (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was performed at 1% agrose/17.5% formaldehyde gels and transferred to Zeta Probe nylon membranes (Bio-Rad Laboratories, Hercules, CA) by capillary action. Membrane-bound RNA was photo cross-linked at 1200 μl (1 min). Membranes were prehybridized for 4 h at 42°C (5x SSPE, 5x Denhardt’s solution, 0.1% formaldehyde, 0.5% SDS, and 100 μg/ml denatured salmon testes DNA), and denatured probes were added in fresh, prewarmed hybridization buffer. After hybridization (42°C overnight), membranes were washed once in 2x SSC, 0.1% SDS (20 min at room temperature [RT]), and twice in 0.2x SSC, 0.1% SDS (15 min for 42°C). To normalize, blots were stripped (3 times at 80°C for 30 min; 2x SSC and 0.5% SDS) and reprobed with a tubulin probe. In all cases, hybridization was analyzed by phosphorimaging using a Typhoon Storm 8600 system (GE Healthcare) with native ImageQuant software.

**Metabolic Radiolabeling and Immunoprecipitation**

Pulse-chase metabolic radiolabeling with [%35S]methionine/cysteine of trypanosomes and immunoprecipitation of labeled reporters have been detailed previously (Triggs and Bangs, 2003; Peck et al., 2008). Unless stated otherwise, lysates for immunoprecipitation were prepared in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS). Pulse times were 2 min (V5), 5 min (BiP: GPI), 10 min (TbICATL), or 15 min (p67). VSG transit to the surface was analyzed as described previously (Bangs et al., 1986; Sutterwala et al., 2008). In brief, transfected cells were briefly washed, and pulse-chased, acid-precipitable, membrane-bound VSG to soluble VSG by endogenous GPI-phospholipase C (PLC). Cell-associated and soluble VSG were then separated by centrifugation and immunoprecipitation under standard conditions. For Tbs24c1, Tbs23c1, and Tbs23c2 constructs, VSG were radiolabeled for 1 h with [35S]methionine/cysteine as appropriate. Tbs24c1 and Tbs23c2 VSG were immunoprecipitated from cell lysates with anti-T7 or anti-VSG. All immunoprecipitates were sepa...
rated on 12% SDS-polyacrylamide gel electrophoresis (PAGE) gels and visualized via the Typhoon system (GE Healthcare). Quantification of phosphorimages was done by ImageQuant software as described previously (Terry et al., 2007).

Sequential immunoprecipitation experiments have been detailed previously (Bangs et al., 1996). In brief, parasites were radiolabeled with [35S]methionine/cysteine for 2.5 h. Cells were lysed in TEN buffer (50 mM Tris-HCl, 150 mM NaCl, and 5 mM EDTA, pH 7.5) with 0.5% NP-40, protease inhibitor cocktail (1 mg/ml each of leupeptin, antipain, aprotinin, and chymostatin), 0.1 mM tosyllysine chloromethyl ketone, and 0.5 mM phenylmethylsulfonyl fluoride. Lysates were cleared of debris by microcentrifugation (5 min at 13,000 rpm) and immunoprecipitated overnight with either anti-HA or anti-Ty antibody bound to protein A-Sepharose beads. Precipitates were washed twice in TEN buffer. Bound material was solubilized in 1% SDS (200 μl) and reconstituted to standard RIPA conditions (1.0 ml). Secondary immunoprecipitates were done with the alternate antibody (anti-HA or anti-Ty as appropriate) bound to protein A-Sepharose beads (6 h at 4°C). Beads were washed twice in TEN buffer, and bound material was solubilized in sample buffer, fractionated on a 12% SDS-PAGE gel, and imaged as described above.

Antibodies

Rabbit anti-BiP, rabbit anti-VSG221, monoclonal mouse anti-p67 (mAb218), and rabbit anti-TbCATL have all been described previously (Bangs et al., 1993; Alexander et al., 2002; Peck et al., 2008). Mouse anti-HA (Invitrogen, Carlsbad CA), rabbit anti-HA (Covance Research Products, Berkeley, CA), and mouse anti-Ty (AB Arcadia, Hybridaoma Facility, Birmingham, AL) were used as in previous publications (Sutterwala et al., 2008). Rabbit anti-T7 (Bethyl Laboratories, Montgomery, TX) was used at the recommended dilutions. Rabbit anti-Golgi reassembly stacking protein (GRASP) (He et al., 2004), and mouse monoclonal antibody (mAb) L382 anti-HA (Kohl et al., 1999) were generously provided by Graham Warren (Max F. Perutz Laboratories, Vienna, Austria) and Keith Gull (University of Oxford, Oxford, United Kingdom), respectively.

Immunofluorescence Microscopy

Immunostaining of formaldehyde-fixed parasites was done as described previously, with minor alterations (Sutterwala et al., 2008). After settling onto poly-L-lysine slides, cells were incubated (1 h at RT) with blocking buffer: phosphate-buffered saline (PBS), 10% normal goat serum (NGS), 1% Triton X-100, 1% bovine serum albumin. Cells were stained with primary antibodies diluted in blocking buffer, washed, and stained with the appropriate secondary antibodies and 4,6-diamidino-2-phenylindole (DAPI) (500 ng/ml). After washing, slides were mounted with PBS/glycerol (1:1). Serial image stacks (0.2-μm Z-increment) were collected with capture times from 100 to 500 ms (100X PlanApo oil immersion 1.4 numerical aperture) on a motorized Axiosplan IIi (Carl Zeiss, Jena, Germany) equipped with a rear-mounted excitation filter wheel, a triple pass (DAPI/Fluorescein isothiocyanate/Texas Red) emission cube, differential interference contrast (DIC) optics, and an Orca AG charge-coupled device camera (Hamamatsu, Bridgewater, NJ).

All images were collected with OpenLab 4.0 software (Improvement, Lexington, MA), and individual channel stacks were deconvolved by a constrained iterative algorithm, pseudocolored, and merged using Velocity 5.0 software (Improvement). This arrangement allows pixel precise localization in x, y and z dimensions (Supplemental Figure S1).

Transmission Electron Microscopy

For immunolocalization at the ultrastructural level, parasites were fixed in 4% paraformaldehyde/0.05% glutaraldehyde in phosphate-buffered saline for 1 h at 4°C. Samples were then embedded in 10% gelatin and infiltrated overnight with 2.3 M sucrose and 20% polyvinyl pyrrolidone in piperoxane-N,N′-bis-(ethanesulonic acid) (PIPES) and MγC3, at 4°C. Samples were trimmed, frozen in liquid nitrogen, and sectioned with an Ultracut UCT cryo-ultramicrotome (Leica Microsystems, Bannockburn, IL). Fifty-nanometer sections were blocked with 5% FBS and 5% NGS for 30 min and subsequently incubated with mouse anti-BiP (1/50) and goat anti-HA (1/200; Sigma-Aldrich, St. Louis, MO) antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. Sections were washed in block buffer and probed with goat anti-mouse (12-nm colloidal gold-conjugated) and goat anti-rabbit (18-nm colloidal gold-conjugated) antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h at room temperature. Sections were washed in PIPES buffer followed by a water rinse and stained with 0.3% uranyl acetate/2% methylcellulose. Samples were viewed with a 1200EX transmission electron microscope (JEOL USA, Peabody, MA). Parallel controls omitting primary antibodies were consistently negative at all time points and with colloidal gold-conjugated secondary antibodies used in these studies.

RESULTS

Identification of Trypanosomal COPII Machinery

Trypanosome COP II coat orthologues were identified by querying the T. brucei genome database (www.sanger.ac.uk/Projects/T_brucei) with the respective S. cerevisiae open reading frames (Supplemental Table S1). Single orthologues were found for Sar1 and Sec31, whereas two paralogous genes exist for Sec23 (TbSec23.1 and TbSec23.2), Sec24 (TbSec24.1 and TbSec24.2), and Sec13 (TbSec13.1 and TbSec13.2) (Supplemental Table S1). We have chosen an unbiased, numbered nomenclature for the T. brucei orthologues (i.e., TbSec23.1, etc.) because the functional relationship to other orthologues over such large phylogenetic distances is dubious. To begin our studies on the early secretory pathway in African trypanosomes, we chose to focus on TbSar1, TbSec23, and TbSec24, because Sar1 initiates the process of COPII vesicle formation and because the Sec23/Sec24 heterodimer is critical to cargo recruitment into the budding vesicle. Alignment of the paralogous TbSec23 and TbSec24 open reading frames reveals that these sequences are modestly similar to each other (59 and 44%, respectively).

Secretory Transport Is COPII Dependent in Trypanosomes

In yeast and mammals, trafficking of all classes of secretory cargo is COPII-vesicle dependent. To confirm this in trypanosomes, we generated a dominant-negative mutant of TbSar1. A single amino acid change in Sar1 (T34N in P. falciparum; T39 in mammals) creates a GDP-dominated-negative mutant, with reduced affinity for GTP. Expression of this mutant inhibits further rounds of COPII vesicle formation (Kuge et al., 1994; Connerly et al., 2005). Using this approach, we made the equivalent TbSar1 mutation (T34N), creating the dominant-negative, GDP-locked form of TbSar1 (TbSar1DN). This construct and a matched wild-type control, both of which contain a C-terminal T7-epitope tag, were introduced into BSF trypanosomes under a tetracycline-inducible promoter, and conditional synthesis was monitored by metabolic radiolabeling and specific immunoprecipitation with anti-T7 antibody (Figure 1A, bottom). Expression of both TbSar1WT and TbSar1DN was detected as early as 1 h of induction (lanes 2 and 5) and increased at 3 h (lanes 3 and 6). Expression of TbSar1WT had no effect on cell growth, but strikingly TbSar1DN expression resulted in arrest of cell growth after only 4 h of induction, followed rapidly by cell death (Figure 1A, top). This catastrophic effect indicates that TbSar1 activity is critical for cell viability.

To confirm that cargo egress from the ER is COPII-dependent in trypanosomes, we determined the effect of TbSar1DN expression on the intracellular transport of three well-characterized, endogenous secretory reporters: TbCATL (soluble), p67 (type I transmembrane), and VSG (GPI anchored). After TbSar1DN induction, ER exit was monitored by pulse-chase analysis using distinct transport-dependent processing events for each reporter. TbCATL, a lysosomal cathepsin-L-like protease (Caffrey and Steverding, 2009), is synthesized in the ER as an immature proenzyme (∼53 kDa, I) and as an additional smaller “precursor” form (∼50 kDa, X) (Figure 1B, Tet−). The exact nature of precursor X is not clear, but our own observations (Sutterwala and Bangs, unpublished) indicate that it is derived from the TbCATL open reading frame. On arrival in the lysosome, both forms are converted by proteolytic cleavage to mature active TbCATL (43 kDa, M). After TbSar1DN induction, there was a 1.9-fold delay in the processing of the immature forms of TbCATL (I+X) (Table 1). p67, a lysosomal associ-

1 In our prior publications, we have used the term “trypanopain” to refer to TbCATL as a generic name for this cathepsin L orthologue in all T. brucei subspecies. In accord with the recent proposal of Caffrey and Steverding (2009), we now adopt this universal nomenclature.
Figure 1. TbSar1DN expression is lethal and delays forward protein trafficking in *T. brucei*. (A–D) Transgenic cell lines containing the inducible TbSar1WT (squares, A only) and TbSar1DN (circles, all panels) constructs were cultured without (Tet−, open symbols) or with tetracycline (Tet+, closed symbols) to induce expression of the wild-type and dominant-negative TbSar1 proteins. (A) Growth of the TbSar1WT and TbSar1DN cell lines were monitored (top, mean ± SD for triplicate cultures), and cells were metabolically radiolabeled (bottom) for 1 h in the absence (lanes 1 and 4) or presence of tetracycline after 0 h (lanes 2 and 5) or 2 h (lanes 3 and 6) of prior tetracycline induction. As indicated, lysates were immunoprecipitated with anti-T7 for TbSar1 proteins (S, top images) or anti-VSG (V, bottom images) as a labeling/loading control. Precipitates were analyzed by SDS-PAGE (10^7 cell equivalents/lane) and phosphorimaging. (B–D) Control induction. As indicated, lysates were immunoprecipitated with anti-T7 for TbSar1 proteins (S, top images) or anti-VSG (V, bottom images) for absence (lanes 1 and 4) or presence of tetracycline after 0 h (lanes 2 and 5) or 2 h (lanes 3 and 6) of prior tetracycline induction. TbSar1WT and TbSar1DN cell lines were monitored (top, mean ± SEM for triplicate cultures), and cells were metabolically radiolabeled (bottom) as disappearance of the initial forms for reporter transport as measured by loss of gp100 decreased 2.6-fold after TbSar1DN induction (Table 1). Finally, we monitored transport of VSG, a GPI-anchored homodimer. GPI anchor

Table 1. Kinetics (t_{1/2}) of reporter transport

<table>
<thead>
<tr>
<th>Reporter</th>
<th>Tet</th>
<th>TbSar1DN</th>
<th>TbSec23.1</th>
<th>TbSec23.2</th>
<th>TbSec24.1</th>
<th>TbSec24.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TbbCATL^b</td>
<td>−</td>
<td>16.5 ± 2.3</td>
<td>10.5 ± 0.5</td>
<td>14.5 ± 0.3</td>
<td>11.5 ± 0.5</td>
<td>11.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>31.2 ± 3.6 (1.9)</td>
<td>10.2 ± 0.9 (1.0)</td>
<td>19.5 ± 0.8 (1.3)</td>
<td>16.0 ± 2.5 (1.4)</td>
<td>13.0 ± 0.8 (1.2)</td>
</tr>
<tr>
<td>p67^c</td>
<td>−</td>
<td>40.7 ± 3.9</td>
<td>43.8 ± 3.3</td>
<td>39.7 ± 4.2</td>
<td>36.5 ± 2.3</td>
<td>37.0 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>107.7 ± 9.3 (2.6)</td>
<td>44.2 ± 7.1 (1.0)</td>
<td>583 ± 3.4 (1.5)</td>
<td>515.6 ± 3.6 (1.4)</td>
<td>410 ± 4.5 (1.1)</td>
</tr>
<tr>
<td>VSG^d,e</td>
<td>−</td>
<td>18.9 ± 2.6</td>
<td>27.5 ± 2.2</td>
<td>23.5 ± 4.4</td>
<td>20.7 ± 4.2</td>
<td>23.2 ± 5.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>219.7 ± 8.5 (11.6)</td>
<td>52.5 ± 2.0 (1.9)</td>
<td>128.7 ± 12.7 (5.5)</td>
<td>81.8 ± 1.3 (4.0)</td>
<td>30.0 ± 5.3 (1.3)</td>
</tr>
<tr>
<td>BiPN:GPI^f</td>
<td>−</td>
<td>19.1 ± 0.4</td>
<td>20.7 ± 0.8</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>28.8 ± 3.9 (1.5)</td>
<td>35.1 ± 9.4 (2.7)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.

^a Kinetics of transport were quantified, as defined below, in control (Tet−) cells and after induction (Tet+) of dominant-negative TbSar1 or specific COPII subunit RNAi, as indicated. Data were analyzed in Prism 4 (GraphPad Software, San Diego, CA). Half-times (t_{1/2}) in minutes were determined graphically and are presented as means ± SEM for triplicate determinations. -Fold differences are indicated in parentheses.

^b TbbCATL was measured as loss of initial species (I ± X).

^c p67 was measured as loss of initial gp100 ER glycoform.

^d VSG was measured as loss of cell-associated VSG after hypotonic lysis.

^e Half-times for VSG transport in induced TbSar1DN and TbSec23.2 RNAi cells were extrapolated and should be considered approximate.

^f BiPN:GPI was measured as loss of initial immature form (I).
attachment occurs immediately after translocation into the ER, and VSG is then rapidly transported to the cell surface via the Golgi and flagellar pocket (Bangs et al., 1985, 1986; Ferguson et al., 1986; Duszenko et al., 1988). The assay for VSG transport relies on the onset of susceptibility to hydrolysis by endogenous GPI-PLC during hypotonic lysis, concurrent with arrival at the cell surface. Newly synthesized VSG en route to the flagellar pocket is resistant to the action of GPI-PLC under these conditions. After TbSar1DN expression, VSG trafficking was drastically reduced (Figure 1D, bottom, Tet/H11002 vs. Tet/H11001), with the rate of transport decreasing 12-fold (Figure 1D and Table 1). Collectively, the severe effect of TbSar1DN expression on VSG transport, in addition to the significant delays seen for both TbbCATL and p67 trafficking, indicates that ER exit of newly synthesized trypanosomal secretory cargo is critically dependent on Tb-Sar1 and the COPII machinery. We attempted to visualize these delayed reporters within the ER but were unsuccessful, probably because the window of time between TbSar1DN induction and cell death is too short to allow sufficient accumulation of newly synthesized proteins for immunodetection.

Silencing of TbSec23 and TbSec24 Paralogues Is Lethal in BSF Trypanosomes

The requirement of TbSar1 for viability and efficient cargo transport led us to investigate the role of the TbSec23 and TbSec24 isoforms. Inducible dsRNA constructs targeting each subunit were introduced individually into BSF trypanosomes and in each case cellular viability was severely affected by RNAi silencing (Figure 2, A–D). Sustained growth arrested as early as 12 h postinduction, and cell death followed beginning at 18–24 h. Consequently, all subsequent RNAi phenotypic analyses were performed at 12 h of dsRNA induction. Northern blot analyses of total RNA isolated from control and silenced cells indicated that TbSec23.1 and TbSec23.2 transcript levels were specifically reduced to 33.2 ± 5.8 and 35.3 ± 6.3%, respectively (mean ± SEM, n = 3), without significantly affecting transcript levels from the paralogous gene (Figure 2, A and B). Similarly, induction of dsRNA targeting TbSec24.1 or TbSec24.2 specifically depleted transcript levels to 30.9 ± 3 0.2 and 23.5 ± 11.4%, respectively (mean ± SEM, n = 3) (Figure 2, C and D). Interestingly, however, knockdown of each TbSec24 transcript led to a modest increase of the paralogous transcript (TbSec24.1 up ~70%; TbSec24.2 up ~40%), suggesting that cells attempt to compensate for the depletion of one TbSec24 subunit message with the upregulation of the other. Collectively, these data indicate that BSF cell viability is dependent on the expression of all TbSec23 and TbSec24 subunits. Consistent with this conclusion is the repeated failure of all attempts to achieve double-targeted gene disruptions for each of the four COPII subunits (data not shown).

VSG Transport Is Preferentially Dependent on TbSec23.2 and TbSec24.1

The presence of duplicate isoforms of TbSec23 and TbSec24 raises the question of whether they are functionally reduct-
dant or whether they play distinct cargo-specific roles in ER exit. To investigate this issue, we analyzed the trafficking of our suite of endogenous secretory reporters after specific RNAi silencing for each COPII subunit. At best minor defects in trafficking of TbbCATL or p67 were observed when any of the four COPII subunits were ablated; -fold decreases in transport rates ranged from 1.0- to 1.5-fold for all combinations of subunits and reporters (Supplemental Figure S2 and Table 1). Likewise, minor defects were seen in VSG trafficking for TbSec23.1 and TbSec24.2 ablation, 1.9- and 1.3-fold, respectively (Figure 3, A and D, and Table 1). In contrast, dramatic and roughly equivalent reductions in VSG trafficking were seen after silencing of TbSec23.2 (5.5-fold) or TbSec24.1 (4.0-fold) (Figure 3, B and C, and Table 1). Together, these data suggest that the paralogous TbSec23 and TbSec24 subunits are redundant for TbbCATL and p67 trafficking. In contrast, VSG is preferentially dependent on TbSec23.2 and TbSec24.1 for efficient egress from the ER.

BiPN:GPI Trafficking Is Preferentially Dependent on TbSec23.2

To determine whether the TbSec23.2/24.1 dependence of VSG trafficking is VSG-specific, or rather applies to all GPI-anchored cargo, we monitored the progression of BiPN:GPI, a single GPI-anchored reporter that was constitutively expressed in the TbSec23.1 and TbSec23.2 RNAi cell lines. We have shown previously that attachment of a GPI anchor to the N-terminal ATPase domain of BiP, an ER-localized molecular chaperone, led to a mixed fate of shedding from the cell surface and degradation in the lysosome (Schwartz et al., 2005). BiPN:GPI is initially synthesized as an ~55-kDa immature form (I) in the ER, which is subject to GPI carbohydrate modification during transit of the Golgi, forming a higher molecular weight mature glycoprotein (M). The fate of BiPN:GPI in the TbSec23.1 and TbSec23.2 cell lines after RNAi silencing was determined by pulse-chase analysis and immunoprecipitation with anti-BiP antibody (Figure 4, A and B). Endogenous BiP (B) was detected in all samples, indicating equivalent loading (lanes 1–6). In addition, as described previously (Bangs et al., 1996), endogenous newly synthesized VSG (V), which transiently associates with BiP during folding, was detected in equivalent amounts in all time zero samples (lanes 1). Depletion of TbSec23.1 message had a minor effect on the transport kinetics of BiPN:GPI as judged by the relative rates of disappearance of the immature ER glycoform (Figure 4A, lanes 2–6, and Table 1). In contrast, knockdown of TbSec23.2 dramatically impaired ER export of the BiPN:GPI precursor and maturation in the Golgi, with the rate of transport delayed by ~2.7-fold (Figure 4B, lanes 2–6, and Table 1). These data indicate that similar to VSG, ER egress of BiPN:GPI is preferentially dependent on TbSec23.2, sug-
**Trypanosome Early Secretory Pathway**

The existence of discrete TbSec23/TbSec24 heterodimers raises the issue of subcellular localization. Are there distinct ERES defined by each pair, or do they all colocalize in the same sites for ER exit? To address these questions, we performed immunofluorescence imaging with the four double-tagged TbSec23:HA/TbSec24:Ty cell lines. Each cell line was stained with an anti-HA and anti-Ty antibody to label the component (TbSec23.1:HA, 106.6 kDa; TbSec23.2:HA, 83.3 kDa; TbSec24.1:Ty, 108.1 kDa; and TbSec24.2:Ty, 100.8 kDa) are indicated (stars). These bands were not immunoprecipitated from untagged control cell lines (data not shown). (B) Physical interactions were determined by sequential immunoprecipitations using the doubly HA/Ty-tagged cell lines as described in Materials and Methods. Each cell line was radiolabeled (2 h), lysed under non-denaturing conditions, and immunoprecipitated first with either anti-HA or anti-Ty antibodies as indicated (1°). Specific immunoprecipitates were solubilized under denaturing conditions and reprecipitated with the alternate antibody (2°, anti-Ty or anti-HA). Final precipitates were analyzed by SDS-PAGE (10° cell equivalents/lane) and phosphorylation. The vertical white lines between lanes 2 and 3 indicate intervening lanes that were digitally excised for the sake of presentation (all remaining lanes from same exposure).

**Figure 5.** TbSec23.1/TbSec24.2 and TbSec23.2/TbSec24.1 Form Obligate Physical Pairs

The matched impact of TbSec23.2 and TbSec24.1 silencing on VSG transport (Figure 3 and Table 1) could be explained if these two subunits form exclusive and obligate heterodimers within the COPII coat. In this scenario, knockdown of either subunit would have the same overall effect. Such associations have been seen in other systems and can have functional consequences for selective trafficking of secretory cargo (Shimoni et al., 2000). To investigate this possibility in trypanosomes, we analyzed the physical associations between the TbSec23 and TbSec24 subunits by epitope tagging and pull-down experiments. Four BSF cell lines were developed with single HA-tagged TbSec23 subunits and single Ty-tagged TbSec24 subunits in all possible combinations: TbSec23.1:HA/TbSec24.1:Ty, TbSec23.1:HA/TbSec24.2:Ty, TbSec23.2:HA/TbSec24.1:Ty, and TbSec23.2:HA/TbSec24.2:Ty. All four doubly HA/Ty-tagged cell lines were metabolically radiolabeled, and standard lysates (containing NP-40, deoxycholate, and SDS) were subjected to immunoprecipitation with either anti-HA or anti-Ty antibodies (Figure 5A). In each case, epitope-tagged polypeptides of the predicted size and immunoreactivity were detected, confirming that the tagged proteins are expressed appropriately.

To investigate the physical pairing of individual TbSec23 and TbSec24 subunits into heterodimers, radiolabeled lysates were prepared under gentler conditions (NP-40 only) to preserve molecular interactions and were then subjected to immunoprecipitation with anti-HA or anti-Ty. These primary pull-downs (Figure 5B, 1°) were solubilized in SDS, readjusted to standard lysate conditions, and then immunoprecipitated again with the other antibody, either anti-HA or anti-Ty as appropriate (Figure 5B, 2°). The results were unequivocal. TbSec23.1:HA was detected in secondary pull-downs only when coexpressed with TbSec24.2:Ty (lane 7 vs. lane 3) and vice versa (lane 5 vs. lane 6). Likewise, TbSec23.2:HA was detected in secondary pull-downs only when coexpressed with TbSec24.1:Ty (lane 4 vs. lane 8) and vice versa (lane 2 vs. lane 1). No evidence was detected for coprecipitation of Sec23.1:HA and Sec24.1:Ty (lanes 1 and 3) or for TbSec23.2:HA and TbSec24.2:Ty (lanes 6 and 8). Collectively, these data indicate that the TbSec23 and TbSec24 subunits have discrete interactions, yielding two exclusive and obligate heterodimers: TbSec23.1/TbSec24.2 and TbSec23.2/TbSec24.1.

This obligate pairing of subunits can be explained by primary sequence differences between paralogues. Crystallographic studies in *S. cerevisiae* defined the interface surfaces involved in Sec33/Sec24 dimerization (Bi et al., 2002), and these regions in the trypanosome subunits display considerable variation (Supplemental Figure S3B). When the *T. brucei* sequences are modeled onto the *S. cerevisiae* crystal structure, subtle and compensatory differences in three critical contact surfaces are revealed (Supplemental Figure S4). These changes suggest a coevolution of matched *T. brucei* subunits such that dimerization is physically restricted, consistent with the pattern revealed by our biochemical analyses.

**COPII-defined ERES Are in Proximity to the Golgi and the FAZ**

The existence of discrete TbSec23/TbSec24 heterodimers raises the issue of subcellular localization. Are there distinct ERES defined by each pair, or do they all colocalize in the same sites for ER exit? To address these questions, we performed immunofluorescence imaging with the four double-tagged TbSec23:HA/TbSec24:Ty cell lines. Each cell line was stained with an anti-HA and anti-Ty antibody to label the indicated TbSec23 and TbSec24 subunits (Figure 6, A–D). Despite their exclusive physical interactions, each TbSec23:HA/TbSec24:Ty combination, and by extension all four TbSec subunits, colocalized precisely in three dimensions to one or two discrete sites in the postnuclear region of postcytokinesis BSF trypanosomes (G1 phase cells with a single kinetoplast and nucleus). These findings indicate that recently divided BSF trypanosomes have one or two ERES per cell, with uniform composition in regard to COPII components. Interestingly, these sites are also consistently proximal to the flagellum in a region called the flagellar attachment zone (FAZ) (Figure 7). This apparent juxtaposition with the FAZ suggests that the ERES may be organized on the FAZ-associated ER (FAZ:ER), an extension of smooth ER that interdigitates longitudinally with a quartet of subpellicular microtubules that subdivide the flagellum along the entire length of the cell body (Figure 9) (Bastin et al., 2000; Vaughan et al., 2008).

The ERES in procyclic trypanosomes have been shown to be in proximity to the Golgi, as defined by TbGRASP (He et al., 2004), and we wished to visualize this architecture in BSF trypanosomes. We began by localizing the ERES in TbSec23.2:HA cells in relationship to the ER with anti-BiP antibody, and in relationship to the Golgi with either an anti-TbGRASP antibody (He et al., 2004) or an anti-Ty antibody to detect a Ty-tagged glycosyltransferase (TbGTS15:Ty). We have previously validated TbGTS15:Ty as a Golgi marker (Sutterwala et al., 2008). In addition, the FAZ was simultaneously localized as a subcellular reference point in all images with a mAb (L3B2) to the FAZ filament (Kohli et al.,
Typical images for all of these antibody combinations in BSF cells containing one (top) or two (bottom) ERES are presented in Figure 7. TbSec23.2:HA colocalized with one or two distinct lobes of the ER, which were in turn closely aligned with the FAZ filament along the entire cell body (Figure 7A), confirming these sites as true ERES, and the FAZ:ER as the site of ERES assembly. Closely juxtaposed with the ERES, but distal to the FAZ, was a zone defined by TbGRASP (Figure 7B), which closely abutted the ERES, but when viewed in the z-dimension was laterally distinct. When imaged relative to TbGT15:Ty, the TbGRASP-defined zone was typically juxtaposed and overlapping (Figure 7C). Notably, of 150 Golgi scored for TbGT15:Ty and TbGRASP staining, only ~18% showed precise colocalization; the re-
Figure 8. Ultrastructural localization of ERES. The TbSec23.2:HA cell line was fixed and prepared for immunoelectron microscopy as described in Materials and Methods. Sections were stained with anti-BiP (12-nm gold) and anti-HA (18-nm gold) to detect ER and ERES respectively. (A and B) Images showing specificity of staining with both reagents. Anti-BiP staining is predominantly associated with peripheral reticular membranes and nuclear envelope (A and B), and anti-HA staining is clustered in association with peripheral ER membranes (B). (C and D) Images of individual ERES demonstrating proximity to the flagellum and confirming the association of ERES with the FAZ:ER. Bars (A–E), 0.25 µm. Nucleus, n; nuclear envelope, ne; peripheral ER, er; ER exit sites, eres; and flagellum, fl. Double-thick surface coats at the interface of cell body and flagellar membranes are indicated by brackets. Asterisks indicate fixation-induced lacunae flanking the flagellum and bounded by the flagellar membrane (arrowheads in C only).

remaining ~82% showed only partial overlap between the two markers; and in the later case, the TbGRASP signal was always proximal to the ERES and FAZ. Finally, when TbGT15:Ty was imaged relative to the ERES, it defined a distinct structure that was distal to the FAZ and showed no lateral overlap with the ERES (Figure 7D). Collectively, these data indicate that the ERES and Golgi are closely associated in BSF trypanosomes and suggest that the entire ERES:Golgi junction, whether there is one or two per cell, is closely associated with the FAZ:ER in the region between the centrally located nucleus and the posterior flagellar pocket.

To confirm that the ERES is assembled on the FAZ:ER, we performed immunoelectron microscopy on the TbSec23.2:HA cell line with anti-BiP to detect ER elements and with anti-HA to detect COPII-containing structures (Figure 8). BiP staining was associated with both the nuclear envelope (Figure 8A) and peripheral ER elements (Figure 8, A and B), whereas HA staining was clustered in association with peripheral ER elements (Figure 8B). In each case, little staining was seen elsewhere over the cell body confirming the specificity of the staining patterns. TbSec23.2:HA staining was typically observed as clusters consistently associated with underlying peripheral ER elements (Figure 8, B–E) and usually in close association with the flagellum (Figure 8, C–E). These results are fully consistent with images acquired by fluorescence microscopy and confirm the FAZ:ER as the site of ERES formation.

DISCUSSION

In this work, we address the mechanistic basis of GPI-dependent trafficking in the early secretory pathway of African trypanosomes. In yeast, the COPII machinery mediates ER exit of all classes of secretory cargo, including soluble, polytopic membrane, and GPI-anchored proteins (Schimmoeller et al., 1995; Kuehn et al., 1996). Conditional expression of a dominant-negative form of TbSar1 confirmed that the COPII machinery affects similar functions in trypanosomes. The failure of newly synthesized p67 to be processed to mature glycoforms, indicates that the TbSar1DN block is in ER-to-Golgi transport. The extremely rapid onset of growth arrest and subsequent cell death upon induction of TbSar1DN synthesis was striking. These results confirm that TbSar1, and by inference the entire COPII machinery, is functional and directly involved with the forward transport of secretory cargo from the ER in trypanosomes, a conclusion supported by the RNAi phenotypes seen with the four TbSec23/24 subunits.

Multiple and redundant COPII subunits within a species are common. The human genome contains two Sar1, Sec23, and Sec31 paralogues each and four Sec24 genes (Shen et al., 1995; Paccaud et al., 1996; Tang et al., 1999; Slankiewich et al., 2006). The Sec24 subunits are redundant and nonessential, with overlapping abilities to recognize transport signals (Lang et al., 2006; Wendeler et al., 2006). S. cerevisiae has three Sec24 paralogues (Sec24, Lst1, and Iss1). Only Sec24 is essential, but loss of Lst1 significantly impacts forward trafficking of the plasma membrane ATPase Pma1 (Kurihara et al., 2000; Shimoni et al., 2000). P. pastoris has two paralogues of Sec23 (PpSec23 and PpShl23) and Sec24 (PpSec24 and PpLst1); yet, only PpSec23 and PpSec24 are required for viability. Interestingly, PpSec23 forms heterodimers with both Sec24 subunits, but PpShl23 only interacts with PpLst1 forming a redundant and nonessential heterodimer (Eski et al., 2006).

Trypanosomes stand in contrast to each of these eukaryotic systems in that both TbSec23 and TbSec24 genes are essential. Furthermore, the TbSec23.1/TbSec24.2 and TbSec23.2/TbSec24.1 subunits form obligate and exclusive heterodimers. There is considerable sequence variation in the trypanosomal subunits (Supplemental Figure S3B) in the regions known to form the dimer interface (Bi et al., 2002) (Supplemental Figure S4), which likely form the basis for dimer exclusivity. These discrete dimers are redundant for
Figure 9. Diagram of ERES:Golgi junctions in BSF trypanosomes. A partial cross section of a cell in the postnuclear region transecting the junction of an ERES and Golgi complex is represented. Major structures include the flagellum containing the 9 + 2 axoneme and paraflagellar rod; the FAZ, including pellicular microtubules, FAZ filament, antiparallel microtubule quartet, and the FAZ:ER; the ERES defined by COPII subunits; and the Golgi defined by TbGT15 and TbGRASP. Diagram inspired by Bastin et al., 2000 and Hill, 2005.

the forward trafficking of soluble (TbhCATL) and transmembrane (p67) cargo. However, trafficking of GPI-anchored cargo is selectively dependent on the TbSec23.2/TbSec24.1 pair. The need for bloodstream form trypanosomes to synthesize a full complement of VSG (10^7 molecules per cell per 6 h cell cycle) may explain the requirement for this heterodimer, but why is the TbSec23.1/TbSec24.2 heterodimer essential? Perhaps it is a simple matter of capacity to handle the total flux of all newly synthesized secretory cargo from the ER. Alternatively, this heterodimer may be preferentially required for transport of some of yet unidentified cargo. These possibilities are not mutually exclusive.

There is precedent in S. cerevisiae for GPI-selective cargo loading at discrete ERES and into distinct COPII vesicle populations (Muniz et al., 2001; Castillon et al., 2009). In trypanosomes, defined Sec23/Sec24 heterodimers present two alternatives for the organization of ER exit. There may be a single class of COPII vesicles, containing both heterodimers and responsible for transport of all cargo. In this case, recruitment of GPI-anchored cargo into nascent vesicles would be mediated by the TbSec23.2/TbSec24.1 dimer. The alternative is two distinct vesicle populations, each containing one Sec23/Sec24 pair or the other. GPI-anchored cargo would be dependent on the TbSec23.2/TbSec24.1 vesicles, and non-GPI cargo would be accommodated by either class.

Whatever the model, a mechanism must exist for coupling GPI-anchored cargo to the cytoplasmic TbSec23.2/TbSec24.1 dimer. Recruitment of secretory cargo, including GPI-anchored proteins, to nascent COPII vesicles can be mediated by p24s, a family of small type I transmembrane adapter proteins abundant in COPII vesicles (Jenne et al., 1995; Dominquez et al., 1998). p24s have cytoplasmic motifs for interaction with COPII components and associate in oligomers with combinatorial potential for recognition of secretory cargo (Jenne et al., 2002). Yeast has eight p24 orthologues; vertebrates have as many as 10 (Schimmoller et al., 1995; Belden and Barlowe, 1996; Marzioch et al., 1999; Straubing et al., 2009). Individual disruption of p24s in yeast, and of p23 in mammalian cells, selectively impacts ER exit of GPI-anchored cargo (Jenne et al., 1995; Schimmoller et al., 1995; Belden and Barlowe, 1996; Marzioch et al., 1999; Takida et al., 2003; Castillon et al., 2009). The T. brucei genome contains six putative p24 orthologues based on homology searches with Emp24 (Supplemental Table S2), and it may be that these proteins mediate GPI recruitment into TbSec23.2/TbSec24.1-containing vesicles. There is also sequence variation between the TbSec24 isoforms in the B-site region (Supplemental Figure S3C), which in ScSec24 is a binding site for exported membrane proteins, including p24s (Miller et al., 2003; Mossessova et al., 2003). B-site variation, in conjunction with cargo-specific combinations of trypanosomal p24 proteins, could account for the selective dependence of GPI-anchored cargo on the TbSec23.2/TbSec24.1 heterodimer.

The physical organization of the early secretory pathway in BSF trypanosomes differs markedly from other model organisms. In postcytokinesis cells, there are one or two ERES closely associated with the Golgi, consistent with the localization of TbSec13 in PCF trypanosomes (He et al., 2004). Nothing is known about ERES:Golgi organization in other kinetoplastids. However, S. cerevisiae typically has 20 or more ERES throughout the ER, with ill-defined Golgi associations, whereas Pichia has fewer ERES that are clearly associated with defined Golgi complexes (Rossanese et al., 1999; Stephens, 2003). In contrast, animal cells have hundreds of ERES, and competing models of Golgi organization (Lippencott-Schwartz et al., 2000; Shorter and Warren, 2002).

Other features of ERES:Golgi organization in BSF trypanosomes merit comment (Figure 9). First and foremost is the exclusive formation of ERES on the FAZ:ER and thus its association with unique elements of the flagellar cytoskeleton. A bilobe structure thought to be involved in nucleating Golgi formation is also closely juxtaposed to the FAZ in PCF cell (Morriswood et al., 2009), but our results indicate for the first time that it is the entire ERES:Golgi complex that is associated with the trypanosome cytoskeleton, probably via the FAZ:ER. This may reflect the need to ensure fidelity in organellar segregation during cytokinesis. The flagellum and the FAZ are intimately involved in this process and probably provide the scaffold for partitioning the ERES: Golgi into each daughter cell. The role of the bilobe structure in this process in BSF trypanosomes remains to be defined. Second is the overlapping localization of TbGRASP with the Golgi, in contrast with PCF trypanosomes in which TbGRASP colocalizes precisely with other Golgi markers (He et al., 2004; Ho et al., 2006). GRASP orthologues act as cytosolic matrix components to maintain Golgi structure (Barr et al., 1997; Feinstein and Linstedt, 2008), and a simple explanation for the observed distribution is that TbGRASP defines an earlier compartment(s) of the BSF Golgi devoid of the TbgT15 glycosyltransferase. However, GRASP may have other roles in secretory trafficking (Kinseth et al., 2007), including physical interactions with the Sec23/Sec24 dimer (Behnia et al., 2007). It may be that TbGRASP has additional functions in trypanosomes such as maintaining the ERES: Golgi junction and/or in facilitating efficient ER-to-Golgi transport. Finally, our results provide preliminary insights into the replication of the entire ERES:Golgi junction in BSF trypanosomes. Approximately 80% of postcytokinesis cells (1 kinetoplast/1 nucleus) have two Golgi (n = 150; Schwartz and Bangs, unpublished observations), indicating that Golgi duplication is an early event in the cell cycle, as was observed for PCF trypanosomes (He et al., 2004). When a single ER:Golgi junction is present it is located forward near the nucleus, whereas in cells with two junctions, the second is proximal to the flagellar pocket and is frequently less prominent, suggesting that it is the new Golgi (Figures 6 and 7). Similar organization is found in PCF cells, but the old and
new ERES-Golgi junctions are spatially much closer along the longitudinal axis of the cell. This may be a simple consequence of repositioning of the flagellar pocket closer to the nucleus in PCF (Matthews, 1999), thereby compressing the organization of the early secretory pathway. Alternatively, these morphological differences may influence replication of these secretory organelles in a stage-specific manner.

ACKNOWLEDGMENTS

We are grateful to laboratory members past and present, to Dr. Ron Peck and Kevin Schwartz for contribution to the early phase of this work, and to Dr. Sebastian Bednarek for thoughtful discussion and comments. We are particularly indebted to Dr. Wandy Beatty (Washington University-St. Louis) for assistance with electron microscopy and to Dr. James Keck (University of Wisconsin-Madison) for assistance with molecular modeling. We also thank Drs. Graham Warren and Keith Gull for generous gifts of antibodies. This work was supported by U.S. Public Health Service grant R01 AI-35739 (to J.D.B.). E.S.S. was supported by National Institutes of Health Microbial Pathogenesis and Host Responses training grant (T32 AI-055397) to University of Wisconsin-Madison.

REFERENCES


