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Deglycosylation systematically improves N-glycoprotein identification in liquid chromatography–tandem mass spectrometry proteomics for analysis of cell wall stress responses in *Saccharomyces cerevisiae* lacking Alg3p

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ABSTRACT

Post-translational modification of proteins with glycosylation is of key importance in many biological systems in eukaryotes, influencing fundamental biological processes and regulating protein function. Changes in glycosylation are therefore of interest in understanding these processes and are also useful as clinical biomarkers of disease. The presence of glycosylation can also inhibit protease digestion and lower the quality and confidence of protein identification by mass spectrometry. While deglycosylation can improve the efficiency of subsequent protease digest and increase protein coverage, this step is often excluded from proteomic workflows. Here, we performed a systematic analysis that showed that deglycosylation with peptide-N-glycosidase F (PNGase F) prior to protease digestion with AspN or trypsin improved the quality of identification of the yeast cell wall proteome. The improvement in the confidence of identification of glycoproteins following PNGase F deglycosylation correlated with a higher density of glycosylation sites. Optimal identification across the proteome was achieved with PNGase F deglycosylation and complementary proteolysis with either AspN or trypsin. We used this combination of deglycosylation and complementary protease digest to identify changes in the yeast cell wall proteome caused by lack of the Alg3p protein, a key component of the biosynthetic pathway of protein N-glycosylation. The cell wall of yeast lacking Alg3p showed specifically increased levels of Cis3p, a protein important for cell wall integrity. Our results showed that deglycosylation prior to protease digestion improved the quality of proteomic analyses even if protein glycosylation is not of direct relevance to the study at hand.

1. Introduction

Asparagine (N)-linked glycosylation of proteins is a common modification of secretory and membrane proteins in eukaryotes that is important for many aspects of glycoprotein function. The initial stages of N-glycoprotein biosynthesis occur in the endoplasmic reticulum (ER), where glycan to be transferred to protein is built on a lipid carrier by the consecutive action of a series of integral membrane glycosyltransferase enzymes [1]. The mature $Glu_3Man_9GlcNAc_2$ glycan is efficiently transferred to asparagines in nascent polypeptides by the multiprotein enzyme complex oligosaccharyltransferase (OTase) [2,3]. OTase preferentially binds asparagines located in 'glycosylation sequons' (N-x-S/T; x ≠ P) at its peptide acceptor binding site, resulting in efficient modification of such sequences [4,5]. The presence of N-glycans on nascent polypeptides assists productive protein folding in the ER directly by increasing the solubility of polypeptides, and indirectly through lectin-chaperone folding and quality control systems [6]. After glycoproteins leave the ER, their glycan structures are often further modified during transit through the Golgi. This can give rise to a very high diversity of glycan structures on mature proteins, which can affect glycoprotein function in many biological processes, including infection, development and cancer [7–9].

N-glycosylation is also often used as an enrichment tag for reducing the complexity of protein samples [10–12]. Studies using such enrichment methods generally focus on the improvements in proteome depth and protein identification achievable through targeted enrichment of N-glycopeptides, rather than the biology of N-glycosylation *per se*. However, in combination with sensitive and semi-quantitative selected reaction monitoring (SRM) detection, such methodologies offer substantial benefits for proteomic studies of a variety of clinically relevant sample types [13–15].

Abbreviation: OTase, oligosaccharyltransferase.

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Because of its importance in diverse biological systems, protein N-glycosylation is of general interest in proteomic identification and protein characterization studies. Changes in glycosylation have been associated with a wide variety of conditions including inherited disease, cancer and infection [7,16–20]. However, relatively specialized sample preparation, detection methods and data analysis outside the scope of many studies are often required for detailed glycosylation analysis [21,22]. In these cases, the N-glycosylation status of components of a sample is generally ignored. Nonetheless, even without a focus on identification or characterization of N-glycosylation, many samples of interest for proteomic analysis are rich in N-glycoproteins. This includes clinical samples, tissue biopsies, cell culture material and many model organisms such as yeast. N-glycosylation can influence proteomic analysis of such samples, as a key biological role of N-glycosylation is to protect glycoproteins from proteolytic cleavage, an effect still present following protein denaturation. It has been reported that removal of N-glycans by glycosidases such as PNGase F can improve MS identification of N-glycoproteins [23–26]. However, a deglycosylation step is not generally included in proteomic workflows. Here, we performed a thorough systems-level analysis of the benefit of inclusion of PNGase F deglycosylation prior to protease digestion with AspN and trypsin, in analysis of the cell wall proteome of Bakers' yeast.

2. Experimental

2.1. Chemicals

All chemicals were obtained from Sigma–Aldrich (St. Louis, Missouri, USA) unless otherwise specified, and were of analytical grade or higher.

2.2. Yeast strains and growth conditions

Saccharomyces cerevisiae strains used were BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and the *Δalg3* mutant derivative thereof (Open Biosystems). Cells were grown to mid log phase as measured by the optical density at 600 nm at 30 °C in YPD (2% Bacto peptone, 1% yeast extract, and 2% glucose) and cell wall protein samples immediately prepared.

2.3. Cell wall protein sample preparation

Preparation of proteins covalently linked to the yeast polysaccharide cell wall was performed as previously described [27,28]. Biological triplicates of cells grown in 50 ml of YPD to mid log phase were harvested by centrifugation at 4000 rcf for 10 min, resuspended in 50 mM Tris–HCl buffer, pH 7.5, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1× complete protease inhibitor cocktail (Roche, Basel, Switzerland) and lysed by agitation using a Mo Bio Laboratories (Carlsbad, CA, USA) Vortex-Genie 2 at maximum speed with 425–600 μm glass beads for 1 h at 4 °C. Covalently linked cell wall material was pelleted by centrifugation at 18,000 rcf for 1 min and washed 4 times with ice-cold 50 mM Tris–HCl buffer pH 7.5. The pellet was resuspended in 50 mM Tris–HCl buffer pH 8.0, 2% sodium dodecyl sulfate (SDS), 7 M urea and 2 M thiourea. Cysteines were reduced and alkylated by addition of dithiothreitol to 10 mM and incubation at 30 °C with agitation at 1500 rpm in a Benchmark Scientific (South Plainfield, NJ, USA) Multi-Therm Heat-Shake for 30 min, followed by addition of acrylamide to 50 mM and further incubation with agitation at 30 °C for 1 h. Non-covalently linked proteins were removed by washing the 18,000 rcf pellet five times with 1 ml 50 mM Tris–HCl buffer, pH 8, 2% SDS, 7 M urea and 2 M thiourea, followed by five washes with 2% SDS. The pellet was resuspended in 100 μl 1% Tergitol-type nonyl phenoxypolyethoxylethanol (NP-40) and 1× G7 buffer (New England Biolabs, Ipswich, Massachusetts, USA) and aliquoted into 50 μl followed by addition of 500 units of PNGase F (New England Biolabs) and incubated at 37 °C with agitation at 1500 rpm in a Multi-Therm Heat-Shake for 16 h. Samples without PNGase F deglycosylation were incubated in the same buffer without PNGase F enzyme. Cell wall material was pelleted at 18,000 rcf for 1 min and washed 5 times in 50 mM Tris–HCl buffer pH 8.0. The final cell wall pellet, equivalent to 12.5 ml of cell culture, was resuspended in 50 mM NH_4HCO_3, and proteins were digested with either trypsin 4 μg/ml (proteomics grade, Sigma) or AspN 1 μg/ml (proteomics grade, Promega) at 37 °C for 16 h with agitation at 1500 rpm in a Multi-Therm Heat-Shake.

2.4. Mass spectrometry

Peptides were desalted using C18 ZipTips (Millipore, Billerica, Massachusetts, USA) and analyzed by LC-ESI–MS/MS using a Prominence nanoLC system (Shimadzu, Kyoto, Japan) direct on-line with a TripleTof 5600 mass spectrometer with a Nanospray III interface (AB SCIEX, Washington, D.C., USA) as previously described [27]. The Prominence nanoLC system consisted of a DGU-20A5 degasser, two LC-20ADnano LC pumps, a SIL-20C HT autosampler, a CTO-20A column oven and a CBM-20A communications bus module. Approximately 2 μg peptides in 100 μl 5% acetonitrile with 0.1% formic acid were desalted on an Agilent Technologies (Santa Clara, California, USA) C18 trap (300 Å pore size, 5 μm particle size, 0.3 mm i.d. × 5 mm) at a flow rate of 30 μl/min for 3 min, and then separated on a Grace (Deerfield, IL, USA) Vydac EVEREST reversed-phase C18 HPLC column (300 Å pore size, 5 μm particle size, 150 μm i.d. × 150 mm) at 45 °C at a flow rate of 1 μl/min. Peptides were separated with a gradient of 10–60% buffer B over 45 min, with buffer A (1% acetonitrile and 0.1% formic acid) and buffer B (80% acetonitrile with 0.1% formic acid). The TripleTof 5600 mass spectrometer was used in positive ion mode for information dependent acquisition (IDA) experiments performed in high-resolution mode with default settings. An MS TOF scan from *m/z* of 350–1800 was performed for 0.5 s followed by information dependent acquisition of MS/MS with automated selection of up to the top 20 peptides from *m/z* of 40–1800 for 0.05 s per spectrum. Nitrogen gas was used, and gas and voltage settings were adjusted as required. Typical values used were: ionsource gas 1, 10 psi; curtain gas, 30 psi; ionspray voltage floating, 2300 V; interface heating temperature, 150 °C, declustering potential, 80 eV; collision energy (CE), 10 eV. Switch criteria for selection of ions for MS/MS were: charge state, 2–5; ion count exceeding 100 cps; excluding isotopes within 4 Da with a mass tolerance of 50 mDa; dynamic exclusion of former target ions for 30 s after 2 occurrences. CE for MS/MS was determined on-the-fly independently for each selected ion using rolling CE with default parameters.

2.5. Data analysis

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, *S. cerevisiae* with common contaminants; ID focus, biological modifications; enzyme, trypsin or AspN; Search effort, thorough ID. False discovery rate analysis using ProteinPilot was performed on all searches. Protein abundance was estimated from ProteinPilot search results using the values calculated by ProteinPilot for score, coverage or the number of peptides identified per protein. Peptides identified with greater than 95% confidence and with a local false

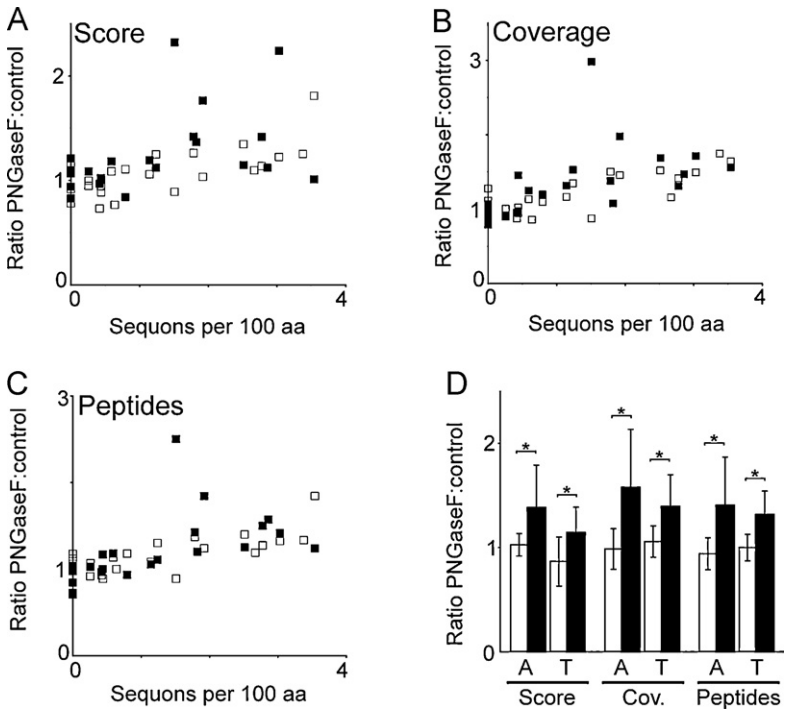

Fig. 1. PNGase F digest improves identification of yeast cell wall glycoproteins using AspN or trypsin. Ratio of (A) score, (B) coverage and (C) number of peptides identified for each protein in PNGase F treated samples relative to nontreated control samples, in combination with AspN (■) or trypsin (□) digest. (D) Average ratio of score, coverage and number of peptides identified in PNGase F treated samples relative to nontreated control samples, in combination with AspN "A" or trypsin "T" digest, for proteins with high (greater than 0.5 glycosylation sequons per 100 amino acids, black) or low (fewer than 0.5 glycosylation sequons per 100 amino acids, white) glycosylation site density. Values show mean. Error bars show standard deviation. $^*P < 0.05$ Students *t*-test.

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). Biological triplicates were analyzed.

3. Results and discussion

3.1. Protein identification

To gauge the benefit of including a PNGase F deglycosylation step prior to protease digestion in a proteomic workflow, we analyzed the proteins covalently linked to the polysaccharide cell wall of Bakers' yeast *S. cerevisiae*. This sample contains many N- and O-glycoproteins, as well as abundant non-glycosylated proteins [27–31]. This analysis robustly identified 26 proteins, with 19 identified after both AspN and trypsin digest, four identified only by trypsin and three only by AspN (Supplementary Table S1 and supplementary material). The proteins identified were consistent with previous proteomic analyses of yeast cell wall fractions [28,32–34]. In addition, AspN digest allowed identification of Egt2p (Supplementary Table S1 and supplementary material), which was not identified in these previous studies. PNGase F treatment significantly improved protein identification in concert with either trypsin or AspN proteolytic digest (Fig. 1 and Supplementary Table S1). Although no additional proteins were identified following PNGase F glycan release than without deglycosylation, the quality of identification of proteins was improved, as measured by protein score, percent peptide coverage and the number of confidently identified peptides (Fig. 1 and Supplementary Table S1).

The extent of the increase in the confidence of protein identification after PNGase F treatment correlated with the density of sites of N-glycosylation in a protein (Fig. 1). That is, the more glycosylation sequons per 100 amino acids in a protein, the greater the improvement in the confidence of identification a protein displayed upon deglycosylation. This association held with use of both trypsin and AspN as protease. Glycoproteins with more than 0.5 glycosylation sequons per 100 amino acids showed significantly more improvement in the confidence of identification with PNGase F treatment than proteins with fewer sequons, as measured by ProteinPilot score, coverage or the number of peptides identified per protein (Fig. 1D). In combination with trypsin, the correspondence between glycosylation site density and improvement in identification was essentially linear. This is consistent with glycan removal 'unmasking' otherwise undetectable tryptic glycopeptides [26], where there would be a linear relationship between the density of glycosylation sites and the number of additional peptides identifiable after glycan removal. In contrast, PNGase F in combination with AspN resulted in a positive but less direct correlation between confidence of identification and glycosylation site density (linear fit for coverage, $R^2 = 0.63$ for trypsin and 0.28 for AspN (Fig. 1)). This is most likely due to PNGase F glycan release converting glycosylated asparagine to aspartate, thereby generating novel AspN cleavage sites at each previously glycosylated asparagine residue. This PNGase F-mediated generation of additional AspN cleavage sites potentially allows identification of two additional peptides per glycosylation site. However, both of these novel AspN peptides may not be of an *m/z* readily identifiable by MS, resulting in a more variable and protein-specific improvement in the confidence of identification. In general, PNGase F deglycosylation resulted in a larger improvement in identification with AspN than with trypsin, but with higher protein-specific variability.

3.2. Protein characterization

Within a given protein, identification of specific stretches of amino acids was highly complementary with AspN and trypsin cleavage. Ecm33p provides an example of this complementary characterization (Fig. 2). Even though Ecm33p was identified with

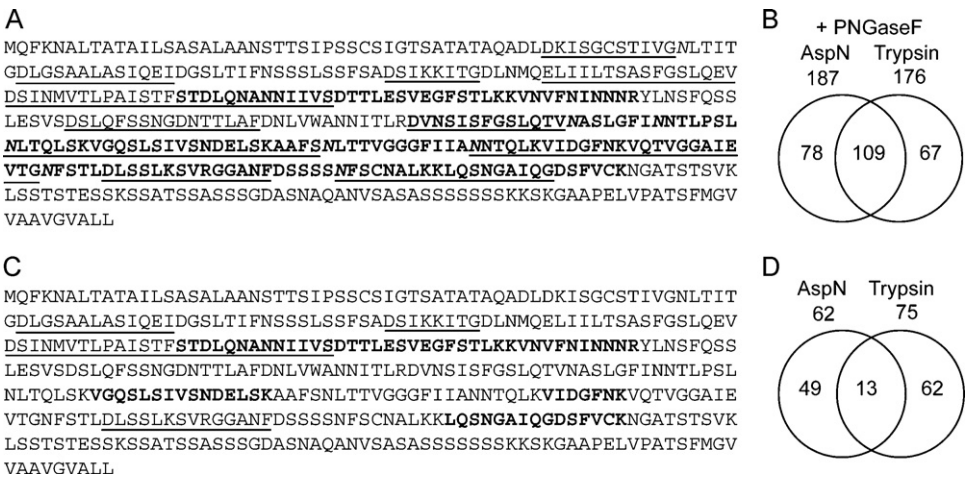

Fig. 2. Coverage of Ecm33p (A) with and (C) without PNGase F digest using AspN or Trypsin. Bold, identified with trypsin. Underlined, identified with AspN. Italic *N*, deamidated asparagine indicating location of a putative N-glycosylation site. (B) Number of amino acids contained in peptides identified after PNGase F deglycosylation and AspN or trypsin digest or (B) without PNGase F deglycosylation.

very high confidence with trypsin or AspN alone, the degree of characterization of the protein was improved with use of both complementary treatments. Of 124 total amino acids present in peptides identified from Ecm33p, only 13 were detected by both AspN and trypsin, with 49 detected only by AspN and 62 only by trypsin (Fig. 2C and D). PNGase F treatment further substantially improved coverage with both proteases. There were 254 amino acids present in peptides identified in total by AspN and trypsin after PNGase F deglycosylation, with 109 detected by both proteases, 78 only by AspN and 67 only by trypsin (Fig. 2A and B). These results clearly show the advantages in protein characterization of use of multiple proteases in conjunction with deglycosylation using PNGase F. Such improved peptide coverage through deglycosylation and protease complementarity may be of particular importance in detailed protein characterization studies. This analysis also allowed putative identification of sites of N-glycosylation, by the conversion of previously glycosylated asparagine to aspartate by PNGase F deglycosylation (Fig. 2) [27]. However, validation of these glycosylation sites requires a negative control lacking PNGase F enzyme to ensure the observed deamidation events are not spontaneous [35], which occurs at asparagines in a sequence-dependent manner [36,37]. However, inclusion of such a control is not required for proteomic identification experiments, where mapping the precise sites of glycosylation is not of direct importance.

3.3. Proteomic comparison of wild type and Δalg3 yeast cell wall composition

We used our method of deglycosylation with protease complementarity to characterize the consequences of loss of the Alg3p protein on the proteome of the yeast cell wall. The Alg3p protein in yeast is an integral membrane glycosyltransferase resident in the ER lumen, which catalyses transfer of a specific mannose monosaccharide to lipid linked glycan during synthesis of the glycan to be transferred to protein in N-glycosylation by OTase [38]. Lack of active Alg3p results in incomplete biosynthesis of this lipid linked glycan, and accumulation of a $Man_5GlcNAc_2$ glycan structure [39]. The activity of OTase is affected by the structure of glycan present, as OTase has high affinity for the mature lipid linked glycan, which limits transfer of the biosynthetic intermediates that are also present on the ER-lumenal face of the ER membrane. This means that OTase has reduced activity in the presence of incomplete glycan, for instance in *Δalg3* cells lacking the Alg3p protein [27,39]. We have previously shown that *Δalg3* cells show site-specific under-glycosylation, with the subset of normally glycosylated asparagine residues that are located in NxS sequons and in secondary structural elements tending to be under-glycosylated, compared with wild type yeast cells [27]. It is likely that this site-specific under-glycosylation phenotype is caused by reduced binding affinity of such protein acceptor sites to the active site of OTase [4]. *Δalg3* Cells also show a slight growth sensitivity to calcofluor white, which is indicative of a cell wall defect [40], and a general reduction in glycosylation efficiency at multiple glycosylation sites in cell wall glycoproteins [27].

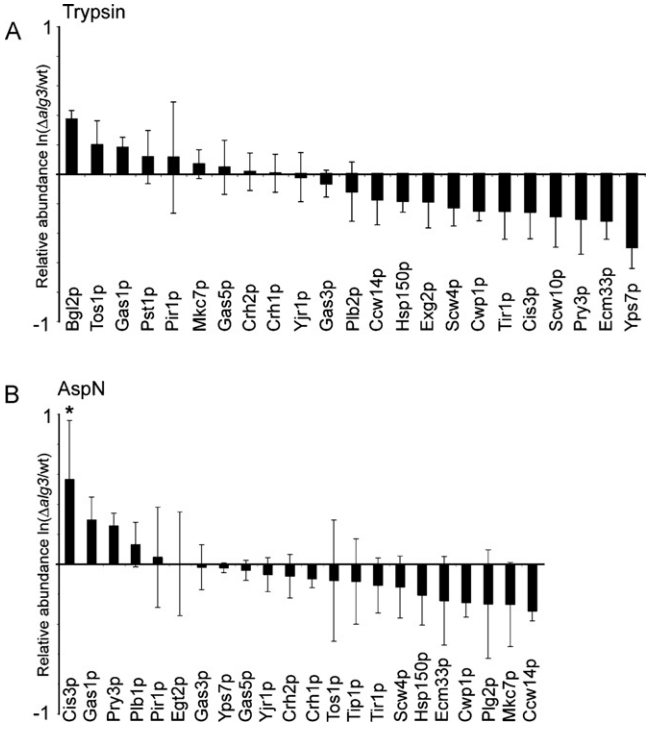

Fig. 3. Relative abundance of cell wall proteins in *Δalg3* and wild type yeast. Natural logarithm of the ratios of protein identification scores from *Δalg3* relative to wild type yeast after PNGase F deglycosylation and protease digest with (A) trypsin or (B) AspN. Values show mean. Error bars show standard deviation. *Significantly upregulated in *Δalg3* cells (ANOVA, $P < 0.05$).

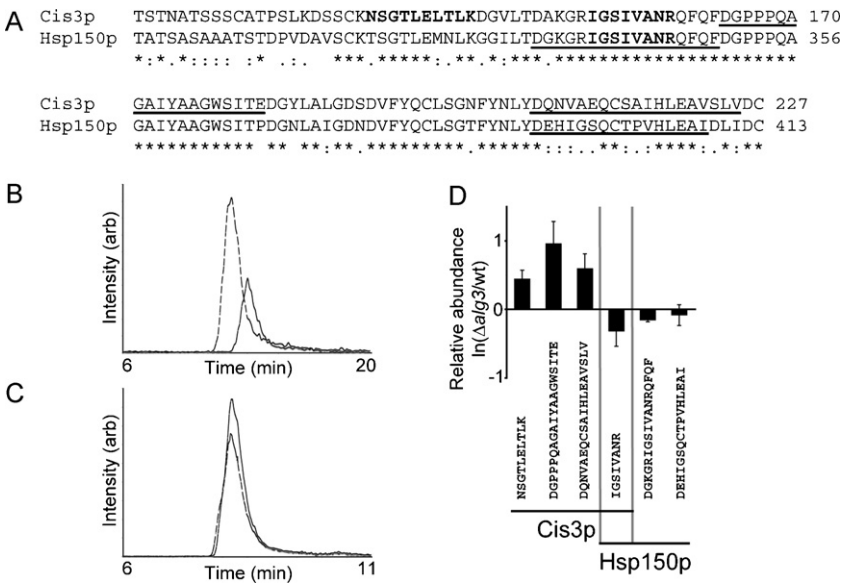

Fig. 4. Relative abundance of the highly similar Cis3p and Hsp150p proteins in *Δalg3* and wild type yeast cell wall preparations. (A) ClustalW alignment of the C-terminal region of yeast Cis3p and Hsp150p. Bold, identified tryptic peptides. Underlined, identified AspN peptides. (B) Extracted ion chromatograms from wild type (solid) and *Δalg3* (dashed) yeast of the $[M+2H]^{2+}$ ion at an *m/z* of 950.95 corresponding to AspN peptide $_{164}$DGPPPQAGAIYAAGWSITE$_{182}$ specific for Cis3p, and (C) the $[M+2H]^{2+}$ ion at an *m/z* of 415.24 corresponding to tryptic peptide IGSIVANR shared by Cis3p (52–59) and Hsp150p (338–345). (D) Natural logarithm of the ratio of abundance of ions corresponding to peptides from Cis3p and/or Hsp150p in *Δalg3* cells relative to wild type cells. Values show mean of biological triplicate. Error bars show standard deviation.

We compared the cell wall proteome of wild type and *Δalg3* yeast cells after PNGase F deglycosylation using either trypsin or AspN protease digest. No significant differences in protein abundance between *Δalg3* and wild type cells were detected using deglycosylated tryptic peptides (Fig. 3A). However, a single protein, Cis3p (YJL158C; UniprotKB accession P47001; also known as Ccw11p, Pir4p, Ccw5p, Scw8p) was upregulated in *Δalg3* cells compared to wild type ($P < 0.05$, ANOVA, Fig. 3B). Cis3p was also identified by tryptic digest, but did not show a significant change in expression between *Δalg3* and wild type cells. To scrutinize this discrepancy, we examined the specific peptides identified from Cis3p by AspN and trypsin. This showed that the amino acid sequence of Cis3p is highly similar to other members of the PIR (protein with internal repeat) protein family, and as such many peptides are shared between members of this family, rather than being specific to a single protein. This is especially difficult for the abundant and highly similar Cis3p and Hsp150p (Pir2p) proteins (Fig. 4A). This makes identification or unambiguous differentiation between these proteins difficult, as has been previously noted [32,34]. Inspection of AspN peptides showed that two Cis3p-specific peptides were confidently identified (D_{164}GPPPQAGAIYAAGWSITE$_{182}$ and D_{207}QNVAEQCSAIHLEAVSLV$_{225}$), both of which showed large and significant upregulation in *Δalg3* cells compared with wild type (Fig. 4). In contrast, only one tryptic peptide was unique to Cis3p (N_{132}SGTLELTLK$_{141}$), with another shared between Cis3p and Hsp150p (I_{152}GSIVANR$_{159}$) (Fig. 4). Of these, only the single Cis3p-unique tryptic peptide (N_{132}-K_{141}) showed large and significant upregulation in *Δalg3* cells (Fig. 4B and D). The tryptic and AspN peptides from Hsp150p showed no significant change. Thus, Cis3p was not confidently identified as upregulated based on tryptic peptide identification, but the complementary analysis using AspN detected several unique peptides which did show significant upregulation in *Δalg3* cells.

Cis3p covalently links cell wall glucan through an alkali labile ester linkage [41] and is also cross linked to other cell wall proteins through disulfide bonds [42,43] to increase the strength of the cell wall [44]. Deletion of *CIS3* results in a mild growth defect compared to wild type cells and substantial changes in cell morphology resulting in increased cell clumping [45,46].

Together with other members of the *PIR* family, gene expression studies have shown upregulation of Cis3p in response to cell wall stress caused by calcofluor white or zymolyase [47], or *via* the cell wall integrity signalling pathway through Mpk1 [48]. A proteomic study showed upregulation of Cis3p and other proteins involved in the cell wall stability response in *Δgas1* cells [34]. Gas1p is an abundant glycosyltransferase which plays a major role in cell wall remodelling during normal vegetative growth, and *Δgas1* cells have a altered cell wall [49]. Hsp150p/Pir2p, a homolog of Cis3p, is upregulated in response to cell wall perturbation in response to beta 1,6-glucan deficiency [50]. In contrast, we did not detect significant changes in the expression of Hsp150p in *Δalg3* cells. It is interesting that we detect specific upregulation of Cis3p, but not other PIR proteins, in *Δalg3* cells compared to wild type (Figs. 3 and 4). This suggests that these proteins, although highly similar in sequence, are not completely functionally redundant, and that Cis3p plays an important role in response to the particular cell wall stresses present in *Δalg3* cells.

Δalg3 cells show underglycosylation at a specific subset of glycosylation sites [27], but do not show large changes in abundance of these cell wall glycoproteins (Fig. 3). However, upregulation of Cis3p suggests that the cell wall is somehow perturbed in *Δalg3* cells, in agreement with previous reports [40]. This may be due to changes in the activity or stability of underglycosylated cell wall glycoproteins resulting in an altered and suboptimal cell wall structure. That is, underglycosylation of these glycoproteins in *Δalg3* cells does not dramatically change their expression, but may affect their function. Alternatively, glycoproteins not covalently linked to the cell wall, but required for its synthesis, may have reduced expression, stability or function in *Δalg3* cells.

4. Conclusions

Standard procedures for proteomic analysis generally do not include deglycosylation with PNGase F, and we show that treatment with PNGase F increases the confidence of identification in accordance with the density of glycosylation sites on proteins. Some proteins may be especially prone to improvements in identification following PNGase F digest. It is therefore advantageous

to deglycosylate with PNGase F even if N-glycosylation is not a focus of a proteomic experiment, in samples which contain moderately or highly glycosylated proteins. This would include most secreted or membrane protein samples in eukaryotic biochemical analysis or clinical biomarker discovery samples. Protease digestion with a combination of complementary specific proteases can allow improved protein characterization, especially AspN in combination with PNGase F. We used these approaches to show that the stress response protein Cis3p is a key component of the yeast cell wall stress response to reduced N-glycosylation with loss of *ALG3* in yeast.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.01.026.

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