Free-oligosaccharide control in the yeast *Saccharomyces cerevisiae*: roles for peptide:N-glycanase (Png1p) and vacuolar mannosidase (Ams1p)

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Free oligosaccharides (fOS) are generated during glycoprotein biosynthesis in mammalian cells. Here we report on the origin and fate of these structures in the yeast *Saccharomyces cerevisiae*. After metabolic radiolabelling with [2-³H]mannose ([2-³H]Man) for 30 min, Man₈GlcNAc₂ was identified as the predominant fOS in this organism, and radioactivity associated with this structure was found to correspond to ≈ 1 % of that associated with the same structure N-linked to glycoprotein. Despite provoking a fourfold increase in radioactivity associated with lipid-linked oligosaccharide, the protein-synthesis inhibitor cycloheximide blocked [2-³H]Man incorporation into both endo-β-D-N-acetylglucosamine H-sensitive N-glycans and fOS. Peptide:N-glycanase, encoded by the *PNG1* gene, was found to

be required for the generation of a large proportion of yeast fOS during, and soon after, protein glycosylation. Use of an *ams1*Δ strain deficient in the vacuolar α-mannosidase revealed this enzyme to be responsible for the slow growth-associated catabolism of fOS. The present paper constitutes the first description of fOS formation in intact *S. cerevisiae*, and, with the demonstration that fOS are degraded by the vacuolar mannosidase, a novel function for this poorly understood enzyme has been identified.

Key words: endoplasmic reticulum, endoplasmic reticulum-associated protein degradation (ERAD), free oligosaccharide, N-glycanase, vacuolar mannosidase, vacuole.

INTRODUCTION

In yeast and mammalian cells, the biosynthesis of glycoproteins bearing N-linked oligosaccharide starts with the assembly of a lipid-linked oligosaccharide (LLO; Glc₃Man₉GlcNAc₂-PP-dolichyl) in the membrane of the endoplasmic reticulum (ER). The oligosaccharide portion of this structure is transferred on to nascent proteins in the lumen of the ER by the oligosaccharyl-transferase complex (OST) [1,2]. After folding, glycoproteins are transported to the Golgi apparatus, where their N-linked oligosaccharides are remodelled. In mammalian cells, N-linked oligoMan-type oligosaccharides are trimmed by Golgi mannosidases prior to being replaced by N-acetylglucosamine (GlcNAc), galactose, fucose and sialic acid residues to form the complex-type N-linked oligosaccharide chains which are known to play important roles in many physiological processes [3]. By contrast, the yeast Golgi apparatus does not contain mannosidase activities, and the elongation of N-glycans is generally restricted to the further addition of Man residues. Accordingly, in *Saccharomyces cerevisiae*, two types of N-linked glycan structure are commonly encountered: oligomannose-type glycans bearing 8–14 Man residues, and polymannose ‘mannan’-type structures bearing several hundred Man residues [4,5]. These mannan-bearing proteins are destined to become part of, or associate with, the yeast cell envelope, and comprise inducible hydrolytic enzymes, such as external invertase, and structural mannoproteins [6,7].

Glycoprotein biosynthesis in mammalian cells is accompanied by the generation of free oligosaccharides (fOS) from both LLO [8] and newly synthesized glycoproteins [9] that undergo ER-

associated protein degradation (ERAD). Accordingly, fOS may be generated by OST-mediated hydrolysis of Glc₃Man₉GlcNAc₂-PP-dolichyl in the lumen of the ER [8,10,11], or peptide N-glycanase (PNGase)-mediated de-N-glycosylation of newly synthesized glycoproteins either in the ER [12,13] or the cytosol [14,15]. Furthermore, phosphorylated fOS have been reported to be liberated from dolichyl-oligosaccharides by a cytosolically orientated ER-situated pyrophosphatase [16]. fOS which are liberated in the lumen of the ER can be transported into the cytosol [17,18], where they are trimmed by an endo-β-D-N-acetylglucosamine H (endo H)-like enzyme [19,20] and an α-mannosidase [21–24] in order to yield an oligosaccharide, Man₅GlcNAc [25], that can be imported directly into lysosomes to be degraded [26,27].

In order to further understand the significance of fOS metabolism, and to characterize the molecular machinery involved in this process, we have initiated a study of this phenomenon in the yeast *S. cerevisiae*. To date there have been no reports on the occurrence of fOS in intact yeast cells, but phosphorylated and neutral fOS were detected when yeast microsomes were radiolabelled with GDP-Man [28]. As the initial, ER-situated, steps of glycoprotein biosynthesis, quality control and degradation are reasonably well conserved between yeast and mammalian cells [1], it is likely that some events leading to the formation of fOS may also be common to these two cell types. Here, for the first time, we demonstrate the presence of fOS in intact *S. cerevisiae*. Experiments show that a major population of fOS is generated by the yeast PNGase Png1p during and shortly after glycoprotein biosynthesis. Furthermore, fOS are degraded by the vacuolar α-mannosidase Ams1p in a slow, growth-associated, manner.

Abbreviations used: Man, mannose; Glc, glucose; GlcNAc, N-acetylglucosamine; fOS, free oligosaccharide(s); LLO, lipid-linked oligosaccharide; ER, endoplasmic reticulum; endo H, endo-β-D-N-acetylglucosamine H; AP, 2-aminopyridine; OST, oligosaccharyltransferase; ERAD, endoplasmic reticulum-associated protein degradation; *PNG1*, gene coding for the yeast peptide:N-glycanase (PNGase) Png1p; CHX, cycloheximide.

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Table 1 Yeast strains used in the present study

Strain	Genotype	Source
CENPK 122-2N	Wild-type	M. Jacquet
BY4742	MAT α ; <i>his3</i> Δ 1; <i>leu2</i> Δ 0; <i>lys2</i> Δ 0; <i>ura3</i> Δ 0	EUROSCARF
MKY114	MAT α ; <i>ams1::LEU2</i> ; <i>ura3-52</i> ; <i>leu2-3,112</i> ; <i>his4-619</i>	A. Herscovics
YFL9.26	MAT α ; <i>ams1::LEU2</i> ; <i>mns1::URA3</i> ; <i>his4-619</i>	A. Herscovics
Y16930	MAT α ; <i>his3</i> Δ 1; <i>leu2</i> Δ 0; <i>lys2</i> Δ 0; <i>ura3</i> Δ 0; <i>mns1::kanMX4</i>	EUROSCARF
Y12156	MAT α ; <i>his3</i> Δ 1; <i>leu2</i> Δ 0; <i>lys2</i> Δ 0; <i>ura3</i> Δ 0; <i>png1::kanMX4</i>	EUROSCARF
Y11861	MAT α ; <i>his3</i> Δ 1; <i>leu2</i> Δ 0; <i>lys2</i> Δ 0; <i>ura3</i> Δ 0; <i>ost3::kanMX4</i>	EUROSCARF
Y13929	MAT α ; <i>his3</i> Δ 1; <i>leu2</i> Δ 0; <i>lys2</i> Δ 0; <i>ura3</i> Δ 0; <i>ost4::kanMX4</i>	EUROSCARF
Y14592	MAT α ; <i>his3</i> Δ 1; <i>leu2</i> Δ 0; <i>lys2</i> Δ 0; <i>ura3</i> Δ 0; <i>ost5::kanMX4</i>	EUROSCARF

EXPERIMENTAL

Yeast strains and culture

S. cerevisiae strains used in the present study are listed in Table 1 and were either purchased from EUROSCARF (Frankfurt, Germany) or were kindly donated by Professor Annette Herscovics (McGill Cancer Centre, McGill University, Montreal, Quebec H3G 1Y6, Canada), and Professor Michel Jacquet (Groupe Information Génétique et Développement, Université de Paris-Sud, Orsay, France). All yeast strains were grown aerobically in YPD [1 % yeast extract/2 % (w/v) peptone/2 % (w/v) glucose (Glc)] medium at 30 °C in a rotary shaker (250 rev./min).

Reagents

Yeast extract and SELECT Peptone 140 were purchased from Gibco BRL (Cergy Pontoise, France). D-Sorbitol was obtained from Fluka (Saint Quentin Fallavier, France). [2-³H]Man (20 Ci/mmol) and En³Hance spray were from PerkinElmer Life Sciences (Zaventem, Belgium). Glc and silica-coated TLC plates were obtained from Merck (Darmstadt, Germany). AG 50-X2 (H⁺ form) and AG 1-X2 (acetate form) resins and Bio-Gel P2 came from Bio-Rad S.A. (Marnes la Coquette, France). Charcoal was from BDH Laboratory Supplies (now VWR International Ltd., Poole, Dorset, U.K.). *Aspergillus saitoi* α 1,2-mannosidase was purchased from Oxford GlycoSciences (through Cogec, Paris, France). Adenine, 2-aminopyridine, the bicinechoninic acid ('BCA') protein assay kit, cycloheximide, lyticase, endo-glucosaminidase H, jack-bean (*Canavalia ensiformis*) α -mannosidase, *p*-nitrophenyl α -D-mannopyranoside, Pronase and Triton X-100 were purchased from Sigma-Aldrich Chimie SARL (Saint Quentin Fallavier, France). X-Omat AR film was from Kodak (New York, NY, U.S.A.).

Metabolic radiolabelling of yeast

For metabolic radiolabelling experiments, large-scale cultures were initiated by inoculating YPD medium with overnight precultures at an attenuation (*D*) at 600 nm of 0.1. The conditions chosen for yeast fOS radiolabelling were as follows. Cells (6×10^8) grown to mid-exponential phase in YPD medium were harvested and resuspended in 700 μ l of fresh YPD medium. A 200 μ Ci portion of [2-³H]Man was added and the cells were incubated for 30 min with vigorous shaking at 30 °C. Radiolabelling was stopped by adding 10 vol. of ice-cold PBS. When chase incubations were performed, the pulse radiolabelled cells were resuspended in 1 ml of fresh YPD medium and incubated as above. In some experiments, cells were preincubated

in the presence of 10 μ g/ml cycloheximide (CHX) [29] for 15 min prior to the addition of [2-³H]Man.

Extraction of fOS, glycoproteins and LLO

Cells were extracted by a modification of a previously described method [30]. Briefly, the cell pellet was suspended in 5 ml of methanol/100 mM Tris/HCl, pH 7.4 (2:1, v/v), containing 4 mM MgCl₂. An equal volume of chloroform was added and the mixture was vigorously shaken. After centrifugation, the lower (chloroform) and upper phases (methanolic) were removed, and the interphase re-extracted. The newly obtained phases were pooled with the previous phases. All procedures were carried out at 4 °C with ice-cold solvents. Neutral fOS were recovered from the methanolic phase as described previously [25]. Briefly, this phase was dried, dissolved in water and after desalting on AG-1-X2/AG-50-X2 columns, neutral material was loaded on to charcoal columns, which were then rinsed with water prior to elution of fOS with 30 % (v/v) ethanol. In one experiment the methanolic phase was dried and submitted to size-exclusion chromatography on a 1 cm $\times$ 28 cm column of Bio-Gel P2 equilibrated with 100 mM acetic acid. Radioactive material eluted at the void volume of the column was recovered and separated into neutral, positively charged and negatively charged species using AG-50 and AG-1 resins. Oligosaccharides were released from LLO as previously reported [31].

Enzyme digestions and assays

Pronase digests of dried delipidated protein pellets were performed in 200 μ l of 100 mM Tris/HCl, pH 8, containing 4 mM CaCl₂, and 5 mg/ml of enzyme at 37 °C for 18 h, and terminated by heating at 100 °C for 5 min. After centrifugation, 100 μ l of the supernatant was dried for endo H digestion. Endo H digestions of glycopeptides, fOS or 2-aminopyridine-derivatized oligosaccharides were performed by incubating the substrate in the presence of 10 munits of enzyme in 50–100 μ l of sodium citrate buffer, pH 5.5, for 15 h at 37 °C. Jack-bean α -mannosidase (1 unit) and *Aspergillus saitoi* α 1,2-mannosidase (5 μ units) digestions were performed in 100 μ l of 40 mM sodium acetate, pH 5.0, overnight at 37 °C. Vacuolar α -mannosidase was assayed [32] in spheroblasts [33] treated with 0.2 % Triton X-100. Protein was measured by the bicinechoninic acid (BCA) method [34].

Chromatographic procedures

Oligosaccharide mixtures were resolved by TLC on silica-coated plastic sheets (0.2 mm thickness) in propan-1-ol/acetic acid/water (3:3:2, by vol.) for 36–48 h. Standard oligosaccharides

Man₅GlcNAc₂ [27], Man₈₋₉GlcNAc₂ [18], and the three isomers of Man₈GlcNAc₂ [31,35] were obtained as previously described. Glc₁Man₉GlcNAc was obtained by resolving the mixture of oligosaccharides released from endo H treatment of glycoproteins derived from pulse-radiolabelled HepG2 cells [25]. Resolved radioactive components were visualized by fluorography and, where indicated, were further quantified by scintillation counting after elution from the silica. For HPLC analysis, fOS were treated with endo H prior to being derivatized with 2-aminopyridine as described by Hase et al. [36]. The aminopyridinyl derivatives were analysed on a Hypersil ODS2 column (Supelco 250 mm × 4.5 mm) as previously described [31], using a Waters 600 solvent-delivery system coupled to a Packard 150 TR flow-scintillation analyser.

RESULTS

Identification of fOS in *S. cerevisiae*

Exponentially growing cells were metabolically radiolabelled with [2-³H]Man in YPD for 30 min. Radioactive material recovered in the methanolic phase of the biphasic solvent system used to extract the cells was dried and submitted to size-exclusion chromatography on a Bio-Gel P2 column as shown in the left panel of Figure 1(A). The bulk of the radioactivity found in the fractions indicated by the horizontal bar in Figure 1(A) was found to be cationic in nature, and was not further characterized. Anionic components, comprising 12.8% of the total, were subjected to alkaline phosphatase digestion, which neutralized less than 10% of the starting radioactivity. Although this alkaline phosphatase-sensitive material may correspond to fOS phosphates, which have previously been shown to be generated by yeast microsomes upon incubation with radioactive sugar-nucleotides [28], the low abundance of this material made further characterization difficult. The neutral radioactive components (19.6% of total) recovered from the Bio-Gel P2 column were analysed by TLC, and as shown in the right-hand panel of Figure 1(A) (method b; Bio-Gel), the predominant structure co-chromatographed with standard Man₈GlcNAc₂. Because the nature of this yeast neutral fOS material resembled that known to occur in mammalian cells [37], the protocol routinely employed to recover neutral fOS from the latter cell type, which includes a charcoal-chromatography step, was tested on yeast extracts, and found to give identical results (right panel of Figure 1A). Accordingly, yeast neutral fOS were routinely recovered using the more rapid protocol c.

In order to optimize radiolabel incorporation into fOS, the effects of cell growth and the Glc concentration in the radiolabelling medium were investigated. Cells were harvested at different times during growth and radiolabelled with [2-³H]Man for 30 min prior to quantifying endo H-sensitive N-glycans and fOS. Inspection of Figure 1(B) demonstrates, first, that incorporation of radioactivity into fOS and N-linked glycans is maximal during exponential growth and, secondly, that, during a 30 min radiolabelling period, the quantity of fOS generated corresponds to ≈ 1% of that of its N-linked counterpart. When cells were radiolabelled in the absence of Glc, there was a twofold increase in radioactivity associated with fOS over that obtained when radiolabelling was carried out in YPD. As demonstrated in Figure 1(C), radiolabelling cells in the absence of Glc provoked the appearance of a fOS that co-chromatographed with Man₅GlcNAc₂. Structural and kinetic experiments indicated that the smaller species was not generated from the larger fOS by a trimming process, but is cleaved from either immature LLO or glycoprotein (results not shown). Furthermore, examination of the structure of LLO showed that in the absence, or presence of low

Figure 1 Identification of fOS in *S. cerevisiae*

(A) Wild-type cells (CENPK) were pulse-radiolabelled with [2-³H]Man for 30 min in YPD. After extraction with organic solvents as described in the Experimental section, the methanolic solvent phase was submitted to Bio-Gel P2 chromatography. The column fractions indicated by the horizontal bar were pooled and analysed by ion-exchange chromatography. Anionic material was eluted from AG 1-X2 (acetate form) resin with 3 M formic acid. This material was digested with alkaline phosphatase, again subjected to AG 1-X2 chromatography, and neutral material was assayed by liquid-scintillation counting (³AP). Cationic structures were eluted from AG 50-X2 (H⁺ form) resin with 2 M pyridine acetate, pH 5. Neutral components did not bind to the coupled ion-exchange columns and were eluted in water. This neutral material was analysed by TLC (method b; Bio-Gel) and compared with fOS that were purified by the charcoal method (method c; charcoal), as described in the Experimental section, from a duplicate incubation. (B) Wild-type cells, harvested at different times of culture, were pulse-radiolabelled as described above. Oligosaccharides released from Pronase-digested proteins by endo H, and fOS prepared by method c, were resolved by TLC. The glycoprotein-derived oligosaccharide migrating as Man₈GlcNAc₂ (GP), and the fOS co-migrating with Man₈GlcNAc₂ (fOS), were eluted from the TLC plates and quantified by liquid-scintillation counting. (C) Wild-type yeast cells grown to mid-exponential phase were radiolabelled as described above in either YP medium (0% Glc) or YPD medium (2% Glc), and fOS purified by method c were analysed by TLC. The migration positions of standard oligosaccharides are shown to the left of the chromatographs, and the abbreviations used are: M₉, Man₉GlcNAc₂; M₈, Man₈GlcNAc₂; M₅, Man₅GlcNAc₂. OD 600 nm, D₆₀₀.

Glc levels, Man₅GlcNAc₂-PP-dolichyl was always more abundant than mature Glc₃Man₉GlcNAc₂-PP-dolichyl (results not shown). In mammalian cells, a high ratio of Man₅GlcNAc₂-PP-dolichyl to Glc₃Man₉GlcNAc₂-PP-dolichyl is characteristic of cellular stress and, in particular, of Glc deprivation [38,39]. Accordingly, in

Figure 2 Characterization of the major fOS occurring in pulse-radio-labelled *S. cerevisiae*

Wild-type yeast cells grown to mid-exponential phase were pulse-labelled with [^3H]Man for 30 min. Total fOS were extracted and resolved by TLC. The major fOS component was then eluted from the TLC plate and submitted to either endo H (A) or *A. saitoi* mannosidase ('M'ase; B) digestions. (C) The endo H-digested oligosaccharide shown in (A) was coupled to 2-aminopyridine and subjected to reverse-phase HPLC. The HPLC column was calibrated with the three isomers of $\text{Man}_8\text{GlcNAc}$ in which the terminal non-reducing Man residue on the $\alpha 1,3$ -linked (A), middle (B), and $\alpha 1,6$ -linked (C) branch of the polyMan unit is missing. A scheme of the structure of the major fOS in yeast is shown. (D) Wild-type yeast (wt), and a mutant lacking ER mannosidase activity (*mns1* Δ), were pulse-radio-labelled as described for (A). fOS were recovered and analysed by TLC. The migration positions of standard oligosaccharides are shown to the left of the chromatograms. The extra abbreviations used are as described in Figure 1. M_8GN , $\text{Man}_8\text{GlcNAc}$; M_8GN_2 , $\text{Man}_8\text{GlcNAc}_2$; AU, arbitrary units.

order to simplify interpretation of our results, subsequent radio-labelling experiments were performed in 2% Glc.

The ER-mannosidase-Mns1p-generated isomer of $\text{Man}_8\text{GlcNAc}_2$ is the major fOS structure generated in [^3H]Man-radiolabelled *S. cerevisiae*

The major fOS generated in *S. cerevisiae* is sensitive to endo H and yielded a structure that co-migrated with $\text{Man}_8\text{GlcNAc}$ during TLC, thereby confirming that the parent fOS possessed the di-*N*-acetylchitobiose moiety at its reducing terminus (Figure 2A). *A. saitoi* $\alpha 1,2$ -mannosidase digestion of the fOS yielded a $\text{Man}_5\text{GlcNAc}_2$ product which co-migrated with that generated by digestion of standard $\text{Man}_9\text{GlcNAc}_2$ with the same enzyme (Figure 2B). These results suggest that the major fOS identified in *S. cerevisiae* comprises one or more of the three

Figure 3 Roles of OST, protein synthesis and PNGase on fOS formation

(A) The BY4742 (isogenic for mutants), *ost3* Δ , *ost4* Δ , *ost5* Δ , and *png1* Δ strains were pulse-radio-labelled with [^3H]Man for 30 min. The CENPK strain (wild-type used for CHX experiment) was preincubated in either the absence or presence of 10 $\mu\text{g}/\text{ml}$ CHX for 15 min before pulse-radio-labelling as described above. LLO, fOS and glycoproteins (GP) were extracted from the cells as described in the Experimental section. fOS and their counterparts released from LLO and GP were resolved by TLC as shown (for the BY4742, and *png1* Δ strains) in (B). Fluorographs of the chromatograms were scanned densitometrically, and the quantity of the predominant oligosaccharide present in each fraction [LLO; $\text{Glc}_3\text{Man}_8\text{GlcNAc}_2$ (G_3M_8), fOS; $\text{Man}_8\text{GlcNAc}_2$ (M_8 , closed arrowhead), GP; $\text{Man}_8\text{GlcNAc}$ (M_8 , open arrowhead)] has been expressed as a percentage of that found to occur in the appropriate wild-type incubation.

isomers of $\text{Man}_8\text{GlcNAc}_2$. However, as shown in Figure 2(C), only one $\text{Man}_8\text{GlcNAc}_2$ species could be detected, and this corresponded to isomer B, which is known to be generated by ER mannosidase I (see Figure 2C for structure). This result was reinforced by the observation (Figure 2D) that the *mns1* Δ yeast strain, deficient in ER mannosidase I, yielded a mixture of fOS whose predominant component co-migrated with standard $\text{Man}_9\text{GlcNAc}_2$.

The PNG1 gene product generates the bulk of fOS in *S. cerevisiae*

Using yeast strains deficient in various subunits of OST, it was noted that fOS generation and protein glycosylation are tightly coupled. Accordingly, as shown in Figure 3(A), where incorporation of [^3H]Man into glycoproteins was observed to

be reduced with respect to that observed in wild-type cells, incorporation of radioactivity into fOS, but not LLO, was also reduced by a similar factor. These results demonstrate that the bulk of fOS formation is consequent upon OST activity. The protein synthesis inhibitor CHX caused a ≈ 4 -fold increase in radioactivity associated with LLO, but blocked the formation of glycoproteins and fOS by greater than 95 %, indicating that the bulk of fOS are derived from the de-N-glycosylation of glycoproteins (Figure 3A). A PNGase activity has been identified in *S. cerevisiae*, and is encoded by the *PNG1* gene [15]. An examination of fOS formation in the *png1* Δ strain revealed that whereas [2- 3 H]Man incorporation into LLO and glycoproteins was somewhat elevated when compared with that observed in the wild-type strain, fOS formation was reduced by 70–80 % in the mutant cells (Figure 3A). TLC of the residual fOS that occur in the *png1* Δ strain reveals that Man₈GlcNAc₂ is also the predominant species in this Png1p-independent fOS pool (Figure 3B). Although the major N-glycan recovered from pulse-radiolabelled wild-type or *png1* Δ cells co-migrates with Man₈GlcNAc during TLC, two other types of structures are also observed. First, those indicated by the curly ('face') bracket in Figure 3(B) may correspond to the Man_{9–14}GlcNAc 'core' structures that are generated by Golgi mannosyltransferases [4]. These structures are known to be released by endo H from total glycoproteins derived from exponentially growing *S. cerevisiae* [40]. In addition, this group of structures may contain glucosylated species, as has previously been described [40]. Secondly, radioactivity was incorporated into a group of components (indicated by the square bracket; also apparent in the LLO fraction in some experiments) that remained close to the origin under our TLC conditions. We have not characterized these components, but they may represent large mannan fragments released from cell-wall constituents [6,7] before or during the sequential Pronase and endo H treatments (see the Experimental section). Whatever the origin of these two families of structures, they are largely absent from the fOS fraction, indicating some selectivity in the type of glycoprotein that is de-N-glycosylated *in vivo*.

fOS are degraded by the vacuolar mannosidase (*AMS1* gene product)

Next, fOS catabolism was examined in yeast strains deficient in either or both of the two mannosidases that have been identified in *S. cerevisiae*. In wild-type cells, pulse–chase studies revealed the disappearance of the larger fOS species (Man_{8–7}GlcNAc₂) with a concomitant appearance of smaller components. Importantly, during the chase periods, the behaviour of fOS is quite different to that of the endo H-sensitive N-linked Man₈GlcNAc, the quantity of which decays ($t_{1/2} \approx 5$ min, results not shown) without the appearance of smaller N-linked intermediates (compare upper- and lower-left panels of Figure 4A). When a similar pulse–chase experiment was performed in the *ams1* Δ strain, deficient in vacuolar α -mannosidase activity, Man₈GlcNAc₂ remains the predominant fOS even after 24 h of chase (Figure 4A, upper middle panel), demonstrating that this glycosidase is involved in yeast fOS catabolism. In contrast, the metabolism of N-glycans in this mutant did not differ from that observed in wild-type cells. Analysis of fOS in *ams/mns1* Δ cells that lack both the ER and vacuolar mannosidase activities, revealed a fOS mixture (labelled a–c in Figure 4A, upper right panel) the predominant component of which co-migrated with Man₉GlcNAc₂. Finally, there is a correlation between fOS catabolism and vacuolar mannosidase expression, which is known to be inversely proportional to cell growth [32]. Thus, in wild-

Figure 4 Catabolism of fOS in mannosidase-deficient yeast strains

Wild-type (CENPK; wt), *ams1* Δ , and *ams1/mns1* Δ strains were pulse-radiolabelled with [2- 3 H]Man for 30 min and chased for the indicated times (0 h, 4 h, 24 h). (A) fOS (upper panels) and the released N-linked oligosaccharides from glycoproteins of the corresponding cells (GP, lower panels) were analysed by TLC. The radioactive components migrating in the regions of the curly ('face') and square brackets are described in the Results section. Whereas all fOS obtained from incubations were loaded on to the TLC plates, only 10 % of their N-linked counterparts were subjected to TLC. For chromatographs of fOS, fluorography was for 1 week, whereas GP chromatographs were subjected to fluorography for 24 h. Open and closed arrowheads indicate the migration positions of oligosaccharides containing respectively one or two residues of N-acetylglucosamine. Radioactive components migrating in the region of the vertical bar were eluted and further analysed (see Figure 5B). (B) fOS catabolism was quantitated during the pulse chase experiment performed with wild type and *ams1* Δ cells. Individual radioactive components were eluted from the TLC plate and assayed by liquid-scintillation counting. fOS trimming is expressed as the ratio of smaller structures (M_{7-3} ; Man_{7–3}GlcNAc₂) to the larger components (M_{8-9} ; Man_{8–9}GlcNAc₂) observed at each incubation time. Wild-type and *ams1* Δ strains were grown and incubated under the conditions in which the pulse–chase studies were carried out. After harvesting the cells at 0, 4 and 24 h of chase, cell extracts were assayed for vacuolar mannosidase activity and protein in order to calculate the specific activity of the *p*-nitrophenyl α -D-mannopyranoside (Man-PNP)-hydrolysing activity.

type cells, there is an approximately sixfold increase in vacuolar mannosidase activity during the chase period which closely parallels the observed increase in fOS trimming (Figure 4B).

The bulk of *S. cerevisiae* fOS are generated during, or soon after, glycoprotein biosynthesis, and have ER-type structures

In order to gain more insight into the mechanism of fOS generation in *S. cerevisiae*, the rate of fOS generation in the *ams1Δ* strain was examined. When the quantities of fOS generated during the 30 min pulse and during 30 min intervals over the 4 and 24 h chase periods are calculated, it can be seen that the rate of fOS generation is highest during the pulse, and declines during the chase periods (Figure 5A).

More information on the origin of fOS was obtained by examining their structures in cells deficient in fOS processing/catabolism. The fOS species recovered from the *ams1/mns1Δ* strain (Figure 4A) were subjected to endo H treatment prior to rechromatography (Figure 5B). Elution and liquid-scintillation counting of the different components revealed that, whereas the species co-migrating with $\text{Man}_9\text{GlcNAc}$ (fOSb) corresponded to 76% of the total radioactivity recovered, larger species, indicated by the curly ('face') bracket (Figure 5B), and the smaller component (fOSc) accounted for, respectively, 19 and 5% of the remaining radioactivity. The species labelled 'a' and 'b' were subjected to $\alpha 1,2$ -mannosidase treatment, as shown in Figure 5(C). fOSb was found to yield a limit digest product identical with that produced with standard $\text{Man}_9\text{GlcNAc}$ (Figure 5C). Treatment of fOSa in the same way generated two components (Figure 5C). The more abundant limit-digest product co-migrated with the $\text{Glc}_1\text{Man}_7\text{GlcNAc}$ that was generated by $\alpha 1,2$ -mannosidase treatment of standard $\text{Glc}_1\text{Man}_9\text{GlcNAc}$, whereas the second, less abundant, digest product, labelled 'a'', migrated as an oligosaccharide possessing six Man residues. Owing to a lack of material, the minor component contained in fOSa mixture could not be further analysed, but these preliminary studies indicated that the original fOSa mixture comprised mainly $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$, and smaller quantities of a fOS possibly bearing ten residues of Man, as shown in Figure 5(D). In summary, although evidence is presented for the presence of small quantities of Golgi-modified structures, the bulk of fOS generated in the *ams1/mns1Δ* strain correspond to ER-type structures.

DISCUSSION

Here we report for the first time on the presence and nature of fOS in intact *S. cerevisiae*. In all wild-type strains tested under our conditions the predominant fOS recovered from a 30 min radiolabelling period was demonstrated to be the ER-mannosidase-generated isomer of $\text{Man}_8\text{GlcNAc}_2$. During 30 min radiolabelling of exponentially growing cells the amount of this fOS recovered corresponds to only 1% of the amount of the equivalent N-glycan releasable from glycoproteins by endo H.

Our studies on fOS in *S. cerevisiae* indicate that there are at least two fOS pools, and demonstrate that one of these pools arises from the deglycosylation of glycoproteins by the action of the Png1p that is thought to de-N-glycosylate glycoproteins during ERAD [15]. We noted that fOS appear during the radiolabelling period, but, after that time, their rate of generation is reduced. This result was surprising in that a previous study [41] had shown that Png1p expression is low during exponential growth, and appears to increase 7-fold during the diauxic shift (movement from anaerobic to aerobic respiration). The time course of the appearance of fOS is therefore at odds with the above observation, but is consistent with other studies indicating that this enzyme is involved in ERAD. Examination of the rate of degradation of a mutant carboxypeptidase Y protein revealed this process to be delayed by about 20 min in *png1Δ* cells when compared with that observed in wild-type cells [42]. However, as no de-

Figure 5 fOS structures and their rate of formation in mannosidase-deficient cells

(A) A pulse-chase experiment using *ams1Δ* cells was performed as described for Figure 4, and, after TLC, total fOS were eluted from the plates and quantified by liquid-scintillation counting. The rate of generation of fOS during the pulse-chase experiment is expressed as c.p.m. formed/30 min. Accordingly, the rates of fOS generation were calculated as follows: during the pulse (P); c.p.m. fOS during pulse, during the 4 h chase (C4); (c.p.m. at 4 h–c.p.m. during pulse)/8, and during the last 20 h of the 24 h chase (C24); (c.p.m. at 24 h–c.p.m. at 4 h)/40. (B) fOS derived from *mns1/ams1Δ* cells were recovered after TLC (indicated by the thick vertical bar shown in the upper right-hand panel of Figure 4A), and treated with endo H. The digestion products (lane 2) were then rechromatographed alongside (lane 1) standard $\text{Man}_9\text{GlcNAc}$ (M_9) and $\text{Man}_8\text{GlcNAc}$ (isomer B, M_8) for 48 h. (C) The species labelled a and b were in turn eluted and submitted, in parallel with standard $\text{Glc}_1\text{Man}_9\text{GlcNAc}$ (G_1M_9) and $\text{Man}_9\text{GlcNAc}$ (M_9), to *A. saitoi* mannosidase digestion, and the digestion products were analysed by TLC. The migration position of oligosaccharides is as described for Figure 4. (D) A scheme indicating structures that may be present in the parent fOS mixture labelled a. The $\text{Man}_9\text{GlcNAc}_2$ is substituted with either a Glc residue (G) or possibly a Man (M) residue linked $\alpha 6$, by Golgi-situated membrane-bound mannosyltransferase Och1p [60], to the core $\alpha 3$ Man of the pentasaccharide core. The $\alpha 2$ linkages are indicated by broken lines.

N-glycosylated carboxypeptidase Y intermediates were observed in these experiments, direct evidence that Png1p is involved in ERAD was not obtained. Indeed, using our experimental system we have been unable to demonstrate a role for either the ER mannosidase (*Mns1p* [43]) or the ER mannosidase-like protein

(Htmplp, [44,45]) in the generation of free $\text{Man}_8\text{GlcNAc}_2$ (Figure 2D; I. Chantret, J.-P. Frénoy and S. E. H. Moore, unpublished work). Both these proteins have been shown to accelerate ERAD in *S. cerevisiae*. Nevertheless, the kinetics of Png1p-generated fOS reported here are compatible with the proposition that Png1p is the enzyme responsible for the deglycosylation of glycoproteins during ERAD. In addition, structural studies carried out on fOS generated in yeast deficient in both the ER and vacuolar mannosidases indicated that the bulk of fOS detected in this strain have ER-type structures ($\text{Glc}_{0-1}\text{Man}_9\text{GlcNAc}_2$), suggesting that they did not transit the Golgi apparatus either prior to or after their release from glycoconjugate. However, we did detect small quantities of a $\text{Man}_{10}\text{GlcNAc}_2$ structure that may have arisen by the addition of an $\alpha 1,6$ -linked Man residue to $\text{Man}_9\text{GlcNAc}_2$ by Golgi-situated Och1p [46]. This may not be surprising, as it has been demonstrated that certain glycoproteins destined for ERAD are subject to early Golgi modification before recycling back to the ER for degradation [47–49]. In addition, studies have shown that some misfolded glycoproteins maybe targeted to the vacuole for degradation, via the Golgi apparatus [50]. In conclusion, although some results reported here suggest that the Png1p-dependent fOS pool is generated during ERAD, further experiments will be required in order to unambiguously demonstrate this proposition.

Whereas, Png1p is responsible for the generation of one fOS pool, the origin of the other pool remains to be established, but several possibilities can be considered. First, previous studies employing GDP-Man-labelled yeast microsomes revealed the liberation of $\text{Man}_8\text{GlcNAc}_2\text{-P}$ [28]. Although we did detect small amounts of negatively charged oligosaccharide material that could be neutralized upon digestion with alkaline phosphatase or endo H, there was insufficient material for further characterization. Presently, therefore, formation of neutral fOS from fOS phosphates in intact *S. cerevisiae* remains to be confirmed. Secondly, it has been proposed that fOS can be generated by the hydrolytic action of OST on surplus LLO [8]. However, examination of fOS formation in CHX-treated cells in which LLO accumulates, did not reveal any evidence for increased fOS formation. Thirdly, the second fOS pool, detected in the *pngp1* Δ strain, could be generated by another as yet uncharacterized PNGase.

We demonstrate that the vacuolar mannosidase is responsible for the catabolism of fOS in *S. cerevisiae*. This enzyme was identified and cloned several years ago [51–54] and much is known about its cell biology. Despite a few contradictory studies on the putative role of the vacuolar mannosidase in glycoconjugate catabolism [40,53,55], the function of this enzyme remains unexplored. In the present study we demonstrate one such function for the *AMS1* gene product. The catabolism of fOS by the vacuolar mannosidase seems to be well separated temporally from the process involved in the formation of these components. Perhaps, in yeast, the recycling of fOS is not required until environmental carbon sources become limiting. The finding that the yeast vacuolar mannosidase is involved in the processing of fOS is intriguing, as the peptide sequence of this enzyme has little in common with those of mammalian lysosomal α -mannosidases, but shows similarity to the sequence of neutral cytosolic mannosidase that is responsible for trimming fOS in the cytosol of mammalian cells [56,57]. Interestingly, whereas the vacuolar mannosidase, after its synthesis in the cytosol, is translocated into the vacuole by the cytosol-to-vacuole targeting ('cvt') pathway [58,59], the mammalian cytosolic mannosidase appears to be translocated into the ER, where it is involved in the processing of N-glycans [56]. Thus the finding that the vacuolar mannosidase of *S. cerevisiae* is also involved in fOS processing reveals another common feature of these two enzymes.

In mammalian cells, fOS are generated in both the ER and cytosol, and are trimmed in the cytosol before being transported into lysosomes, where they are further degraded [37]. As it is known, on the one hand, that yeast Png1p is mainly localized to the cytosol, and on the other, that the cytosolic form of the yeast vacuolar mannosidase is active [58], it is possible that the Png1p-generated fOS may both be generated and processed in the cytosol. Such a scheme of events could eliminate the requirement for the ER and lysosomally situated fOS transport processes that have been identified in mammalian liver cells. An examination of the subcellular localization of the events involved in the metabolism of yeast fOS is currently underway in our laboratory.

To summarize, our results demonstrate, for the first time, the presence of fOS in intact yeast, and show that one population of these structures is generated during glycoprotein biosynthesis by PNGase. Studies conducted on fOS catabolism have allowed us to specify a novel function for the yeast vacuolar mannosidase.

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