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MINI-REVIEW

Engineering of glycosylation in yeast and other fungi: current state and perspectives

Karen De Pourcq · Kristof De Schutter · Nico Callewaert

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Abstract With the increasing demand for recombinant proteins and glycoproteins, research on hosts for producing these proteins is focusing increasingly on more cost-effective expression systems. Yeasts and other fungi are promising alternatives because they provide easy and cheap systems that can perform eukaryotic post-translational modifications. Unfortunately, yeasts and other fungi modify their glycoproteins with heterogeneous high-mannose glycan structures, which is often detrimental to a therapeutic protein's pharmacokinetic behavior and can reduce the efficiency of downstream processing. This problem can be solved by engineering the glycosylation pathways to produce homogeneous and, if so desired, human-like glycan structures. In this review, we provide an overview of the most significant recently reported approaches for engineering the glycosylation pathways in yeasts and fungi.

Keywords N-glycosylation engineering · O-glycosylation engineering · Yeast · Fungi · Biopharmaceuticals · Recombinant proteins

K. De Pourcq · K. De Schutter · N. Callewaert (✉)
Unit for Molecular Glycobiology,
Department for Molecular Biomedical Research,
VIB-Ghent University,
Technologiepark 927, 9052, Ghent-Zwijnaarde, Belgium
e-mail: nico.callewaert@dmbr.vib-UGent.be

K. De Pourcq · K. De Schutter
Unit for Molecular Glycobiology,
Department of Biomedical Molecular Biology, Ghent University,
Technologiepark 927, 9052, Ghent, Belgium

N. Callewaert
Unit for Molecular Glycobiology,
Department of Biochemistry and Microbiology, Ghent University,
K.L.-Ledeganckstraat 35, 9000, Ghent, Belgium

Introduction

The use of recombinant proteins as biopharmaceuticals has been increasing, and high performance expression systems are needed to meet the demands. Most therapeutic proteins require glycosylation to ensure proper folding, function, and activity. Therefore, they cannot be produced in standard prokaryotic expression systems, which lack proper glycosylation machinery. Since glycosylation and other post-translational modifications are essential for these therapeutic glycoproteins, most of them are produced in mammalian cells. However, due to the potential spread of infectious diseases through the use of contaminated protein products and potential shortages in bovine serum supply for large-scale production, there is a growing shift to serum-free expression systems. In particular, yeasts and fungi are being developed as excellent alternative protein expression systems. Yeast and fungi have a more rigid cell wall compared to mammalian and insect cells, which makes them more resistant to shear stress and allows them to grow to high cell densities, which facilitates industrial scale up. In addition, fungal expression systems can grow in simple, chemically defined media, which makes the production costs much lower than in mammalian and insect cells.

This, together with the ease of genetic manipulation of microbial organisms and the availability of established molecular tools and know-how, has made yeasts and fungi the expression system of choice when manufacturing costs, speed, and yield are of great importance. Moreover, as eukaryotic organisms, yeast and fungi are able to perform post-translational modifications, such as N- and O-glycosylation, disulfide bond formation, and oligomerization, which are often essential for protein quality and functionality. However, protein glycosylation in microorganisms can be quite different from that in mammalian cells. N-glycans in yeast, for example, are of a heterogeneous high-mannose type.

In contrast, human N-glycans are mainly of the complex or hybrid type. When glycoproteins are produced in yeast, this difference can be detrimental for downstream processing efficiency and also for subsequent therapeutic use. High-mannose type N-glycans attached to recombinant glycoproteins can be cleared rapidly from the human bloodstream due to interaction with human mannose receptors, and they can cause immunogenic reactions in humans, as in the case of α-1,3-linked mannose modifications typical of *Saccharomyces cerevisiae* (Yip et al. 1994).

To overcome these problems, the possibility of reengineering the N-glycosylation pathway has been explored, especially in the species most frequently used for the production of heterologous proteins (*S. cerevisiae*, *Pichia pastoris*, *Yarrowia lipolytica*, *Hansenula polymorpha*, and Aspergilli species).

The N-glycosylation pathway and engineering strategies

N-glycosylation

Over the past decade, several attempts have been made to engineer the yeast N-glycosylation pathway for the production of "humanized" glycoproteins. The first efforts were made in the model yeast *S. cerevisiae*, in which the N-glycosylation pathway was originally characterized (Dean 1999; Kukuruzinska et al. 1987) (Figs. 1 A and 2 A).

In yeast, as in man, the initial steps of N-glycosylation take place at the cytoplasmic side of the endoplasmic reticulum (ER) (Fig. 1 A). Through the sequential addition of *N*-acetylglucosamine (GlcNAc) and mannose (Man) residues, a dolichol-linked glycan precursor is synthesized. This intermediate $Man_5GlcNAc_2$-PP-Dol structure is flipped to the lumen of the ER, where it is further elongated

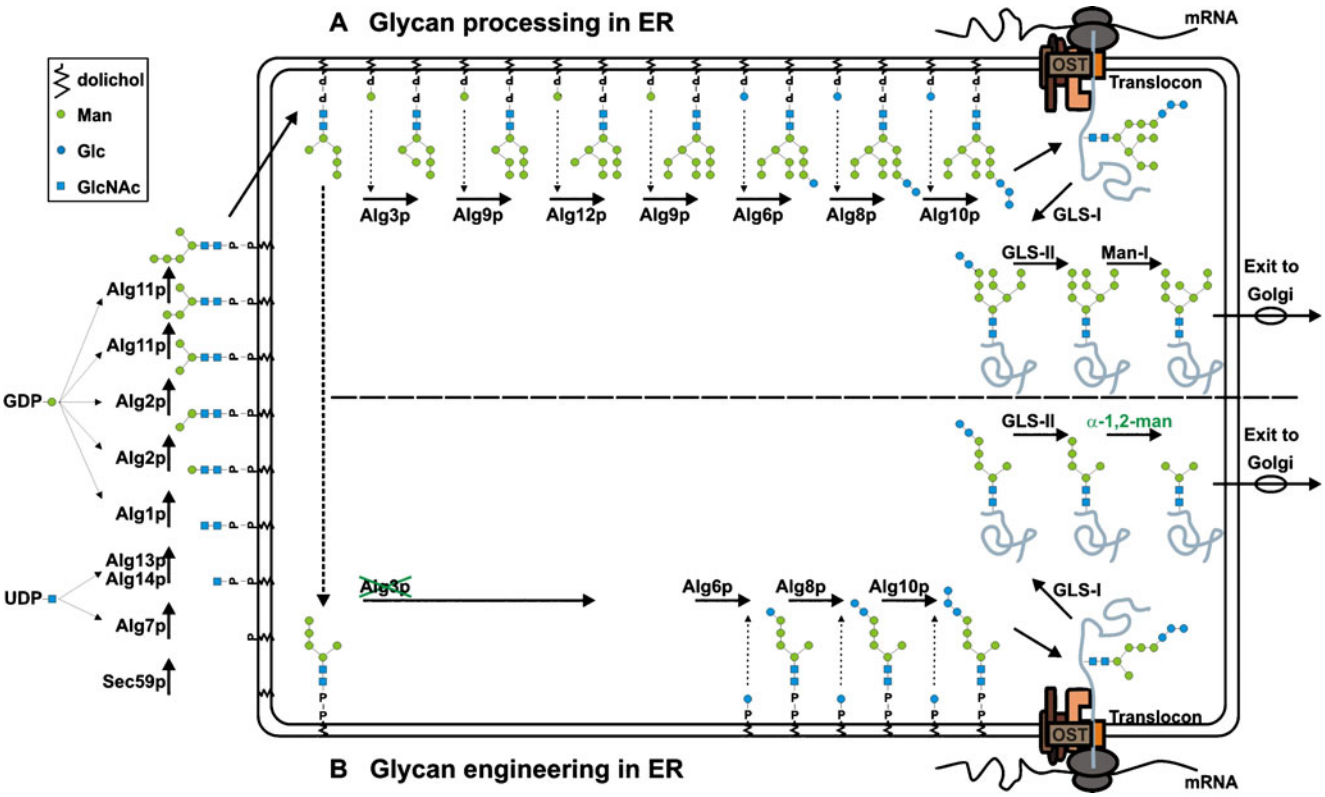

Fig. 1 N-glycosylation and engineering in the endoplasmic reticulum. *A* Endogenous N-glycosylation pathway (as modeled in *S. cerevisiae*). The dolichol-linked oligosaccharide precursor is assembled by the stepwise addition of 14 monosaccharides. The first seven (two GlcNAc residues and five mannose residues) are added on the cytosolic side of the ER membrane. The resulting dol-PP-$GlcNAc_2Man_5$ is then flipped to the ER lumen where four more mannoses and three glucoses are added. The dol-PP-$GlcNAc_2Man_9Glc_3$ precursor is transferred to an asparagine residue in the N-X-S/T consensus of the nascent protein by the oligosaccharyltransferase (*OST*). Immediately after transfer, the glucose residues are trimmed by the glucosidases GLS-I and GLS-II. Upon removal of one mannose residue by mannosidase-I (Man-I), the glycoproteins exit the ER. *B* N-glycosylation engineering in the ER. Disruption of Alg3p activity prevents the addition of more mannoses in the lumen of the ER. Oligosaccharyltransferase does not recognize the truncated precursor glycans as efficiently as the fully built precursor, which results in under-occupancy of the N-glycosylation sites. After processing of the transferred N-glycan by GLS-I and GLS-II, the protein-linked N-glycan is trimmed by the introduced α-1,2-mannosidase (of *A. saitoi* or *T. reesei*) to $Man_3GlcNAc_2$ before exiting the ER

by the sequential activity of the α-1,3-mannosyltransferase *ALG3* and other mannosyltransferases, until $Man_9GlcNAc_2$ is formed. Subsequently, this dolichol-linked sugar is glucosylated by the α-1,3-glucosyltransferase *ALG6*, after which two more glucoses are added. The resultant $Glc_3Man_9GlcNAc_2$ oligosaccharide is transferred to asparagine residues of the nascent polypeptide chain and subsequently trimmed to $Man_8GlcNAc_2$. The newly formed glycoconjugate is then transported to the Golgi apparatus (Kornfeld and Kornfeld 1985; Kukuruzinska et al. 1987). Exceptions are, e.g., *Schizosaccharomyces pombe* and *Kluyveromyces lactis*, which appear to transfer $Man_9GlcNAc_2$ glycoproteins to the Golgi. Further processing of the carbohydrate chain in the Golgi differs between yeasts and higher eukaryotes. In animal cells, the N-glycans of glycoproteins are processed to complex-type glycan structures that may contain sialic acid, galactose (Gal), fucose, and *N*-acetylglucosamine, and additional branches on the $Man_3GlcNAc_2$ glycan core. In yeast, on the other hand, processing in the Golgi starts with the addition of an α-1,6-mannose residue by Och1p (Fig. 2 A). This branch is elongated by stepwise addition mainly of mannose residues, which can lead to a very complicated hypermannosylated glycoform mixture. Abolishing this hypermannosylation, which is common to yeast and very prominent in *S. cerevisiae*, is generally the first engineering target. When

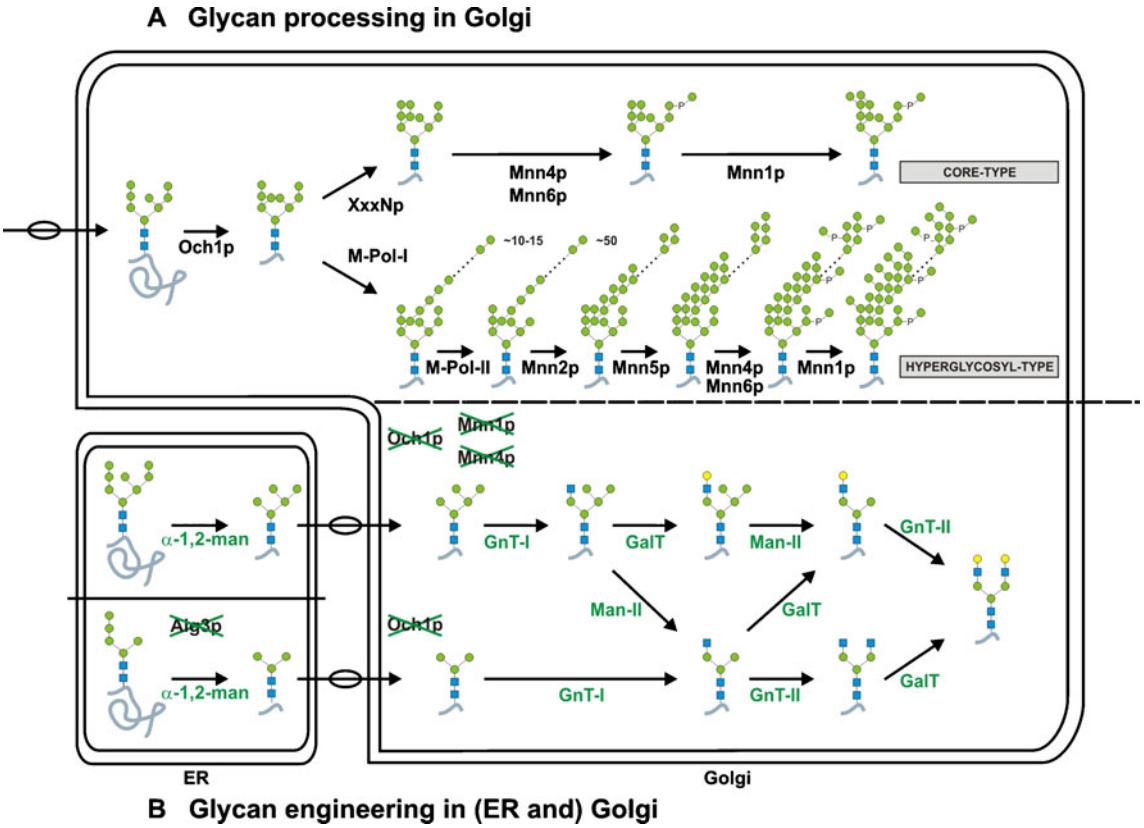

Fig. 2 N-glycosylation and engineering in the Golgi apparatus. *A* Endogenous N-glycosylation pathway in yeasts (as modeled in *S. cerevisiae*). Protein-bound $Man_8GlcNAc_2$ N-glycans imported from the ER are modified by the α-1,6-mannosyltransferase Och1p, which adds an "initiating" α-1,6-mannose to the α-1,3-mannose of the trimannosyl core. To form hyperglycosyl-type N-glycans, elongation of the outer chain is achieved by the sequential action of two protein complexes, mannan polymerase I (Mnn9p, Van1p) and mannan polymerase II (Mnn9p, Anp1p, Mnn10p, Mnn11p, Hoc1p). The α-1,6-mannose backbone is further modified by the addition of α-1,2-mannoses by Mnn2p and Mnn5p, mannosylphosphate residues by Mnn4p and Mnn6p, and capping α-1,3-mannoses by Mnn1p. Core-type N-glycans, found on glycoproteins of cellular organelles, have no outer chain elongation but do show α-1,2-mannose, mannosylphosphate, and α-1,3-mannoses capping modifications. *B* Humanized Golgi processing in yeast. Introduction of α-1,2-mannosidase (from *A. saitoi* or *T. reesei*) in the ER results in the import of $Man_5GlcNAc_2$ structures into the Golgi complex. In addition, disruption of Alg3p in the ER together with the α-1,2-mannosidase activity results in the import of $Man_3GlcNAc_2$ N-glycans in the Golgi. Disruption of Och1p activity prevents the addition of an initiating α-1,6-mannose. The sequential action of introduced human GlcNAc transferase I (GnT-I), human galactosyltransferase (GalT), *Drosophila melanogaster* mannosidase II (Man-II), and rat GlcNAc transferase II (GnT-II) leads to modification of N-glycans to complex-type human-like glycan structures. In some engineering strategies, it is necessary to disrupt Mnn1p and Mnn4p activity to obtain the desired glycosylation. The fusion partners required for correct localization of the proteins are not indicated in the figure, nor the genes involved in the biosynthesis or transport of the activated sugars used in some engineering strategies

this heterogeneity has been overcome, the second engineering target is to divert the yeast N-glycosylation pathway to an intermediate common to the pathway in mammalian cells, which can then be used as a substrate for higher eukaryotic glycosyltransferases to build human-type glycans.

Strategies

So far, almost all successful endeavors to homogenize yeast or fungal N-glycosylation pathways have focused on two engineering approaches. One approach relies on deletion of certain yeast-specific genes involved in hypermannosylation, especially the gene coding for the Och1p mannosyltransferase (Fig. 2 B). Depending on the species and the engineering goal, the *OCH1* disruption is combined with deletion of other aforementioned genes, or with the overexpression of an α-1,2-mannosidase. The other approach focuses on deletion of genes involved in earlier steps of the glycosylation pathway, especially the *ALG3* gene (Fig. 1 B). Both approaches can be further complemented by the overexpression of several glycosyltransferases and glycosidases derived from various species to produce humanized oligosaccharides on the produced glycoproteins (Fig. 2 B).

Interference with the hypermannosylation pathway

Proteins modified with the hypermannosylated glycan structures, which are characteristic of yeasts and very prominent in *S. cerevisiae*, are rapidly cleared from the bloodstream and contain epitopes known to be immunogenic in man. Therefore, most engineering approaches in yeast start by targeting the α-1,6-mannosyltransferase Och1p, which initiates the synthesis of the "outer branch" in these hypermannosyl structures (Nakayama et al. 1992) (Fig. 2 B).

OCH1 disruption in *S. cerevisiae* was shown to be viable, although the strain has severe growth defects (Nagasu et al. 1992). In addition, the glycosylation was not homogeneous because the $Man_8GlcNAc_2$ N-glycans coming from the ER were further modified with α-1,3-mannose and mannosylphosphate residues (Nakanishi-Shindo et al. 1993). Therefore, the next logical step was to disrupt the genes responsible for these additions, i.e., *MNN1* and *MNN4* (Chiba et al. 1998; Nakanishi-Shindo et al. 1993), which generated a strain producing homogeneous $Man_8GlcNAc_2$ N-glycans.

The subsequent expression of an *Aspergillus saitoi* α-1,2-mannosidase tagged with HDEL in the triple knockout (*och1mnn1mnn4*) mutant strain resulted in a significant decrease in α-1,2-linked mannoses and partial conversion of $Man_8GlcNAc_2$ oligosaccharides into $Man_5GlcNAc_2$ (Chiba et al. 1998) (Fig. 2 B). This is the first step in the formation of hybrid and complex-type sugar chains. Although the introduced α-1,2-mannosidase activity was not sufficient to convert all $Man_8GlcNAc_2$, the importance of this work lies in the use and necessity of a proper localization signal (HDEL) for the α-1,2-mannosidase in the ER.

Abe et al. (2009) were able to overcome the growth phenotype and decreased protein productivity associated with the loss of Och1p activity. By applying a mutagenesis on the glyco-engineered strains, they succeeded in circumventing the growth defect in these strains. In addition, some mutants produced more protein (Abe et al. 2009). Though deletion of the outer chain of the N-glycans reduces the strength of the cell wall which results in reduced cell growth, the mutants suppress this phenotype by altering the cell wall or plasma membrane components (Abe et al. 2009). Analysis showed an increase in glucose content, which suggests increased glucan levels and therefore enhanced strength of the cell wall of these mutants (Abe et al. 2009).

The methylotrophic yeast *P. pastoris* has a glycosylation machinery generally similar but different in the details from that of *S. cerevisiae*. It is the main yeast expression system used to produce recombinant proteins. Hyperglycosylation in *P. pastoris* was found to be much less elaborate than in *S. cerevisiae* (Gemmill and Trimble 1999).

The first engineering attempts involved the overexpression of an α-1,2-mannosidase from *Trichoderma reesei* to immediately trim the $Man_8GlcNAc_2$ glycan structure coming from the ER to $Man_5GlcNAc_2$ before it gets hyperglycosylated (Maras et al. 2000; Martinet et al. 1998) (Fig. 2 B). As in *S. cerevisiae*, localization of the enzyme proved critical. Therefore, in subsequent work, the α-1,2-mannosidase from *T. reesei* was targeted to the ER–*cis* Golgi boundary with a C-terminal HDEL retrieval tag (Callewaert et al. 2001). This resulted in a decrease of more than 85% in the number of α-1,2-linked mannose residues. Nevertheless, since larger N-glycans were also still present, the inactivation of the *OCH1* gene proved necessary. Hence, a knockin strain in the *OCH1* locus was generated and combined with the overexpression of the *T. reesei* α-1,2-mannosidase–HDEL (Fig. 2 B). This combined engineering resulted in almost complete conversion to $Man_5GlcNAc_2$ (Vervecken et al. 2004).

Contrary to *S. cerevisiae*, *OCH1* disruption in *P. pastoris* did not severely impede growth (Vervecken et al. 2004), which makes this yeast especially suitable for glyco-engineering. The same results were obtained when the *OCH1* gene was deleted by double homologous recombination (Choi et al. 2003). This *och1* mutant strain did not have severe growth defects, but it exhibited temperature

sensitivity and increased flocculation similar to that observed in *S. cerevisiae* (Choi et al. 2003). However, the N-glycan profile of their *och1* strain was different from the one obtained by Vervecken et al. (2004). This might have been due to the difference in the parent strain used (JC308 versus GS115) or in the nature of the analyzed glycoproteins. After abolishing yeast-specific hyperglycosylation by inactivating the *OCH1* ortholog, Choi et al. tackled the next step of the N-glycan engineering, which was trimming $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ by introducing α-1,2-mannosidase I. This Golgi-resident glycosidase has a domain architecture similar to glycosyltransferases. First, they created a combinatorial array consisting of known Golgi and ER-localized transmembrane domains from *S. cerevisiae* and *P. pastoris* enzymes, along with a collection of catalytic domains from α-1,2-mannosidases derived from various species. Transformation of this genetic library of chimeras with potential mannosidase activity in the *och1* mutant strain resulted in the isolation of several yeast strains that produce almost homogeneous N-glycans of the $Man_5GlcNAc_2$ type (Choi et al. 2003).

As in *P. pastoris*, the hyperglycosylation observed in the methylotrophic yeast *H. polymorpha* is less pronounced than in *S. cerevisiae*. Deletion of *OCH1* in *H. polymorpha* completely blocked the addition of the first α-1,6-mannose residue onto the core oligosaccharide $Man_8GlcNAc_2$ and did not alter growth under normal growth conditions (Kim et al. 2006). Simultaneous overexpression of an *A. saitoi* ER-targeted (HDEL-tagged) α-1,2-mannosidase resulted in the predominant synthesis of $Man_5GlcNAc_2$ N-glycans (Kim et al. 2006) (Fig. 2 B).

Whereas *S. cerevisiae*, *P. pastoris*, and *H. polymorpha* are the most frequently used yeasts for recombinant protein production, there is a growing interest in the dimorphic yeast *Y. lipolytica*. Although it was suggested that the *Y. lipolytica* Och1p α-1,6-mannosyltransferase might play a minor role in the outer-chain elongation of N-glycosylation (Barnay-Verdier et al. 2004), it was shown that knocking out the *Y. lipolytica OCH1* gene results in glycoproteins predominantly modified with $Man_8GlcNAc_2$ glycans (Song et al. 2007; De Pourcq et al., unpublished results). Subsequent introduction of the *T. reesei* α-1,2-mannosidase–HDEL construct used also for *P. pastoris* engineering generated a strain producing $Man_5GlcNAc_2$ glycans on its glycoproteins (De Pourcq et al., unpublished results).

The fission yeast *S. pombe* synthesizes large outer chains on the N-linked oligosaccharides which, unlike their counterparts in *S. cerevisiae*, consist mainly of D-Gal next to D-Man residues. As for the other yeast *OCH1* genes, *Sp*Och1p has α-1,6-mannosyltransferase activity and is the key enzyme in the initiation of outer chain elongation. Structural analysis by HPLC and ^1H NMR of *S. pombe och1* mutants showed that the glycans were heterogeneous ($Hex_{11–15}GlcNAc_2$). This heterogeneity arose mainly from the addition of α-1,2- and α-1,3-Gal residues to the $Man_9GlcNAc_2$ core structure of the cell surface glycoproteins (Ohashi et al. 2009). Subsequent disruption of *GMS1*, a gene encoding the UDP-galactose transporter, resulted in N-glycans consisting of core oligosaccharides extended with α-1,2-linked mannose residues ($Man_{8-12}GlcNAc_2$) from which the fission yeast-specific α-linked galactose residues were completely absent (Ohashi and Takegawa 2010).

K. lactis, another host for protein production, modifies its glycoproteins with heterogeneous hypermannosylated N-glycans, with a size of $Man_{(>30)}GlcNAc_2$. Deletion of the *OCH1* ortholog in *K. lactis* renders the strain unable to add mannose to the core oligosaccharide and thereby abolishes hyperglycosylation. The N-linked oligosaccharides assembled on secreted glycoproteins are of the $Man_{13–14}GlcNAc_2$ type (Liu et al. 2009). Knocking out the *MNN1* gene in addition to the *OCH1* gene was beneficial because it resulted in the production of glycoproteins containing $Man_{9–11}GlcNAc_2$ (Liu et al. 2009). Quantitative analysis of the cell wall components in the *Kloch1* mutant strain indicates a noticeable increase of chitin and β-1,6-glucans and a severe decrease of mannoproteins (Uccelletti et al. 2006), which indicates that a compensatory mechanism ensures stability of the cell wall. In addition, it was found that the *och1* mutation altered mitochondrial morphology and calcium homeostasis (Zanni et al. 2009).

Interference earlier in the N-glycosylation pathway

A second engineering approach to generate fungal expression systems for the production of homogeneously glycosylated recombinant proteins is to interfere early in its endogenous N-glycosylation pathway, i.e., during biosynthesis of the dolichol-linked glycan precursor in the endoplasmic reticulum (Fig. 1 A). Disrupting the Alg3p α-1,3-mannosyltransferase function in *S. cerevisiae* prevents the addition of α-1,3- and α-1,6-mannosyl residues on the α-1,6-arm of the trimannosyl core (Verostek et al. 1991), and the lipid–oligosaccharide pool accumulates $Man_5GlcNAc_2$-PP-Dol (Huffaker and Robbins 1983) (Fig. 1 B). However, the used *alg3* mutant was leaky, which complicated the analysis. Because of this, additional types of protein-bound carbohydrate structures were found, including some that contained glucose. Glucose retention appeared to be due to inefficient glucosidase II trimming of minor glucosylated intermediates (Verostek et al. 1993a, b). It was subsequently reported that the initiating α-1,6 mannosyltransferase Och1p of *S. cerevisiae* displays very low activity towards the *alg3* $Man_5GlcNAc_2$ structure both in vivo and in vitro, as compared with its activity toward a

$Man_8GlcNAc_2$ or $Man_9GlcNAc_2$ structure (Nakayama et al. 1997; Verostek et al. 1993a, b). The *S. cerevisiae och1 mnn1 alg3* mutants proved to accumulate $Man_5GlcNAc_2$ and $Man_8GlcNAc_2$ in total cell mannoproteins, lacked mannose outer chain addition, and displayed a leaky phenotype of the *alg3* mutation, which explains the generation of $Man_8GlcNAc_2$ as well as $Man_5GlcNAc_2$ (Nakanishi-Shindo et al. 1993).

An analogous engineering strategy was followed in *P. pastoris*. In addition to disruption of Alg3p functionality, it was found necessary to delete *OCH1*. *Pp*Och1p, when compared to the *Sc*Och1p, showed a broader substrate specificity with respect to the *alg3* $Man_5GlcNAc_2$ structure versus the wild-type $Man_8GlcNAc_2$ (Davidson et al. 2004) (Fig. 2 B). This double mutant strain produced mainly $Man_5GlcNAc_2$ glycans that can easily be trimmed to $Man_3GlcNAc_2$ structures by an α-1,2-mannosidase. However, several larger glycans ranging from $Hex_6GlcNAc_2$ to $Hex_{12}GlcNAc_2$ were also observed, and these could not be digested with different mannosidases. Further analysis revealed that some structures were modified with mannosylphosphate, whereas at least some of the $Hex_6GlcNAc_2$ species carry a terminal glucose residue. The larger structures are likely the result of Golgi-residing (possibly β-1,2-) mannosyltransferase activity that acts independently of the Och1p-initiated outer chain which can act on a substantially truncated substrate, without the requirement of complete $Man_8GlcNAc_2$ core formation for activity (Davidson et al. 2004).

Interfering with endogenous core N-glycosylation was also explored in *Y. lipolytica*. By deleting Alg3p activity and at the same time overexpressing an α-1,2-mannosidase, we wanted to obtain a homogeneous $Man_3GlcNAc_2$ structure on glycoproteins (Fig. 1 B). This is the ideal starting point for constructing human-like sugars. However, as in *P. pastoris*, disruption of the *ALG3* gene led not only to the $Man_5GlcNAc_2$ glycan structure, but also to larger glycan structures. Further analysis revealed that they were glucose-capped $Man_5GlcNAc_2$ oligosaccharides (De Pourcq et al., unpublished results). Further engineering is ongoing to generate the desired $Man_3GlcNAc_2$ glycan structures.

H. polymorpha alg3 mutants seem to synthesize very little, if any, glucosylated glycan structures (Oh et al. 2008). Deleting the *ALG3* gene in *H. polymorpha* leads to predominantly $Man_5GlcNAc_2$ glycan structures on model glycoproteins. The fraction of $Hex_{6-8}GlcNAc_2$ that is observed can be almost completely converted to $Man_3GlcNAc_2$ in vitro with α-1,2- and α-1,6-mannosidases. Deletion of the endogenous Och1p outer chain α-1,6-mannosyltransferase results in the disappearance of the $Hex_{6-8}GlcNAc_2$ sugar structures. Only a minor fraction of $Hex_6GlcNAc_2$ remains. This fraction is also seen in *alg3* mutants digested in vitro with α-1,2- and α-1,6-mannosidases and cannot be trimmed completely to $Man_3GlcNAc_2$ in vivo by overexpression of an ER-targeted α-1,2-mannosidase. It is therefore not excluded that this $Hex_6GlcNAc_2$ glycan structure, even though low in abundance, could account for glycan structures containing a capping glucose (Oh et al. 2008).

In the filamentous fungi *Aspergillus nidulans* and *Aspergillus niger*, knockout of *ALGC*, the *ALG3* ortholog, leads to production of $Man_{3-6}GlcNAc_2$ glycans (Kainz et al. 2008). In vitro digestion with α-1,2-mannosidase leads to almost exclusively $Man_3GlcNAc_2$. Hence, contrary to *P. pastoris* and *Y. lipolytica*, the larger glycan structures recalcitrant to in vitro α-1,2-mannosidase treatment were not detected in *Aspergillus* species (Kainz et al. 2008).

A very important remark is that *ALG3* disruption leads to under-occupancy of the N-glycosylation site, as the oligosaccharyltransferase (OST) does not transfer the truncated dolichol-linked precursors with equal efficiency as the normal $Glc_3Man_9GlcNAc_2$ precursor (Aebi et al. 1996; Cueva et al. 1996). This aspect of the engineering strategy is underdocumented (e.g., Is the defect equally penetrant in different yeast species and can it be modulated by the cultivation conditions?) and will require further work to solve. A possibility to overcome the problem with N-glycosylation site occupancy was found in the use of the STT3 protein from trypanosomatid parasites.

The OSTs in yeast and fungi are hetero-oligomeric protein complexes consisting of eight subunits (Kelleher and Gilmore 2006), of which the STT3 protein is the catalytic subunit (Yan and Lennarz 2002; Kelleher et al. 2003; Nilsson et al. 2003). The genomes of most multicellular eukaryotes encode two homologues of the STT3 protein, which leads to different OST isoforms with distinct enzymatic properties. These isoforms have different specificities for the peptides and precursor substrate and act sequentially to maximize the efficiency of N-glycosylation (Kelleher et al. 2003).

Based on phylogenetic analysis, the simplest eukaryotic OST would be a single subunit STT3 protein. In trypanosomatid genomes, only this *STT3* ortholog of the OST subunits could be found, and this subunit is also present in multiple copies. In *Leishmania major* for instance, in which N-glycosylation is similar to that of bacteria, the OST forms a homodimeric enzyme consisting of the catalytic active single subunit STT3 protein encoded in the genome by four paralogues (Nasab et al. 2008). Also in *Trypanosoma brucei*, OST activity is catalyzed by single subunit enzymes and these are encoded by three paralogous genes (Berriman et al. 2005).

In these trypanosomatid parasites, N-glycosylation mainly follows the pathway described for fungal and animal cells, but different structures are transferred to the

protein (McConville et al. 2002). In *Leishmania* species, for instance, the proteins are modified with Man_6- or Man_7-$GlcNAc_2$ structures (Parodi 1993). Expression of the four *Leishmania major* STT3 paralogues in *S. cerevisiae* showed that the *Lm*STT3 paralogues were active enzymes that did not incorporate into the OST complex but instead formed dimers, of which three complemented the yeast *stt3* deletion (Nasab et al. 2008). Of *T. brucei*, two out of three STT3 paralogues could complement the yeast *stt3* deletion. Although unable to compensate the yeast *stt3* deletion, the *Tb*STT3A protein was shown to selectively transfer biantennary $Man_5GlcNAc_2$ (Izquierdo et al. 2009). Further, the *Tb*STT3B protein was found to selectively transfer triantennary $Man_9GlcNAc_2$ (Izquierdo et al. 2009).

Thus, the expression of the single subunit STT3 OST protein from trypanosomatid parasites in the yeast heterologous expression system could increase the efficiency of N-glycosylation. In addition, selection of specific *STT3* gene paralogues could offer selective transfer of the "required" glycan structures.

Further humanization of the N-glycans into hybrid and complex-type sugars

To further mimic the human N-linked glycosylation machinery in yeast or fungal hosts, it is necessary to introduce several glycosidases, glycosyltransferases, and possibly accessory proteins, such as sugar transporters (Fig. 2 B). As N-glycosylation processing is sequential, proper targeting of these enzymes is important.

Early work focused on studies of Golgi glycosyltransferase expression in yeast

The earliest experiments in this field involved attempts to express mammalian glycosyltransferases in *S. cerevisiae*. The human full-length β-1,4-galactosyltransferase and the full-length α-2,6-sialyltransferase were successfully expressed but were retained as enzymatically active enzymes in the ER (Krezdorn et al. 1994). However, since their action is required in the Golgi apparatus, a new strategy had to be applied. Hence, the cytoplasmic and transmembrane domains were removed from the coding sequence of the human Golgi β-1,4-galactosyltransferase 1 (GalT-I), and the remaining sequences (coding for the stem and catalytic domains of this glycosyltransferase) were fused to sequences coding for the signal sequence of yeast invertase. This construct yielded secreted, enzymatically active enzyme (Kleene et al. 1994).

Subsequently, a chimeric protein consisting of the membrane anchor of the yeast α-1,2-mannosyltransferase Mnt1p fused to the soluble human β-1,4-galactosyltransferase 1 domain was expressed in a *S. cerevisiae alg1* background and shown to be localized in the early Golgi (Schwientek and Ernst 1994; Schwientek et al. 1995, 1996). The *alg1* mutation results in the glycosylation of proteins with a single beta-linked GlcNAc residue, which can act as a substrate for the galactosyltransferase. Expression of Mnt1-GalT-I in this strain resulted in the incorporation of galactose in the N-glycans, which demonstrates for the first time that β-1,4-galactosyltransferase 1 activity can be functionally localized to the Golgi apparatus of a yeast by fusing the gene to the targeting signals of a yeast Golgi protein. This strategy was later followed repeatedly in the humanization of yeast N-glycosylation (see below). Moreover, this early result demonstrated the presence of UDP-galactose in the *S. cerevisiae* Golgi apparatus, and thus the likely presence of a UDP-galactose transporter in the *S. cerevisiae* Golgi, even though its proteins usually have no galactose residues. Further proof for the existence of a *S. cerevisiae* UDP-galactose transporter was provided by the overexpression of a *S. pombe* α-1,2-galactosyltransferase in an *mnn1* background (Roy et al. 1998). However, in both cases, galactosylation was not complete. A study to analyze the limiting factors in galactosylation revealed that the UDP-Gal transporter rather than α-1,2-galactosyltransferase played a key role in efficient galactosylation in *S. cerevisiae* (Kainuma et al. 1999).

The intracellular expression of sialyltransferases in yeast has been described multiple times (Borsig et al. 1995; Krezdorn et al. 1994; Schwientek et al. 1995), but yeasts do not naturally produce the CMP-sialic acid sugar nucleotide substrate. In another approach, sialyltransferase was expressed on the yeast cell wall, and provided that the cells were supplied with CMP-sialic acid, sialic acid was transferred to the N-glycans of a substrate glycoprotein (Mattila et al. 1996; Salo et al. 2005; Sievi et al. 1998). Since *N*-acetylglucosaminyltransferase I (GnT-I) is essential in the humanization of N-glycans in yeast, rat GnT-I expression was attempted in *S. cerevisiae* (Yoshida et al. 1999). However, the enzyme was localized in all organelles, which indicates that the mammalian Golgi retention signal did not function in yeast (Yoshida et al. 1999).

Biotechnology-focused N-glycan engineering: the last decade

About the year 2000, the available information hinted at the necessity of properly locating the catalytic domains of the enzymes that are overexpressed within the Golgi cisternae to build up a functional "humanized" pathway in yeast. At about that time, engineering in *S. cerevisiae* and *P. pastoris* also enabled the production of glycoproteins with the $Man_5GlcNAc_2$ N-glycan substrate for the mammalian Golgi N-glycosylation pathway.

Further efforts were initiated to prove that the typical branch structure of mammalian N-glycans (LacNAc structure) can be synthesized in the *Pichia* Golgi apparatus. This was achieved by introducing two additional glycosyltransferases, i.e., human GlcNAc transferase I (GnT-I) and human β-1,4-galactosyltranferase I (GalT-I) (Fig. 2 B). Both catalytic domains were fused to the localization domain of *S. cerevisiae* α-1,2-mannosyltransferase Kre2p, yielding a Kre2–GnT-I and a Kre2–GalT-I fusion construct (Vervecken et al. 2004). About 90% of the available $Man_5GlcNAc_2$ N-glycans were converted to $GlcNAcMan_5GlcNAc_2$ structures upon introduction of the Kre2–GnT-I hybrid protein. This indicated that sufficient UDP-GlcNAc is present in the *P. pastoris* Golgi apparatus and that higher eukaryotic GlcNAc transferases can be functionally expressed in the yeast (*Pichia*) Golgi apparatus, which means that practically useful modification levels could be achieved. Work to increase the branching level of N-glycans with GlcNAc transferases was therefore obvious (Hamilton et al. 2003). The *P. pastoris* strain producing $GlcNAcMan_5GlcNAc_2$ structures reported by researchers at Glycofi (Choi et al. 2003) was obtained by a different strategy. Instead of using one localization and catalytic domain combination, they screened a library of combinations to identify the most successful fusion constructs not only for α-1,2-mannosidase activity but also for GlcNAc transferase I activity, which identified a $GlcNAcMan_5GlcNAc_2$ strain (Choi et al. 2003). It is remarkable that complete conversion required an increase in the availability of the glycosyl donor UDP-GlcNAc in the Golgi. This was done by incorporating the UDP-GlcNAc transporter of *K. lactis* in the $Man_5GlcNAc_2$ strain, along with the chimeric library of GnT-I enzymes. As mentioned above, Vervecken et al. (2004) obtained >90% GnT-I-mediated modification without UDP-GlcNAc transporter co-expression. This is consistent for many test proteins ranging in expression level from low to several 100 mg/L (Jacobs et al. 2009). Therefore, we suspect that the Kre2–hGnT-I fusion is exceptionally active.

Further engineering of this strain to add galactose by introducing the Kre2–GalT-I fusion construct resulted in a $GlcNAcMan_5GlcNAc_2$ to $GalGlcNAcMan_5GlcNAc_2$ conversion efficiency of about 10% of the N-glycans from cell wall glycoproteins (Vervecken et al. 2004), which demonstrates the feasibility of producing LacNAc branches in the *Pichia* Golgi. Later, analysis of the secreted protein N-glycans demonstrated about 50% conversion efficiency (unpublished data)—this was the first time in vivo galactosylation of N-glycans in *P. pastoris* was reported. To increase the galactosylation efficiency, researchers at Glycofi used a strategy that was successful in *Escherichia coli*-based oligosaccharide synthesis (Blixt et al. 2001). In this method, the *S. pombe* UDP-galactose 4-epimerase and the catalytic domain of human β-1,4-GalT (Bobrowicz et al. 2004). It is believed that the UDP-galactose 4-epimerase part of the protein converts UDP-Glc to UDP-Gal in the Golgi apparatus, which increases the availability of UDP-Gal as a substrate for the human GalT-I. Indeed, Jacobs et al. reproduced these results in 2009 and confirmed that this construct enables galactosylation of up to 85% of terminal beta-GlcNAc residues in *P. pastoris*. However, how this works remains somewhat unclear. UDP-Gal 4-epimerase is natively a cytosolic enzyme that requires an NAD^+ cofactor (Nayar et al. 2004). To our knowledge, no system for importing NAD^+ into the eukaryotic secretory system has been reported. UDP-Gal 4-epimerase is only active as a dimeric molecule and it can dimerize without binding NAD^+ (Scott and Timson 2007). Therefore, an intriguing possibility is that the fusion of this enzyme to the hGalT-I catalytic domain merely dimerizes the latter, perhaps increasing its catalytic activity. hGalT-I is a natively dimeric protein. Alternatively, perhaps only a fraction of the fusion enzyme molecules is translocated and that the rest provides UDP-Gal 4-epimerase activity in the cytosol. Several of these hypotheses are experimentally testable but this has not yet been done.

The next step in generating human-like complex glycans is the trimming of the two mannose residues on the α-1,6-arm of the $Man_5GlcNAc_2$ core (Fig. 2 B). In the higher eukaryotic N-glycosylation pathway, this is catalyzed by mannosidase II (ManII), which localizes in the same Golgi compartment as GnT-I. This mannosidase functionality can be introduced by following the same combinatorial approach used in the previous steps. The $GlcNAcMan_5GlcNAc_2$ strains were transformed with chimeric constructs in which catalytic ManII domains are fused to fungal Golgi-localized transmembrane domains. The best performing strain was one that was transformed with a *Drosophila melanogaster* ManII and produced mainly $GlcNAcMan_3GlcNAc_2$ as N-glycans, though also a fair amount of $GlcNAcMan_4GlcNAc_2$ and $GlcNAcMan_5GlcNAc_2$ N-glycans were present (Hamilton et al. 2003). In vitro enzymatic analysis revealed that this was not due to incomplete trimming by ManII, but resulted from the activity of a native *Pichia* α-1,2-mannosyltransferase, which recognizes the newly formed $GlcNAcMan_3GlcNAc_2$ structure, and does not occur naturally in *Pichia*. However, when combined with the introduction *N*-acetylglucosaminyltransferase II (GnT-II) activity (again selected from a library of targeting signal-catalytic domain fusions), it resulted in predominant modification with $GlcNAc_2Man_3GlcNAc_2$ N-glycans on a test protein (Hamilton et al. 2003). In this manner, the undesired action of the native *Pichia* α-1,2-mannosyltransferase at the point of ManII introduction could be largely avoided. No doubt, the introduced GnT-II enzyme competes for substrate with the endogenous α-1,2-manno-

syltransferase, either due to overwhelming catalytic activity (expression is driven by a very strong promoter) or due to localization in the Golgi earlier than the endogenous enzyme.

At that point in time, all technology was in place to produce biantennary human-like, beta-1,4-galactosylated N-glycans. These are the type of glycans found on the Fc part of human IgG, where this particular non-fucosylated structure has great advantages over the naturally occurring core-fucosylated counterpart: it increases the potential of the antibody for antibody-dependent cytotoxicity, which is desirable in many anti-tumor and anti-viral therapeutic antibodies (Rothman et al. 1989; Umana et al. 1999; Shields et al. 2002; Shinkawa et al. 2003).

For some therapeutic glycoproteins, however, it is desirable to obtain sialylation of the terminal beta-galactoses, mainly when long serum half-life is desired (such as for erythropoietin). This is particularly difficult in yeasts because they lack the synthetic pathway for CMP-*N*-acetylneuraminic acid, the donor sugar nucleotide for the most common type of sialylation. Hamilton and colleagues successfully introduced the sialic acid biosynthetic pathway in *P. pastoris* by expressing human UDP-GlcNAc-2-epimerase/ManNAc kinase (GNE), human *N*-acetylneuraminate-9-phosphate synthase (SPS), human CMP-sialic acid synthase (CSS), mouse CMP-sialic acid transporter (CST), and a fusion protein (ST) comprised of the mouse α-2,6-sialyltransferase catalytic domain and the leader peptide from *S. cerevisiae* mannosyltransferase 1 (Mnt1) (Hamilton et al. 2006). The parental strain used is far more heavily engineered than a similar strain described before by the same group (Bobrowicz et al. 2004). Additional *Pichia* genes had been inactivated, resulting in an *och1 pno1 mnn4B bmt2* background. Och1p is the initiating alpha-1,6-mannosyltransferase discussed above. *S. cerevisiae PNO1* and *MNN4* are involved in mannosylphosphorylation of N-glycans. *PpBMT2* was later characterized by the same group as a beta-mannosyltransferase (Mille et al. 2008). Indeed, Jacobs et al. also observed significant beta-mannosyltransferase product formation on processing intermediates of the heterologous complex-type N-glycan biosynthetic pathway, in very particular cases (unpublished results). Moreover, the strain also expresses the enzymes needed for building the $Gal_2GlcNAc_2Man_3GlcNAc_2$ substrate glycan for sialylation, as well as UDP-GlcNAc and UPD-Gal transporters. It is noted that the authors here switched from their previous hGalT-UDP-Gal 4-epimerase fusion construct to two independent expression constructs for both enzymes (Hamilton et al. 2006).

Jacobs et al. (2009) recently integrated what was judged as the most efficient parts of these technologies into the GlycoSwitch system for *Pichia* N-glycan engineering. In all cases tested so far, application of this five-vector system resulted in the production of predominantly $Gal_2GlcNAc_2Man_3GlcNAc_2$ glycans. If required, sialylation can easily be achieved in vitro with recombinant sialyltransferases and CMP-NANA (Cheng et al. 2010; Yu et al. 2006) or with *Trypanosoma cruzi trans*-sialidase and a suitable sialic acid donor (Ryckaert et al. 2005). In that way, the complex sialylation pathway does not have to be integrated in the yeast. This has the added advantage that in vitro conversion can be driven to completion.

Building on the "*alg3* knockout" strategy of guiding the yeast N-glycosylation pathway to a homogeneous core structure that can function as a substrate for the mammalian Golgi enzymes (see "Interference earlier in the N-glycosylation pathway"; Figs. 1 B and 2 B), Glycofi researchers knocked out *ALG3* in a $GlcNAcMan_5GlcNAc_2$ strain (Bobrowicz et al. 2004; Choi et al. 2003; Hamilton et al. 2003). This $GlcNAcMan_5GlcNAc_2$ strain has an och1 deletion and contains the catalytic domains of mouse α-1,2-mannosidase and human GnT-I fused to the yeast localization signals from Sec12p and Mnn9p, respectively, and a UDP-GlcNAc transporter from *K. lactis* (Choi et al. 2003; Hamilton et al. 2003). Upon disruption of *ALG3*, the resulting strain secreted proteins modified mainly with $GlcNAcMan_3GlcNAc_2$ glycan structures. In addition, N-glycans of higher molecular mass were observed. Further analysis revealed that they contain one terminal β-linked GlcNAc residue (as expected) and up to four α-1,2-mannose residues, resulting from endogenous Golgi-resident α-1,2-mannosyltransferases (as already described above). To further engineer this strain, GnT-II was introduced by the previously reported combinatorial library strategy of fusion constructs. As before, introduction of the rat GnT-II–Mnn9p fusion protein eliminated the N-glycan heterogeneity, and resulted in a strain producing quite homogeneous biantennary complex N-glycans with terminal GlcNAc residues, i.e., $GlcNAc_2Man_3GlcNAc_2$ (Bobrowicz et al. 2004). However, as described above, the *alg3* mutation generally results in under-occupancy of N-glycosylation sites and this was underdocumented in these reports.

Engineering N-glycosylation pathways in filamentous fungi

Filamentous fungi are distinct from the yeasts discussed above. They possess two types of α-1,2-mannosidases, one of which trims $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ in the ER (Yoshida et al. 2000), while the other resembles mammalian Golgi α-1,2-mannosidases that trim $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ (Ichishima et al. 1999). However, the latter enzyme is often not strongly expressed and/or is secreted from the cells, and early research into these fungi showed

that only a minor part of their N-glycans were of the $Man_5GlcNAc_2$ type, the substrate for the GlcNAc transferase I (Maras et al. 1997a). In addition, the N-glycans of fungi are frequently substituted with terminal glucose or galactose residues or phosphomonoesters and diesters (De Bruyn et al. 1997; Garcia et al. 2001; Harrison et al. 2002; Maras et al. 1997b; Nakao et al. 1987). Attempts were made to convert the N-glycans present on the cellobiohydrolase I produced by *T. reesei* into more mammalian-like oligosaccharides by performing in vitro glycosylation using recombinant *N*-acetylglucosaminyltransferase I, human β-1,4-galactosyltransferase, and rat α-2,6-sialyltransferase (Maras et al. 1997a). Only 0.25% to 1.8% of the N-glycans of cellobiohydrolase I of *T. reesei* could be modified to a hybrid structure. Nevertheless, these researchers provided the first proof of in vivo transfer of a GlcNAc residue to fungal *Trichoderma* N-glycans (yielding $GlcNAcMan_5GlcNAc_2$) by overexpression of recombinant human β-1,2-GlcNAc transferase I (Maras et al. 1999). Earlier attempts to introduce an active β-1,2-GlcNAc transferase I in the filamentous fungus *A. nidulans* were unsuccessful as no modification of the N-glycans could be observed (Kalsner et al. 1995). Later, in a similar experiment, expression of rat GnT-I in *Aspergillus oryzae* resulted in successful in vivo transfer of a GlcNAc residue to some of the N-glycans of alpha-amylase (Kasajima et al. 2006). It is clear that, as in yeast, enough substrate must be produced for the GlcNAc transferase I, i.e., $Man_5GlcNAc_2$ or $Man_3GlcNAc_2$.

A. niger was engineered by introducing the α-1,2-mannosidase of *T. reesei* fused to the HDEL domain, which resulted in a strain modifying its glycoproteins mainly with $Man_{5-6}GlcNAc_2$ glycan structures (Vervecken et al., unpublished data).

As mentioned above, good progress was made in 2008 by Kainz and colleagues, who created *Aspergillus* strains producing $Man_3GlcNAc_2$ N-glycans. To achieve this, they first overexpressed a fusion protein composed of the *P. pastoris* Sec12p leader and the *Caenorhabditis elegans* α-1,2-mannosidase catalytic domain. This strain produced increased amounts of $Man_5GlcNAc_2$-type N-glycans. Subsequent introduction of a human GlcNAc transferase I yielded a strain producing a fraction of the $GlcNAcMan_5GlcNAc_2$ N-glycan. In the same study, to produce fairly homogeneous $Man_3GlcNAc_2$ N-glycans, the *Aspergillus ALG3* ortholog (*ALGC*) was deleted (Kainz et al. 2008).

O-glycosylation engineering in yeast

In addition to N-glycosylation, yeasts also O-glycosylate secreted proteins with oligomannosyl-glycans that differ from the mucin-type O-glycosylation in humans (Strahl-Bolsinger et al. 1999). In contrast to N-glycosylation, O-glycans are synthesized by the stepwise addition of monosaccharides. In yeast, the first step in O-glycosylation is catalyzed by a family of protein mannosyltransferases in the ER that use dolichol-phosphate mannose as donor (Fig. 3 A). Further elongation takes place in the Golgi apparatus by several mannosyltransferases using GDP-mannose as donor (Goto 2007) (Fig. 3 A). In contrast, mucin-type O-glycan synthesis in mammalian cells starts with the attachment of a *N*-acetylgalactosamine (GalNAc) residue to specific Ser/Thr residues (Lis and Sharon 1993). Further elongation of these initiating residues with a variety of monosaccharides results in a highly diverse collection of oligosaccharides. In contrast to yeasts, this reaction is catalyzed by a family of GalNAc transferases in the Golgi apparatus using UDP-GalNAc as donor. O-glycans introduce unpredictable and sometimes hard to detect heterogeneity in recombinant glycoproteins. However, engineering of the O-glycosylation pathway is not as advanced as N-glycosylation engineering and no satisfactory, biotechnologically useful strategies have yet been found.

When IgG was expressed in the methylotrophic yeast *Ogataea minuta*, it was found to be O-glycosylated, but this was not so when the same IgG was expressed in mammalian systems (Kuroda et al. 2008). To overcome this O-glycosylation, the rhodanine-3-acetic acid derivative was added to the culture (Fig. 3 B) because it inhibits Pmt protein activity in *Candida albicans* and induces a phenotype associated with the loss of Pmtp activity (Orchard et al. 2004). Addition of moderate concentrations of this inhibitor during cultivation of the strain expressing the antibody had no major effects on cell growth and led to a decrease of heterogeneity and molecular weight of the antibody, which indicates that O-glycosylation was partly suppressed (Kuroda et al. 2008). Moreover, the suppression of O-glycosylation was associated with an increase in the amount of assembled antibody and enhanced the antigen-binding activity of the secreted antibody (Kuroda et al. 2008). The necessity of adding a drug during large-scale cultivation could be problematic.

Attempts were made to introduce a mucin-type O-glycosylation pathway in the model yeast, *S. cerevisiae*, while suppressing the yeast's own O-mannosylation (Fig. 3 B). Amano et al. (2008) created a strain capable of generating O-linked *N*-acetylgalactosamine (GalNAc) structures. To this end, they engineered *S. cerevisiae* with the pathway required for the synthesis and transport of UDP-GalNAc and for GalNAc-modification of mucin-type O-glycosylation sites of the protein (*Bacillus subtilis* UDP-Gal/GalNAc-4-epimerase, human UDP-Gal/GalNAc transporter, human ppGalNAc-T1, and *D. melanogaster* core1 β1-3 GalT; both latter genes were fused to the *ScMNN9* transmembrane domain) (Fig. 3 B). The yeast's own O-mannosylation was suppressed in this strain by the addition of the rhodanine-3-acetic acid derivative Pmt

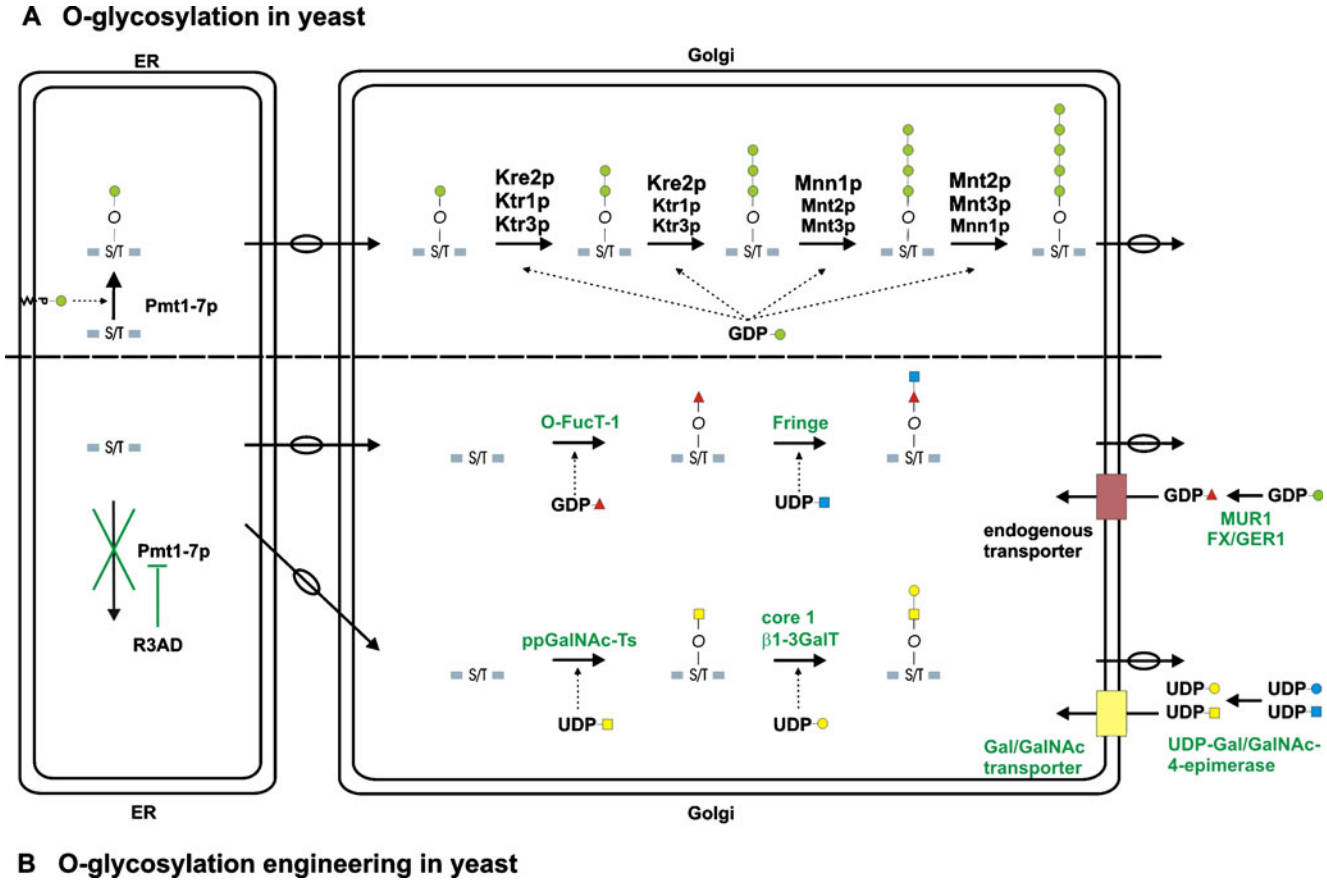

Fig. 3 O-glycosylation and engineering in yeast. *A* Endogenous O-glycosylation in yeast (as modeled in *S. cerevisiae*). O-glycans are sequentially synthesized on the side chain of Ser or Thr residues. In the ER, the first mannose residue is added by a family of protein-O-mannosyltransferases (Pmt1-7p). Further elongation in the Golgi apparatus is catalyzed by several mannosyltransferases. *B* O-glycosylation engineering in yeast. Two examples to generate mucin-type O-glycans had been described in *S. cerevisiae*. Providing a biosynthetic pathway for the production of UDP-GalNAc and UDP-Gal (UDP-Gal/GalNAc-4-epimerase) in addition to its transporter (Gal/GalNAc transporter), a protein-O-GalNAc transferase (ppGalNac-Ts) and a β-1,3 Gal transferase (core 1 β-1,3-GalT) allows yeast to generate mucin-type GalGalNAc O-glycans. In the second example, O-FucGlcNAc mucin-type O-glycans were generated through introduction of a biosynthetic pathway for GDP-Fuc (MUR1 and FX/GER1), a GDP-fucose transferase (O-FucT-1) and a UDP-GlcNAc transferase (Fringe). In both examples, inhibition of the yeast's own O-glycosylation pathway was achieved by adding rhodanine-3-acetic acid (R3AD) to the culture

inhibitor. This strain modified the MUC1a peptide and the human glycoprotein podoplanin mainly with O-glycans of the Galβ1-3GalNAcα1-O-Ser/Thr type (Amano et al. 2008). As in *O. minuta*, the rhodanine-3-acetic acid derivative Pmt inhibitor was unable to completely suppress O-mannosylation. However, complete suppression is likely not feasible because O-mannosylation is required for survival (Gentzsch and Tanner 1996).

Chigira et al. (2008) created a strain generating another type of mucin-type O-glycans. By expression of human O-fucosyltransferase 1, along with genes whose protein products convert cytoplasmic GDP-mannose to GDP-fucose (*Arabidopsis thaliana* GDP-mannose-4,6-dehydratase *MUR1* and GDP-4-keto-6-deoxy-mannose-3,5-epimerase/4-reductase *FX/GER1*) in an EGF-domain expressing *S. cerevisiae* strain, they were able to produce O-fucosylated EGF domains (Chigira et al. 2008) (Fig. 3 B). This showed that *S. cerevisiae* can transport GDP-fucose. Further, some of the O-FucT-1 and GDP-fucose was released from the cells into the medium by secretion or cell lysis, which resulted in O-fucosylation in the medium (Chigira et al. 2008). The introduction of human Manic Fringe into the O-fucosylating strain resulted in elongation of O-fucose with *N*-acetylhexosamine (HexNAc) (Chigira et al. 2008). These engineering approaches show the possibility of introducing mucin-type O-glycosylation in yeast and that these systems can be used to produce glycoproteins containing many types of glycoforms.

The remaining main goal is to engineer yeast strains which are completely deficient in their own O-mannosylation, in an approach not requiring drug addition, and keeping the strain not only viable, but robust enough for large-scale protein manufacturing. It remains to be seen whether such engineering is possible.

Discussion

To date, only *P. pastoris* has been glyco-engineered to provide an array of individual strains in which each host strain can produce homogeneous glycoproteins with a specific human-like glycoform at a high degree of homogeneity. This created an expression system in which we can analyze the effect of the different glycoforms on protein function and optimize the glycosylation intended for specific therapeutic functions (Hamilton and Gerngross 2007). For instance, Li et al. utilized the various engineered *P. pastoris* strains to create six N-glycoforms of monoclonal IgG antibodies (anti-CD20, Rituxan) and analyze their specific binding affinities and their corresponding antibody-dependent cell cytotoxicity (Li et al. 2006). This study showed that specific glycoforms displayed 100-fold higher binding affinity to the low affinity variant FcgRIIIa receptor and exhibited improved in vitro human B-cell depletion (Li et al. 2006).

Another example is the production of recombinant lactoferrin with two humanized glycoforms, sialylated and non-sialylated, to evaluate the effects of terminal N-glycan structures on the functionality of the protein (Choi et al. 2008).

N-glycosylation engineering in yeast and filamentous fungi has generated a number of strains in different species in which the endogenous N-glycosylation pathway is abolished and a humanized pathway is introduced. With further development, these strains will become a valuable platform for the production of (pharmaceutical) glycoproteins.

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