Preferential transfer of the complete glycan is determined by the oligosaccharyltransferase complex and not by the catalytic subunit

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Most eukaryotic cells show a strong preference for the transfer *in vivo* and *in vitro* of the largest dolichol-P-P-linked glycan ($Glc_3Man_9GlcNAc_2$) to protein chains over that of biosynthetic intermediates that lack the full complement of glucose units. The oligosaccharyltransferase (OST) is a multimeric complex containing eight different proteins, one of which (Stt3p) is the catalytic subunit. Trypanosomatid protozoa lack an OST complex and express only this last protein. Contrary to the OST complex from most eukaryotic cells, the Stt3p subunit of these parasites transfers in cell-free assays glycans with $Man_{7-9}GlcNAc_2$ and $Glc_{1-3}Man_9GlcNAc_2$ compositions at the same rate. We have replaced *Saccharomyces cerevisiae* Stt3p by the *Trypanosoma cruzi* homologue and found that the complex that is formed preferentially transfers the complete glycan both *in vivo* and *in vitro*. Thus, preference for $Glc_3Man_9GlcNAc_2$ is a feature that is determined by the complex and not by the catalytic subunit.

N-glycosylation | *Saccharomyces cerevisiae* | *Trypanosoma cruzi*

Glycosylation of asparagine residues is one of the main posttranslational modifications in eukaryotic cells, because >80% of proteins that follow the secretory pathway carry N-linked glycans. Depending on the specific glycoprotein, this modification may be essential for the acquisition of the tertiary structure within the endoplasmic reticulum lumen, for the quality control of folding in the same subcellular location, for the correct sorting of lysosomal enzymes, or for the many roles that glycoproteins have either in the plasma membrane or in the external milieu.

The substrate donor used in the earliest description of the *en bloc* transfer of a glycan to protein from a lipid derivative was a dolichol-P-P-linked, glucose-labeled oligosaccharide that contained mannose and *N*-acetylglucosamine units ($Glc_3Man_9GlcNAc_2$, as was determined later) (1). Further reports showed that glycans lacking glucoses were also transferred in cell-free assays. Because glucose residues are not normally found in glycoproteins, it was initially believed that the glucose-free compounds were the true precursors in *in vivo* transfer reactions and that the glucose-containing compound was an oddity of no biological significance. A major advance in the field was the report that the glucose-containing lipid derivative was by far the main one occurring in normal cells, that its glycan was transferred to proteins at rates between 20- and 25-fold higher than those of compounds lacking the full complement of glucose units, and that both high-mannose- and complex-type glycans in mature glycoproteins were produced by intracellular processing of the fully glucosylated glycan (2, 3).

The enzyme involved in the transfer reaction (the oligosaccharyltransferase or OST) appeared to be a membrane-bound complex that was closely associated to the translocon and was formed in *Saccharomyces cerevisiae* by eight subunits, five of which appeared to be essential for viability of the microorganism (4–6). Several lines of evidence indicate that one of the essential proteins (Stt3p) is the catalytic subunit and is actually responsible for the transfer of the glycan. (*i*) Cross-linking between Ost1p, Ost3, and Stt3p and an acceptor polypeptide chain was obtained when a photoreactive reagent was engineered close to a N-glycosylation site (7); further, under certain experimental conditions, exclusive cross-linking to the last protein subunit was observed (8). (*ii*) An Stt3p homologue (PglB protein) was found to be encoded by the bacteria *Campylobacter jejuni* genome (9). Homologues to other OST complex components were absent. The PglB protein catalyzed the transfer of a variety of undecaprenol-P-P-linked glycans to asparagine residues in the canonical consensus sequence N-X-T/S. The structures of the transferred glycans differed widely from those transferred in eukaryotic cells (10). (*iii*) Point mutations in the sequence WWDYG, a motif present in all members of the Stt3p family, eliminated or sharply reduced OST activity (11). (*iv*) Genomic analysis showed that Stt3p is the only protein of the entire OST complex encoded by the genomes of trypanosomatid protozoa (6).

Trypanosomatid protozoa synthesize and transfer to protein unglucosylated glycans ($Man_9GlcNAc_2$, $Man_7GlcNAc_2$, or $Man_6GlcNAc_2$, depending on the species) (12). In addition to species-specific deficiencies in certain dolichol-P-Man-dependent mannosyltransferases, all trypanosomatid species are unable to synthesize dolichol-P-Glc, which is the glucosyl donor in the synthesis of the complete glycan (13). Cell-free assays showed that, irrespective of the largest lipid-linked glycan synthesized and transferred *in vivo*, OSTs from all trypanosomatid protozoa tested transfer to protein $Man_{7-9}GlcNAc_2$ and $Glc_{1-3}Man_9GlcNAc_2$ at the same rate (14).

Is the capacity to transfer all glycans at the same rate a feature determined specifically by trypanosomatid Stt3p, or, alternatively, is it the complex that determines the preferential transfer of $Glc_3Man_9GlcNAc_2$ in mammalian, plant, fungal, and other eukaryotic cells? To answer this question, we have replaced *S. cerevisiae* Stt3p by the *Trypanosoma cruzi* homologue in the fungal OST complex. It is worth mentioning that *T. cruzi* and *S. cerevisiae* Stt3ps (799 and 719 aa, respectively) show a 29% identity and 48% similarity according to the Blast-2-Seq program. Results obtained indicate that it is the complex, not the catalytic subunit, that determines the preferential specificity of the OST for the complete glycan.

Results

Incorporation of *T. cruzi* Stt3p in the Yeast OST Complex. Yeast Stt3p was replaced in the OST complex by its *T. cruzi* homologue as described in *Materials and Methods*. Briefly, a plasmid encoding yeast Stt3p (pScSTT3) was introduced into a diploid strain harboring a disrupted *STT3* allele, and sporulation was then

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Abbreviations: CPY, carboxypeptidase Y; OST, oligosaccharyltransferase; SC, synthetic complete; YPD, yeast extract/peptone/dextrose.

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induced. A haploid colony bearing the plasmid and the disrupted gene was selected and transformed with a plasmid encoding *T. cruzi* Stt3p (pTcSTT3). Resulting cells were then cured of the yeast plasmid. The plasmid shuffling approach was followed, because transfection of diploid yeast cells directly with pTcSTT3 followed by sporulation yielded, in all cases, only two viable spores per tetrad. None of the viable cells had a disrupted yeast *STT3* gene. The most plausible explanation of the requirement of the plasmid shuffling approach for obtaining viable cells expressing the parasite subunit is that, as it will be explained below, the OST complex bearing *T. cruzi* Stt3p is rather inefficient in the transfer reaction. Underglycosylation of a glycoprotein that is absolutely required either for proper sporulation or for germination might result in its misfolding or in its inability to be properly processed beyond the endoplasmic reticulum.

To check that the protozoan and not the yeast protein was encoded in the resulting yeast cells, total DNA was prepared from four of the colonies, and *Escherichia coli* DH5α cells were electroporated and plated on LB Amp (LB supplemented with 200 μg/ml ampicillin). Plasmidic DNA was prepared from six colonies. Restriction analysis and complete sequencing revealed in all cases complete pTcSTT3 plasmids without rearrangements. The sequences coding for *T. cruzi* Stt3p were identical with those reported in the gene bank. In addition, PCR analyses were performed on total DNA from several strains to confirm disruption of *S. cerevisiae STT3* in cells carrying plasmid pTcSTT3. Combinations of primers from outside both ends of the disrupted gene (ScProm and ScTerm) yielded the exclusive presence of either 2,936- or 2,355-bp fragments when template DNA was derived from strains carrying intact yeast *STT3* (Fig. 1*A*, lanes 1 and 4) or a disrupted yeast *STT3* plus plasmid pTcSTT3 (Fig. 1*A*, lanes 3 and 6), respectively. On the other hand, both fragments were obtained when DNA had been obtained from cells carrying a disrupted chromosomal *S. cerevisiae STT3* and plasmid pScSTT3 (Fig. 1*A*, lanes 2 and 5). Primers ScProm and KanB, located outside the disrupted gene and inside the replacing one, respectively, yielded only the expected 571-bp fragment when DNA was from strains harboring a disrupted yeast *STT3* (Fig. 1*B*, lanes 2, 3, 5, and 6). Further, primers specific for intact *S. cerevisiae STT3* (ScSTT3F and ScSTT3R) gave a negative result with plasmid pTcSTT3 or with DNA from strains carrying a disrupted yeast *STT3* and this last plasmid (Fig. 1*C*, lanes 3, 6, and 8), but the expected 564-bp fragment was synthesized with plasmid pScSTT3 or with DNA from strains harboring it or an intact chromosomal *STT3* (Fig. 1*C*, lanes 1, 2, 4, 5, and 7).

That the protozoan protein was actually forming part of the complex was shown by using a HA-tagged version of it in a pull-down experiment in which material immunoprecipitated with Ost1p antiserum was run on gels under dissociating conditions. This antiserum was chosen for immunoprecipitation to ensure that the eventual detection of Stt3p in the immunoprecipitate would actually indicate that the catalytic subunit was forming part of the entire complex, because Stt3p and Ost1p belong to different subcomplexes within the three that form the entire yeast OST complex (15, 16). Western blot analysis with HA antiserum revealed the presence of Stt3p in the tagged OST complex but not in the wild-type yeast version (Fig. 1 *D* and *E*). A second immunoprecipitation that was performed, as in the first one, with Ost1p antiserum on the resulting supernatant revealed that all HA-tagged material associated with Ost1p had been recovered in the first immunoprecipitation (not shown); furthermore, no HA signal appeared upon SDS/PAGE-Western blot analysis of the 10% trichloroacetic acid precipitate obtained from the supernatant of the first immunoprecipitation (Fig. 1*E*). This last result showed that all HA-tagged *T. cruzi* Stt3p expressed in *S. cerevisiae* was forming part of the complex.

The complexes in cells expressing both *S. cerevisiae* and *T. cruzi*

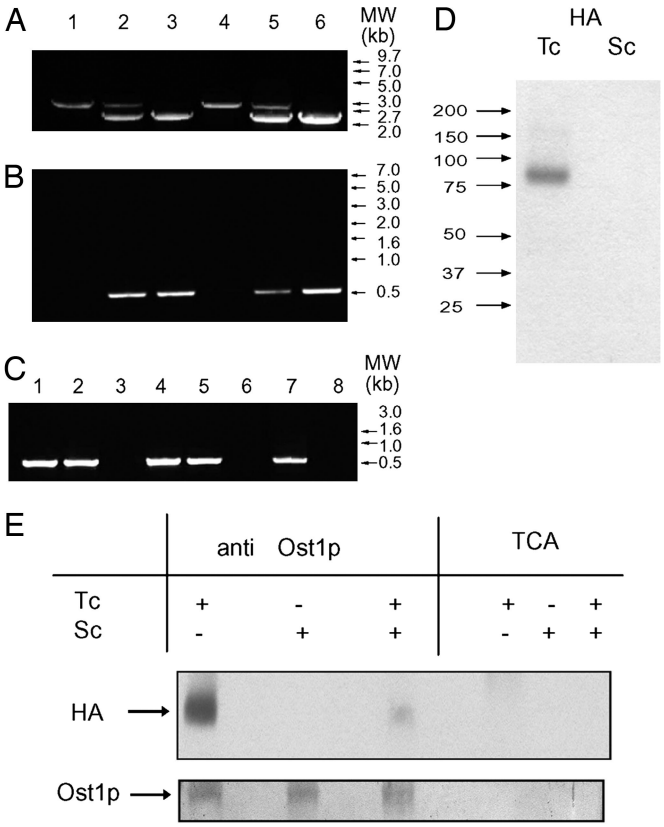

Fig. 1. Characterization of *S. cerevisiae* cells expressing *T. cruzi* Stt3p. (*A–C*) PCR fragments were synthesized by using primers ScProm and ScTerm (*A*), ScProm and KanB (*B*), or ScSTT3F and ScSTT3R (*C*) and total DNA as a template from the following strains or plasmids: lane 1, wild type; lane 2, *stt3*::*KanMX4* pScSTT3; lane 3, *stt3*::*KanMX4* pTcSTT3; lane 4, alg5::*HIS3*; lane 5, *alg5*::*HIS3 stt3*::*KanMX4* pScSTT3; lane 6, *alg5*::*HIS3 stt3*::*KanMX4* pTcSTT3; lane 7, pScSTT3; lane 8, pTcSTT3. (*D* and *E*) Cell detergent-soluble extracts from yeast cells expressing plasmid-coded *T. cruzi* Stt3p having a HA tag (Tc), *S. cerevisiae* Stt3p (Sc), or both were treated with Ost1 antiserum, and the precipitated material was run on SDS/PAGE and submitted to Western blot analysis with HA or Ost1p (OS) antisera. The 10% trichloroacetic acid precipitates of the supernatants of immunoprecipitations (TCA) were submitted to similar SDS/PAGE-Western blot analysis with HA antiserum. The methodology used was as described in ref. 17.

Stt3p mainly contained the yeast subunit despite the fact that the parasite protein was encoded in a high-copy-number expression vector with a very strong promoter, whereas the yeast subunit was coded in a centromeric expression vector with the subunit's own promoter (Fig. 1*E*; compare the HA signal of immunoprecipitates of extracts obtained from cells expressing either the parasite or both subunits). This result probably indicates a higher stability of the complex formed with the endogenous subunit than with the foreign catalytic subunit. Furthermore, no HA signal was observed on SDS/PAGE-Western blot analysis of the 10% trichloroacetic acid precipitates obtained from the supernatants of the immunoprecipitation of extracts of cells expressing both subunits (Fig. 1*E*). Results shown in Fig. 1*E* indicate, therefore, that orphan subunits, in contrast to assembled parasite subunits, were not stably inserted into membranes and probably rapidly degraded. Western blots performed with Ost1p antiserum showed that similar amounts of complex components had been loaded into the wells (Fig. 1*E*).

Cell-Free Transfer of Glycans. The transfer assays contained as the substrate donor a mixture of dolichol-P-P derivatives containing $Glc_{1-3}Man_9GlcNAc_2$ and $Man_9GlcNAc_2$ (Fig. 2*A*) and contained

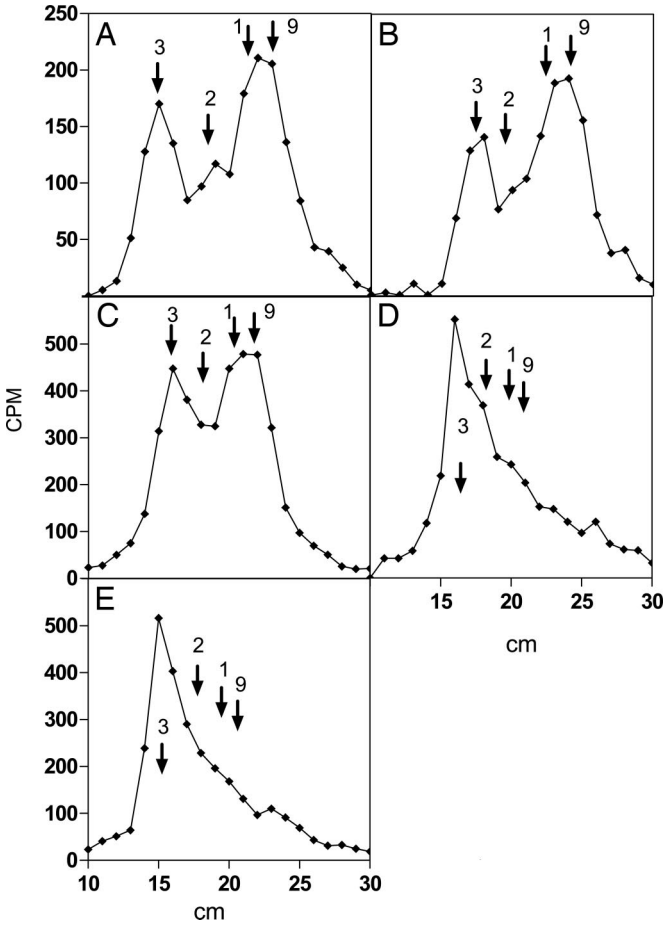

Fig. 2. Cell-free transfer of glycans. A mixture of dolichol-P-P-linked oligosaccharides was incubated with microsomes and an acceptor hexapeptide. Results are shown for glycans of the substrate used (*A*), glycans transferred by *T. cruzi* microsomes (*B*), glycans remaining linked to dolichol-P-P after the last transfer reaction (*C*), and glycans transferred by *S. cerevisiae* OST having *T. cruzi* (*D*) or yeast (*E*) Stt3p. Results of paper chromatographies of glycans are shown. Standards were as follows: 9, $Man_9GlcNAc$; 1, $Glc_1Man_9GlcNAc$; 2, $Glc_2Man_9GlcNAc$; 3, $Glc_3Man_9GlcNAc$.

as the substrate acceptor a hexapeptide containing a consensus glycosylation signal and *T. cruzi* or yeast microsomes as the enzyme source. As previously reported, the protozoan membrane preparation transferred all glycans at the same rate in the presence of detergents (Fig. 2*B*), whereas under identical conditions, both *S. cerevisiae* and rat liver cell microsomes preferentially transferred the largest glycan (14). Analysis of the nontransferred glycans revealed that their pattern was similar to that of the substrate (Fig. 2*C*), thus ensuring that results shown in Fig. 2*B* represent glycans transferred at the initial stages, not the last stages. Contrary to results obtained for the protozoan preparation, microsomes derived from yeasts bearing either *T. cruzi* or *S. cerevisiae* Stt3p, both coded in plasmids, preferentially transferred $Glc_3Man_9GlcNAc_2$ (Fig. 2 *D* and *E*, respectively). Although not absolutely identical, the profiles in Fig. 2 *D* and *E* clearly support the above conclusion.

***In Vivo* Transfer of Glycans.** Carboxypeptidase Y (CPY) has four N-glycans in wild-type *S. cerevisiae* cells grown under normal conditions. The same result was obtained with cells harboring a disrupted chromosomal *STT3* and yeast Stt3p coded in a plasmid (Fig. 3*A*, lane 1). A different result was obtained when the yeast protein was replaced by its *T. cruzi* homologue, also coded in a

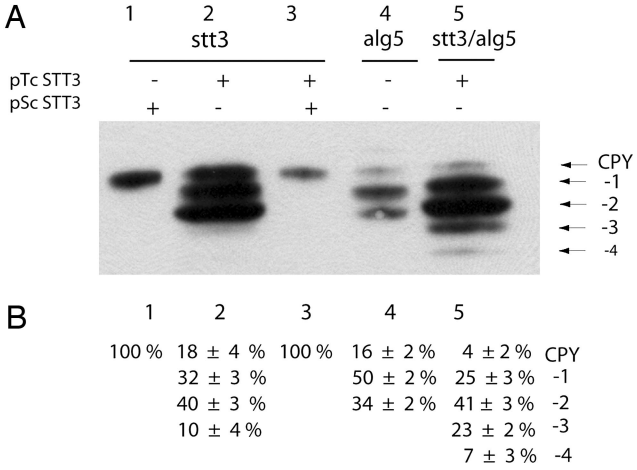

Fig. 3. CPY glycosylation. (*A*) Total soluble yeast proteins were submitted to SDS/PAGE, followed by Western blotting with CPY antiserum. Strains used were those harboring a disrupted chromosomal *STT3* plus yeast Stt3p (lane 1) or *T. cruzi* Stt3p (lane 2), or both proteins (lane 3) (both proteins coded in plasmids) and strains with a disrupted *ALG5* but with wild-type chromosomal *STT3* (lane 4) or with disrupted *ALG5* and chromosomal *STT3* but expressing *T. cruzi* Stt3p coded in a plasmid (lane 5). −1, −2, −3, and −4 refer to the number of N-glycans absent from CPY molecules. (*B*) Quantification of signal intensity in *A*.

plasmid, because underglycosylated (that is, molecules containing a lower number of glycans) CPY molecules were visualized (Fig. 3*A*, lane 2). This result indicates that the OST complex containing the protozoan catalytic subunit was rather inefficient in the transfer of $Glc_3Man_9GlcNAc_2$. *In vivo* glycosylation occurs when the Asn unit in the glycosylation sequence is precisely 10–12 aa from the inner endoplasmic reticulum membrane surface (18). Inefficient transfer, then, results in nonoccupied glycosylation sites. The possibility that the CPY underglycosylation that was observed could be due to fewer OST complexes being formed and not to an intrinsic property of the complex seems unlikely because the latter is intimately associated to translocons. Presence of translocons lacking OST complexes would have resulted in CPY molecules totally devoid of glycans, a feature that was not detected. On the other hand, cells expressing both yeast and protozoan Stt3p subunits synthesized fully glycosylated CPY molecules (Fig. 3*A*, lane 3). This result agrees with the fact that, under those conditions, the OST complex primarily contained the yeast subunit (Fig. 1*E*).

Because *ALG5* codes for the enzyme that is responsible for dolichol-P-Glc synthesis, *alg5* mutants synthesize $Man_9GlcNAc_2$-P-P-dolichol as the largest derivative, and it is this glycan that is (inefficiently) transferred to proteins (19). As a consequence, some CPY molecules in *alg5* mutants are underglycosylated (Fig. 3*A*, lane 4). If *T. cruzi* Stt3p transferred *in vivo* $Glc_3Man_9GlcNAc_2$ and $Man_9GlcNAc_2$ at the same rate when in a complex, it should be expected that yeast *alg5* mutants bearing the protozoan protein would show a degree of CPY underglycosylation similar to that observed in *ALG5* cells expressing *T. cruzi* Stt3p. On the contrary, if this last protein, when in a complex, preferentially transferred $Glc_3Man_9GlcNAc_2$, a higher degree of CPY underglycosylation would be expected to appear in *alg5* mutants because they would display two additive inefficiency factors, the protozoan Stt3p and the *alg5* mutation. Comparison of CPY patterns of cells expressing *T. cruzi* Stt3p and bearing either *ALG5* or *alg5* genotypes revealed that the latter alternative was what actually occurred. Whereas in the former case (*T. cruzi* Stt3p, *ALG5*) a substantial proportion of CPY molecules were fully glycosylated and no totally unglyco-

sylated species were detected (Fig. 3*A*, lane 2), in the latter case (*T. cruzi* Stt3p, *alg5*), almost no signal corresponding to molecules containing four glycans appeared, and, in addition, completely glycan-free CPY molecules were visualized (Fig. 3*A*, lane 5). This difference indicated that glycan transfer in cells expressing the parasite subunit and forming $Man_9GlcNAc_2$ as the largest compound was more inefficient than that in cells able to synthesize the fully glucosylated glycan and confirmed that, *in vivo*, as in cell-free assays, preference for $Glc_3Man_9GlcNAc_2$ depends on the OST complex, not on the catalytic subunit. The experiment shown in Fig. 3*A* was performed four times. Quantification of the average intensity of signals ± SD is depicted in Fig. 3*B*. The gel shown in Fig. 3*A* corresponds to one of the samples.

Discussion

We have shown that the specificity of the *T. cruzi* Stt3p homologue with respect to glucosylated and unglucosylated glycans depends on whether the protein is forming part of a multiprotein complex. In protozoan-derived microsomes (that is, in a context lacking the other components of the OST complex of higher eukaryotes), *T. cruzi* Stt3p transferred glucosylated and unglucosylated glycans at the same rate, but when forming part of the *S. cerevisiae* OST multimeric complex, the catalytic subunit preferentially used $Glc_3Man_9GlcNAc_2$ as a substrate.

Early reports showed that higher eukaryotic OSTs did not show significant differences in K_m values for the dolichol-P-P derivatives containing fully glucosylated ($Glc_3Man_9GlcNAc_2$) or unglucosylated ($Man_9GlcNAc_2$) glycans (20). A more recent study showed that saturation curves for both $Glc_3Man_9GlcNAc_2$-P-P-dolichol and $Man_9GlcNAc_2$-P-P-dolichol are sigmoidal, not hyperbolic (21). Because the saturation curve for the acceptor substrate yielded linear Lineweaver–Burk plots, consistent with a single binding site for the acceptor peptide, the existence of sigmoidal saturation curves for both glucosylated and unglucosylated donor substrates is compatible with the occurrence of two dolichol-P-P-derivative binding sites: one regulatory and the other catalytic. Based on extensive kinetic experiments performed with proteoliposomes containing yeast OST and almost homogeneous donor substrates, R. Gilmore and coworkers (21) developed an interesting model by which binding of $Glc_3Man_9GlcNAc_2$-P-P-dolichol to the regulatory site would enhance the relative affinity of the catalytic one for the same substrate by a factor of ≈1.5, whereas binding of $Man_9GlcNAc_2$-P-P-dolichol to the regulatory site would result in a 7- to 10-fold enhancement of the catalytic site relative affinity for the fully glucosylated derivative. That is, even under conditions when the fully glucosylated substrate is a relative minor component of the dolichol-P-P-derivative pool, as happens, for instance, in the leaky *alg3* yeast mutant, binding of unglucosylated compounds to the regulatory site would result in the transfer of $Glc_3Man_9GlcNAc_2$ (22) (Alg3p catalyzes the transfer of the sixth mannose unit to the dolichol-P-P derivative). Although the catalytic site has been localized to the Stt3p subunit, the location of the regulatory site is unknown. The present report supports the proposed model (21); it shows that a site not entirely localized to Stt3p (that is, different from the catalytic one) may influence the choice of the glycan transferred by the OST. It was further shown that two different mammalian OST complexes containing either the so-called STT3-A or STT3-B isoform of the catalytic subunit displayed a different preference for the transfer of glycans (STT3-A is shorter than STT3-B, and both forms share a 59% amino acid identity) (23). Thus, OST-I and OST-III complexes that contained STT3-B or STT3-A, respectively, showed a 2.6 or 14.7 times higher transfer of $Glc_3Man_9GlcNAc_2$ over $Man_9GlcNAc_2$. Our present work suggests that this result is probably caused not by the different catalytic subunits but by the different architecture of the resulting complexes.

What is the advantage for cells of transferring $Glc_3Man_9GlcNAc_2$ and not a biosynthetic intermediate? First, transfer of the larger glycan would ensure uniformity in the complex- and high-mannose-type glycan structures of individual mature glycoproteins. Because processing reactions require a precise substrate structure to proceed, transfer of a variety of different glycans to the same Asn unit might result in different glycans in the fully processed glycoprotein, which would result in less efficient or even useless protein species because many of glycoprotein roles depend on precise glycan structures. Concerning transfer of a glycan containing three glucose units, it has been speculated that release of the outermost glucose unit, which occurs immediately after transfer to a peptide, likely promotes release of the substrate from the OST complex (19). The middle glucose unit thus exposed constitutes a negative signal for binding to the nonconventional chaperones calnexin and calreticulin, thus allowing certain glycoprotein molecules to attain their final conformation without assistance. Only exposure of the innermost glucose would allow initiation of the glycan-dependent cycle of glycoprotein folding (24).

Although not essential for single-cell viability, preferential transfer of the largest glycan confers subtle advantages that determine its occurrence in almost all eukaryotes. Most eukaryotic cells display two security devices to ensure the preferential transfer of the largest glycan that they are able to synthesize: an active synthetic machinery of dolichol-P-P derivatives resulting in the accumulation of the largest glycan and the existence of the OST complex. Trypanosomatid cells display only the former feature.

Materials and Methods

Cloning of the Gene Encoding *T. cruzi* Stt3p (pTcSTT3). The *T. cruzi* Stt3p homologue-encoding gene (2,397 bp, Tc 00.1047053505163.80/5150.t0008, http://tcruzidb.org/index.shtml) was cloned in a high-copy-number yeast expression vector (p425GPD; LEU) with a very strong promoter. A plasmid bearing a strong promoter was used to ensure a high expression level of the parasite subunit because trypanosomatids and yeasts partially differ in the use of codons. Cloning was performed by PCR using genomic DNA from *T. cruzi* CL Brener isolate as a template. Primers used were as follows: TCDBATG, 5′-ATGGACACAGCACAATTAACAC-3′; TCDBTAA, 5′-TTAGCCACGCCCTTCATTG-3′. The fragment ends were filled with T4 DNA polymerase, treated with T4 PN kinase, and blunt-cloned in the SmaI vector site. The cloned gene was sequenced, and it seemed to be identical with that reported in the above-mentioned gene bank. The gene was also cloned with a *Haemophilus influenzae* agglutinin tag at its C terminus. In this case, the second primer used was TCDBHATAA, 5′-GCCCTAAGCGTAGTCTGGGACGTCGTATGGGTAG-CCACGCCCTTCATTGCGGAT-3′.

Cloning of the Gene Encoding *S. cerevisiae* Stt3p (pScSTT3). The entire gene was cloned with its own promoter and terminator in the centromeric expression vector pRS316 (URA). A 3,207-bp fragment containing the entire gene (2,157 bp) plus 461 bp and 589 bp at the 5′ and 3′ ends, respectively, was cloned by PCR using chromosomal DNA as a template and primers that had SacI and XhoI sites at their ends.

***S. cerevisiae* Strain.** The diploid heterozygous Y24390 strain (EUROSCARF, Frankfurt, Germany) was used. This strain has the *STT3* locus (*YGLO22w*) disrupted by a kanamycin resistance gene (*KanMX4*) in one of the alleles. It is derived from the strain whose genome was fully sequenced (BY4743) and has the following genotype: *Mata/α; his3Δ1/his3Δ1;*

leu2Δ0/leu2Δ0; Lys-2/lys2Δ0; Met-15/met15Δ0; ura3Δ0/ura3Δ0; YGLO22w::*KanMX4/YGLO22w*.

Expression of *T. cruzi* Stt3p in *S. cerevisiae*. Plasmid pScSTT3 was introduced into the above strain by electroporation. The diploid strain (URA^+;LEU^-;Kan^R) was sporulated, and several asci were dissected; in all cases, the four spores were viable. A haploid spore was selected that carried the following genotype: *MATα; LYS2; his3Δ1; met15Δ0; ura3Δ0; leu2Δ0; stt3*::*KanMX4* pScSTT3. This strain was transformed with plasmid pTcSTT3, and haploids carrying both plasmids were selected in synthetic complete (SC) medium without uracil and leucine. This strain was grown in liquid yeast extract/peptone/dextrose (YPD) medium and plated in SC medium with 5′fluorotic acid for curing the strain of pScSTT3. Several colonies were picked and analyzed for growth on YPD plus kanamycin, SC medium without leucine, and SC medium without uracil and leucine. All of the colonies were resistant to kanamycin and grew in SC medium without leucine, but none of them grew in SC medium lacking uracil.

***S. cerevisiae* Mutant in *STT3* and *ALG5* Genes.** Strains *MATα; LYS2; his3Δ1; met15Δ0; ura3Δ0,leu2Δ0; stt3*::*KanMX4 ura3–52; lys2–801; ade2–101; his3Δ200; trp1-Δ1; leu2-Δ1; alg5*::*HIS3* were crossed in liquid medium, and diploids were selected in plates containing SC medium minus histidine, lysine, and uracil. Cells were sporulated and dissected; in all cases, the four spores were viable. Spores were then analyzed for mating type, kanamycin resistance, and growth on SC without uracil and histidine. Several spores had the genotype *stt3*::*KanMX4; alg5*::*HIS3* pScSTT3. To confirm the absence of a functional *ALG5* gene (genotype *alg5*::*HIS3*), total proteins were prepared, and CPY underglycosylation was checked by Western blotting. The plasmid pTcSTT3 was introduced into *stt3*::*KanMX4; alg5*::*HIS3; leu2-Δ1* or *leu2Δ0* pScSTT3 cells by electroporation. Transformants were selected in SC medium without uracil, leucine, and histidine, and viability in YPD plus kanamycin was checked. Selected transformants were grown in YPD with 0.6 M sorbitol and plated on 0.6 M sorbitol SC medium with 5′fluorotic acid for curing this strain of pScSTT3. Selected colonies grew in YPD without sorbitol plus kanamycin and SC medium without histidine and leucine, and absence of growth in the same medium but lacking uracil was checked. Total DNA was prepared and electroporated into *E. coli* DH5α. Plasmid DNA prepared was shown to be pTcSTT3 by restriction analysis and sequencing of both *STT3* gene ends. Resulting cells were then *stt3*::*KanMX4; alg5*::*HIS* pTcSTT3.

Primers Used for Checking Yeast *STT3* Disruption. The following primers were used for checking disruption of yeast *STT3*. Primers from outside the gene: ScProm, 5′-GACACACTATG-CAACGCAG3-′; ScTerm, 5′-TGCATCAGACTCCCTCATC-3′. These primers are located 321–303 and 440–458 bp upstream and downstream of *STT3*, respectively. Primer KanB, 5′-CTGCAGCGAGGAGCCGTAAT-3′ is homologous to the noncoding strand and is located 250 bp downstream of the 5′ end of the *KanMX4* module (1,576 bp). Primers specific for yeast *STT3*: ScSTT3F, 5′-CCTATCATTGCCTCCGTT-3′; ScSTT3R, 5′-TGGTTCTGTCTGCCATGC-3′. These primers are located 1,027–1,044 and 1,591–1,574 bp within *STT3* (2,157 bp).

Antisera. CPY and OST1p antisera were generous gifts from Reid Gilmore (University of Massachusetts, Worcester, MA). HA affinity-purified antiserum was from Roche (Penzberg, Germany).

***T. cruzi* and Yeast Microsomes.** These microsomes were prepared as described in ref. 14. In the case of *S. cerevisiae*, a complete antiprotease mixture was included from the onset of the preparation. *T. cruzi* cells were from the CL Brener isolate.

OST Assay. This assay was performed in the presence of detergents by using a mixture of [^{14}C]$Man_9GlcNAc_2$ and [^{14}C]$Glc_{1-3}Man_9GlcNAc_2$ dolichol-P-P derivatives as donor substrates and the hexapeptide Tyr-Asn-Leu-Thr-Ser-Val as an acceptor as described in ref. 14 except that 1 mM 1-deoxynojirimycin was included in the incubation mixtures.

Other Methods. Paper chromatographies were performed on no. 1 chromatography paper from Whatman (Maidstone, U.K.) with 1-propanol/nitromethane/water (5:2:4) as a solvent.

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