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Glycoprotein Biosynthesis in a Eukaryote Lacking the Membrane Protein Rft1*

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Background: The membrane protein Rft1 was proposed to flip Man₅GlcNAc₂-PP-dolichol (M₅-DLO) to the ER lumen for N-glycoprotein biosynthesis.

Results: Rft1-null Trypanosoma brucei has a normal steady-state level of mature dolichol-linked oligosaccharide (mDLO) and significant N-glycosylation.

Conclusion: Rft1 is not required for M₅-DLO flipping in vivo but aids conversion of M₅-DLO to mDLO by another mechanism.

Significance: The M₅-DLO flippase remains to be identified.

Mature dolichol-linked oligosaccharides (mDLOs) needed for eukaryotic protein N-glycosylation are synthesized by a multistep pathway in which the biosynthetic lipid intermediate Man₅GlcNAc₂-PP-dolichol (M₅-DLO) flips from the cytoplasmic to the luminal face of the endoplasmic reticulum. The endoplasmic reticulum membrane protein Rft1 is intimately involved in mDLO biosynthesis. Yeast genetic analyses implicated Rft1 as the M₅-DLO flippase, but because biochemical tests challenged this assignment, the function of Rft1 remains obscure. To understand the role of Rft1, we sought to analyze mDLO biosynthesis in vivo in the complete absence of the protein. Rft1 is essential for yeast viability, and no Rft1-null organisms are currently available. Here, we exploited Trypanosoma brucei (Tb), an early diverging eukaryote whose Rft1 homologue functions in yeast. We report that TbRft1-null procyclic trypanosomes grow nearly normally. They have normal steady-state levels of mDLO and significant N-glycosylation, indicating robust M₅-DLO flippase activity. Remarkably, the mutant cells have 30–100-fold greater steady-state levels of M₅-DLO than wild-type cells. All N-glycans in the TbRft1-null cells originate from mDLO indicating that the M₅-DLO excess is not available for glycosylation. These results suggest that rather than facilitating M₅-DLO flipping, Rft1 facilitates conversion of M₅-DLO to mDLO by another mechanism, possibly by acting as an M₅-DLO chaperone.

Protein N-glycosylation is ubiquitous in eukaryotes. N-Linked oligosaccharides direct folding, quality control, and degradation of most proteins that enter the secretory pathway and also influence their subsequent trafficking. The importance of N-linked oligosaccharides is evinced by the discovery of human congenital disorders of glycosylation (2), in which errors in oligosaccharide synthesis or assembly result in developmental, neurological, and metabolic dysfunction, often with life-threatening consequences. Consequently, detailed knowledge of the mechanisms of N-linked oligosaccharide assembly is critically important for understanding human physiology.

The N-linked oligosaccharide moiety is assembled on a dolichyl diphasphosphate lipid carrier before being transferred en bloc to glycosylation sequons (Asn-Xaa-(Ser/Thr)) in nascent proteins as they enter the lumen of the endoplasmic reticulum (ER) (3-5). Synthesis of the dolichol-linked oligosaccharide (DLO) is a multistep process that initially generates Man₅GlcNAc₂-PP-dolichol (M₅-DLO) on the cytoplasmic side of the ER (Fig. 1). M₅-DLO is then flipped across the membrane to the ER lumen where it is elaborated in a series of reactions to mature DLO (mDLO). Metazoan mDLOs have a Glc₃Man₅GlcNAc₂ glycan, although mDLOs in other eukaryotes have smaller glycans. For example, mDLO in procyclic forms of the parasitic protozoan Trypanosoma brucei has a Man₅GlcNAc₂ glycan (6) that, after transfer to protein, is trimmed by mannosidases to generate “processed” N-glycans (Fig. 1).

The molecular machinery required for DLO assembly has been largely identified, except for the lipid flippases responsible for translocating M₅-DLO and other dolichol-based lipids...
across the ER membrane (Fig. 1) (3, 5, 7). This impasse appeared to be broken when Ng et al. (8) and later Helenius et al. (9) working with the yeast Saccharomyces cerevisiae, concluded that the ER membrane protein Rft1 is required directly for membrane translocation of M5-DLO. These investigators showed that yeast cells in which Rft1 expression was reduced to levels much lower than normal (though not nil) accumulate M5-DLO and hypoglycosylate proteins as would be expected in the absence of Rft1. Levels much lower than normal (though not nil) accumulate M5-DLO and underglycosylate proteins. Our results show that M5-DLO flipping occurs in the complete absence of Rft1 in vivo but that Rft1 nonetheless influences the conversion of M5-DLO to M9-DLO.

**Experimental Procedures**

**Materials**—Unless otherwise stated, all reagents were of analytical grade and purchased from Sigma or Merck. Restriction enzymes were from Fermentas (St. Leon-Rot, Germany) and antibiotics from Sigma, Invitrogen (Nunningen, Switzerland), or Invitrogen. EasyTag® Expres35S35S protein labeling mix was from PerkinElmer Life Sciences. BioMax MS and MBX films were from GE Healthcare.

**Trypanosome Cultures**—T. brucei strain Lister 427 procyclic forms were cultured at 27 °C in SDM-79 containing 5% inactivated fetal bovine serum. Rft1 knock-out clones were grown under the same condition, in the presence of 15 µg/ml G418 for the single allele knock-out and an additional 10 µg/ml blasticidin for the double allele knock-out clones.

**Generation of TbRft1-null Cells**—Constructs to replace the endogenous TbRft1 genes were based on the pKO plasmid containing resistance cassettes consisting of the following elements (5’ to 3’): the EP1-EP2 procyclin intergenic region, a G418 or blasticidin resistance gene, and the tubulin βa intergenic region (16). A 397-bp 5’ and a 427-bp 3’ recombination sequence flanked the resistance cassettes. Recombination sequences adjacent to the Tbrft1 open reading frame were obtained by PCR amplification using primers 5’_forward gcccaaggtcatgtcgcttaagtccgc and 5’_reverse gccaatcccac-
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caaaggagccggtgctgc for the 5'-recombination sequence and 3'_forward gcaaggagccggtgctgc and 3'_reverse gcggagccggtgctgc for the 3'-recombination sequence (HindIII, EcoRI, Xbal, and SacI restriction sites are underlined). Prior to transfection into T. brucei 427 procyclic forms, plasmids were digested upstream and downstream of the recombination sequences using HindIII and SacI. Clones were obtained by limiting dilution under antibiotic selection using 15 μg/ml G418 and/or 10 μg/ml blasticidin. Clones were PCR-tested for correct integration using primer 5'_UTR_control gcaaggagccggtgctgc and TbrRft1_reverse gcaaggagccggtgctgc and cloned into a pLEW100-based expression vector (17) (HindIII and XhoI restriction sites are underlined). Clones were obtained by limiting dilution under antibiotic selection using 2 μg/ml puromycin.

Southern Blot Analysis—For Southern blot analysis, 1.2 μg of Agel or SacII-Smal-digested genomic DNA from wild-type and mutant cells was separated on a 1% agarose gel and transferred to Hybond-N+ nylon transfer membrane (GE Healthcare) using 10× SSC buffer (150 mM Na+-citrate, pH 7.0, containing 1.5 mM NaCl). The membrane was probed with a 427 bp 32P-labeled PCR product of the TbrRft1 3' recombination sequence generated with the primers gcaaggagccggtgctgc and cgctctagagtggtgaaggcgctggttc, and cloned into a pLEW100-based expression vector (17) (HindIII and XhoI restriction sites are underlined). Clones were obtained by limiting dilution under antibiotic selection using 2 μg/ml puromycin.

Flow Cytometry Analysis—Trypanosomes at mid-log phase (107 parasites) were harvested by centrifugation at 1500 × g for 10 min at 4 °C and resuspended in 200 μl of cold SDM-79. Concanavalin A-FITC conjugate was added to a final concentration of 3 μg/ml. After 1 h of incubation in the dark on ice, 1 ml of cold SDM-79 was added, and parasites were centrifuged as above. After washing once in cold SDM-79, trypanosomes were resuspended in 2 ml of cold SDM-79 and analyzed by flow cytometry (BD FACSCalibur or FACSscan) at a concentration of 5 × 105 cells/ml. Data were analyzed using flow cytometry analysis software FlowJo.

RESULTS

T. brucei Rft1 Is Functionally Equivalent to S. cerevisiae Rft1—We identified an Rft1 orthologue in T. brucei by BLAST searching the predicted proteome of T. brucei strain TREU 927 using human and S. cerevisiae Rft1 sequences as queries. We retrieved a single orthologous sequence, Tbl11.01.3540, that was annotated as a 598-amino acid hypothetical protein. Topology prediction programs indicated that Tbl11.01.3540 is a membrane protein with multiple transmembrane spans. To determine whether Tbl11.01.3540 could functionally substitute for yeast Rft1 (ScRft1), we used a haploid yeast strain (YG1137) (9) in which ScRft1 is expressed under the control of the glucose-repressible GAL1–10 promoter. On shifting YG1137 cells from galactose- to glucose-containing medium, ScRft1 expression is repressed and the cells fail to grow (Fig. 2A). At the same time, carboxypeptidase Y (CPY), a vacuolar protease with four N-glycans, becomes hypoglycosylated, with ever fewer glycosylation sites on the protein being occupied as the incubation time in cold PBS. Methanol (room temperature) was added to the tube, and the contents were disrupted by vigorously vortexing. The suspensions were dried and processed for FACE analyses as described (21, 22). In brief, after most lipids were removed by extraction with chloroform/methanol (2:1, by volume) and aqueous components were removed by extraction with water, DLOs were obtained by extraction with chloroform/methanol/water (10:10:3, by volume). N-Linked glycoproteins remained in the residual material. The glycans units of DLOs were released with weak acid. N-Glycans were released with PNGase F and further purified by ion exchange. All glycan was conjugated with 7-amino-1,3-naphthalenedisulfonic acid (ANDS) and resolved on an oligosaccharide profiling gel, with all loads normalized to total protein in the chloroform/methanol/water (10:10:3) residue. Due to necessary, the fluorescently conjugated glycans were incubated in 50 mM sodium citrate buffer, pH 5.5, for 18 h at 37 °C with 100 units of Endo H (New England Biolabs) and/or 0.05 milliunits of α-1,2-mannose-specific mannosidase (Aspergillus saitoi, Prozyme). Fluorescently labeled oligosaccharides were detected with a Bio-Rad Fluor-3 scanner and quantified with Quantity-One software.

Fluorophore-assisted Carbohydrate Electrophoresis (FACE) Analysis of Trypanosomal Glycoconjugates—Trypanosomes were collected by centrifugation and washed twice with ice-cold PBS. Methanol (room temperature) was added to the tube, and the contents were disrupted by vigorously vortexing. The suspensions were dried and processed for FACE analyses as described (21, 22). In brief, after most lipids were removed by extraction with chloroform/methanol (2:1, by volume) and aqueous components were removed by extraction with water, DLOs were obtained by extraction with chloroform/methanol/water (10:10:3, by volume). N-Linked glycoproteins remained in the residual material. The glycans units of DLOs were released with weak acid. N-Glycans were released with PNGase F and further purified by ion exchange. All glycan was conjugated with 7-amino-1,3-naphthalenedisulfonic acid (ANDS) and resolved on an oligosaccharide profiling gel, with all loads normalized to total protein in the chloroform/methanol/water (10:10:3) residue. Due to necessary, the fluorescently conjugated glycans were incubated in 50 mM sodium citrate buffer, pH 5.5, for 18 h at 37 °C with 100 units of Endo H (New England Biolabs) and/or 0.05 milliunits of α-1,2-mannose-specific mannosidase (Aspergillus saitoi, Prozyme). Fluorescently labeled oligosaccharides were detected with a Bio-Rad Fluor-3 scanner and quantified with Quantity-One software.
glucose-containing media is increased and ScRft1 levels decrease (Fig. 2B, lanes indicated TbRft1 −) (9–11). Heterologous expression of Tb11.01.3540 enabled YG1137 cells to grow on glucose-containing media (Fig. 2A) and prevented CPY hypoglycosylation (Fig. 2B, lanes indicated TbRft1 +) indicating that the trypanosome protein, henceforth TbRft1, is functionally equivalent to ScRft1.

TbRft1 Is Nonessential in Procyclic Form Trypanosomes—We used homologous recombination to replace the TbRft1 alleles in diploid procyclic cells with G418 and blasticidin resistance genes, eliminating the ability to express any form of TbRft1 protein. We recovered viable clones of TbRft1-null trypanosomes that grew nearly normally (Fig. 3A). The generation time of two independent TbRft1-null clones was 15.3 ± 0.9 and 14.4 ± 0.2 h, compared with 11 ± 0.2 and 13.1 ± 0.5 h for wild-type cells and single knock-out cells, respectively. These data suggest that growth rate is sensitive to TbRft1 gene dosage (in contrast to effects on glycosylation, see below). Southern blot analyses (Fig. 3B) confirmed that we had indeed disrupted both alleles of TbRft1 and that the growth of the cells was not due to inappropriate integration of the drug resistance genes elsewhere in the genome rather than at the TbRft1 locus. We conclude that TbRft1 is a nonessential protein in T. brucei procyclic forms in culture.

To determine whether the TbRft1-null trypanosomes had a glycosylation defect, we incubated the cells with FITC-ConA, a fluorescent conjugate of the mannose-binding lectin concanavalin A, and quantified cell surface fluorescence by flow cytometry. TbRft1-null trypanosomes bound ~75% less FITC-ConA than wild-type cells (Fig. 3C). This was specifically due to the lack of Rft1, as FITC-ConA binding could be restored by ectopic expression of TbRft1 (Fig. 3D, histogram labeled rescue). Thus, despite their robust growth characteristics, the Rft1-null cells show decreased levels of cell surface glycosylation.

Dolichol-linked Oligosaccharide Synthesis and Protein N-Glycosylation in TbRft1-null Cells—To examine the glycosylation phenotype of the Rft1-null trypanosomes in more detail, we used FACE (21), a sensitive method to quantify steady-state levels of oligosaccharides. The cells were treated with organic solvent to extract DLOs and precipitate proteins. Oligosaccharides were released from DLOs and N-glycoproteins with mild acid and peptide: N-glycosidase F (PNGase F) treatment, respectively. N-Glycans were further purified by ion exchange chromatography to enrich neutral glycans, including high mannose structures. All glycans were conjugated with AND5 fluorophore and analyzed on an oligosaccharide profiling gel.

The profile of DLOs (Fig. 4A) in TbRft1-null trypanosomes was striking. The steady-state level of M9-DLO in the TbRft1-null cells (24.2 ± 2.2 pmol/mg) was similar to that in wild-type cells (22.8 ± 0.2 pmol/mg) indicating that the null cells have an intact DLO assembly pathway. However, the steady-state level of M5-DLO in the TbRft1-null trypanosomes was ~40-fold higher than that in wild-type cells (382.7 ± 34 versus 8.7 ± 0.9 pmol/mg). Also apparent was the accumulation of M6-DLO, M7-DLO, and M8-DLO (Fig. 4A), whose consumption has no obvious requirement for the M5-DLO flippase. Cumulative results obtained from independent experiments and individual TbRft1-null clones (Table 1) indicate that this pattern is highly reproducible; TbRft1-null cells have a 30–100-fold higher steady-state level of M5-DLO compared with wild-type cells but an essentially normal level of M9-DLO (Table 1). Although a reduction in TbRft1 gene dosage had a mild effect on the rate of growth (Fig. 3A), steady-state levels of the various glycoconjugates were similar in wild-type and heterozygous trypanosomes (Table 1).
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We considered whether the accumulated M5-DLO is a direct product of the DLO biosynthetic pathway ("biosynthetic M5" (Fig. 4E)) or whether it is derived from mature M9-DLO as a result of the action of cellular mannosidases ("processed M5" (Fig. 4E)). To distinguish these possibilities, we determined the susceptibility of the accumulated M5-DLO to Endo H and α1,2-mannosidase (α1,2m). As outlined schematically in Fig. 4E, biosynthetic M5 is susceptible to α1,2m yielding Man$_{9}$GlcNAc$_{2}$-ANDS but resistant to Endo H; in contrast, processed M5 is susceptible to Endo H (the GlcNAc-ANDS product is not retained within the resolving gel) but resistant to α1,2m. The glycosidase digests shown in Fig. 4B indicate clearly that the accumulated M5-DLO is "biosynthetic," i.e. it corresponds to a DLO biosynthesis intermediate.

We next analyzed neutral N-glycans released by PNGase F. T. brucei procaryotic forms almost exclusively make high mannose type N-glycans (25). The profile of N-glycans seen in TbRft1-null trypanosomes was identical to that in wild-type cells except that the total level of N-glycans was, on average, ~67% lower (Fig. 4C and Table 1), consistent with the ~75% lower surface reactivity of TbRft1-null cells toward ConA (Fig. 3C). The full range of N-glycans was observed in TbRft1-null cells, from Man$_{6}$GlcNAc$_{2}$ to Man$_{9}$GlcNAc$_{2}$. Glycosidase digests (Fig. 4D, illustrated schematically in Fig. 4E) indicated that the Man$_{9}$GlcNAc$_{2}$ species recovered in the N-glycan fraction correspond to processed M5 (Fig. 4E). This result indicates that in Rft1-null cells a higher order oligosaccharide such as M9 is transferred to proteins and subsequently trimmed by cellular mannosidases to processed M5. The cumulative data reveal that TbRft1-null trypanosomes have a complete N-glycosylation pathway but nevertheless accumulate large amounts of the M5-DLO biosynthetic intermediate that is not directly transferable to protein.

**N-Glycosylation of p67 in TbRft1-null Cells**—To refine our analyses of N-glycosylation in the TbRft1-null trypanosomes, we focused on a specific protein and chose p67, a well-characterized lysosomal N-glycoprotein with 14 N-glycosylation sites (19). We immunoprecipitated p67 from trypanosomes that had been metabolically labeled with [35S]methionine/cysteine and analyzed the protein by SDS-PAGE and fluorography. Fig. 5A shows the expected ~100-kDa band for fully glycosylated p67 in wild-type cells and a lower molecular weight (~72 kDa) diffuse band in TbRft1-null cells (Fig. 5A, compare lane 2 with lane 1). Treatment with either Endo H or PNGase F converted the diffuse band to an ~67-kDa product (Fig. 5A). Thus, p67 in

**TABLE 1**

Steady-state levels of M9-DLO, M5-DLO, total DLOs and total neutral N-glycans in wild-type, TbRft1-null, and single knock-out trypanosomes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Glycoconjugate</th>
<th>A3 (+/+)</th>
<th>B1(2) (+/-)</th>
<th>3F (+/+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M9-DLO</td>
<td>22.8 ± 0.2</td>
<td>24.2 ± 2.2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>M5-DLO</td>
<td>8.7 ± 0.9</td>
<td>382.7 ± 34</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Σ DLO</td>
<td>32.2 ± 1.4</td>
<td>423.7 ± 36</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Σ N-glycans</td>
<td>687 ± 16</td>
<td>294 ± 36</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>M9-DLO</td>
<td>17.4 ± 0.6</td>
<td>6.4 ± 0.8</td>
<td>14.2 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>M5-DLO</td>
<td>8.4 ± 0.4</td>
<td>248.2 ± 2.2</td>
<td>840.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Σ DLO</td>
<td>25.8 ± 1</td>
<td>254.6 ± 3</td>
<td>855 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Σ N-glycans</td>
<td>857 ± 100</td>
<td>323 ± 27</td>
<td>454 ± 16</td>
</tr>
</tbody>
</table>

Mean ± range of duplicate measurements is quoted as picomoles of glycoconjugate per mg of protein; ND means not determined. Two different TbRft1-null clones (A3 and B1(2)) were analyzed, alongside single knock-out (3F) and wild-type (427) cells. Raw data for experiment 1 are depicted in Fig. 3, A and C.
TbRft1-null cells is \( N \)-glycosylated, and the glycans are fully Endo H-sensitive indicating that they have the expected oligomannose structure.

We observed that Endo H-treated p67 from TbRft1-null trypanosomes migrates slightly faster than the corresponding sample from wild-type cells (Fig. 5A, compare lane 4 with lane 3). This is likely because fewer \( N \)-glycosylation sites are occupied in p67 from TbRft1-null cells, and therefore, there are fewer GlcNAc residues left on the protein after Endo H cleavage. Differences in migration are also observed in PNGase F-treated p67 samples (Fig. 5A, compare lane 6 with lane 5). In this case, lower site occupancy would result in fewer Asn residues being converted into negatively charged Asp residues after PNGase F digestion of p67 from TbRft1-null trypanosomes, making this protein migrate slightly faster than its counterpart from wild-type cells. We conclude that fewer \( N \)-glycosylation sites are occupied in p67 from TbRft1-null cells compared with wild-type cells, thus explaining its faster and more diffuse migration on SDS-PAGE. Based on gel mobility, we estimate that p67 in the TbRft1-null trypanosomes has 2–4 \( N \)-glycans.

Despite being underglycosylated, p67 is correctly localized to the lysosome in TbRft1-null cells. Immunofluorescence microscopy (Fig. 5B) revealed a single organelle in the region between the nucleus and the posterior flagellar pocket that was stained with anti-p67 antibodies as well as with antibodies against TbCatL, a trypanosomal cathepsin L orthologue (24).

**DISCUSSION**

We generated an Rft1-null cell, making it possible for the first time to characterize quantitatively the consequences of complete Rft1 absence by measuring the steady-state levels of key DLO intermediates and \( N \)-glycoproteins. In contrast, previous analyses of Rft1 function \textit{in vivo} were done on cells in which the protein was acutely depleted but not eliminated (9). Our results clearly show that TbRft1-null procyclic trypanosomes can execute the entire ER \( N \)-glycosylation pathway, \textit{i.e.} synthesis of M9-DLO, transfer of Man\(_n\)GlcNAc\(_2\) to proteins, and trimming of \( N \)-glycans. However, measurements of the steady-state levels of DLO intermediates by FACE show that although M9-DLO levels are unaffected, the mutant cells accumulate large amounts of the M5-DLO biosynthetic intermediate. Accumulation of the downstream intermediates M6-DLO, M7-DLO, and M8-DLO was also detected. These data indicate that TbRft1 impacts the multistep conversion of M5-DLO to M9-DLO.

Our results are inherently inconsistent with the assignment of Rft1 (8, 9) as the M5-DLO flipase. Another protein, yet to be identified, very likely plays this role because spontaneous flip-flop of M5-DLO (26) is too slow to supply the DLO biosynthetic pathway in TbRft1-null trypanosomes; the \( t_{1/2} \) for M5-DLO flipping in synthetic bilayers is predicted to be \( \gg 100 \) h, \textit{i.e.} much slower than that for common phospholipids (27). It is formally possible that M5-DLO flipping in the mutant cells is the result of a nonspecific process, involving membrane factors that do not normally serve this function. However, given our earlier demonstration of a chromatographically discrete protein fraction with structure-specific M5-DLO flipping activity \textit{in vitro} (11–13), there is little justification for invoking such a model.

For insights into the observed steady-state levels of M5-DLO and M9-DLO in wild-type and TbRft1-null cells, we modeled the DLO pathway as a sequential, irreversible series of reactions in which a source \((S)\) is converted to M5-DLO, then to M9-DLO, and finally to product\(\)\((P)\). This simplification is possible because in steady state, a series of reactions can be replaced by a single reaction without invoking a rate-limiting step. Thus, in Reaction 1,

\[
S \rightarrow M5 - DLO \rightarrow M9 - DLO \rightarrow P
\]

where the rate constant \( k_1 \) describes the set of reactions leading to the production of M5-DLO, the rate constant \( k_2 \) characterizes the multistep conversion of M5-DLO to M9-DLO, and the rate constant \( k_3 \) indicates the conversion of M9-DLO to products. The steady-state levels of M5-DLO and M9-DLO are shown in Equation 1,

\[
M5 - DLO_{ss} = (k_1 / k_2)S
\]

\[
M9 - DLO_{ss} = (k_2 / k_3)M5 - DLO_{ss} = (k_1 / k_3)S \quad \text{(Eq. 1)}
\]

Thus, the simplest explanation for the \(~100\)-fold higher level of M5-DLO\(_{ss}\) in TbRft1-null cells is that \( k_2 \) is \(~100\)-fold lower in these cells compared with wild-type cells. This change does not affect M9-DLO\(_{ss}\), which does not depend on \( k_2 \).

How does TbRft1 affect \( k_2 \), \textit{i.e.} conversion of M5-DLO to M9-DLO? The processes encapsulated in \( k_2 \) are minimally the M5-DLO flipping event and the four luminal mannosyl transfer reactions. As the cumulative biochemical data reported earlier...
(10–12) and the in vivo results presented here demonstrate that M5-DLO flipping does not require Rft1, and the luminal mannosyltransferases are known (Alg3, Alg9, and Alg12, with Alg9 carrying out two of the four mannosylations (7)), we conclude that Rft1 carries out its role separately from the core machinery carrying out two of the four mannosylations (7), we conclude that Rft1 plays an accessory albeit important role in M9-DLO biosynthesis. For example, it may control k₂ by functioning as a DLO chaperone to supply M5-DLO to the flipase or mannosyltransferases or to increase the chemical activity of M5-DLO by preventing its possible aggregation. Similar considerations apply to M₆-, M₇-, and M₈-DLO as these lipids also accumulate in the TbRft1-null cells. Alternatively, it could stabilize a complex of the luminal mannosyltransferases, thus enabling substrate channeling. Complex formation involving components of the DLO biosynthetic machinery has been previously reported (28, 29). In this context, it is interesting to note that disease-causing mutations in human Rft1 are located in hydrophilic loop regions of the protein that are predicted to be in the ER lumen (30); these functionally important loops could conceivably be involved in Rft1’s proposed role as luminal M5-DLO chaperone or its proposed role as a partner of luminal mannosyltransferases. As discussed previously (11, 12), Rft1’s function may resemble that of Lec35/MPDU1, a metazoan protein that is involved in luminal mannosyl transfer reactions in vivo (31). Lec35/MPDU1 was originally thought to be an excellent candidate for the mannose-phosphate dolichol flippase (Fig. 1), but the current consensus is that it acts as a dolichyl-lipid chaperone (31, 32).

Why is N-glycosylation reduced even though M₉-DLOₘₙₙ levels are normal in TbRft1-null cells? Steady-state measurements do not give information about flux. Thus, although M₉-DLOₘₙₙ levels are normal, the rates of M₉-DLO synthesis and consumption may be coordinately reduced by factors (yet to be determined) secondary to the absence of TbRft1. M₉-DLO is subject to at least two known fates (collectively described by the rate constant k₃ in Reaction 1), consumption by oligosaccharyltransferase for protein N-glycosylation and turnover, possibly by regulated cleavage of its pyrophosphate linkage (22). The balance between the two fates can be altered without affecting k₂ or M₉-DLOₘₙₙ. Thus, with less M₉-DLO being consumed for N-glycosylation, greater turnover could be accommodated in the TbRft1-null strain. However, it is unlikely that mannose 6-phosphate-dependent hydrolysis of DLOs described by Gao et al. (22, 33) could contribute to DLO turnover because M₉-DLO appears not to be a substrate for this process. Alternatively, M₉-DLO levels could be limiting in both wild-type and TbRft1-null trypanosomes. In this event, the reduced rate of production of M₉-DLO in the TbRft1-null cells could account for the observation of fewer glycans per protein.

Where does M₅-DLO accumulate in TbRft1-null cells? Wild-type procyclic trypanosomes normally transfer M₉ oligosaccharides to proteins and process these to triantennary M₅ structures (Fig. 1). When biosynthetic M₅-DLO is the only donor available to oligosaccharyltransferase in procyclic forms of T. brucei, proteins are underglycosylated and N-glycans are aberrantly processed to biantennary complex structures (34). The M₅-DLO that accumulates in TbRft1-null cells could potentially compete with mature M₉-DLO in the oligosaccharyltransferase reaction, resulting in biantennary complex N-glycans that would be resistant to Endo H digestion. This is not the case; analysis of total N-glycans (Fig. 4D), as well as N-glycans on p67 (Fig. 5A), indicates structures that are completely susceptible to Endo H. The M₅ structures that we detected in N-glycans were also Endo H-sensitive; they were derived by normal processing of mature M₉ N-glycans and did not originate by direct transfer of biosynthetic M₅. Thus, M₅-DLO does not compete with M₉-DLO and therefore must not have access to the site of oligosaccharyl transfer.

Either of two contrasting models could account for the segregation of accumulated M₅-DLO from the site of oligosaccharyl transfer in the ER: model 1, M₅-DLO accumulates on the cytoplasmic side of the ER, and model 2, M₅-DLO accumulates on the luminal side of the ER, perhaps in an aggregated form with low chemical activity or in a region of the ER that is laterally segregated from the oligosaccharyltransferase. Examples of lateral segregation of reactions within a single pathway in the ER have been previously noted (35, 36). The models have implications for possible functions of Rft1. In model 1, Rft1 would function as an accessory protein to supply M₅-DLO to the flipase on the cytoplasmic side of the ER, and in model 2, Rft1’s function would be on the luminal side of the ER, possibly to prevent M₅-DLO aggregation and increase its chemical activity, or chaperone M₅-DLO between ER domains.

The two models predict different transbilayer orientations for M₅-DLO; in model 1, M₅-DLO would be mainly oriented toward the cytoplasm, and in model 2, M₅-DLO would be able to flip bidirectionally between leaflets. Current biochemical approaches, e.g. capturing M₅-DLO on the cytoplasmic side of intact ER preparations with a lectin (11, 14), would not be able to distinguish between these models as M₅-DLO would be quantitatively captured in both cases. Indeed, unless it is known that the transbilayer distribution of M₅-DLO is stable during the analysis, it would be difficult to make any conclusions about its orientation. Development of suitable techniques to distinguish between these models remains an objective for future work.

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