Free glycans derived from O-mannosylated glycoproteins suggest the presence of an O-glycoprotein degradation pathway in yeast

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ABSTRACT

In eukaryotic cells, unconjugated oligosaccharides that are structurally related to N-glycans, i.e. free N-glycans (FNGs), are generated either from misfolded N-glycoproteins destined for the endoplasmic reticulum (ER)-associated degradation (ERAD), or from lipid-linked oligosaccharides, donor substrates for N-glycosylation of proteins. The mechanism responsible for the generation of FNGs is now well understood, but the issue of whether other types of free glycans present remains unclear. Here, we report on the accumulation of free, O-mannosylated glycans in budding yeast that were cultured in media containing mannose as the carbon source. A structural analysis of these glycans revealed that their structures are identical to those of O-mannosyl glycans that are attached to glycoproteins. Deletion of the cyc8 gene, which encodes for a general transcription repressor, resulted in the accumulation of excessive amounts of free O-glycans, concomitant with a severe growth defect, a reduction in the level of an O-mannosylated protein, and compromised cell wall integrity. Our findings provide evidence in support of a regulated pathway for the degradation of O-glycoproteins in yeast and offer critical insights into the catabolic mechanisms that control the fate of O-glycosylated proteins.

N- and O- glycosylations are major forms of co- and post-translational modifications in eukaryotes. The explosive advances in the field of glycobiology during past two decades have revealed not only the pathways for the biosynthesis of glycans but also how glycans function in various biological processes, such as protein quality control,
protein targeting, signal transduction, cell–cell interactions, immune responses, cell differentiation, and cancer metastasis (1,2).

In the ER of budding yeast, *Saccharomyces cerevisiae*, N-glycosylation reactions are initiated by transferring an oligosaccharide composed of 14 sugars (Glc₃Man₉GlcNAc₂) from lipid-linked oligosaccharides (LLOs) to Asn residues in the consensus sequence, Asn-Xaa-Ser/Thr (where Xaa is any amino acid except Pro), of nascent polypeptide chains (3,4). The immature N-glycoproteins are then assisted by various ER-resident molecular chaperones such as BiP, calnexin, and peptide disulfide isomerase (PDI) to achieve the correct protein folding and/or the formation of protein complexes. In this process, N-glycans are recognized as “Tags” that dictate the folding status of the carrier proteins (5-8).

Several studies of budding yeast have shown that O-mannosylation is involved in these two major biological processes. One is related to cell wall integrity. The rigid and protective layer of the cell wall, composed of heavily O-mannosylated and/or N-glycosylated proteins, is essential for cell viability and correct cell division during vegetative growth as well as under conditions of environmental stress, e.g., heat stress and proteolysis (9,10). Mutants that are defective in the key O-mannosylation enzyme therefore exhibit slow growth due to the lack cell wall integrity (11,12). The other pivotal function of O-mannosylation in the biological process is the quality control of newly synthesized proteins in the ER. It has been reported that some O-glycosylated model ERAD substrates including α-factor, Gas1*, and KHNt undergo excessive O-mannosylation by protein O-mannosyltransferases 1 and 2 (Pmt1 and Pmt2) (13,14) and that Pmt1 and Pmt2 interact with the component of the luminal surveillance complex for ERAD, Hrd1 to facilitate the degradation of excessive O-mannosylated misfolded proteins (15). Moreover, in a recent study, Xu *et al.* clearly showed that O-mannosylation executed by Pmt1 and Pmt2 terminates attempts of long-retained misfolded proteins to undergo folding in the ER and facilitates their degradation. It therefore appears that both N- and O-glycosylation are critical for the recognition and elimination of misfolded glycoproteins in the ER (16).

In addition to N- and O-glycans on proteins in the secretory pathway, it is known that “free”, unconjugated forms of oligosaccharides related to N-glycans, (free N-glycans; FNGs) can accumulate in the cytosol in eukaryotic cells (17-26). There are two major pathways for the generation of FNGs in budding yeast (21,25). First, in the peptide:N-glycanase (PNGase)-dependent pathway, FNGs are generated from misfolded glycoproteins, by the action of a cytosolic PNGase (Png1). In the second, the oligosaccharyltransferase (OST)-dependent pathway, mature forms of DLO (dolichol-linked oligosaccharides: Glc₃Man₉GlcNAc₂) are liberated in the luminal side of the ER by OST. The FNGs that accumulate in the ER are then processed by glycosidases in the ER, and are eventually transported to the cytosol by an unidentified mechanism. The biological relevance of the formation/degradation of FNGs is, however, poorly understood.

During the course of our studies dealing with the functional importance of FNG-generation, we analyzed the free oligosaccharides that had accumulated in yeast cells under various culture conditions. Surprisingly, novel forms of
free oligosaccharides were observed when the cells were cultured in media containing mannose as the carbon source. Extensive structural analyses clearly indicated that these oligosaccharides are structurally identical to the O-mannosyl glycans that are covalently attached to yeast glycoproteins. We hypothesized that the free O-glycans (FOGs) are probably liberated from O-glycoproteins by a putative endo-α-mannosidase-like activity. We also showed that the formation of FOGs is regulated by the general transcription repressor Cyc8, and further analyses suggested that the hyper-activation of the FOG generation-pathway may result in the dysregulation of protein kinase-C (PKC)-mediated cell wall-integrity. Our results thus imply that a novel mechanism exists for regulating the catabolism of O-mannosyl glycans in yeast.

Results

Generation of the novel free oligosaccharides produced in yeast

The findings reported in our previous studies suggested that the yeast cytosolic/vacuolar α-mannosidase, Ams1, plays a pivotal role in the catabolism of free-N-glycans (FNGs) that accumulate in the cytosol (22,25,33). Several studies have shown that the intrinsic expression level and enzymatic activity of Ams1 is significantly increased under certain specific culture conditions such as cell wall stress, deprivation of the carbon source, and starvation caused by insufficient nitrogen sources (22,25,38). To understand the molecular mechanisms for the activation of the Ams1-dependent degradation of FNGs, we cultured yeast cells in various culture media for 3 hr at 30˚C and profiled the structure of the PA-labeled FNGs by size-fractionation HPLC analysis. In normal culture media, YPGA, the major form of FNGs were Hex7:0HexNAc2 as reported previously (22,39). In contrast, the generation of FNGs was reduced when certain types of culture media, such as YPGal, YPGly, and YP were used, probably due to the intracellular activation of Ams1. This result is consistent with previous observations that Ams1 activity is increased when the level of glucose in the culture media is reduced (25,33). On the other hand, we unexpectedly discovered that large amounts of novel free oligosaccharides were produced only when the cells were grown in the YPMan media (Figure 1A, peaks a–d). The oligomers corresponding to these peaks were structurally altered on treatment with jack bean α-mannosidase (Figure 1B), suggesting that they are α-mannosylated glycans. These results suggest that, in budding yeast, the generation of novel α-mannosylated free oligosaccharides can be induced by using YPMan as a medium.

Biosynthesis pathway of the novel structure for free-glycans

We next attempted to determine the structure of these novel free oligosaccharides. For this purpose, the compounds corresponding to the collected peaks a-d were subjected to structural and linkage analyses. The findings indicated that the MS values for peaks a–d corresponded to those of PA-Hex2:5. Moreover, analyses of these peaks after digestion with various glycosidases clearly indicated that the structures of the PA-Hex3:5 glycans (peaks b-d) were identical to those of the O-mannosyl glycans in Saccharomyces cerevisiae (See Table II and III; Figure 1C for the structures of O-mannosyl glycans). We therefore hypothesized that these free glycans are derived from the O-mannosyl glycans on glycoproteins. To validate this
hypothesis, the total amounts of free-glycans in the two mutants that were defective in the first and second steps of mannose transfer reactions, \( \text{pmt1}\Delta \text{pmt2}\Delta \) and \( \text{ktr1}\Delta \text{kre2}\Delta \text{ktr3}\Delta \), respectively (see Figure 1C for the transferase responsible for each mannose residue) were quantified. As shown in Figure 1C and D, a substantial reduction in the level of Man2–5 free-glycans was observed in the case of the \( \text{ktr1}\Delta \text{kre2}\Delta \text{ktr3}\Delta \) cells. This result clearly suggests that the enzymes responsible for the addition of the second \( \alpha \)-mannose of \( O \)-mannosyl glycans on glycoproteins is also required for the formation of Man2–5 free oligosaccharides, now designated as free \( O \)-glycans (FOGs). There are, however, two possible scenarios for how these FOGs could be formed; (1) they are released from \( O \)-mannosylated glycoproteins by the action of an endo-\( O \)-\( \alpha \)-mannosidase, or (2) they are formed by the same machinery that is involved in \( O \)-mannosylation by using free mannose as a donor. To distinguish between these two possibilities, cells defective in the first mannose transfer, \( \text{pmt1}\Delta \text{pmt2}\Delta \), were tested for the formation of FOGs. As shown in Figure 1C and D, a significant reduction in the generation of Man2–5 glycans was observed for the \( \text{pmt1}\Delta \text{pmt2}\Delta \) cells, indicating that the formation of \( O \)-mannosyl glycans is likely a prerequisite for the efficient formation of FOGs. This result therefore favors the first scenario, i.e. FOGs are formed from protein-linked \( O \)-mannosyl glycans by the action of an endo-\( O \)-\( \alpha \)-mannosidase. Since the \( O \)-linked sugar chains are elongated starting at Man1 and proceed to Man5 in early-to-late Golgi, we suspected that the de-\( O \)-mannosylation of glycoproteins occurs in a late stage of the secretory pathway. To verify this hypothesis, we examined the generation of FOGs in a \( \text{sec18–1} \) mutant, which is deficient in ER-to-Golgi trafficking at a non-permissive temperature (37°C). At the permissive temperature, significant amounts of Man2–5 FOGs were generated in \( \text{sec18–1} \) cells. On the other hand, negligible generation of the Man2–5 of FOGs was observed at the non-permissive temperature (37°C) (Figure 1E), suggesting that functional ER-to-Golgi trafficking is required for the efficient production of FOGs. We also examined the time-course for the generation of \( N \)- and \( O \)- free glycans. The rate of generation of FNGs was essentially unaltered during the time course analysis after changing the media from YPGlc to YPMan, whereas the total amount of the FOGs in the cells reached a maximum at around 3 hr (Figure 1F). These results suggest that the concentration of mannose is a key trigger for the specific activation of the FOGs generation pathway.

**FOGs were accumulated in the post-Golgi compartment**

While it has been clarified that most of the FNGs in the wild type are released from misfolded glycoproteins that are destined for ERAD by the action of Png1 (a cytosolic peptide: \( N \)-glycanase) \((21,25)\), the subcellular site for the generation of FOGs remains unknown. To clarify this issue, we utilized a reporter protein, \( \text{Prc1pp-ManLe-FLAG} \). This reporter protein was composed of three functional domains; (I) the catalytic domain of an \( \alpha \)-mannosidase derived from tomato (ManLe), which was successfully expressed by yeast cells as an active, secreted enzyme \((30)\); (II) ER-targeting signal (\( \text{Prc1-pre} \)) and (III) vacuole-targeting signals (\( \text{Prc1-pro} \)) (Figure 2A). The 2\textsuperscript{nd} and 3\textsuperscript{rd} domains were derived from a vacuolar protease, Prc1, and these domains were expected to
allow this protein to be delivered to the vacuole via the Golgi-to-vacuole sorting receptor, Vps10. The Prc1pp-ManLe-FLAG expressed in ams1Δ or vps10Δ ams1Δ was N-glycosylated, indicating that this reporter protein is at least correctly targeted to the ER (Figure 2B). We then proceeded to verify the intracellular amount of FOGs with or without the expression of Prc1pp-ManLe-FLAG. As shown in Figure 2C, the expression of Prc1pp-ManLe-FLAG caused a significant reduction in the levels of FOGs in ams1Δ cells. On the other hand, in vps10Δ ams1Δ cells, the total amount of FOGs in the cell was comparable to that for ams1Δ cells with a vector control, possibly due to the defective transport of Prc1pp-ManLe-FLAG and/or its potential substrates to post-Golgi compartments. This result thus indicates that FOGs are, in sharp contrast to the case for most FNGs, generated in the lumenal side of the vesicles, and most likely accumulated in a post-Golgi compartment, such as vacuoles.

**Intracellular pools of nucleotide sugars and N- and O- glycosylation in YPMan culture conditions**

Having confirmed the source of the FOGs, we next examined the issue of how the YPMan culture conditions affect the N- and O-glycosylation status in yeast. To this end, the levels of nucleotide-sugars, as well as the extent of N- and O-glycosylation on glycoproteins, were quantitated. The biosynthesis of nucleotide sugars is a crucial step for the synthesis of donor substrates for N-glycosylation and O-mannosylation, DLO and dolichyl-phosphate-mannose (Dol-P-Man) (Figure 3A) (4). As shown in Figure 3B, a significant increase of the level of GDP-Man was observed when YPMan was used as the culture medium. In contrast, no increase in the levels of other nucleotides sugars, which are important for the biosynthesis of N-glycans, was observed, i.e. UDP-Glc and UDP-GlcNAc (Figure 3B). In addition, despite the increase in GDP-Man levels, the total level of N- and O-glycans on glycoproteins was comparable between the YPGlc and YPMan culture media (Figure 3C). From these data, we conclude that, in the case of YPMan media, the levels of both GDP-Man and FOGs were increased, while no significant increase in the levels of FNGs, N-glycosylation or O-glycosylation were observed. It can therefore be safely assumed that the increase in FOGs is not simply due to the overloaded synthesis of O-glycans, but rather a regulated event that is controlled by media conditions, including the enhanced catabolism of O-glycans.

**The generation of FOGs is negatively regulated by the general transcription repressor, Cyc8.**

To gain additional insights into the regulation of the formation of FOGs under YPMan culture conditions, we examined the effect of glucose concentration in the culture media. As shown in Figure 4A, small but significant amounts of FOGs were generated when a medium containing 1% glucose and 1% mannose was used. Most importantly, when a medium containing 0.2% glucose and 2% mannose was used, the generation of FOGs was significantly inhibited compared with a medium containing only 2% mannose (compare the 1st and 2nd column of Figure 4A). This fact led us to assume that the generation of FOGs is not only induced by the presence of mannose but is also strictly repressed by the presence of glucose in the media. We therefore further investigated the issue of
whether the transcription factors involved in the regulation of catabolite repression also regulate the generation of FOGs. As shown in Figure 4B, a deletion strain of one component of the general transcription repressor complex for glucose repression, i.e. cyc8Δ cells, exhibited a drastic increase (~10 fold) in the generation of the FOGs. However, the deletion of other components of the repressor complex, i.e. mig1Δ and tup1Δ, showed no dramatic increase in the generation of FOGs, suggesting that Cyc8 is specifically involved in regulating the generation of FOGs.

During the analysis of the FOGs in cyc8Δ cells, we also found that cyc8Δ grew more slowly than usual in YPMan media. To dissect this phenotype further, cyc8Δ cells were cultured on plates containing several different carbon sources. As shown in Figure 4C, attempts to grow cyc8Δ cells on YPMan plates resulted in severely retarded growth, while this was not observed for YPGlc or YPSuc plates, suggesting that the growth defect phenotype of cyc8Δ is specific for YPMan media. We further examined the detailed phenotype of the growth defect of cyc8Δ under various conditions, e.g., high temperature and the addition of cell wall-perturbation reagents, Calcofluor white (CFW) or Congo red (CR). When cultured under YPMan at 30 and 36°C, the cyc8Δ cells exhibited a strong growth defect (Figure 4D, second and third panels). Furthermore, the cyc8Δ cells showed a strong sensitivity to YPMan supplemented with CFW or CR (Figure 4D, fourth and fifth panels) even at 25°C, indicating that the integrity of the cell walls in these cells had been compromised. Notably, these growth defect phenotypes were mitigated in YPGlc media, leading us to hypothesize that the hyperactivation of FOG formation and the loss of cell wall integrity could be a related event, possibly through the excessive de-O-mannosylation of cell wall proteins and/or secretory proteins.

**Hyper activation of the generation of FOGs disrupts the cell wall integrity pathway (PKC signaling pathway).**

Based on the fact that cyc8Δ cells show a cell wall defect-phenotype in YPMan media, we suspected that the cell wall integrity pathway (PKC pathway) in these cells had been compromised. To validate this hypothesis, the phosphorylation status of Mpk1, an intermediate in the PKC pathway, was examined using a phosphorylated Mpk1-specific antibody (Figure 5A). As shown in Figure 5B, Mpk1 was phosphorylated only when cells were exposed to the cell wall stressor reagent, CFW, in wild-type cells. In contrast, when cyc8Δ cells were cultured in YPMan medium, Mpk1 was found to be constitutively phosphorylated. Moreover, no further increase in the levels of phosphorylated Mpk1 was observed in these cells upon CFW-treatment, in YPMan medium. Those results clearly suggest that PKC signaling is dysregulated in cyc8Δ cells by YPMan media. We further examined O-mannosylation status as well as the expression level of the cell wall-sensor protein, Wsc1, which is known to be a heavily O-mannosylated protein through a serine/threonine cluster in their stem region that is located in the extracellular space (40). As expected, a substantial reduction in the total amount of Wsc1 was observed in the cyc8Δ cells cultured with YPMan (Figure 5C). It should be noted that, by the RNA-Seq analysis for profiling gene expression patterns among the different carbon sources, we found that no significant changes in the
transcription level of \( WSC1 \) in WT and \( cyc8\Delta \) in the YPMan culture for 180 min, as compared with culturing these cells with YPGlc for 180 min (Figure 5D and E), suggesting that the reduction of the expression level of Wsc1 in \( cyc8\Delta \) cells cultured with YPMan is not due to the transcriptional suppression of \( WSC1 \) but rather the post-transcriptional regulation of Wsc1 proteins. Collectively, these results indicate that in budding yeast the Wsc1 protein in \( cyc8\Delta \) cells cultured with YPMan might cause an instability and dysfunction in the Wsc1 protein. It could therefore be assumed that the de-mannosylation of Wsc1-HA, presumed to be upregulated in \( cyc8\Delta \) cells cultured with YP Man media, may be involved in the destabilization of the Wsc1-HA protein, which could be a mechanistic basis for the dysregulation of the PKC pathway in \( cyc8\Delta \) cells.

**Discussion**

The generation and catabolism of free oligosaccharides derived from glycoproteins as well as LLOs are fundamental phenomena that occur from bacteria to mammalian cells (17,18,41). However, most studies have focused on the generation of FNGs, *i.e.* free oligosaccharides that are structurally related to \( N \)-glycans. Although Iwatsuka *et al.* reported the detection of extracellular FOGs with structures similar to mucin-type \( O \)-glycan chains in human sera (42), the occurrence of intracellular FOGs have not been reported in any system. In this study, we serendipitously discovered that a series of novel free-glycans that are structurally related to \( O \)-mannosyl glycans are produced only when the yeast cells were cultured in media containing mannose as the sole carbon source. Structural analyses of the FOGs showed that they are structurally identical to \( O \)-mannosyl glycans that are attached to yeast glycoproteins. Consistent with this assumption, PA-Man\(_{2-5}\) prepared from yeast \( O \)-mannosyl glycans were found to co-migrate with the PA-Man\(_{2-5}\) peaks of FOGs when compared by dual-gradient reversed-phase HPLC (43) (unpublished data). We conclude that these FOGs are generated by the action of a putative endo-\( O-\alpha\)-mannosidase to \( O \)-mannosyl glycoproteins because (1) the \( pmt1\Delta \ \ pmt2\Delta \) mutant, which is defective in the first mannose transfer to Ser/Thr residues in acceptor proteins, also showed a significantly reduced generation of FOGs (Figure 1D), indicating that \( O \)-mannosylation is prerequisite for the formation of FOGs; and (2) the addition of pNP-\( \alpha \)-Man to the YPMan media did not result in the lengthening of the oligomannose structures on this compound (unpublished observation), implying that elongation of \( O \)-mannosyl glycans likely occurs on the glycoprotein itself. At present, however, the possibility of the direct lengthening of oligomannose structures with free mannose as an acceptor cannot be completely excluded. Irrespective of their sources, when we induce ER stress in yeast, FOGs were not observed, indicating that the mere ER stress is not sufficient to induce the formation of FOGs (25).

It has been reported that, in yeast, FNGs accumulate in the cytosol and are processed by \( \alpha \)-mannosidases, Ams1, that are localized in cytosol/vacuoles (25,26,33). In contrast, FOGs were predicted to be localized in the lumenal side of vesicles, since they are susceptible to digestion by the lumenal \( \alpha \)-mannosidase, Prc1pp-ManLe-FLAG. Since the digestion reaction was found to be Vps10-dependent, it was assumed that either substrates (\( O \)-mannosylated proteins) or the ManLe enzyme needed to
be delivered to post-Golgi compartments, such as vacuoles, thus facilitating the demannosylation reaction (Figure 2). This result is further supported by the observation that the overexpression of Ams1 in \textit{atg19Δ} cells, which causes Ams1 to be exclusively localized in the cytosol, did not result in a reduction of the levels of FOGs, whereas a significant reduction in FNGs was observed (unpublished observation). However, the precise location where FOGs are generated currently remains unclear. Efforts are currently underway to identify factors, including a putative de-\textit{O-}mannosylation enzyme (\textit{endo-\textit{O-\alpha-}mannosidase}) that might be involved in the generation of FOGs, in order to clarify this issue.

GDP-Man levels were increased when YPMan medium was used, suggesting that the intracellular pool of mannose is the rate limiting step in the synthesis of GDP-Man (Figure 3B). On the other hand, despite the fact that substantial amounts of FOGs were generated when YPMan medium was used, the total amounts of \textit{N-} and \textit{O-}glycans on glycoproteins were not significantly altered (Figure 3C). It should also be noted that the total amount of FOGs observed after a 3 hr culture with YPMan was about one-tenth that of the \textit{O-}glycans on glycoproteins (compare Figures 1E and 3C), indicating that formation of FOGs is likely to be a regulated event and only a subset of the \textit{O-}mannosyl glycans are released, even under specific conditions such as in YPMan media. Collectively, the increase in the total amount of intracellular FOGs was not only associated with the overloaded synthesis of the donor substrate, GDP-Man, but also with the enhanced release of \textit{O-}mannosylated glycans from proteins, both of which would be regulated by media conditions.

It was also found that, in \textit{cyc8Δ} cells, a marked increase (~10 times) in the generation of FOGs was observed when cultured in YPMan medium, suggesting that the generation of FOGs is strictly regulated by Cyc8 (Figure 4B). However, no FOGs were observed to be produced in \textit{cyc8Δ} cells when they were cultured in YPGlc medium (Figure 4B), strongly indicating that at least two factors are essential for the generation of FOGs in yeast; (i) transcriptional de-repression of the genes (by removing glucose from the media) involved in the generation of FOGs, and (ii) sensing of the presence of mannose in the media. It is noteworthy that the deletion of other binding partners of Cyc8, \textit{i.e.} Mig1 and Tup1, which are also involved in the transcriptional repression by glucose, did not result in a significant increase in the generation of FOGs (Figure 4B). Further study will be required for an understanding of the detailed mechanism by which Cyc8 controls the generation of FOGs. One potential explanation is that unidentified binding partners for Cyc8 could be involved in regulating the generation of FOGs. This hypothesis is consistent with the fact that each deletion mutant of any of the 12 known binding partners for Cyc8 (Mig1, Crt1, Rox2, Nrg1, Sko1, Cup9, Sut1, Aft1, Cin5, Skn7, Pdh1, and Yap6) (44) still did not result in any significant increase of the generation of FOGs (unpublished observation). An alternative (but distant) possibility is that the intrinsic prion-like aggregation property of Cyc8, which is caused by an internal Q-rich sequence (45), might somehow regulate the formation of FOGs that are produced in YPMan media.

In addition to the excessive generation of FOGs, \textit{cyc8Δ} cells also exhibited some severe growth defects in YPMan, consistent with a cell wall defect,
implying that the hyperactivation of the de-O-mannosylation pathway may affect the integrity of the cell wall. To the best of our knowledge, this is the first report of a yeast mutant showing a sensitivity against YPMAn media. It was also clearly shown that the PKC pathway in cyc8Δ cells was dysregulated, accompanied by constitutive activation and no further upregulation upon CFW-treatment in YPMAn media (Figure 5B). These data lead us to propose that the dysfunction of Wsc1 may accelerate the development of severe cell wall defects and cell death, and it is also tempting to speculate that the excessive de-O-mannosylation may be involved in the malfunction of Wsc1. In this connection, it should be noted that mutants defective in O-mannosylation were also unstable in Wsc1 and accordingly, would fail to inactivate the PKC pathway (29).

In summary, in this study we report on the production of novel types of free oligosaccharides, when yeast cells are cultured in YPMAn medium. We identified the structures of those glycans and found that they are structurally identical to those of O-mannosyl glycans. FOGs are most likely formed by the de-O-mannosylation of glycoproteins, and the reaction appears to be regulated by the Cyc8 transcriptional repressor. cyc8Δ cells exhibited a sensitivity towards YPMAn media, with a compromised cell wall integrity, implying the existence of a link between those phenotypic consequences and FOG formation. Clarification of detailed mechanism responsible for FOG formation will provide answers to the biological and physiological relevance of the generation/catabolism of FOGs.

**Experimental procedures**

**Yeast strains and culture conditions.**

The yeast strains used in this study are summarized in Table I. Disruption of genes was performed by one step PCR methods (27). Yeast cells were transformed by the LiOAc method (28). Yeast cells were grown in YPGlc (2% peptone, 1% yeast extract, and 2% glucose) medium. For the generation of FOGs, yeast cells were grown in YPMAn (2% peptone, 1% yeast extract, and 2% mannose) medium for 3 hr. For the culture of cells harboring plasmids, CASC media (0.67% yeast nitrogen base without amino acid, 0.5% casamino acid, and 2% glucose) were used. Incubations were carried out at 30°C unless noted otherwise.

**Plasmids used in this study**

pRS416-WSC1-HA was a generous gift from Dr. Sabine Strahl (Heidelberg University, Germany) (29). For the construction of pRS416GAP-Prc1pp-ManLe-FLAG, the cDNA sequence coding tomato (Lycopersicon esculentum) acidic α-mannosidase, ManLe (Genbank, AC209509.1) (30) was chemically synthesized and cloned into pTAC2 by Fasmac (Kanagawa, Japan) as a PCR template. A pair of following DNA fragment was then amplified; (I) 3012bp of ManLe fused with FLAG by PCR from pTAC2-ManLe using a pair of following primers, pRS416GPD-pp-SmaI-ML1-F (5′-AACAAGGGATCCCCGGAAAT ATATGGTCTACAACACTT-3′) and pRS416GPD-pp-EcoRI-ML1-flag-R (5′-GCTTGATATCGAATTCTCATTTAATCTAAGG ACATGTGAGATTTC-3′); (II) 336bp of Prc1-pre/pro signal sequence by PCR from genomic DNA of the BY4741 strain using a pair of following primers, pRS4x6-SpeI-Prc1pp-F (5′-AACAAGGGATCCCCGGAAAT ATATGGTCTACAACACTT-3′) and pRS416GPD-pp-EcoRI-ML1-flag-R (5′-GCTTGATATCGAATTCTCATTNA CATGAGGATTC-3′); (I) 336bp of Prc1-pre/pro signal sequence by PCR from genomic DNA of the BY4741 strain using a pair of the following primers, pRS4x6-SpeI-Prc1pp-F (5′-AACAAGGGATCCCCGGAAAT ATATGGTCTACAACACTT-3′) and
Those two DNA fragments were fused by overlap-extension PCR using pRS4x6-SpeI-Prc1pp-F and pRS416GPD-pp-EcoRI-ML1-flag-R primers. The Prc1pp-ManLe-FLAG fragment was then cloned into pRS416GPD digested with SpeI and EcoRI using an In-Fusion HD cloning kit (TaKaRa, Kyoto, Japan) according to the manufacturer’s protocol.

**Preparation of pyridylaminated FOGs**

Free oligosaccharides including FOGs were prepared as described previously with minor modifications (25). After growing the cells overnight, 10 OD₆₀₀ units of cells were re-inoculated into 10 ml of YPMan media and incubated for 3 hr at 30˚C to induce the generation of FOGs. The cells were then collected and washed with PBS. For the disruption of the cells, the collected cells were resuspended in 1 ml of 70% ethanol and centrifuged at 20,000 × g for 5 min at 4˚C. The resultant supernatant was collected and evaporated to dryness in a Speed-Vac concentrator. For desalting the sample, the dried samples were dissolved in water, and applied to a column of ion exchange chromatography resin, *i.e.* AG1-X2 (resin volume, 500 μl; 200–400 mesh; acetate form) followed by a column of AG50-X8 (resin volume, 500 μl; 200–400 mesh; H’form) (Bio-Rad), and the flow-through fractions containing neutral free oligosaccharides were evaporated to dryness. Pyridylation of the isolated FOGs was performed as described previously (25).

**Preparation of pyridylaminated N- and O-glycans derived from glycoproteins**

For the isolation of N- and O-glycans from glycoproteins, whole glycoproteins were extracted by a hot citrate extraction method as described previously (31). N-glycans and O-glycans were released by a PNGaseF treatment and hydrazinolysis, respectively, as described previously (31,32). The desalting and PA-labeling of the glycans were performed as described in “Preparation of pyridylaminated FOGs”.

**Digestion of free glycans with a glycosidase**

Digestion of the FOGs by glycosidases was carried out as described previously (25). Briefly, for the digestion of FOGs with jack bean α-mannosidase (40 mU, Seikagaku corp., Tokyo, Japan) or *Aspergillus saitoi* α-1,2 mannosidase (0.5 mU, Seikagaku corp., Tokyo, Japan), the samples, in 20 μl of the 50 mM of sodium acetate buffer (pH 5.0) at 37˚C, were incubated with the enzymes for 6 hr. The digestion of FOGs with *Xanthomonas manihotis* α-1,2/3,-mannosidase (60 U; New England Biolabs, Beverly, MA) was carried out in a 20 μl of reaction buffer supplied by the manufacturer at 37˚C for 6 hr. The enzyme reaction was terminated by adding 100 μl of 100 % ethanol to the incubation. After centrifuging the samples at 20,000 × g for 5 min at 4˚C to remove insoluble materials, the supernatant was evaporated to dryness and the residue was then resuspended in water for HPLC analysis.

**HPLC analysis of glycans**

Size fractionation HPLC with a Shodex NH2P-40 3E column (3.0 mm I.D. × 250 mm, Shodex, Tokyo, Japan) was performed as described previously (33). The elution involved the use of two solvent gradients, solvent A (93% acetonitrile in 0.3% acetate (pH 7.0)) and solvent B (20% acetonitrile in 0.3%
acetate (pH 7.0)). The column temperature was set at 25°C and the flow rate was 0.45 ml/min. The gradient program was as follows: 0-0.5 min, 1-10% solvent B; 0.5-45 min, 10-55% solvent B; 45.1-47 min isocratic 70% solvent B; 47.1-67.1 min isocratic 1% solvent B. For the quantification of the peak areas, each peak area was normalized by that of PA-labeled glucose hexamer (2 pmol/area) in PA-Glucose oligomer standard (degree of polymerization: 3-15, TaKaRa, Kyoto, Japan). The peaks corresponding to Man2,5 were measured as the total amount of FOGs through all experiments and normalized by 1.0 OD600 units of cells (approximately 2.0 x 10^7 cells).

MALDI-ToF-MS analysis
MALDI-ToF-MS analyses were carried on an AXIMA-QIT instrument (Shimadzu, Kyoto, Japan) using 2,5-dihydroxybenzoic acid (Shimadzu, Kyoto, Japan) as the matrix as described previously (34).

Quantitative analysis of nucleotide sugars
The preparation and Ion-Pair Reversed-Phase HPLC of the nucleotide sugars were carried out as described previously (35). Each peaks derived from UDP-Glc, UDP-GlcNAc, and GDP-Man were assigned by comparing its elution position with that of standards (Sigma Aldrich, St Louis, MO, USA).

Protein extraction and immunoblot analysis
For immunoblot analysis, cells were grown in appropriate media overnight. After the saturated cells were re-inoculated into fresh media, they were grown to the mid-log phase. Five OD600 units of cells were collected and washed with distilled water. Whole protein was extracted by treating cell pellets with 50 µl of 200 mM of NaOH for 20 min on ice, after which 50 µl of 2 × sample buffer (0.25 M Tris-HCl (pH 6.8), 8% SDS, 40% glycerol, 0.02% B PB, and 0.4 M DTT) was added, and the samples were boiled for 5min. Aliquots of 5 µl of the supernatant were subjected to SDS-PAGE analysis. The separated proteins were transferred to a polyvinyl dene difluoride (PVDF) membrane and the membrane was blocked with TTBS (25mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% (v/v) Tween 20) containing 0.5% (w/v) skim milk. The blot was incubated with F-7, an anti-HA mouse monoclonal antibody (for detection of Wsc1-HA; 1: 10,000; Santa Cruz Biotechnology, Inc. Santa Cruz, CA), an anti-phospho-p44/42 MAPK polyclonal rabbit antibody (for detection of phosphorylated Mpk1; 1:5000; Cell Signaling Technology Japan, (Tokyo, Japan)), or an anti-Pgk1 mouse monoclonal antibody,22C5 (1: 10,000; Thermo Fisher Scientific, Waltham, MA), followed by incubation with an HRP conjugated anti-mouse IgG antibody (1:5,000; GE Healthcare, Piscataway, NJ) or an HRP conjugated anti-rabbit IgG antibody (1:5,000; GE Healthcare) both in the TTBS containing 0.5% (w/v) skim milk. Bands were visualized using LAS3000 mini (Fujifilm co, Tokyo, Japan) using Immobilon Western Reagents (Millipore).

RNA-Seq
Yeast cells were harvested using membrane filters (mixed cellulose esters, 0.45 µm, Merck), and directly transferred into liquid nitrogen. Frozen cells were mixed with frozen droplets of 600 µL lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl, 1 mM DTT, 100 µg/ml cycloheximide, and 1% Triton X-100), and lysed by Multi-beads Shocker
Lysates were treated with 25 U of TURBO DNase (Thermo Fisher Scientific) and cleared by centrifugation at 20,000 \( \times \) g for 10 min at 4°C. TRIzol LS reagent (Thermo Fisher Scientific) and Direct-zol RNA Kit (Zymo research, Irvine, CA) were used for the extraction of total RNA. Ribosomal RNAs were depleted using the Ribo-Zero Gold rRNA Removal Kit (Yeast) (Illumina, San Diego, CA) and the preparation of RNA-seq library was performed using TruSeq Stranded mRNA Library Prep Kit (Illumina) following the manufacturer's instructions. The libraries were sequenced on a HiSeq 4000 (Illumina). Reads were mapped to yeast transcriptome using TopHat v2.0.9 (36). We counted the number of reads within coding sequence for each mRNA, and performed the differential expression analysis using the DESeq package (37). The custom R scripts will be available upon requests.

**Accession number**

RNA-Seq data (GSE130332) used in this study were deposited in NCBI.

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**Conflict of interest**

The authors declare that they have no conflicts of interest with the contents of this article.

**Author contributions**

HH and TS designed the experiments. HH, TM, YT, and JS performed the HPLC experiments and analyzed the results. RO, CH analyzed the extracellular/intracellular protein level of Wsc1 by immoblot. KN analyzed/quantified the intracellular pools of nucleotide sugars. YN performed study of the localization of fOGs using ManLe-FLAG and analyzed results. YS and SI helped RNA-Seq analysis. HH wrote the paper with TS.
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Figure legends

Figure 1 Unknown source of the free oligosaccharides are generated in cells cultured in mannose-containing media. **A**, HPLC profile of the free oligosaccharides derived from the yeast cells cultured with various carbon sources (YPSuc, YPGal, YPMAn, YPGly, and YPGlc contain 2% sucrose, galactose, mannose, glycerol, and glucose, respectively). YP media contains no carbon source. Asterisks and Hex7–9 represent non-specific peaks and those of PA-labeled Hex7,9HexNAc2, respectively. **B**, Unknown peaks are sensitive to a jack bean α-mannosidase treatment. **C**, HPLC profile of the fOGs in pmt1Δ pmt2Δ and ktr1 Δkre2 Δktr3Δ mutants and the structures of the O-glycan in yeast. First, a mannose on Ser/Thr residues on glycoproteins is transferred via the action of the protein O-mannose transferase, Pmt1-6. The Golgi-resident α-1,2 mannosyltransferases Ktr1, Kre2, and Ktr3, are involved in the transfer of the second and third mannose units. Two outer mannoses are transferred via the action of α-1,3-mannosyltransferases, Mnn1, Mnt2, and Mnt3. Mannosyltransferases with bold letters are primary enzymes in each transfer step (11,12). Mutants used this analysis, pmt1Δ pmt2Δ and ktr1Δ kre2Δ ktr3Δ, are all in ams1Δ background. **D**, Quantitative analysis of the generation of FOGs of Figure1C. **E**, HPLC profile of the FOGs/FNGs in sec18-1 mutant defective in the ER-to-Golgi trafficking. **F**, Time course for the generation of FOGs in the cells cultured with YPMan. Error bar, mean ±S.D. from three independent experiments. For statistical analysis, Student’s t-test was applied. The symbols * and ** represent p<0.05 and p<0.01, respectively.

Figure 2 FOGs accumulate in the compartment where the proteins are sorted by Vps10. **A**, Schematic representation of the primary structure of the ManLe-FLAG fused with the vacuole targeting signals of Prcl, the Prcl-prepro sequence. **B**, Expression level and glycosylation status of ManLe-FLAG in ams1Δ and vps10Δ ams1Δ are analyzed by immunoblot analysis. **C**, Accumulated FOGs are sensitive to the Prclpp-ManLe-FLAG that is targeted to the late secretory pathway in Vps10 dependent fashion. Error bar, mean ±S.D. from three independent experiments. For statistical analysis, Student’s t-test was applied. The symbol ** represents p<0.01.

Figure 3 Quantitative analysis of the total amount of nucleotide sugars, N- and O-glycans on glycoproteins. After cultivating the cells in YPGlc or YPMan for 3 hr, the levels of nucleotide sugars, N- and O-glycans on glycoproteins were determined. **A**, Schematic representation of the biosynthetic pathway for nucleotide sugars, cell wall polymers, and glycans. For statistical analysis, Tukey-Kramer test was used to calculate difference between the groups. **B**, Total amount of GDP-Man is significantly increased in the case of cells cultured in YPMan. For statistical analysis, Student’s t-test was used to calculate differences between groups. **C**, the generation of N- and O-glycans on glycoproteins are comparable in the both culture conditions, i.e. YPGlc and YPMan. Error bar, mean ±S.D. from three independent experiments. The symbols n.s., *, **, and *** represent “not significant”, p<0.05, p<0.01, and, p<0.005, respectively.

Figure 4 Generation of FOGs is strictly controlled by the general transcriptional co-repressor, Cyc8. **A**, Generation of FOGs is suppressed by the addition of glucose in the YPMan medium. **B**, Disruption of CYC8 gene causes hyper activation of the FOGs generation
pathway. C, Growth phenotype of cyc8Δ cells on the plates containing several carbon sources. D, Spotting assay of cyc8Δ cells. Serial diluted cells (∗5) were spotted on the plates. Error bar, mean ±S.D. from three independent experiments. For statistical analysis, Student’s t-test was applied. The symbols n.s., *, **, and *** represent “not significant”, p<0.05, p<0.01, and, p<0.005, respectively.

Figure 5 Aberrant processing of O-glycosylation in the cell surface sensor protein for the cell wall integrity pathway, Wsc1, may causes the constitutive activation of Mpk1 in cyc8Δ cells. A, Schematic representation of the cell wall integrity signaling pathway. B, Immunoblot analysis of phosphorylated Mpk1 by an anti-phosphorylated Mpk1 antibody with or without activation of the cell wall integrity pathway by 20 µg/ml of CFW in YPGlc of YPMan media. C, Immunoblot analysis of Wsc1-HA in wild-type and cyc8Δ cells culture with YPGlc or YPMan. The relative amount of Wsc1-HA is indicated at the bottom of each lane. D, Analysis of transcriptional regulation of Wsc1 under YP Man culture. MA plot for differential expression analysis in wild-type and cyc8Δ cells. The mean of read counts normalized to library size versus the log2 fold-change between YPMan and YPGlc media was shown. The WSC1 gene is highlighted as a black dot. E, The relative transcription level of WSC1 in WT and cyc8Δ cells shown in Figure5D.
Table I, Yeast strains used in this study

| Strain name  | Plasmid          | Genotype                                                                 | Source |
|--------------|------------------|--------------------------------------------------------------------------|--------|
| BY4741       | –                | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0                                       | Lab strain |
| W303-1A      | –                | MATα leu2-3,112 his3-11 ade2-1 ura3-1 trpl-1 can1-100                    | Lab strain |
| YS63-1C      | –                | MATα sec18-1 leu2 ura3 trpl his3                                        | Lab strain |
| YME2093      | –                | MATα amsΔ1::hphNT1 pmt1Δ::kanMX4 pmt2Δ::His3MX6 BY4741                   | This study |
| YME2090      | –                | MATα amsΔ1::His3MX6 ktr1Δ::hisG kre2Δ::hisG ktr3Δ::hisG W303             | This study |
| ‡A. saitoi   | –                | MATα cye8Δ::kanMX4 BY4741                                              | Open Biosystems |
| YHH027       | pRS416-WSC1-HA   | MATα BY4741                                                            | This study |
| YHH031       | pRS416-WSC1-HA   | MATα cye8Δ::kanMX4 BY4741                                              | This study |
| YHH120       | pRS416GPD-Prec1pp-ManLe | MATα amsΔ1::kanMX6 BY4741                                      | This study |
| YHH121       | pRS416GPD-Prec1pp-ManLe | MATα amsΔ1::His3MX6 vps10Δ::kanMX4 BY4741                           | This study |
| YHH130       | pRS316           | MATα amsΔ1::kanMX6 BY4741                                              | This study |

Table II, MALDI-ToF MS analysis of size-fractionated glycans

| Peak | Observed mass (m/z) | Composition |
|------|---------------------|-------------|
| a    | 443.0 [M+Na]+      | Hex2-PA     |
| b    | 605.3 [M+Na]+      | Hex3-PA     |
| c    | 767.3 [M+Na]+      | Hex4-PA     |
| d    | 929.5 [M+Na]+      | Hex5-PA     |

Table III, Linkage analysis of unidentified glycans

| Peak | α-Man’ ase | α-1,2-Man’ ase | α-1,2/3-Man’ ase | α-1,2-Man’ ase | Predicted structure |
|------|------------|---------------|-----------------|---------------|---------------------|
| a    | +          | −             | +               | +             | ＠Man2              |
| b    | +          | +*            | +               | +             | ＠Man3              |
| c    | +          | −             | +               | +             | ＠Man4              |
| d    | +          | −             | +               | +             | ＠Man5              |

‡A. saitoi α-1,2-Man’ase cannot digest PA-labeled α-1,2-mannobiose, which has ring-opening mannose moiety in their reducing end.

*Man3 was converted to Man2 due to the substrate specificity of A. saitoi α-1,2-Man’ase as mentioned above.
Figure 2, Hirayama et al.
Figure 3, Hirayama et al.
Figure 4, Hirayama et al.
Figure 5, Hirayama et al.
Free glycans derived from O-mannosylated glycoproteins suggest the presence of an O-glycoprotein degradation pathway in yeast

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