Oxidoreductase activity of oligosaccharyltransferase subunits Ost3p and Ost6p defines site-specific glycosylation efficiency

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Edited by Carolyn R. Bertozzi, University of California, Berkeley, CA, and approved May 7, 2009 (received for review December 10, 2008)

Asparagine-linked glycosylation is a common posttranslational modification of diverse secretory and membrane proteins in eukaryotes, where it is catalyzed by the multiprotein complex oligosaccharyltransferase. The functions of the protein subunits of oligosaccharyltransferase, apart from the catalytic Stt3p, are ill defined. Here we describe functional and structural investigations of the Ost3/6p components of the yeast enzyme. Genetic, biochemical and structural analyses of the luminal domain of Ost6p revealed oxidoreductase activity mediated by a thioredoxin-like fold with a distinctive active-site loop that changed conformation with redox state. We found that mutation of the active-site cysteine residues of Ost6p and its paralogue Ost3p affected the glycosylation efficiency of a subset of glycosylation sites. Our results show that eukaryotic oligosaccharyltransferase is a multifunctional enzyme that acts at the crossroads of protein modification and protein folding.

crystal structure | mass spectrometry | *N*-glycosylation | protein folding | thioredoxin-like protein

Asparagine linked (*N*-)glycosylation is a common and essential co- and posttranslational modification of proteins in eukaryotes, and also occurs in archaea and some bacteria (1). In eukaryotes, the presence of *N*-glycans assists protein folding in the endoplasmic reticulum (ER) (2), and subsequent modification in the Golgi results in a diverse range of glycan structures on mature proteins. The specific glycan structures present are important in modulating protein function in processes including development, inflammation, cancer, and the immune response (3–5).

In eukaryotes, *N*-glycosylation is catalyzed in the ER lumen by the multiprotein complex oligosaccharyltransferase (OTase), which consists of up to 8 protein subunits (6). Glycan from the mature lipid-linked oligosaccharide donor (typically Glucose₃Mannose₉*N*-acetylglucosamine₂-pyrophosphatidolchol) (7) is transferred en bloc to asparagines in selected glycosylation sequons (N-x-S/T; x≠P) in protein substrates (6). In some divergent eukaryotes, archaea and *N*-glycosylating bacteria, OTase is a single protein, homologous to the catalytic STT3 protein subunit of the eukaryotic enzyme (8).

The protein subunits of OTase, in addition to the catalytic STT3 protein, are possibly involved in regulation of activity, including influencing glycan and protein substrate specificity. It is emerging that several protein subunits of the eukaryotic OTase are required for efficient glycosylation of particular subsets of protein substrates. For example, mammalian ribophorin I and II are required for glycosylation of selected membrane proteins and P-glycoprotein, respectively (9–11). The catalytic STT3 and the homologous OST3/6 proteins are also present in multiple isoforms in many organisms (STT3A/B and OST3/6). This results in the presence of OTase complexes with different protein isoform compositions, which in turn have been reported to affect OTase glycan and protein substrate-specific

activities. The mammalian STT3A/B isoforms are differentially expressed in various tissues, and result in OTases with altered kinetic characteristics (12) and preferences for co- or posttranslational glycosylation (13). The OTase of trypanosomatids consists of a single STT3 protein, but often multiple isoforms are present. These single protein OTase isoforms have different substrate specificities in *Trypanosoma brucei* (14, 15) and *Leishmania major* (16). These organisms therefore appear to have increased the range of glycoprotein substrates through duplication and divergence of STT3 protein activity, rather than addition of accessory protein subunits to OTase. In yeast, Ost3p and Ost6p are products of paralogous genes, and have the same predicted topology of an N-terminal domain located in the ER lumen followed by 4 transmembrane helices. Incorporation of either Ost3p or Ost6p results in the presence of 2 alternative OTase complexes (17–19), which have different protein substrate-specific activities at the level of individual glycosylation sites (20). Additionally, the human homologues of Ost3/6p (IAP and N33) are required for correct brain development (21, 22), suggesting that they also facilitate glycosylation of a subset of protein substrates.

The ER luminal domain of Ost3/6p is predicted to have a thioredoxin-like fold with a characteristic CxxC active-site motif (23). Thioredoxin-like folds are commonly present in proteins with disulfide oxidoreductase activity and in the ER are specifically involved in disulfide oxidation and isomerization during protein oxidative folding (24). Oxidative protein folding and *N*-glycosylation are linked processes in eukaryotic cells. Disulfide bond formation can compete with glycosylation in some substrates (25), in accordance with the requirements of eukaryotic OTase for a flexible substrate. Somewhat in contradiction to this, *N*-glycans play an essential role in the oxidative folding of proteins by locally recruiting the protein disulfide isomerase ERp57 through the lectin calnexin to regions of proteins requiring disulfide isomerase activity for correct folding (26–29). The presence of a thioredoxin-like fold in a subunit of the eukaryotic OTase suggested a multifunctional enzyme, in which disulfide oxidoreductase activity could directly influence the folding of protein substrates and affect their glycosylation.

Author contributions: B.L.S., R.G., and M.A. designed research; B.L.S., C.U.S., J.P.A.G., M.S.B., F.F., and E.M. performed research; B.L.S., C.U.S., J.P.A.G., M.S.B., E.M., and G.C. analyzed data; and B.L.S., C.U.S., J.P.A.G., M.S.B., F.F., E.M., G.C., R.G., M.G.G., and M.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: Atomic coordinates and structure factors have been deposited with the Protein Data Bank, www.pdb.org (PDB ID codes 3G7Y, 3G9B, and 3GA4).

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This article contains supporting information online at www.pnas.org/cgi/content/full/0812515106/DCSupplemental.

Fig. 2. In vitro characterization of Ost6L. (A) Determination of the redox potential of Ost6L from its redox equilibrium with DTT_{red}/DTT_{ox}. (B) Guanidium-induced equilibrium unfolding indicated that Ost6L_{ox} (circles) was more stable than Ost6L_{red} (triangles). (C) The apparent pK_a of the active-site cysteines of Ost6L as determined from its reactivity with iodoacetamide. (D) Stoichiometric reduction of an oxidized model peptide [NRCSQSGSCWN (44)] by reduced Ost6L (triangles), *E. coli* thioredoxin (circles), or no protein (squares). (E) Reduction of insulin (115 μM) by various concentrations of Ost6L (triangles), *E. coli* thioredoxin (circles), or no protein (squares). (F) Disulfide isomerase activity as determined by reactivation of scRNase by the *E. coli* disulfide isomerase DsbC (circles), Ost6L (squares), or no protein (open triangles), relative to native RNaseA (triangles).

synchrotron radiation (Ost6L_{ox}*) at 1.3 Å resolution; and the reduced state (Ost6L_{red}) at 1.96 Å resolution. In agreement with our biochemical data, Ost6L indeed exhibited a thioredoxin-like core fold, but several additional features unexpected for a protein belonging to the thioredoxin-like superfamily were observed (Fig. 3B): an N-terminal αβ-motif (D₈-L₃₀); a conspicuous loop preceding the CxxC active-site motif (R₄₆GTNSNGMS₅₄); two 3₁₀-helices between β-strands 4 and 5 (S₁₁₄-Q₁₁₉, 3₁₀A), and between β-strand 5 and α-helix 5 (P₁₃₄-N₁₃₆, 3₁₀B); and a C-terminal linker connecting the luminal domain with the first predicted transmembrane helix of Ost6p.

In contrast to classical thioredoxin-like proteins, Ost6L had a distinctive loop adjacent to its CxxC motif (see Fig. 3 and Fig. S2). A similar insertion before the active-site motif corresponding to the Ost6L loop is present in all Ost3/6p sequences, suggesting that this is a general, functionally important feature of this protein subclass (Fig. S3). In both Ost6L_{ox} and Ost6L_{ox}*, this active-site loop adopted an essentially fixed conformation, stabilized by several main-chain hydrogen bonds (see Fig. 3C). The main-chain amide of C₅₅ hydrogen-bonded the carbonyl

Fig. 3. Ost6L has a thioredoxin-like fold with unique features. (A) Schematic overview of the yeast OTase complex. The ER luminal domain of Ost6p (Ost6L) is boxed. (B) Ribbon diagram of the structure of Ost6L. The active-site cysteines are shown in yellow. (C) Superimposed active-site regions of Ost6L_{ox} (brown) and Ost6L_{red} (cyan). In Ost6L_{ox}, the loop was stabilized by hydrogen bonds (black dashed lines); and in Ost6L_{red}, helix α3 was extended by S₅₄ and C₅₅, and the loop was flexibly disordered (dotted cyan line, no electron density was visible). (D) Surface representation of Ost6L_{ox}* showing the proposed peptide-binding groove adjacent to the exposed active-site C₅₅, colored by electrostatic surface potential (red, acidic; white, neutral; blue, basic).

group of G₄₇ via a well-ordered water molecule. Other stabilizing interactions were provided by hydrogen bonds formed by the main-chain amide of T₄₈ with the carbonyl of M₅₃, the carbonyl of N₄₉ with the main-chain amide of N₅₁, and by the carboxamide of N₄₉ with the main-chain amide moieties of G₅₂ and M₅₃.

Upon reduction of the Ost6L disulfide, the active-site loop became flexible, with no electron density observed for residues G₄₇ to G₅₂ (see Fig. 3C). The loop flexibility allowed the active-site cysteine C₅₅ to adopt a conformation different from other reduced thioredoxin-like proteins (37, 38). In classical thioredoxin-like proteins, reduction mainly leads to variation in the χ₁ torsion angle of the N-terminal cysteine C₅₅, with only marginal main chain rearrangements. In Ost6L, reduction instead led to conformational changes in both the C₅₅ side and main chain, resulting in an elongated active-site helix. Extension of the active-site helix forced the main chain amino group of C₅₅ to point away from G₄₇, and thus prevented the formation of the hydrogen bond network that presumably kept the loop ordered in the oxidized form.

The ordered loop in Ost6L_{ox} created a distinct groove near the active-site motif (see Fig. 3D). This groove was a strong candidate for a peptide-binding site, as it possessed slightly basic and hydrophobic regions, an acidic patch, and contained several residues that could easily adapt to various substrate polypeptides (M₄₅, N₄₉, M₅₃ and N₈₉). The surface of Ost6L also featured a distinctive hydrophobic patch (Fig. S4). The residues in the patch were also the only continuous area of the surface of Ost6L under strong purifying selection (see Fig. S4B) and the corresponding residues in Ost3p are also hydrophobic (see Fig. S3). This hydrophobic patch may be involved in interactions with substrate polypeptides or with other proteins in a multiprotein complex.

Previous genetic experiments (18, 20) and our in vivo and structural data (see Figs. 1 and 3) suggested that Ost3/6p might be

Fig. 4. Noncovalent peptide binding by Ost6L_{ox}. Relative enrichment of the Cwp1p^{21–31} versus the H5150p^{338–345} peptide, by columns with covalently attached Ost6L_{ox} (circles), Ost6L_{red} (p), or no protein (squares). Data are average of triplicates. Error bars indicate the range.

involved in substrate recognition mediated by the redox-dependent active-site groove. We therefore tested if Ost6L_{ox} and Ost6L_{red} could bind peptides noncovalently *in vitro*. A complex mixture of peptides from yeast cell-wall proteins (a rich source of *N*-glycoproteins, and hence potential Ost6L substrates) was applied to columns containing covalently bound Ost6L_{ox} or Ost6L_{red}. Retained peptides were then identified by mass spectrometry. Ost6L_{ox} but not Ost6L_{red} differentially enriched specific peptides (Fig. 4, Fig. S5, and Table S2), in accordance with the putative peptide-binding site present only in Ost6L_{ox} (see Fig. 3).

Discussion

The thioredoxin-like fold of the Ost3p/Ost6p subunit predicted a CxxC active-site motif essential for oxidoreductase activity. This prompted us to hypothesize that a potential oxidoreductase activity of Ost3/6p directly affects the oxidative folding of OTase protein substrates to assist their efficient glycosylation. We made use of our recently described method to measure the *N*-glycan occupancy of many sites in proteins covalently linked to the yeast polysaccharide cell wall (20). Glycosylation site occupancy in cells with both active-site cysteines of Ost3p and Ost6p replaced by serines revealed that these replacements did not completely abolish the function of Ost3/6p, as there was still a subunit-specific glycosylation phenotype of these variant forms: a subset of sites requiring Ost3p was not affected in the Ost3p CxxC variant strain. However, we observed site-specific underglycosylation in the SxxS variants, pointing to a functional role of the CxxC motif in specific glycosylation site selection. These 2 classes of sites were exemplified by N₂₅₃ in Gas1p (GAS1.253) and N₁₇₇ in Crh1p (CRH1.177) (see Fig. 1 *B* and *C*). CRH1.177 required Ost3p, either wild-type or CxxC variant, for complete glycosylation, while GAS1.253 required the presence of Ost6p as well as a CxxC motif. Interestingly, the GAS1.253 glycosylation site is present in a domain with 3 predicted disulfide bonds. Furthermore, sites that were affected by mutations in the CxxC motif of either Ost3p or Ost6p (such as GAS1.253) were more proximal in primary sequence to cysteines than sites that were not affected (see Fig. 1*D*). Our analysis monitored the glycosylation status of multiple *N*-glycosylation sites and only a subset of these was affected by mutation in the CxxC motif. We therefore conclude that the CxxC motif has an impact on efficient glycosylation. Irrespective of the number of sites affected, our results were best explained by the hypothesis that these OTase subunits have a dual function in site recognition: one function required the CxxC motif, the other was independent of this motif.

The CxxC-dependent glycosylation of defined sites in substrate proteins supported the hypothesis that the potential oxidoreductase activity of the Ost3p/Ost6p subunit affected OTase function *in vivo*. To investigate this putative oxidoreductase activity, we performed various *in vitro* assays using the purified ER luminal thioredoxin-like domain of Ost6p (Ost6L). Surprising for a protein present in

Fig. 5. Proposed model of Ost3/6p function in *N*-glycosylation. See text for details. (A) Nascent polypeptide chain with reduced cysteine residues passes through the translocon into the ER lumen. (B) Nucleophilic attack performed by the reduced polypeptide cysteine residue leads to (C) binding of the polypeptide to Ost6p via a mixed disulfide. Alternatively, polypeptide may be bound through noncovalent interactions with the peptide-binding groove. Translocation of the nascent polypeptide continues, with folding inhibited because of Ost6p binding constraints. (D) The catalytic site of OTase transfers glycan to the asparagine of a glycosylation sequon. (E) Resolution of the mixed disulfide releases the captured polypeptide chain. (F) Translocation and folding of the glycosylated polypeptide chain continues. (G) Glycosylated and folded protein.

the oxidizing environment of the ER, the redox potential, apparent active-site cysteine pK_a, and relative stability of the oxidized and reduced forms of Ost6L were similar to cytoplasmic Trx. Ost6L_{red} could stoichiometrically reduce a disulfide bond in a model peptide (see Fig. 2*D*) and showed activity as a catalyst of insulin reduction (see Fig. 2*E*), supporting oxidoreductase activity of this OTase subunit. However, these activities were lower than that of Trx. Although it is possible that the reductase activity of Ost6L allows Ost3/6p to locally reverse oxidative folding in substrate polypeptides, thereby providing unfolded substrate to the catalytic site of OTase, we favor an alternative model that is based on our finding that Ost6L is oxidized under the redox conditions of the ER (see Fig. S1). We propose that reduced cysteines of nascent polypeptide chains can perform a nucleophilic attack on the active-site disulfide bond of Ost6L, forming a mixed disulfide (Fig. 5). This mixed disulfide could tether the nascent polypeptide to Ost6p and prevent oxidative folding of the protein, thereby allowing efficient glycosylation of nearby N-x-S/T sequons by the catalytic center of OTase. The tethered nascent polypeptide chain would be released after resolution of the mixed disulfide by the free cysteine of the Ost6p CxxC motif, leaving the polypeptide reduced and Ost6L oxidized. The slow kinetics we observed for Ost6L redox reactions relative to Trx may act to prolong substrate tethering to Ost6p, while the exceptionally reducing redox potential of the Ost6L active-site

cysteines would thermodynamically favor release of reduced substrate polypeptide and re-oxidized Ost6L. Folding of the glycosylated polypeptide chain could continue after its release, including N-glycan/ERp57-mediated oxidative folding.

The structural change in the loop and groove close to the CxxC active-site motif of Ost6L (see Fig. 3C), and accompanying redox-state dependent peptide binding (see Fig. 4), could provide an additional, noncovalent, mechanism controlling temporary recruitment of specific polypeptide stretches. Specific polypeptide binding, both noncovalent and disulfide-mediated, may thus slow the early stages of substrate protein folding, thereby increasing the accessibility of specific sites and, hence, N-glycosylation efficiency. Bacterial OTase, a single protein homologous to the catalytic subunit of the eukaryotic enzyme, glycosylates sites in intrinsically flexible regions of folded substrate protein (39). Ost3/6p may thus allow eukaryotic OTase to actively maintain substrate protein in an accessible, unfolded state to increase the range of potential glycosylation sites.

To our knowledge, Ost6L is unique among thioredoxin-like proteins in showing such a dramatic redox-dependent structural change. Polypeptide substrate recognition mediated by the redox-dependent active-site groove could also explain the presence of Ost3/6p homologues in plants lacking a CxxC active-site motif, but containing a loop insertion at this position (see Fig. S3). The high-resolution structures we present here are unique in representing soluble, biochemically active domains of the eukaryotic OTase. In combination with the previously reported structures of the small transmembrane protein Ost4p from yeast (40), the soluble C-terminal domain of archaeal Stt3p from *Pyrococcus furiosus* (41), and the cryo-EM structure of the yeast OTase complex (42), our results provide a significant step toward a unified structural understanding of the mechanisms controlling N-glycosylation activity and substrate selection by the eukaryotic OTase.

Materials and Methods

Yeast Strains. Yeast strains used: YG889 (MATa *ade2-101 ura3-52 his3Δ200 tyr1 Δost3::HIS3 Δost6::kanMX4*) (18) and S5330 (MATa *ade2-101 ura3-52 his3Δ200 tyr1*) (43). Standard protocols were followed for yeast manipulation. Cells were grown as described (20).

Cell-Wall Protein Sample Preparation and Mass Spectrometry. Analysis of N-glycan site-specific occupancy was performed as described (20).

Plasmid Constructs. For expression in *E. coli*, the DNA sequence corresponding to the luminal domain of Ost6p (from the signal peptidase cleavage sites to the beginning of the first transmembrane helix as predicted in the UniProt Knowledgebase: residues 1–166 of mature Ost6p) was cloned into a derivative of the pBAD *E. coli* expression vector with the sequence coding for a myc-His₆ tag replaced by one coding for a His₁₀ tag, using PCR primers incorporating NcoI and BamHI restriction sites (pBAD-Ost6L-His-10). For expression in yeast, the following plasmids were used: pOST3 (18), pOST6 (18), pOST3_{C-ss},

pOST6_{C-ss}. Variants with amino acid replacements were constructed from the previously reported pOST3 and pOST6 plasmids using overlap PCR, using oligonucleotide pairs incorporating the desired sequence changes and PstI-SalI restriction endonucleases.

Protein Expression and Purification. Protein expression in *E. coli* cells harboring the vector pBAD-ost6L-His-10 was induced by addition of L-arabinose. Ost6L was purified from cell extracts by anion exchange-, immobilized metal ion- and size exclusion chromatography. See the *SI Text* for full details.

In Vitro Protein Analysis. The redox potential of the active-site disulfide of Ost6L was determined through equilibration with an excess of DTT_{ox} and DTT_{red} at different ratios. The free energies of stabilization (ΔG°) of oxidized and reduced Ost6L were determined from guanidinium-induced equilibrium unfolding curves based on the 2-state model of protein folding. The pH-dependent reactivity of the active-site thiols of Ost6L with iodoacetamide (35) was used to determine their apparent pK_a. A previously described synthetic peptide (NRCSQGSCWN) (44) was used as substrate in an in vitro reductase assay. The peptide contains 2 cysteines, and served as a model of an unfolded, oxidized polypeptide. Ost6L-catalyzed reduction of insulin by DTT was determined essentially as described (45). Protein disulfide isomerase activity was measured by reactivation of disulfide-scrambled RNaseA upon catalyzed disulfide shuffling. See the *SI Text* for full details.

Protein Crystallization and Structure Determination. Purified Ost6L_{ox} was crystallized using the sitting drop vapor diffusion method with 50 mM Na-citrate pH 3, 18% PEG 3350 as the precipitation solution. Crystals were incubated with 10 mM Tris(2-carboxyethyl)phosphine to obtain Ost6L_{red}. For structure solution, crystals of Ost6L_{ox} were soaked for 45 s in a cryo-protecting solution containing 0.5 M NaBr and a single wavelength anomalous dispersion dataset at the Br-peak wavelength ($\lambda = 0.9193 \text{ \AA}$; Ost6L_{peak}) to 2.0 Å resolution was collected and used for phasing. Three other datasets were collected: native protein (2.21 Å resolution, Ost6L_{ox}), protein with the active-site disulfide partially reduced by synchrotron radiation (1.3 Å resolution, $\lambda = 0.8551 \text{ \AA}$; Ost6L_{ox}*), and TCEP-reduced protein (1.96 Å resolution; Ost6L_{red}). Crystallographic statistics are given in Table S3. See the *SI Text* for full details.

Sequence and Structural Analyses. Electrostatic surface calculations at 310 K were carried out solving the linearized Poisson-Boltzmann equation with APBS [dielectric constants: 2 (protein) and 80 (solvent); 0.144 M monovalent salt; surface probe radius: 1.4 Å] (46) integrated in Pymol v0.99. Electrostatic surface potential representations were interpolated between acidic, –45 kT and basic, +45 kT.

Ost6L Peptide-Binding Determination. Tryptic peptides from deglycosylated cell wall proteins (20) were applied to CNBr-activated Sepharose 4B beads coupled with Ost6L_{red}, Ost6L_{ox} or no protein, and eluted in PBS with 1 mM EDTA. Peptides in each fraction were analyzed by MALDI-TOF/TOF-MS. See the *SI Text* for full details.

ACKNOWLEDGMENTS. Data collection was performed at the Functional Genomics Center Zurich (FGCZ) and at Swiss Light Source, Paul Scherrer Institut, Villigen, Switzerland. We thank the staff of FGCZ and of the beamline X06SA for support in MS and X-ray data collection, respectively. This project was funded by the Swiss National Science Foundation, the Eidgenössische Technische Hochschule Zürich, and the Universität Zürich within the framework of the National Center of Competence in Research Structural Biology program and the Glycolnit program.

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