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Mixed disulfide formation *in vitro* between a glycoprotein substrate and yeast oligosaccharyltransferase subunits Ost3p and Ost6p

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ABSTRACT

Oligosaccharyltransferase (OTase) glycosylates selected asparagine residues in secreted and membrane proteins in eukaryotes, and asparagine (N)-glycosylation affects the folding, stability and function of diverse glycoproteins. The range of acceptor protein substrates that are efficiently glycosylated depends on the action of several accessory subunits of OTase, including in yeast the homologous proteins Ost3p and Ost6p. A model of Ost3p and Ost6p function has been proposed in which their thioredoxin-like active site cysteines form transient mixed disulfide bonds with cysteines in substrate proteins to enhance the glycosylation of nearby asparagine residues. We tested aspects of this model with a series of *in vitro* assays. We developed a whole protein mixed disulfide interaction assay that showed that Ost6p could form mixed disulfide bonds with selected cysteines in pre-reduced yeast Gas1p, a model glycoprotein substrate of Ost3p and Ost6p. A complementary peptide affinity chromatography assay for mixed disulfide bond formation showed that Ost3p could also form mixed disulfide bonds with cysteines in selected reduced tryptic peptides from Gas1p. Together, these assays showed that the thioredoxin-like active sites of Ost3p and Ost6p could form transient mixed disulfide bonds with cysteines in a model substrate glycoprotein, consistent with the function of Ost3p and Ost6p in modulating N-glycosylation substrate selection by OTase *in vivo*.

1. Introduction

Asparagine (N-)linked glycosylation is a common co- and post-translational modification that affects the structure and function of secretory and membrane bound proteins in eukaryotes [1], archaea [2] and some bacteria [3]. In eukaryotes, nascent polypeptide is N-glycosylated by the enzyme oligosaccharyltransferase (OTase) after entry into the endoplasmic reticulum (ER) lumen through the translocon [4–6]. N-glycans enhance the efficiency of glycoprotein folding in the ER by increasing the solubility of folding nascent polypeptide and by recruiting the disulfide isomerase ERp57 through the lectins calnexin and calreticulin [7–10]. Glycan structures are modified during glycoprotein traffic through the Golgi, and the final structures present on mature glycoproteins can play important roles in regulating protein function [1,11]. Changes in the structure of glycans attached to proteins are also associated with numerous diseases [12–15].

OTase catalyses the key step of N-glycosylation, transfer of oligosaccharide from a dolicholpyrophosphate carrier to asparagines in nascent polypeptides translocated into the ER lumen [16]. OTase is a multiprotein complex consisting of a catalytic subunit, Stt3p, and varying numbers of accessory protein subunits in different organisms [5,17,18]. The acceptor peptide-binding site of Stt3p specifically recognizes asparagine residues in glycosylation sequons (Asn-Xaa-Ser/Thr; Xaa≠Pro) [19], substantially increasing the efficiency of glycosylation at these sites [20]. The accessory subunits are also important for determining the protein acceptor specificity of OTase, as several interact with acceptor nascent polypeptide to influence N-glycosylation substrate selection [20–26]. In *Saccharomyces cerevisiae*, there are two isoforms of OTase defined by incorporation of either of the homologous proteins Ost3p or Ost6p [27–29], which have an ER lumenal thioredoxin-like domain [23,30] followed by four transmembrane helices. These OTase isoforms have different acceptor protein specificities [27,31,32] at the level of individual glycosylation sites [22,23].

A model of Ost3p and Ost6p function in site selection by OTase has been proposed [23], in which nascent polypeptide transiently binds to the peptide-binding groove in their thioredoxin domain, which slows protein folding and presents nearby asparagine residues to the active site of OTase for efficient glycosylation. This transient binding of stretches of nascent polypeptide is proposed to occur through noncovalent interactions and also through the formation of a transient mixed disulfide between a cysteine in

Abbreviation: O Tase, oligosaccharyl transferase.

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the nascent polypeptide and the active site CxxC motif of Ost3p or Ost6p [23]. Hydrophobic stretches of polypeptide do bind to the peptide-binding grooves of both Ost3p and Ost6p *in vitro*, consistent with this model [33]. The CxxC motif of Ost3p and Ost6p is also important for glycosylation of a subset of sites *in vivo* in yeast, where mutation of the Ost3p or Os6p CxxC cysteines to serines results in inefficient glycosylation at several asparagines [23]. One such affected site is Asn253 in Gas1p. Here, we performed a series of *in vitro* assays to test if Ost3p and Ost6p were capable of sequestering the model substrate polypeptide Gas1p through transient mixed disulfide bonds.

2. Materials and methods

2.1. Protein expression and purification

The malE gene was deleted from existing pMAL-c2x-OST3 and pMAL-c2x-OST6 vectors [33] and replaced with an N-terminal His10 tag by site-directed PCR mutagenesis [34] leaving the DNA sequence corresponding to the lumenal domain of Ost3p or Ost6p as predicted in UniProt Knowledgebase (position 23–185 of Ost3p/P48439/ YOR085W; position 25–188 of Ost6p/Q03723/YML019W) to give pHis10-OST3 and pHis10-OST6. TOP10 *E. coli* cells carrying pHis10-OST3 or pHis10-OST6 were grown in LB media supplemented with 100 mg/L ampicillin. Protein expression was induced at an OD600 nm of 0.7 by addition of IPTG to a final concentration of 1 mM and incubation for 4 h at 37 °C, after which the cells were harvested by centrifugation. Cells were resuspended in ice cold 50 mM potassium phosphate buffer pH 7.5, 150 mM NaCl, 10 mM imidazole, 1× complete mini EDTA free protease inhibitor cocktail (Roche) and 1 mM PMSF, and lysed with a One Shot cell disrupter (Constant Systems). Protein was purified from clarified whole cell extracts using TALON resin (Clontech) and eluted in 50 mM potassium phosphate buffer pH 7.5, 150 mM NaCl and 200 mM imidazole. Proteins were buffer exchanged with PD-10 columns (GE Healthcare) into 50 mM potassium phosphate buffer pH 7.5, 150 mM NaCl and 1 mM EDTA. *S. cerevisiae* Ost3p, Ost6p and Gas1p were expressed and purified from *E. coli* as maltose-binding protein fusions (MBP-Ost3, MBP-Ost6 and MBP-Gas1) as previously described [33]. MBP-Gas1 was eluted in 50 mM Tris–HCl buffer pH 7.5, 150 mM NaCl, 1 mM EDTA and 0.1% SDS. MBP-Ost3 and MBP-Ost6 were not eluted but were retained on amylose–agarose beads as described [33].

2.2. Whole protein mixed disulfide formation analysis

Purified MBP-Gas1 in 50 mM Tris–HCl buffer pH 7.5, 150 mM NaCl, 1 mM EDTA and 0.1% SDS was reduced by addition of DTT to a final concentration of 10 mM and incubation at 95 °C for 10 min. SDS and DTT were removed from reduced MBP-Gas1 by buffer exchange using PD-10 columns into 50 mM Tris–HCl buffer pH 7.5, 150 mM NaCl and 1 mM EDTA. In order to allow the *in vitro* formation of mixed disulfide bonds between reduced cysteines in MBP-Gas1 and the oxidized CxxC active site motif of Ost3 or Ost6, purified and reduced MBP-Gas1 was incubated with either purified Ost3, Ost6 or no additional protein in 50 mM potassium phosphate buffer pH 7.5, 150 mM NaCl and 1 mM EDTA at 25 °C for 1 h (Fig. 1). Sulfide reactivity was quenched by cysteine alkylation with addition of acrylamide to a final concentration of 25 mM and SDS to a final concentration of 0.1%, and incubation for 30 min. Protein was precipitated by addition of four volumes of 1:1 methanol:acetone, incubation at −20 °C for 16 h and centrifugation at 18,000 rcf for 10 min. The protein pellet was resuspended in 50 μL of 50 mM ammonium acetate, 10 mM DTT and 1 μg trypsin (proteomics grade, Sigma–Aldrich) and incubated at 37 °C for 16 h.

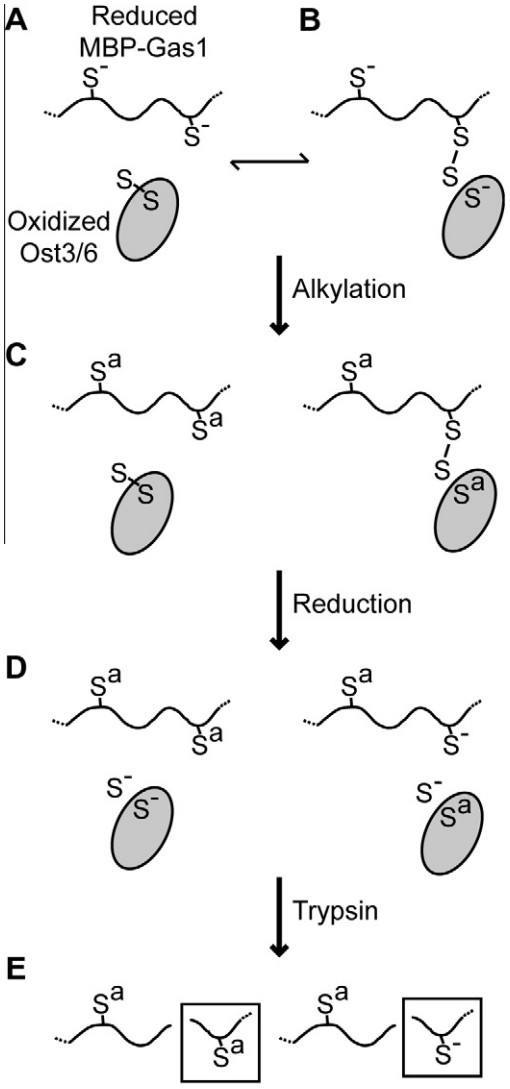

Fig. 1. Experimental design for detecting *in vitro* whole protein mixed disulfide formation with Ost3 or Ost6. (A) Reduced MBP-Gas1 is incubated together with oxidized Ost3 or Ost6. (B) A cysteine in Gas1 performs nucleophilic attack on the Ost3/6 cystine and forms a mixed disulfide bond. This reaction is reversible [23]. (C) Alkylation of free cysteines prevents further mixed disulfide shuffling. (D) Disulfide bonds are reduced with DTT. (E) Trypsin digestion and LC–ESI–MS/MS analysis allows detection of alkylated or reduced forms of the same cysteine-containing peptide (boxed). Peptides detected with an alkylated cysteine were originally free as in (A), and peptides detected with a reduced cysteine originally formed a disulfide bond as in (B). See Section 2 for details.

2.3. Peptide mixed-disulfide affinity chromatography

Purified MBP-Gas1was precipitated by addition of four volumes of 1:1 methanol:acetone and incubation at −20 °C for 16 h. Protein was resuspended in 50 μL of 50 mM ammonium acetate, 10 mM DTT and 1 μg trypsin and incubated at 37 °C for 16 h. Residual trypsin activity was inhibited by addition of PMSF to a final concentration of 1 mM and incubation at 25 °C for 30 min, followed by incubation at 95 °C for 5 min. Peptides were desalted using tC18 Sep-Pak cartridges (Waters) and eluted with 70% acetonitrile in 50 mM Tris–HCl buffer pH 7.5 with 1 mM EDTA to prevent re-oxidation of cysteines. Desalted and reduced tryptic peptides from MBP-Gas1 were applied to purified MBP-Ost3 or MBP-Ost6 still

Table 1
Identification of reduced and alkylated versions of cysteine-containing tryptic peptides from Gas1p detected by LC–ESI–MS/MS used to measure MBP-Gas1 whole protein mixed disulfide formation with Ost3 or Ost6.

Peptide sequence	*m/z*	*z*	Mass	Score
G_{48}VAYQADTANETSGSTVNDPLANYES**C**$_{74}$SR_{76}	1007.43	3	0.001	17
G_{48}VAYQADTANETSGSTVNDPLANYES<u>C</u>$_{74}$SR_{76}	1031.11	3	0.004	18
N_{253}LSIPVFFSEYG**C**$_{265}$NEVTPR$_{271}$	724.67	3	−0.001	16
N_{253}LSIPVFFSEYG<u>C</u>$_{265}$NEVTPR$_{271}$	748.35	3	−0.001	10
S_{338}YSATTSDVA**C**$_{348}$PATGK$_{353}$	779.84	2	−0.002	15
S_{338}YSATTSDVA<u>C</u>$_{348}$PATGK$_{353}$	815.36	2	−0.000	17
V_{91}YAINTTLDHSE**C**$_{103}$MK$_{105}$	575.59	3	−0.001	15
V_{91}YAINTTLDHSE<u>C</u>$_{103}$MK$_{105}$	599.27	3	−0.001	17
M_{210}TDYFA**C**$_{216}$GDDDVK$_{222}$	740.28	2	−0.003	15
M_{210}TDYFA<u>C</u>$_{216}$GDDDVK$_{222}$	775.80	2	−0.006	13
Y_{415}GAYSF**C**$_{421}$TPK$_{424}$	568.74	2	−0.003	10
Y_{415}GAYSF<u>C</u>$_{421}$TPK$_{424}$	604.26	2	−0.005	14

Reduced cysteines are bold. Alkylated cysteines are underlined. Peptide numbering is from sequence of native Gas1p.

bound to amylose-agarose beads (Fig. 3). These bead slurries were incubated in 50 mM potassium phosphate buffer pH 7.5, 150 mM NaCl and 1 mM EDTA with gentle agitation at 25 °C for 1 h. Reduced cysteines were then alkylated by addition of acrylamide to a final concentration of 25 mM and incubation for 30 min. Bead slurries were transferred to gravity flow columns and the beads were washed with 25 column volumes of 50 mM Tris–HCl buffer pH 7.5, 150 mM NaCl and 1 mM EDTA. Peptides bound to MBP-Ost3 or MBP-Ost6 were eluted with this same buffer containing in addition 10 mM DTT.

2.4. Mass spectrometry and data analysis

Peptide samples were desalted using C18 ZipTips (Milipore) prior to mass spectrometry analysis. Peptides were analysed by LC–ESI–MS/MS using a Prominence nanoLC system (Shimadzu) and TripleTof 5600 mass spectrometer with a Nanospray III interface (AB SCIEX), as previously described [35,36]. Peptides were identified using ProteinPilot (AB SCIEX), searching the LudwigNR database (downloaded from http://apcf.edu.au as at 27 January 2012; 16,818,973 sequences; 5,891,363,821 residues) with standard settings: Sample type, identification; Cysteine alkylation, acrylamide; Instrument, TripleTof 5600; Species, not limited; ID focus, biological modifications; Enzyme, trypsin; Search effort, thorough ID. False discovery rate analysis using ProteinPilot was performed on all searches. Peptides identified with greater than 99% confidence and with a local false discovery rate of less than 1% were included for further analysis, and MS/MS fragmentation spectra were manually inspected. Extracted ion chromatograms were obtained using PeakView 1.1 (AB SCIEX).

3. Results

3.1. Ost6p can form mixed disulfides with a model substrate protein

A key prediction of the proposed model of Ost3p and Ost6p function in N-glycosylation [23] is that the CxxC active sites in these proteins are capable of forming transient disulfide bonds with cysteine residues in substrate polypeptides. We tested if Ost3p and Ost6p had this activity with two complementary *in vitro* assays. For these assays we used the thioredoxin-like ER lumenal domains of Ost3p and Ost6p, and yeast Gas1p, a model glycoprotein substrate, as Asn253 in Gas1p requires the CxxC motif of either Ost3p or Ost6p for efficient glycosylation *in vivo* [22,23]. Selected peptides from Gas1p can also transiently bind non-covalently to the peptide-binding groove of both Ost3p and Ost6p *in vitro* [33]. We therefore tested if cysteine residues of Gas1p could form mixed-disulfide bonds with the active site cysteines of Ost3p and Ost6p. We expressed and purified Gas1p as a periplasmically targeted MBP-fusion protein from *E. coli*, reduced its disulfide bonds by incubation in SDS and DTT, and desalted the protein to obtain reduced MBP-Gas1 protein. We then measured the extent to which each cysteine residue was free, or alternatively involved in a disulfide bond in a cystine (Fig. 1). For this, we first alkylated free cysteines by incubation with acrylamide and then reduced the disulfide bonds with DTT. The MBP-Gas1 protein was subsequently digested to peptides with trypsin in the presence of DTT and EDTA to prevent re-oxidation of cysteines. When MBP-Gas1 protein alone was subjected to this analysis, we identified six cysteine-containing peptides from Gas1p, all of which were detected in two forms – with the cysteines either reduced or alkylated (Table 1). Reduction of MBP-Gas1 was therefore partial, mimicking incompletely oxidized polypeptide still in the process of folding, and a likely substrate of OTase. To test if Ost3 or Ost6

Table 2
LC–ESI-MS/MS identification of tryptic peptides from MBP-Gas1 in load and DTT elution fractions in peptide mixed-disulfide affinity chromatography with MBP-Ost3.

Protein	Sequence	*m/z*	*z*	ΔMass	Score
Load					
MBP	T_{371}AVINAASGR$_{380}$	480.27	2	0.001	14
MBP	G_{42}YNGLAEVGK$_{51}$	504.26	2	0.001	10
MBP	D_{322}KPLGAVALK$_{331}$	506.31	2	0.002	15
MBP	I_{343}AATMENAQK$_{352}$	538.77	2	0.002	16
MBP	T_{154}WEEIPALDK$_{163}$	601.31	2	0.000	14
MBP	V_{266}NYGVTVLPTFK$_{277}$	669.38	2	−0.001	12
MBP	E_{304}FLENYLLTDEGLEAVNK$_{321}$	699.69	3	0.003	23
MBP	F_{93}GGYAQSGLLAEITPDK$_{109}$	883.95	2	0.004	23
Gas1p	Y_{415}GAYSF**C**TPK$_{424}$	568.76	2	0.000	15
Gas1p	V_{91}YAINTTLDHSE**C**MK$_{105}$	575.60	3	−0.002	16
Gas1p	Y_{303}GLVSIDGNDVK$_{314}$	640.33	2	−0.002	18
Gas1p	M_{210}TDYFA**C**GDDDVK$_{222}$	740.29	2	−0.005	18
Gas1p	A_{106}LNDADIYVIADLAAPATSINR$_{127}$	763.07	3	0.007	27
Gas1p	S_{338}YSATTSDVA**C**PATGK$_{353}$	779.86	2	0.008	25
Gas1p	I_{194}PVGYSSNDDEDTR$_{207}$	784.35	2	0.012	17
Gas1p	T_{315}LDDFNNYSSEINK$_{328}$	830.38	2	0.002	22
Gas1p	D_{128}DPTWTVDLFNSYK$_{141}$	850.89	2	0.000	18
Gas1p	E_{468}IGSMGTNSASGSVDLGSGTESSTASSNASGSSSK$_{502}$	1065.47	3	0.002	23
Elution					
Gas1p	G_{48}VAYQADTANETSGSTVNDPLANYES**C**$_{74}$SR_{76}	1007.44	3	−0.010	25

All cysteines are reduced and bold. Peptide numbering is from sequence of native *E. coli* MBP and native *S. cerevisiae* Gas1p.

could form mixed disulfides with the cysteines in Gas1 we incubated reduced MBP-Gas1 together with either Ost3 or Ost6 and measured the extent of alkylation at each detectable cysteine in Gas1. Peptides containing the CxxC active site cysteines of Ost3p or Ost6p, which are the only cysteines in the ER lumenal domains of these proteins, were not detected in this analysis. Some cysteines, such as Cys348 in tryptic peptide Ser338-Lys353, showed no change in the extent of alkylation in the presence or absence of Ost3 or Ost6 (Fig. 2A–C). However, the extent of alkylation of some peptides did change. For example, Cys74 in tryptic peptide Gly48-Arg76 was significantly less alklyated when MBP-Gas1 had been incubated in the presence of Ost6 than when MBP-Gas1 was incubated either alone or together with Ost3 ($P < 0.05$, ANOVA) (Fig. 2D–F). This indicated that during the incubation of Ost6 and reduced MBP-Gas1, Cys74 in some MBP-Gas1 proteins was not alkylated because it formed a disulfide bond with a cysteine in the CxxC motif of Ost6 (Fig. 1B). Of the six cysteine-containing Gas1 tryptic peptides, two formed mixed disulfide bonds with Ost6 detectable with this analysis ($P < 0.05$, ANOVA) but none with Ost3 (Fig. 1G and H). This showed that the cysteines in the CxxC thioredoxin-like active site of Ost6 could form mixed disulfides with cysteines in a physiological substrate polypeptide *in vitro*.

3.2. Ost3p can form mixed disulfides with tryptic peptides from a model substrate protein

Formation of disulfides between cysteines in whole Gas1 protein and the active site cysteines of Ost3 or Ost6 may have been limited by the folding status of the partially reduced MBP-Gas1 protein. Cysteines are commonly associated with hydrophobic regions, are typically buried in folded proteins, and may be inaccessible even in a fully reduced protein. Some cysteines may therefore have not been able to form a disulfide with Ost3 or Ost6 in the context of the whole MBP-Gas1 protein (Fig. 1 and 2 and Table 1). However, interaction between substrate polypeptide and Ost3/6p *in vivo* is proposed to occur directly upon entry of the nascent polypepide from the translocon into the ER lumen [23]. To mimic this form of nascent polypeptide we tested if reduced tryptic peptides from Gas1 could form disulfide bonds with the active site cysteines of Ost3 or Ost6 (Fig. 3). We applied reduced peptides to amylose-agarose beads with pre-bound MBP-Ost3 or MBP-Ost6, alkylated free cysteines to freeze disulfide reactivity, washed away non-bound peptides and eluted peptides bound through a disulfide to Ost3/6p with DTT. We then detected peptides in the load and elution fractions using LC–ESI–MS/MS (Table 2). We detected 18 peptides from MBP-Gas1 in the load sample applied to the amylose–agarose beads with MBP-Ost3 or MBP-Ost6 (Table 2). No peptides were detected in the elution from beads with MBP-Ost6. However, one peptide, Gly48-Arg76, was detected in the DTT elution from beads with MBP-Ost3 (Table 2). This peptide contained Cys74, the same residue identified as forming a mixed disulfide with Ost6 in our whole protein mixed disulfide assay (Fig. 2D–F and H).

4. Discussion

S. cerevisiae OTase subunits Ost3p and Ost6p modulate the acceptor protein substrate range of OTase *in vivo* [22]. Both pro-

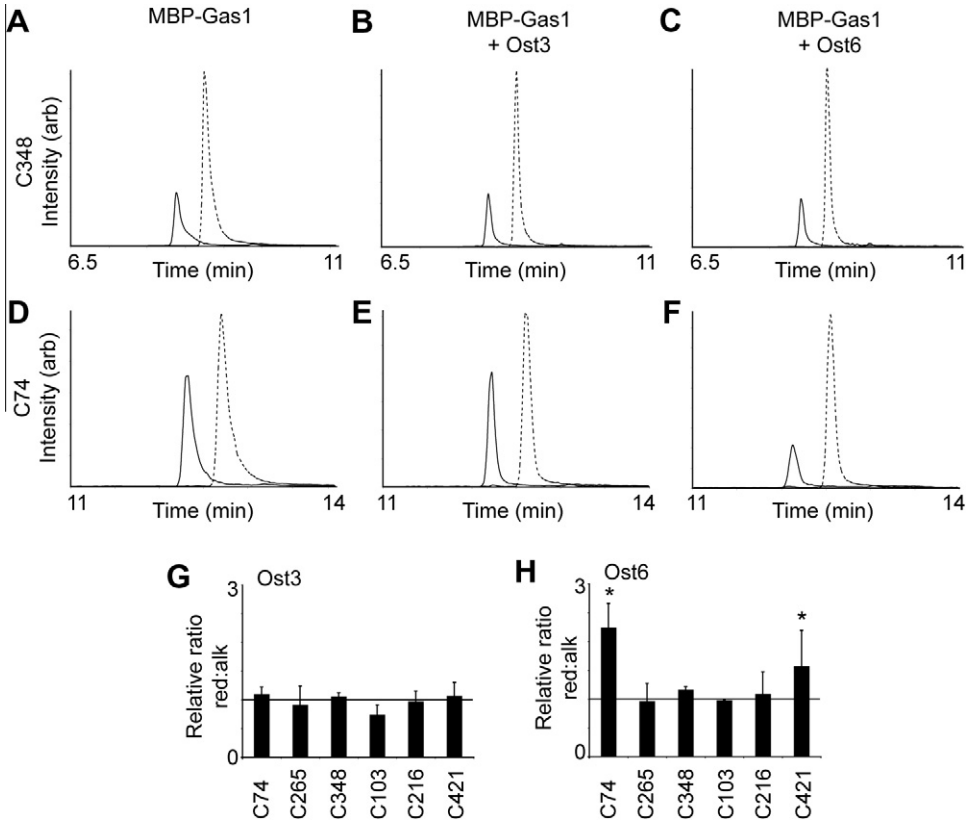

Fig. 2. *In vitro* mixed disulfide formation between MBP-Gas1 whole protein and Ost3 or Ost6. Extracted ion chromatograms of the $[M+2H]^{2+}$ ions corresponding to peptide Ser338-Lys353 with Cys348 reduced at an *m/z* of 779.84 (dashed) or alkylated at an *m/z* of 815.36 (full) from incubation of (A) MBP-Gas1 alone, (B) MBP-Gas1 with Ost3, and (C) MBP-Gas1 with Ost6. Extracted ion chromatograms of the $[M+3H]^{3+}$ ions corresponding to peptide Gly48-Arg76 with Cys74 reduced at an *m/z* of 1007.43 (dashed) or alkylated at an *m/z* of 1031.11 (full) from incubation of (D) MBP-Gas1 alone, (E) MBP-Gas1 with Ost3, and (F) MBP-Gas1 with Ost6. Ratios of the intensity of reduced and alkylated versions of peptides containing Cys74, Cys265, Cys348, Cys103, Cys216 or Cys421 from MBP-Gas1 (Gas1p amino acid numbering, Table 1) when incubated together with (G) Ost3 or (H) Ost6, relative to incubation of MBP-Gas1 alone. Values show mean of biological triplicates. Error bars show s.e.m. $^{*}P < 0.05$, ANOVA. Horizontal lines in (G) and (H) at a value of 1 are equivalent to no change.

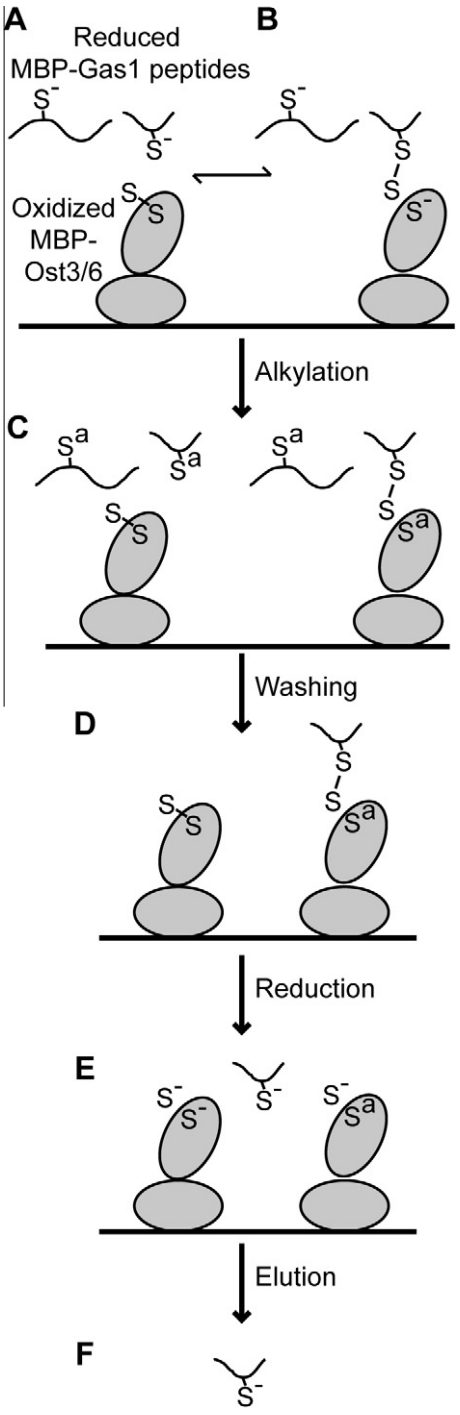

Fig. 3. Experimental design for *in vitro* peptide mixed-disulfide affinity chromatography with Ost3 or Ost6. (A) Reduced MBP-Gas1 tryptic peptides are incubated together with amylose-agarose beads with bound purified oxidized MBP-Ost3 or MBP-Ost6. (B) A cysteine in a Gas1 peptide performs nucleophilic attack on the Ost3/6 cystine and forms a mixed disulfide bond. This reaction is reversible [23]. (C) Alkylation of free cysteines prevents further mixed disulfide shuffling. (D) Non-bound peptides are removed by thorough washing. (E) Reduction with DTT releases peptides bound to Ost3 or Ost6 through a disulfide bond. (F) LC–ESI–MS/MS analysis allows detection of peptides. See Materials and Methods for details.

teins have an ER lumenal thioredoxin-like domain, whose CxxC active site residues are required for efficient *in vivo* glycosylation of a subset of glycosylation sites, including N253 in Gas1p [23]. It has been proposed that the active site cysteines of Ost3p and Ost6p can form transient mixed disulfides with nascent polypeptide substrate [23]. To test this, we developed two independent *in vitro* assays to identify cysteines in *S. cerevisiae* Gas1p that could form transient mixed disulfide bonds with the thioredoxin-like active site cysteines of Ost3p or Ost6p. One of these assays tested interactions using Gas1p as a reduced but intact MBP fusion protein (Fig. 1), and the other used this same protein after it had been digested to peptides with trypsin (Fig. 3). The combination of these approaches identified that the Cys74 cysteine residue in Gas1p could form a mixed disulfide bond with both Ost3p and Ost6p (Fig. 2, Table 2). In addition, the whole protein interaction approach identified that Cys421 could form a mixed disulfide bond with Ost6p (Fig. 2). While efficient glycosylation of Asn253 in Gas1p *in vivo* requires the CxxC motif of either Ost3p or Ost6p [23], it is not clear if this requirement is due to the formation of a mixed disulfide bond between Ost3p or Ost6p and either Cys74 or Cys421 in Gas1p, that our analyses here detected could bind *in vitro*. Both Cys74 and Cys421 are distant from Asn253 both in amino acid sequence and in the predicted final folded protein structure of Gas1p [37,38]. It is not clear if the peptides that we detected forming mixed disulfides with Ost3p and Ost6p *in vitro* also form such transient bonds *in vivo*, or if these particular bonds are key for glycosylation at Asn253 of Gas1p. Although we detected six peptides from Gas1p that contain cysteine residues (Table 1), there are eight additional cysteine residues in Gas1p that we did not detect with our analytic approach. Some of these cysteine residues may also form mixed disulfide bonds with Ost3p and/or Ost6p relevant to N-glycosylation of Asn residues in Gas1p *in vivo*.

In summary, our results validated a key prediction of the proposed model of Ost3/6p function in N-glycosylation [23]. It has been previously shown that the CxxC motifs of Ost3p and Ost6p are important for glycosylation *in vivo* [23]. Here, we showed that the ER lumenal domains of both Ost3p and Ost6p could form mixed disulfides with cysteines in their physiological protein substrate Gas1p, using a peptide and a whole-protein assay. Ost3p and Ost6p showed differences in their activities in these assays, consistent with their different peptide-binding activities *in vitro* [33] and different range of substrates *in vivo* [22]. The assays we developed may also be applicable to *in vitro* identification of transient mixed disulfide bonds in other systems.

Acknowledgments

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