SWATH-MS Glycoproteomics Reveals Consequences of Defects in the Glycosylation Machinery*

Lucia F. Zacchi‡§ and Benjamin L. Schulz‡¶

Glycan macro- and microheterogeneity have profound impacts on protein folding and function. This heterogeneity can be regulated by physiological or environmental factors. However, unregulated heterogeneity can lead to disease, and mutations in the glycosylation process cause a growing number of Congenital Disorders of Glycosylation. We systematically studied how mutations in the N-glycosylation pathway lead to defects in mature proteins using all viable Saccharomyces cerevisiae strains with deletions in genes encoding Endoplasmic Reticulum lumenal mannosyltransferases (Alg3, Alg9, and Alg12), glucosyltransferases (Alg6, Alg8, and Die2/Alg10), or oligosaccharyltransferase subunits (Ost3, Ost5, and Ost6). To measure the changes in glycan macro- and microheterogeneity in mature proteins caused by these mutations we developed a SWATH-mass spectrometry glycoproteomics workflow. We measured glycan structures and occupancy on mature cell wall glycoproteins, and relative protein abundance, in the different mutants. All mutants showed decreased glycan occupancy and altered cell wall proteomes compared with wild-type cells. Mutations in earlier mannosyltransferase or glucosyltransferase steps of glycan biosynthesis had stronger hypoglycosylation phenotypes, but glucosyltransferase defects were more severe. ER mannosyltransferase mutants displayed substantial global changes in glycan microheterogeneity consistent with truncations in the glycan transferred to protein in these strains. Although ER glucosyltransferase and oligosaccharyltransferase subunit mutants broadly showed no change in glycan structures, Ost3Δ cells had shorter glycan structures at some sites, consistent with increased protein quality control mannosidase processing in this severely hypoglycosylating mutant. This method allows facile relative quantitative glycoproteomics, and our results provide insights into global regulation of site-specific glycosylation.

Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.056366, 2435–2447, 2016.

From the ‡School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, Queensland, 4072, Australia; §Fundacion Instituto Leloir, Avenida Patricia Argenztinas 435, Ciudad Autonomia de Buenos Aires, 1405, Argentina

Received October 21, 2015, and in revised form, January 25, 2016 Published, MCP Papers in Press, April 19, 2016, DOI 10.1074/mcp.M115.056366

Author contributions: L.F.Z. and B.L.S. designed research; L.F.Z. and B.L.S. performed research; L.F.Z. and B.L.S. analyzed data; L.F.Z. and B.L.S. wrote the paper.

Protein glycosylation is a highly conserved co- and post-translational modification of proteins that influences protein folding, stability, solubility, and function (1, 2). N-glycosylation of Asparagine (Asn) residues occurs in eukaryota, archaea, and some bacteria, although the biosynthetic pathways and glycan structures are diverse in these organisms (3). In eukaryotes, nascent polypeptides in the Endoplasmic Reticulum (ER) are the protein acceptor substrates for N-glycosylation, as folded proteins cannot be efficiently N-glycosylated and N-glycosylation is critical for efficient protein folding. After protein folding, glycan structures can be further truncated or extended by Golgi-resident glycosyltransferases. Glycan biosynthesis is inherently inefficient, resulting in structural diversity of mature glycoproteins. Diversity in the presence or absence of glycans on glycoproteins is termed macroheterogeneity, whereas diversity in the structures of glycans at a specific site is termed microheterogeneity. This structural diversity is key for the regulation of many biological functions of glycoproteins.

Eukaryotic N-glycosylation is catalyzed in the ER lumen, where the enzyme oligosaccharyltransferase (OTase) transfers donor glycans en bloc from a dolichol pyrophosphate (DolP) carrier (Lipid-linked oligosaccharide; LLO) to selected Asn in nascent polypeptides. Approximately 80% of secretory proteins are N-glycosylated, making this modification of high fundamental, medical, and biotechnological relevance (4, 5). In Bakers' yeast Saccharomyces cerevisiae, the LLO is synthesized in a defined, sequential, stepwise process by a series of enzymes integral or attached to the ER membrane: Alg1–14 (Fig. 1A) (6). LLO biosynthesis begins on the cytosolic face of the ER membrane, where Alg7 and the Alg13-Alg14 complex transfer the first and second N-acetylglucosamine (GlcNAc) residues to DolP. This is followed by the sequential addition of Mannose (Man) residues by Alg1, Alg2, and Alg11 to synthesize the Man2GlcNAc2 structure. This LLO is then flipped into the ER lumen by a process requiring Rft1 (7). Man residues are then successively added by Alg3, Alg9, Alg12, and again Alg9.

1 The abbreviations used are: Asn, Asparagine; ER, Endoplasmic Reticulum; Glc, Glucose; GlcNAc, N-acetyl-glucosamine; LLO, Lipid-linked oligosaccharide; Man, Mannose; OTase, Oligosaccharyltransferase; Ser, Serine; SWATH, Sequential window acquisition of all theoretical fragment ion spectra; Thr, Threonine.
to construct the \( \text{Man}_9\text{GlcNAc}_2 \) structure (Fig. 1A). The LLO structure is completed by addition of three Glucose (Glc) residues to branch A through the sequential action of Alg6, Alg8, and Die2/Alg10 (Fig. 1A). This final Glc,\text{Man}_9\text{GlcNAc}_2 structure is the preferred LLO donor transferred to proteins by OTase, compared with biosynthetic intermediates. Because of the sequential nature of LLO biosynthesis, defects in LLO biosynthesis because of deficiency of an Alg enzyme result in accumulation of the precursor LLO structure (8–10). For instance, in \textit{alg6a} yeast the unglucosylated \text{Man}_9\text{GlcNAc}_2 LLO structure accumulates and is transferred to protein (11). Importantly, OTase transfers these truncated LLOs to proteins with reduced efficiency, resulting in hypoglycosylation of diverse proteins (11). In particular, the terminal \( \alpha1,2 \) Glc on the A branch of the LLO is critical for efficient \textit{in vivo} glycosylation (12). The Man content of the B and C branches also influences OTase function (13). Thus, although the glycan normally transferred by OTase has a canonical structure, OTase can transfer a variety of truncated glycans to polypeptides. This capability of OTase can increase the glycan heterogeneity of mature proteins and ensures that the essential process of protein glycosylation can continue even with perturbation of the glycan biosynthetic pathway.

OTase catalyzes the transfer of glycans from LLO to selected Asn residues in nascent polypeptides. Asn are glucosylated with high efficiency if they are located in glycosylation sequons (\( NxS/T; x \neq P \), as this is the peptide acceptor-binding motif of Stt3, the catalytic subunit of OTase (14). The yeast OTase is a hetero-oligomeric complex composed of essential (Ost1, Ost2, Wbp1, Stt3, and Swp1) and nonessential (Ost3, Ost4, Ost5, and Ost6) subunits. There are two OTase isoforms in yeast containing one of either of the paralogous Ost3 or Ost6 subunits, which have different protein substrate specificity at the level of individual glycosylation sites (15–18). The function of the other OTase subunits is less well defined. However, lack of any of the nonessential subunits or mutations in essential subunits leads to inefficient \textit{N}-glycosylation of diverse glycoproteins (15, 19, 20).

The structures of \( N \)-glycans are substantially processed after transfer to protein. Immediately after the glycan is transferred to nascent polypeptides by OTase, the terminal \( \alpha1,2 \) Glc is trimmed by Glucosidase I, whereas the remaining Glc residues and some Man residues are removed during protein folding (21). \( N \)-glycans are further modified in a protein-, cell-, and species-specific manner as glycoproteins progress through the Golgi Complex. In yeast this results in high mannose \( N \)-glycans with extended polymannose structures (22, 23).

Despite the critical function of the presence and structure of \( N \)-glycans on key aspects of protein maturation, \( N \)-glycosylation by the OTase can proceed even with immature LLO structures. Remarkably, yeast is viable in the absence of genes encoding several Alg enzymes and OTase subunits (9). However, many reports have shown that these mutations result in inefficient glycosylation of diverse proteins (8, 11, 12, 20, 24–26). Here, we describe a simple, semi-quantitative, automated, and sensitive SWATH-mass spectrometry method to simultaneously measure macro- and microheterogeneity of diverse glycoproteins. We use this method to analyze the cell wall glycoproteome of the complete set of viable yeast deletion mutants in \( N \)-glycan biosynthesis to understand how OTase activity is affected by sub-optimal glycan donor structure at a systems level.

METHODOLOGY

Yeast Strains and Growth Conditions—All yeast strains used in this study derive from the BY4741 wild-type strain and were obtained from the genome deletion collection (Open Biosystems) (Table I). Yeast strains were grown in YPAD medium (1% yeast extract, 2% dextrose, 2% Bactopeptone) at 30 °C.

Cell Wall Sample Preparation—Yeast were grown in 50 ml YPAD in an orbital shaker at 30 °C and 200 rpm, harvested at mid-log phase (OD\text{600 nm} 1.0) by centrifugation, and frozen at −20 °C. Proteins covalently linked to the polysaccharide cell wall were prepared following previously published protocols (15), but excluding endoglycosidase treatment. Briefly, cells were completely lysed, cysteines were reduced and alkylated with acrylamide, the insoluble cell wall polysaccharide with covalently attached proteins was thoroughly washed in strongly denaturing conditions, and proteins were digested with trypsin. Peptides and glycopeptides were desalted using C18 ZipTips (Millipore) prior to analysis by LC-ESI-MS/MS.

Mass Spectrometry and Data Analysis—Desalted peptides were analyzed by LC-ESI-MS/MS using a Prominence nanoLC system (Shimadzu) and TripleTof 5600 mass spectrometer with a Nanospray III interface (SCIEX) as previously described (27). Peptides were separated with buffer A (1% acetonitrile and 0.1% formic acid) and buffer B (80% acetonitrile with 0.1% formic acid) with a gradient of 10–60% buffer B over 45 min. Gas and voltage setting were adjusted as required. For information-dependent acquisition (IDA), an MS TOF scan from m/z 350–1800 was performed for 0.5 s followed by IDA of MS/MS in high sensitivity mode with automated CE selection of the top 20 peptides from m/z of 40–1800 for 0.05 s per spectrum and dynamic exclusion of peptides for 5 s after 2 selections. Identical LC conditions were used for SWATH-MS, with an MS-TOF scan from an m/z of 350–1800 for 0.05 s followed by high-sensitivity information-independent acquisition with 26 m/z isolation windows with 1 m/z window overlap each for 0.1 s across an m/z range of 400–1250. Collision energy was automatically assigned by the Analyst software (SCIEX) based on m/z window ranges.

Peptide identification was performed essentially as previously described (27) using ProteinPilot 4.1 (SCIEX), searching the UniProt database (downloaded from http://uniprot.org on 15 January 2015; 16,818,973 sequences) with standard settings: sample type, identification; cysteine alkylation, acrylam-
and microheterogeneity in mature cell wall proteins. To generate different LLO structures in vivo we utilized the full set of viable mutants in Alg enzymes responsible for the ER luminal portion of the LLO biosynthetic pathway (Fig. 1A). The strains selected carry single mutations in genes encoding the ER luminal mannosyltransferases (Alg3, Alg9, and Alg12) and glucosyltransferases (Alg6, Alg8, and Die2/Alg10) (Fig. 1A). Lack of these enzymes causes accumulation of the LLO precursor substrate of the enzyme, which is inefficiently transferred to proteins by OTase (8, 11, 12, 25, 26, 30). We focused our analysis on proteins covalently linked to the yeast cell wall, a well-defined subcellular fraction enriched in glycoproteins that has been previously extensively characterized by MS (glyco)proteomics (15, 24, 27, 31). To measure glycan macro- and microheterogeneity we designed and used a novel SWATH-MS glycoproteomic workflow to measure cell wall protein site-specific glycan structure and occupancy, and cell wall proteome abundance.

**Defects in ER Mannosyltransferases lead to alterations in the structure of N-glycans on mature proteins**—The structure of glycans on mature proteins can have profound impacts on glycoproteins’ activity and stability. To be able to directly measure site-specific glycan occupancy and structure on peptides in a complex mixture we employed a variation of our previously published SWATH-MS glycoproteomic protocol (27), omitting the deglycosylation step to allow direct detection and relative quantification of glycopeptides. Peptide libraries of nonglycosylated but sequon-containing peptides were generated from Information Dependent Acquisition analysis of hypoglycosylating strains. Glycopeptides were not confidently identified by standard CID MS/MS and database searching, preventing automated identification and SWATH-MS quantification. To overcome this, we generated additional glycopeptide libraries containing experimentally determined fragment ions from nonglycosylated versions of sequon-containing peptides and parent ion masses corresponding to all theoretical glycopeptide masses. To allow use of the same fragment ions for glycosylated and nonglycosylated versions of the same peptide, we selected b- and y- ions that did not contain the glycosylated Asn residue. Although CID fragmentation of glycopeptides typically results in abundant glycan fragment ions, with few detectable peptide fragment ions (32, 33), we were able to use this approach to measure glycopeptides corresponding to eight glycosylation sites (Fig. 1, 2, and 3). Parent glycopeptide masses were constrained based on yeast glycan biosynthesis and instrument m/z detection limits. During N-glycosylation, OTase transfers a glycan with a maximum number of 14 monosaccharides (GlcNAc2-Man3Glc3, Fig. 1A). All the Glc and some Man residues are trimmed during ER protein folding quality control, leaving glycans with a maximum of 11 monosaccharides (GlcNAc2-Man3) as proteins exit the ER (21). If a protein is delayed in folding it will be retained in the ER and subjected to extensive Man trimming on the B and C branches, resulting in truncated glycan struc-

**RESULTS**

N-glycosylation is an enzymatic process catalyzed by the multiprotein subunit OTase enzyme. OTase displays preferences both for the acceptor protein to be glycosylated and the LLO glycan donor to be transferred to the protein (15, 29). Our goal was to quantify how differences in LLO structure affect the ability of OTase to glycosylate substrates in vivo and identify the repercussions of these changes on glycan macro-
**A.** Schematic representation of the ER and Golgi luminal glycan biosynthetic pathways. Monosaccharides are indicated in colors: blue squares, N-acetylglucosamine (GlcNAc); green circles, Mannose (Man); and blue circles, Glucose (Glc). The dolichol-pyrophosphate (DolP) lipid carrier to which the glycan is attached is shown in black. The glycan is transferred by oligosaccharyltransferase to an Asn in a sequon in a protein acceptor (gray). Glycans are processed in the ER by glucosidases and mannosidases (21), and the glycoprotein is then exported to the Golgi Complex, where 1,6 mannosyltransferases such as Och1 poly-mannosylate the glycan.

**B.** Eight selected peptides used for the measurements presented in this figure.

| No. | Peptide sequence | Protein |
|-----|------------------|---------|
| 1   | FFYSNNLSGQYYR   | Gas1    |
| 2   | GVAYGADTANPETGSTYNPLANYESCIPPAjSR | Gas1 |
| 3   | YAINPPTTLHSECIPPAjMK | Gas1 |
| 4   | NPSLSVPVFSEYGCIPPAjNEVTPR | Gas1 |
| 5   | LNH53STFEAHYLIIFSEYGCIPPAjNKK | Gas3 |
| 6   | NPSVSLPTTK | Gas3 |
| 7   | FHNYTDLAWMD | Chr1 |
| 8   | VQTVGGAIEVTQ53FSTLDLSSK | Ecm33 |

**C.** Average fraction of glycosylated peptides 1–8 containing glycans with 5–15 Man residues and 2 GlcNAc residues in each strain.

**D.** Fraction of glycosylated Gas1p NOSTGS peptide containing glycans with 5–10 Man residues and 2 GlcNAc residues in each strain. Data is the mean ± S.E. of biological triplicates. *p < 0.05. Data from supplemental Table S2.

**Fig. 1.** ER LLO glycosyltransferase defects alter glycan microheterogeneity on mature cell wall proteins. A, Schematic representation of the ER and Golgi luminal glycan biosynthetic pathways. Monosaccharides are indicated in colors: blue squares, N-acetylglucosamine (GlcNAc); green circles, Mannose (Man); and blue circles, Glucose (Glc). The dolichol-pyrophosphate (DolP) lipid carrier to which the glycan is attached is shown in black. The glycan is transferred by oligosaccharyltransferase to an Asn in a sequon in a protein acceptor (gray). Glycans are processed in the ER by glucosidases and mannosidases (21), and the glycoprotein is then exported to the Golgi Complex, where α1,6 mannosyltransferases such as Och1 poly-mannosylate the glycan. B, Eight selected peptides used for the measurements presented in this figure. C, Average fraction of glycosylated peptides 1–8 containing glycans with 5–15 Man residues and 2 GlcNAc residues in each strain. D, Fraction of glycosylated Gas1p NOSTGS peptide containing glycans with 5–10 Man residues and 2 GlcNAc residues in each strain. Data is the mean ± S.E. of biological triplicates. *p < 0.05. Data from supplemental Table S2.
Fig. 2. Early defects in LLO ER mannosylation or glucosylation lead to more severe hypoglycosylation defects. A, Fraction of unglycosylated peptide to the sum of un- and glycosylated forms of that peptide for each of the eight selected peptides from Fig. 1B in each strain. Data is the mean ± S.E. of biological triplicates. * p < 0.05. Data from supplemental Table S2. B, Heatmap showing the fraction of unglycosylated peptide to whole protein in each mutant strain relative to the wild-type strain. Each square represents the mean of biological triplicates. Only significant differences are displayed (p < 0.05). The data was clustered with Cluster 3.0 and presented using TreeView. Data from supplemental Table S3. C, Number of sequon-containing peptides measured in each mutant strain with significantly (p < 0.05) increased (in white) or decreased (in black) glycosylation occupancy compared with wild type.
Fig. 3. Simultaneous measurement of global site-specific glycan macro- and microheterogeneity. Heatmaps depicting the fraction of unglycosylated peptide (U) or peptide glycosylated with glycans containing two GlcNAc and an increasing number of Man (5–15), for each of the eight selected peptides in Fig. 1B. Fractions were calculated as in Figs. 1 and 2. The data was presented using TreeView. Each square represents the mean from biological triplicates. Data from supplemental Table S2.

atures on the mature glycoprotein (21) (Fig. 1A). This means that proteins exiting the ER may contain a range of glycans with short B and C branches. These same branches are biosynthetically truncated in algl mutants (Fig. 1A). Secretory glycoproteins can be further mannosylated as they traffic through the Golgi complex (22). In yeast, Golgi polymannosylation of N-glycans occurs at branch A (34) (Fig. 1A). Because large glycoproteins may exceed the maximum detectable m/z for a given mass spectrometer instrument, the glycan size that can be measured is limited by the length of a particular glycopeptide. We therefore designed a matrix of glycopeptide masses considering that the glycans attached will have a Man0-XGlcNAc2 structure, where X was limited by the size of the particular peptide, up to a maximum total glycopeptide m/z of 1250, ranging from 9 to 15.

We used our extended glycopeptide library in conjunction with SWATH-MS detection to determine the impact of defects in the LLO biosynthetic pathway on site-specific glycan structures of mature cell wall proteins. For these analyses, we selected eight peptides from four cell wall glycoproteins for which we could robustly detect glycopeptide microheterogeneity (Fig. 1B, supplemental Table S2). We then measured the effects of the alg mutations compared with the wild-type strain on the glycan microheterogeneity of the mature proteins. The glycopeptides detected in the wild-type strain had an average distribution of glycan structures ranging from 8 to 15 Man (Fig. 1C). Absence of any of the enzymes responsible for ER luminal LLO mannosylation (Alg3, Alg9, or Alg12) had a profound effect on the Man content of glycans on mature glycoproteins (Fig. 1C). Deletion of any of the mannosyltransferases led to a decrease in the number of hexoses per glycan, consistent with the expected truncations in the LLO structures accumulating in these strains (Fig. 1A). This phenotype was more severe in the alg3Δ mutant than in the alg9Δ mutant, and in the alg9Δ mutant compared with the alg12Δ mutant (Figs. 1C, 1D, and 3). For example, for the Gas1 N40GS glycopeptide (Figs. 1B and 3) the Man9 glycan was the most abundant form in wild-type cells, Man6 in alg12Δ cells, Man5 in alg9Δ cells, and Man4 in alg3Δ cells (Figs. 1B, 1D, and 3). These results indicate that even after glycan extension in the Golgi complex, mannosyltransferase defects early in LLO biosynthesis lead to shorter glycan structures on mature glycoproteins (Figs. 1 and 3).

The nonessential OTase subunits Ost3 and Ost6 define two isoforms of OTase with different peptide acceptor substrate specificities, and deletion of the more abundant Ost3 paralog produces a substantially more severe hypoglycosylation defect than loss of Ost6 (15). Little is known about the precise function of Ost5, but this small protein links Stt3 to the other subcomplexes within the OTase multiprotein complex (20, 35).
(Fig. 1A). Because mutations in the OTase are not likely to impact LLO biosynthesis, we predicted that glycans in the ost3Δ, ost5Δ, and ost6Δ strains would have similar structures to the wild-type strain. Indeed, the ost3Δ, ost5Δ, and ost6Δ strains showed the same average glycan structure distribution as wild-type cells (Fig. 1C). The same result was observed for the mutants in any of the three ER LLO glucosyltransferases (Fig. 1C). However, close inspection of site-specific glycan microheterogeneity revealed strain-specific differences at some glycosylation sites. For example, Man10 and Man9 glycans in Gas1 N304GS were significantly less abundant in ost3Δ, ost5Δ, and ost6Δ strains compared with the wild-type strain. We observed that within mannosyltransferase mutants or within glucosyltransferase mutants, defects early in the biosynthetic pathway resulted in quantitatively more severe hypoglycosylation. For example, absence of the first (Alg3), second (Alg9), or third (Alg12) ER LLO mannosyltransferase resulted in underglycosylation of 78%, 63%, or 42% respectively at Gas1 N57ET (Fig. 2A, supplemental Table S2). Similarly, absence of the first (Alg6), second (Alg8), or third (Die2) ER LLO glucosyltransferase resulted in underglycosylation of 61%, 44%, or 23% respectively at Crh1 N177YT (Fig. 2C, supplemental Table S2). Thus, defects in both LLO mannosylation and glucosylation negatively impacted the efficiency of protein glycosylation by OTase.

To gain a more global perspective on the effect of alg mutations on macroheterogeneity, we compared glycan occupancy at all identifiable peptides containing sequons that were N-glycosylated in wild-type cells. Instead of comparing the unglycosylated peptide to the pool of glycosylated peptides for that glycosylation site (Fig. 2A), we calculated the ratio of the intensity of each unglycosylated peptide to the intensity of its respective whole protein as measured by SWATH-MS (27) (Figs. 2B and 2C and supplemental Table 3). Using this approach, glycosylation defects that lead to decreased glycan occupancy increase the unglycosylated peptide:protein ratio.

To determine how the alg mutations impact sequon occupancy, we measured 46 robustly detectable sequon-containing peptides belonging to 15 cell wall proteins (supplemental Table S3). In some cases, more than one sequon-containing
peptide was identified per protein, and some of the peptides were detected with multiple charge states (supplemental Table S3). Although not strictly quantitative, the degree of hypoglycosylation measured by both our two approaches correlated well (supplemental Fig. S1). Again, most sequons were efficiently glycosylated in the wild-type strain, with low unglycosylated peptide:protein ratios (Figs. 2C and 3). Deletion of ER LLO mannosyltransferases significantly affected glycosylation of at least 1/3rd (19.0 ± 5.2 peptides) of the measured peptides compared with the wild-type strain (Figs. 2B and 2C). As observed above, deletion of ALG3 had the stronger phenotype, leading to the hypoglycosylation of 25 peptides, compared with 16 peptides for the mutants in ALG9 and ALG12 (Fig. 2C). Thus, accumulation of the Man$_{n}$GlcNAc$_{2}$ LLO led to a more severe hypoglycosylation defect than accumulation of the Man$_{n}$GlcNAc$_{2}$ LLOs. This result suggests that the ER LLO glucosyltransferases or OTase more efficiently recognize LLO structures with more Man in the B and C branches. All three mutants in the ER glucosyltransferases of Ost6 (15) and that deletion of OST5 leads to a milder glycosylation defect (20). The hypoglycosylation levels observed in the ost3Δ strain were similar to the levels observed in the alg3Δ and in the alg6Δ strains (Fig. 2). Collectively, these results indicate that defects in LLO mannosylation and LLO glucosylation led to defects in peptide glycosylation as severe as defects in select OTase subunits. Thus, overall OTase glycosylation efficiency is similarly affected by absence of the Ost3 subunit or by the sole availability of Man$_{n}$GlcNAc$_{2}$ and Man$_{n}$GlcNAc$_{2}$ LLO structures as donors.

Mutations at different stages in the glycans biosynthetic pathway or in the glycosylation machinery had a wide impact on the glycan macroheterogeneity of mature proteins. Although the effect of each mutation on each specific glycosylation site varied, mutations that prevented the first ER lumenal mannosyltransferase step (Alg3), the first glycosylation step (Alg6), or that eliminated Ost3 led to the largest hypoglycosylation phenotypes across many sites (Fig. 2 and 3). Mutants in OTase subunits showed clear site-specific hypoglycosylation (Figs. 2 and 3), consistent with previous studies showing that Ost3-OTase and Ost6-OTase have distinct protein substrate preferences at the level of individual glycosylation sites (15, 18, 36). In contrast, alg mutations led to a consistent pattern of underglycosylation across different sites (Figs. 2 and 3). That is, in general the same sites were qualitatively affected in the alg strains, albeit with different quantitative degrees of hypoglycosylation. It appears that this effect is driven by general protein acceptor substrate binding affinity to OTase, as hypoglycosylated sites in alg3Δ mutants were enriched in the less efficiently modified NxS sequons, compared with the more efficiently modified NxT sequons (p < 0.01, Fisher’s exact test).

Defects in LLO Biosynthesis and Glycosylation Lead to Changes in the Cell Wall Proteome—Global hypoglycosylation and alterations in glycan microheterogeneity would be expected to result in defects in glycoprotein folding, secretion, and function. To measure the severity of these effects we used the data from our SWATH-MS analyses to perform relative quantification of the abundance of cell wall proteins in our suite of alg and ost mutant strains. Changes in the cell wall proteome in these strains may be because of direct effects on the folding efficiency or stability of hypoglycosylated glycoproteins, or indirect effects compensating for cell wall damage because of defects in hypoglycosylated glycoprotein function. In either case, changes in the cell wall proteome can be used as a quantitative measure of the severity of defects in the glycosylation biosynthesis pathway. We selected peptides from 26 cell wall proteins that were confidently identified (Table II) and determined their relative abundance in each of
the yeast strains (supplemental Table S4). The levels of 14 out of the 26 measured proteins were not significantly altered in any mutant strain compared with the wild type strain (at least twofold compared with wild-type, $p < 0.05$) (Table II and Fig. 4). The remaining 12 proteins showed either a significant increase in abundance (Bgl2, Gas1, Yps1, Plb1, Yjr1, Exg2, and Mkc7) (Table II, in bold, and Fig. 4B and 4C) or a significant decrease in abundance (Uth1, Tos1, Scw4, Plb2, and Pst1) in selected mutants (Table II and Fig. 4B and 4C). These results indicate that the cell wall proteome does not change uniformly in response to alterations in the glycosylation machinery.

Each alg and ost mutant showed quantitatively different changes in cell wall proteome (Fig. 4). The largest differences were observed in the alg6Δ and alg8Δ glucosyltransferase mutants, and in the ost3Δ mutant, with a total of 9, 7, and 12
proteins with significant differences in abundance compared with wild type, respectively (Fig. 4). These mutants resulted in similar overall effects on cell wall protein abundance across the 27 proteins measured, and the proteomes of these mutants clustered in heatmap analysis (Fig. 4C). Consistent with severe hypoglycosylation (Fig. 2 and 3), these changes in cell wall proteome suggest that early defects in LLO ER glucosylation have particularly severe consequences for OTase function (Figs. 1 and 2). However, also as observed above, the ost3Δ mutant showed the most severe defects both in terms of the number of proteins affected and the intensity of the defect (Fig. 4). The alg3Δ, alg9Δ, alg12Δ, die2Δ, ost5Δ, and ost6Δ mutants showed smaller changes in cell wall proteome, with the alg3Δ mutant being the most severely affected of these (Fig. 4).

Changes in abundance of proteins with similar function, similar sequence, or encoded by paralogous genes were often inversely correlated in the different mutants. For example, the abundance of phospholipase B Plb1 was significantly increased in the alg6Δ, alg8Δ, and ost3Δ mutants compared with wild type, whereas the abundance of the phospholipase B Plb2 was significantly decreased in these mutants (Fig. 4B). Similarly, the abundance of Tos1 was significantly reduced in the alg6Δ and ost3Δ mutants, whereas the level of Toh1/Yjr1 (Tos One Homolog) was increased in these same strains (Fig. 4C). In other examples, Gas1 was significantly more abundant in the alg3Δ, alg6Δ, alg8Δ, die2Δ, and ost3Δ mutants, whereas Gas3 and Gas5 remained unchanged in these strains. Similarly, the abundance of Scw4 and Pst1 were significantly affected in at least one of the mutants tested, but
the expression levels of their respective paralogs Scw10 and Ecm33 showed no significant change (Fig. 4B and 4C). These differences cannot be simply explained by the number of glycosylation sequons present in each protein. For example: Scw4 and Scw10 have both 1 sequon; Pst1 and Ecm33 are both heavily N-glycosylated; Tos1 has two sequons but Toh1/ Yjr1 has 11; and whereas Gas1 has 10 sequons, Gas3 and Gas5 have 6. These results also indicate that the number of sequons in a protein does not predict how defects in the glycosylation machinery will affect the abundance of the mature protein.

**DISCUSSION**

Biosynthesis of the LLO to be transferred to protein by OTase occurs through a series of sequential enzymatic reactions with high substrate specificity (4, 6) (Fig. 1A). Lack of any given LLO glycosyltransferase leads to the accumulation of the product of the preceding step, and transfer of this incomplete glycan structure to protein by OTase (Fig. 1A) (30). Our analysis of glycan microheterogeneity in the suite of N-glycan biosynthetic mutants confirmed this model, and further demonstrated that defects in LLO mannosylation are retained in N-glycan structures on mature glycoproteins (Figs. 1 and 3). Although strains lacking functional ER LLO glucosyltransferases or OTase subunits showed no overall change in glycan microheterogeneity compared with wild type, some site-specific differences were apparent (Fig. 1D and 3, and supplemental Table S2). In particular, several glycosylation sites had shorter glycans with fewer Man residues in ost3Δ cells compared with wild type. As ost3Δ cells showed the most severe hypoglycosylation phenotype of any strain (Figs. 2, 3, and 4), glycoprotein folding was likely particularly compromised in these cells. This would increase glycoprotein residence in the ER during folding, and result in increased mannosidase trimming, producing the shorter glycan structures at some sites in ost3Δ cells. Alternatively, hypoglycosylation in ost3Δ cells may affect Golgi glycosyltransferase function and result in less efficient polymannosylation. Analysis of global site-specific glycan macro- and microheterogeneity may be a valuable tool to investigate details of glycoprotein quality control.

Inhibition of the early stages of the LLO biosynthetic pathway either genetically (by deletion of ALG1, ALG2, ALG7, ALG13, or ALG14) or chemically (with tunicamycin) severely impacts cell growth (37–39), whereas mutations later in the pathway have milder effects. We therefore expected that more severe hypoglycosylation phenotypes would be observed in the biosynthetically early viable alg mutants. Indeed, we found that lack of Alg3 led to a more severe hypoglycosylation phenotype and shorter glycans on mature proteins than lack of Alg9 and Alg12 (Figs. 1–3). Similarly, lack of Alg6 led to a more severe hypoglycosylation phenotype than lack of Alg8 or Die2 (Figs. 1–3). Surprisingly, however, deletion of the glucosyltransferases led to a more severe defect than deletion of the mannosyltransferases. These results suggest that LLO glucosylation can proceed even when the B and C branches of the LLO are incompletely mannosylated (Man_3,4GlcNAc_2) (Figs. 1B and 2A). This is consistent with previous reports that hypoglycosylation in alg3Δ cells can be rescued by overexpression of the Alg6 glucosyltransferase (6). These data are also in agreement with previous reports showing that the OTase can transfer a wide range of LLO glycans with diverse numbers of Man (Fig. 3) (9). However, the observation that less LLO mannosylation led to more severe hypoglycosylation suggests that the Man structures on the B and C branches do affect LLO recognition by the ER LLO glucosyltransferases and/or by OTase. Thus, recognition of the donor LLO glycan by the Alg enzymes and OTase appears to involve many aspects of LLO glycan structure besides the integrity of the A branch or the presence of the terminal Glc residue. Specifically, our results show that recognition of donor LLO substrate by OTase is based on a combination of all three A-branch glucoses, and B- and C-branch mannoses.

Mutations of any of the ER lumenal mannosyltransferases or glucosyltransferases caused a qualitatively similar pattern of hypoglycosylation (Fig. 2). That is, in general the same sequons were hypoglycosylated in the different alg mutants, but to quantitatively different extents (Fig. 2B). This hypoglycosylation phenotype was especially strong for NxS sequons, which have lower affinity for the OTase peptide-binding site (14). These results suggest that the OTase activity toward any glycosylation site is similarly affected by deviations from the canonical Glc_3Man_3GlcNAc_2 LLO structure. This is in contrast to known defects in OTase, which show strong site-specificity.

Of the nonessential OTase subunits, deletion of OST3 led to the most severe hypoglycosylation (Fig. 2B). Deletion of OST6 on the other hand showed a minor hypoglycosylation phenotype, and resulted in hypoglycosylation of few sites, including the Gas1 N253LS sequon, a site previously observed to require Ost6, but not Ost3, for efficient glycosylation (15). Interestingly, this same sequon was also hypoglycosylated in the ost5Δ mutant. Lack of Ost5 also resulted in a limited effect on glycosylation, although its absence impacted glycosylation at more sequons than Ost6 (Fig. 2). As with Ost3 and Ost6, Ost5 is not a catalytic subunit of OTase, and it appears to be a small linker subunit tethering other OTase subunits. Ost5 appears to form a subcomplex with Ost1, and this subcomplex associates with the catalytic Stt3 subunit of OTase via Ost5 (20, 35). Ost5 is required for optimal OTase function *in vitro*, but OST5 deletion has no effect on the stability of Ost1 or Stt3 subunits, and has been previously reported to cause only a minor hypoglycosylation phenotype in yeast (20, 35). Our results here suggest a role for Ost5 in determining some aspect of site-specific glycosylation by OTase, as not all sites were equally affected by its deletion.

Inefficient protein glycosylation leads to ER stress and induction of the unfolded protein response. In yeast, glycos-
lation defects also lead to the activation of the cell wall integrity pathway, leading to alterations in the expression of certain cell wall proteins (40–46). Consistent with this, our SWATH-MS relative quantification of the cell wall proteome showed qualitatively consistent changes in protein abundance across the glycosylation mutants, but with larger changes in protein abundance in strains with severe hypoglycosylation defects (Figs. 2 and 4). Lack of glycosylation at specific sites can affect the folding and traffic of glycoproteins, and can lead to the premature degradation of hypoglycosylated folding intermediates (21, 47). However, we only measured mature proteins that had successfully trafficked to the cell wall. Importantly, our results nonetheless indicate that the majority of the mature proteins present at the cell wall in the alg and ost mutant strains were hypoglycosylated and may thus be partially functional or have an altered stability, in addition to changes in protein abundance. Glycosylation defects are likely to lead to similar global effects on glycoprotein macroheterogeneity, microheterogeneity, abundance, and function in other systems including human CDGs. Further, we observed that the strains with the most severe defects in microheterogeneity (the mannosyltransferase mutants) did not show the most severe hypoglycosylation defects across all strains nor the largest changes in cell wall proteome compared with wild type (Figs. 1–4). These results demonstrate that defects in microheterogeneity do not necessarily correlate with defects in macroheterogeneity or in the levels of mature proteins at their final destination. This emphasizes the importance of analytical approaches that consider macroheterogeneity, microheterogeneity, and protein abundance, as these have profound physiological effects and may fluctuate independently.

In this work we describe a sensitive SWATH-MS based glycoproteomic workflow that allows simultaneous characterization and relative quantification of global site-specific glycan macro- and microheterogeneity in complex protein mixtures. We have successfully used this approach to measure global changes in glycan macro- and microheterogeneity in the full set of viable yeast mutants in the N-glycosylation biosynthesis pathway. We also anticipate that the logic and rationale of the workflow we describe can be adapted to the study of other post-translational modifications.

Acknowledgments—We thank Julio J. Caramelo for helpful discussions.

* This work was supported by Australian Research Council Discovery Project grant DP160102766 to BLS. LFZ holds a Post-doctoral Fellowship from CONICET, Argentina. BLS holds an Australian National Health and Medical Research Council RD Wright Biomedical (CDF Level 2) Fellowship APP1087975.

† To whom correspondence should be addressed: School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, Queensland, 4072, Australia. Tel.: +61 7 336 54875; Fax: +61 7 3365 4273; E-mail: b.schultz@uq.edu.au.

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