nature biotechnology

© 2004 Nature Publishing Group http://www.nature.com/naturebiotechnology

Advances in the production of human therapeutic proteins in yeasts and filamentous fungi

Tillman U Gerngross

Yeast and fungal protein expression systems are used for the production of many industrially relevant enzymes, and are widely used by the research community to produce proteins that cannot be actively expressed in *Escherichia coli* or require glycosylation for proper folding and biological activity. However, for the production of therapeutic glycoproteins intended for use in humans, yeasts have been less useful because of their inability to modify proteins with human glycosylation structures. Yeast glycosylation is of the high-mannose type, which confers a short *in vivo* half-life to the protein and may render it less efficacious or even immunogenic. Several ways of humanizing yeast-derived glycoproteins have been tried, including enzymatically modifying proteins *in vitro* and modulating host glycosylation pathways *in vivo*. Recent advances in the glycoengineering of yeasts and the expression of therapeutic glycoproteins in humanized yeasts have shown significant promise, and are challenging the current dominance of therapeutic protein production based on mammalian cell culture.

Protein-based therapeutics are emerging as the largest class of new chemical entities being developed by the drug industry[1]. Unlike small molecules, which typically are synthesized by chemical means, most proteins are sufficiently complex to necessitate their production in living systems, mostly by recombinant DNA technology. As such, the choice of recombinant expression hosts has been the subject of ongoing evaluation and much effort has been directed at developing novel protein expression systems with improved characteristics. The five primary metrics for evaluating such novel protein expression hosts are: (i) the cost of manufacturing and purification (ii) the ability to control the final product including its post-translational processing (iii) the time required from gene to purified protein (iv), the regulatory path to approve a drug produced on a given expression platform and (v) the overall royalties associated with the production of a recombinant product in a given host.

Yeasts and filamentous fungi have been extensively used in the industrial enzyme industry for the production of recombinant proteins—and most companies (DSM (Heerlen, The Netherlands), Genencor (Palo Alto, CA, USA), Novozymes (Bagsveard, Denmark) and others) have developed large-scale fermentation capacity around yeasts and/or fungi. The ability of these organisms to grow in chemically defined medium in the absence of animal-derived growth factors (e.g., calf serum) and to secrete large amounts of recombinant protein, together with the ease of scale-up, have made these hosts the system of choice for producing many industrial enzymes, where cost of goods is a primary concern[2]. The research community has relied on yeasts for the production of recombinant proteins that cannot be obtained from *E. coli* because of folding problems or the requirement for glycosylation. Yeasts have been used to express proteins at very high protein titers, including mammalian proteins, as exemplified by the production of 14.8 g/l of gelatin in *Pichia pastoris*[3]. Expression kits based on yeasts are available from almost all major research tool companies and their ease of use has made them an attractive solution for many protein expression needs.

However, the choice of protein expression system is most often dictated by the ultimate use of the product and not necessarily the ease of initial production. Proteins intended for use in humans impose the most significant challenges in this context, and the aberrant nature or absence of post-translational processing associated with a given host, often becomes an overriding consideration. In this article, we review the challenges involved in the production of therapeutic glycoproteins and the current state of development of engineered yeast strains that are able to produce humanized glycoproteins.

Glycosylation of therapeutic proteins

There are approximately 140 therapeutic proteins approved in the United States and Europe, and an additional 500 in clinical trials[1]; with an even larger number in preclinical development. The therapeutic protein market can be roughly divided into two segments—proteins that are not post-translationally modified and those that require post-translational processing (mostly N-glycosylation) to attain full biological function. Nonglycosylated proteins are typi-

Thayer School of Engineering, the Department of Biological Sciences and the Department of Chemistry, Dartmouth College, Hanover, New Hampshire 03755, USA and GlycoFi Inc., 21 Lafayette Street, Lebanon, New Hampshire 03766, USA. Correspondence should be addressed to T.U.G. (Tillman.gerngross@dartmouth.edu).

Published online 4 November 2004; doi:10.1038/nbt1028

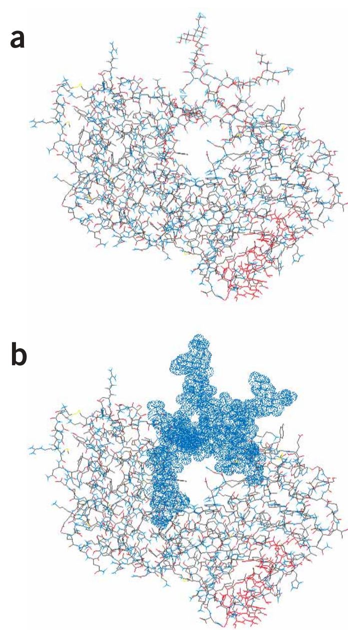

Figure 1 Protein structure of interferon-β illustrates the impact of glycosylation on hydrodynamic volume; glycosylated interferon-β (top) and glycosylated interferon-β with glycosylation highlighted in blue (bottom). Source: http://www.dkfz.de/spec/glycosciences.de/modeling/glyprot/php/main.php

© 2004 Nature Publishing Group http://www.nature.com/naturebiotechnology

cally expressed in either *E. coli* or the yeast *Saccharomyces cerevisiae* and currently constitute about 40% of the therapeutic protein market with an annual growth rate of about 12%[4]. Some aglycosylated proteins are expressed in both systems, for example, recombinant insulin is produced in *E. coli* (Humulin, Eli Lilly, Indianapolis, IN, USA) and yeast (Novolog, Novo Nordisk, Bagsvaerd, Denmark).

The production of recombinant proteins that are N-glycosylated in their native state has in most cases required mammalian expression hosts that have the ability to mimic human glycosylation. Prokaryotic hosts such as *E. coli* do not glycosylate proteins, and lower eukaryotic expression systems such as yeast and insect cells are typically unable to provide mammalian glycosylation. Aglycosylated forms of glycoproteins tend to be misfolded, biologically inactive or rapidly cleared from circulation[5]. Yeasts are able to glycosylate proteins, yet the nonhuman nature of yeast glycans negatively impacts protein half-life because of the affinity of these glycans to high-mannose receptors present on macrophages and endothelial cells[6]. For these reasons, mammalian cells, in particular Chinese hamster ovary (CHO) cells, have emerged as the most widely used expression system for the production of complex human glycoproteins—currently about 60% of the therapeutic protein market are glycoproteins with an annual growth rate of approximately 26%[4]. CHO cells are typically able to express glycoproteins with human-like glycosylation patterns, although it is commonly recognized that glycan structures produced from CHO cell lines differ from those produced in human cells and sometimes have to be modified to meet therapeutic efficacy[7,8]. A few natively glycosylated proteins are produced in nonglycosylating hosts (e.g. Betaferon (interferon-β-1b); Schering-Plough, Kenilworth, NJ, USA) yet the lack of glycosylation can compromise half-life and other clinically relevant properties of the molecule such as renal clearance, which is affected by the hydrodynamic volume of the protein (**Fig. 1**).

It has to be recognized that glycoproteins produced by mammalian cell culture are inherently heterogenous mixtures of glycoforms, and that scale-up and regulatory approval have been a matter of maintaining a reproducible composition and ratio of glycoforms. Because it is well understood that certain glycoforms are more active than others[9], the relative distribution of individual glycoforms has to be kept constant during preclinical manufacturing and post-approval production—often representing a major bioprocessing challenge. A purified protein preparation derived from a mammalian cell culture process is essentially a mixture of individual drugs, each with its own pharmacokinetic, pharmacodynamic properties and efficacy profile[10]. For example, human erythropoietin (EPO), a glycoprotein that plays a major role in regulating the level of circulating erythrocytes, has found wide use in the treatment of anemia. Natural EPO contains three N-glycans (glycans linked to asparagine residues of the glycoprotein), which are critical to its therapeutic activity, and one *O*-glycosylation site which has no known function; removal of the N-glycans from EPO results in a protein with a very short half-life and virtually no erythropoietic function *in vivo*[10]. Whereas glycosylation of EPO appears to be essential, it is not sufficient to ensure full biological activity. Recent work has shown that the production of recombinant EPO is highly sensitive to the production process. Different process conditions have been shown to yield material that has a fivefold difference in erythropoietic function *in vivo*, and a detailed analysis of this effect demonstrated that variations in the glycosylation are responsible for these differences[11]. Recent work by Erbayraktar and colleagues has shown that by modifying the glycosylation of EPO, one can create a molecule that entirely lacks erythropoietic function while retaining the neuroprotective function of the protein[12].

Some therapeutic proteins require specific glycosylation to ensure proper cellular targeting. For example, glucocerebrosidase, the enzyme used for replacement therapy in patients with Gaucher disease, is based on a recombinant enzyme produced in mammalian cells[7]. However, the therapeutically active form of glucocerebrosidase requires terminal mannose sugars, which are responsible for delivery of the protein to macrophages and subsequent incorporation into lysozomes[13]. As discussed, glycoproteins from CHO cells contain complex N-glycans which contain: *N*-acetyl glucosamine (GlcNAc), galactose (Gal) and terminal sialic acid (NANA) (**Fig. 2**). It is therefore necessary to remove all terminal nonmannose sugars after isolation of the glycoprotein. To do so, *in vitro* treatment with neuraminidase (to remove sialic acid), galactosidase (to remove galactose) and hexosaminidase (to remove GlcNAc) is required. The process thus involves a mammalian cell culture step to produce the protein of interest and a purification step followed by three enzymatic processing steps to yield a glycan structure that is appropriate for therapeutic use.

Collectively, these examples are intended to support the view that glycosylation plays an essential role in the function of many therapeutically relevant proteins and that controlling glycosylation can be used to modulate and improve therapeutic function, or even eliminate certain undesirable functions.

Because mammalian cells typically express a heterogenous mixture of glycoforms, several approaches have been developed to overcome this shortcoming including modulating glycosylation pathways in mammalian host cells or using *in vitro* enzymatic methods to modify glycosylation after purification of the protein. For example, Umana and coworkers at the Institute of Biotechnology (ETH, Zurich) showed that CHO cells that overexpress UDP-glucosaminyl transferase III, produce IgGs with increased bisecting

GlcNAc and elevated antibody-dependent cell-mediated cytotoxicity (ADCC) activity[14]. Methods to engineer glycosylation in mammalian cells have been reviewed elsewhere[15,16].

Yeast and fungal expression systems

The growing demand for improved biopharmaceutical expression hosts has led several companies to return to yeast and fungal expression systems, which provide high protein titers (>1 g/l) in fermentation processes that last a few days, are scalable to the 100 m^3 scale, allow for rapid turnaround from gene to protein and offer an array of genetic tools to manipulate the host organism. Decades of research and the production of many industrial enzymes in both yeast and fungi have resulted in robust, scalable processes that allow the low-cost production of many recombinant enzymes. In addition, about a sixth of all currently approved therapeutics are made in yeasts[1], and thus fewer regulatory hurdles are expected when comparing yeast-based production to entirely new expression platforms, such as transgenic animals or plants.

All yeast-based therapeutic proteins are currently produced in the Baker's yeast *S. cerevisiae* (**Table 1**), but other yeasts have been developed to make therapeutic proteins. The yeast *P. pastoris* was originally developed as a single-cell protein-production system by Philips Petroleum (Bartlesville, OK, USA) but was subsequently adapted for heterologous protein expression. More than 120 recombinant proteins have been expressed in this host—many of which are of human or mammalian origin[17]. More recently, *P. pastoris* has been used to express therapeutic proteins that have entered clinical trials (**Table 1**), although some proteins expressed in this host have been shown to contain *O*-mannosylation not present on the native protein[18].

Genencor (Palo Alto, CA, USA) has used the filamentous fungi *Aspergillus niger* and *Trichoderma reesei* for the large-scale production of recombinant industrial enzymes. Recent efforts to express therapeutically relevant proteins have focused on the expression of full-length IgGs in *A. niger*[19]. This antibody is produced at titers just under 1 g/l, is correctly assembled and binds antigen. Ogunjimi *et al.*[20] have had similar success with the expression of intact antibody in *P. pastoris*; however, titers were below 40 mg/l. The structure of the glycans added to the antibodies by *A. niger* and *P. pastoris* are of the high-mannose type, as expected from fungi and yeasts. Ward and colleagues[19] demonstrated that the antibody produced in *A. niger* had similar pharmacokinetic behavior and ADCC activity when compared with a mammalian cell–derived antibody, although the glycan structures differed from mammalian glycosylation and not all glycosylation sites were occupied with glycans. Although this is promising, concern remains that these nonhuman aberrant sugar structures may be immunogenic in humans upon long-term administration.

Berna Biotech, formerly Rhein Biotech (Bern, Switzerland), has developed a proprietary protein expression platform based on the methylotrophic yeast *Hansenula polymorpha* that is currently being further developed for the production of recombinant vaccines. Although yeast systems (e.g., *H. polymorpha, S. cerevisiae* and *P. pastoris*) are often ideal for producing high titers of recombinant proteins, their nonhuman glycosylation patterns have in the past precluded them from consideration for the production of glycoproteins intended for therapeutic use in humans.

Humanizing glycosylation pathways in yeasts and fungi

Given the otherwise attractive attributes of yeasts and filamentous fungi, several groups have investigated the possibility of humanizing glycosylation pathways in these hosts to produce human-like glycoproteins. Early attempts to replace mammalian cell lines with fungal expression hosts were based on the observation that the filamentous fungus *T. reesei* was able to secrete glycoproteins containing intermediates of human glycosylation that were amenable to *in vitro* processing by mammalian glycosyltransferases[21]. In this approach, a fungal precursor protein serves as a substrate for one or several chemoenzymatic glycosylation steps that result in hybrid glycoproteins, albeit it at low yield[22]. Although encouraging, this approach requires several *in vitro* glycosylation reactions, which are costly and cumbersome, and it became apparent that a more attractive solution for high-titer production of human glycoproteins in yeast and filamentous fungi would be to genetically engineer the human glycosylation pathway into the host organism itself.

Although conceptually straightforward, the technical complexity of replicating human glycosylation pathways in fungal hosts is daunting. First, any attempt to reengineer a protein expression host

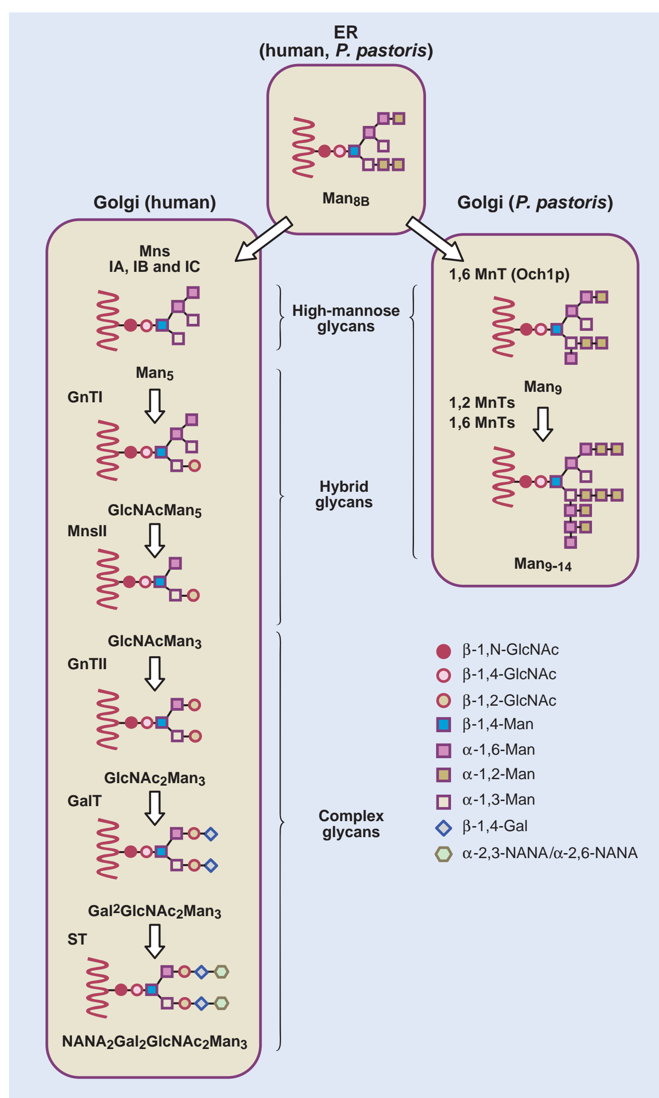

Figure 2 Glycosylation pathways in humans and yeast. Mns, α-1,2-mannosidase; MnsII, mannosidase II; GnT1, β-1,2-*N*-acetylglucosaminyltransferase I; GnTII, β-1,2-*N*-acetylglucosaminyltransferase II; GalT, β-1,4-galactosyltransferase; ST, sialyltransferase; MnT, mannosyltransferase.

© 2004 Nature Publishing Group http://www.nature.com/naturebiotechnology

© 2004 Nature Publishing Group http://www.nature.com/naturebiotechnology

to perform human glycosylation requires a complete inventory of all glycosylation reactions known to occur in a given host, including *O*-glycosylation (i.e., the glycome). By the early 1990s, much was known about the principal glycosylation pathways in humans and several yeasts[23,24]. Whereas human N-glycosylation is of the complex type, built on a tri-mannose core extended with GlcNAc, galactose, and sialic acid (**Fig. 2**), yeast N-glycosylation is of the high-mannose type containing up to 100 or more mannose sugars (hypermannosylation)[25,26].

Early N-glycan processing, involving (i) the assembly of the core oligosaccharide, (ii) its site-specific transfer onto the protein, and (iii) its trimming by glucosidase I, II and an endoplasmic reticulum (ER) resident α-1,2-mannosidase, is highly conserved in mammals and yeast. In fact, up to the formation of the Man_8 glycan intermediate (Man_8B) in the ER both pathways are identical (**Fig. 2**). However, following transport of the protein to the Golgi apparatus, the two pathways diverge significantly. It is at this juncture that mammalian cells rely on additional α-1,2-mannosidases to trim mannose residues[27], whereas yeasts initiate a series of mannosyltransferase reactions to yield the above-mentioned hypermannosylated glycan structures. To date, all successful efforts to humanize yeast glycosylation pathways have focused on the deletion of specific yeast genes involved in hypermannosylation, and the introduction of genes catalyzing the synthesis, transport, and addition of human sugars.

Schachter's group[28] at the University of Toronto was the first to report an attempt to introduce a mammalian glycosylation step in the filamentous fungus *A. nidulans* by expressing β-1,4-*N*-acetylglucosaminyltransferase I (GnTI), the key enzyme responsible for the formation of hybrid glycans in mammals. However, although high transcription levels of the enzyme were observed, and active enzyme could be assayed in cell-free extracts, no impact on the N-glycans was observed[28].

Table 1 Therapeutic proteins produced in the yeasts *S. cerevisiae* and *P. pastoris*

Products on the market

Commercial name	Recombinant protein	Company	Expression system
Actrapid	Insulin	NovoNordisk	*S. cerevisiae*
Ambirix	Hepatitis B surface antigen	GlaxoSmithKline	*S. cerevisiae*
Comvax	Hepatitis B surface antigen	Merck	*S. cerevisiae*
Elitex	Urate oxidase	Sanofi-Synthelabo	*S. cerevisiae*
Glucagen	Glucagon	Novo Nordisk	*S. cerevisiae*
HBVAXPRO	Hepatitis B surface antigen	Aventis Pharma	*S. cerevisiae*
Hexavac	Hepatitis B surface antigen	Aventis Pasteur	*S. cerevisiae*
Infanrix-Penta	Hepatitis B surface antigen	GlaxoSmithKline	*S. cerevisiae*
Leukine	Granulocyte-macrophage colony stimulating factor	Berlex	*S. cerevisiae*
Novolog	Insulin	Novo Nordisk	*S. cerevisiae*
Pediarix	Hepatitis B surface antigen	GlaxoSmithKline	*S. cerevisiae*
Procomvax	Hepatitis B surface antigen	Aventis Pasteur	*S. cerevisiae*
Refuldan	Hirudin/lepirudin	Hoechst	*S. cerevisiae*
Regranex rh	Platelet-derived growth factor	Ortho-McNeil Phama (US), Janssen-Cilag (EU)	*S. cerevisiae*
Revasc	Hirudin/desirudin	Aventis	*S. cerevisiae*
Twinrix	Hepatitis B surface antigen	GlaxoSmithKline	*S. cerevisiae*

Products under development

Protein	Indication	Company	Expression system
Angiostatin	Antiangiogenic factor	EntreMed	*P. pastoris*
Elastase inhibitor	Cystic fibrosis	Dyax	*P. pastoris*
Endostatin	Antiangiogenic factor	EntreMed	*P. pastoris*
Epidermal growth factor analog	Diabetes	Transition Therapeutics	*P. pastoris*
Insulin-like growth factor-1	Insulin-like growth factor-1 deficiency	Cephalon	*P. pastoris*
Human serum albumin	Stabilizing blood volume in burns/shock	Mitsubishi Pharma (formerly Welfide)	*P. pastoris*
Kallikrein inhibitor	Hereditary angiodema	Dyax	*P. pastoris*

Includes approved products and those in clinical development. Sources: (G. Walsh, 2003)[1] or personal communication.

One of the critical steps in obtaining complex glycoproteins in a fungal host requires the generation of Man_5 in high yields, because this substrate is the precursor for all subsequent downstream processing steps (**Fig. 2**). Early attempts to engineer active α-1,2-mannosidases into yeasts were rather discouraging, mostly because *P. pastoris* appeared to compensate for mannose trimming by upregulating mannosyltransferase activity leading to even higher molecular weight glycan structures[29]. Jigami's group[30] at Japan's National Institute of Bioscience and Human Technology (Tsukuba, Ibaraki, Japan), in collaboration with researchers at Kirin (Yokohama, Japan), showed that after systematically eliminating yeast-specific glycosylation reactions by deleting *och1*, *mnn1* and *mnn4* in the yeast *S. cerevisiae*, and expressing an α-1,2-mannosidase targeted to the ER, some Man_5 N-glycans could be generated on an intracellular reporter protein[30]. However, the paucity of Man_5 obtained (less than 30% of total glycans), despite the expression of an α-1,2-mannosidase from a multicopy plasmid (yeast 2μ) and a strong constitutive promotor (glyceraldehyde-3-phosphate, GAPDH), was of concern. These initial attempts in fungi and yeasts showed some promise; however, they also revealed that genetically humanizing glycosylation pathways in these hosts was not trivial and that merely expressing enzymes involved in mammalian glycosylation pathways was unlikely to be successful.

The secretory pathway is a cellular assembly line

In yeasts and humans, host-specific glycosyltransferases and glycosidases line the luminal surface of the ER and Golgi apparatus and thereby provide catalytic surfaces that allow the sequential process-

ing of glycoproteins as they proceed from the ER through the Golgi network. As a glycoprotein proceeds from the ER through the secretory pathway, it is sequentially exposed to different mannosidases and glycosyltransferases. An essential step in recreating human glycosylation pathways in lower eukaryotes is recreating the sequential nature of complex N-glycan processing. In 2000, I initiated a research program with three main focal points: (i) overcoming the uncertainty related to the targeting of heterologous glycosylation enzymes across different species and developing methods to target enzymes to predetermined locations in the secretory pathway of yeast and filamentous fungi, (ii) developing methods to identify enzymes that are in fact active at the site of localization (since the different organelles of the yeast secretory pathway provide different processing environments with different pHs and sugar nucleotide pools, one has to consider how a recombinant enzyme targeted to a particular organelle will behave in that nonnative environment), and (iii) develop efficient screens to identify cell lines that have acquired the ability to process recombinant glycoproteins in a human-like fashion.

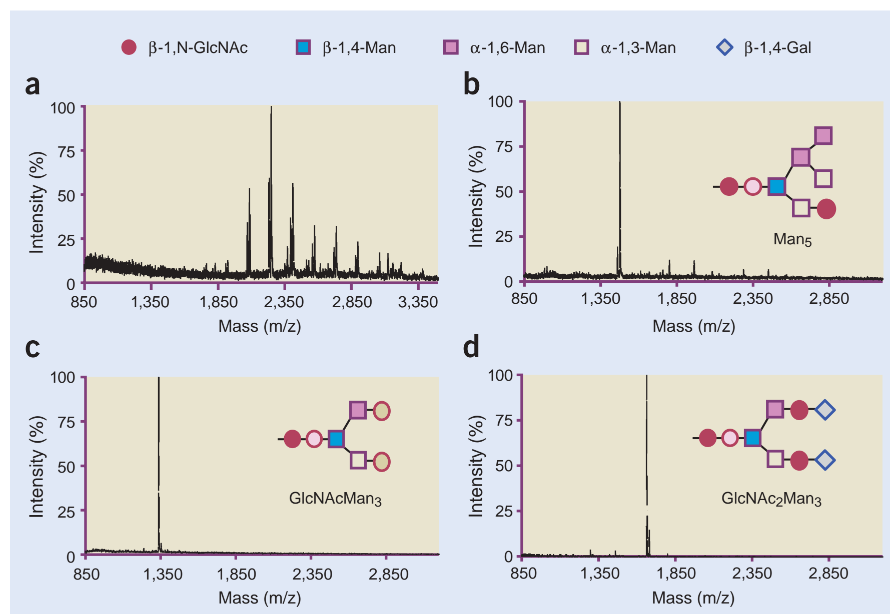

Figure 3 MALDI-TOF mass spectrometry of oligosaccharides released from purified kringle 3 protein produced in wild-type *P. pastoris* and glycoengineered strains of *P. pastoris*. (**a**–**d**) MALDI-TOF mass spectra positive mode: Wild type (**a**), and increasingly humanized glycan structures (see ref. 31, (**b**); ref. 34, (**c**); and ref. 35, (**d**)).

By 2003, my team had shown that many of the problems previously encountered by other researchers could be overcome by generating large-scale combinatorial genetic libraries of glycosylation enzymes fused to libraries of yeast-targeting peptides[31]. By screening a library of over 600 fusion constructs consisting of catalytic domains of α-1,2-mannosidases, and yeast type II leader peptides, we were able to find several specific combinations of catalytic domains and leader peptides that displayed efficient mannosidase activity *in vivo* in *P. pastoris*. Screening was done by purifying a recombinant reporter protein secreted by each strain, and analyzing its glycans by digestion with protein N-glycanase followed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The entire procedure of purification and analysis was carried out by an automated liquid handler allowing the processing of several thousand samples per week.

Glycoproteins expressed in these engineered strains contain over 90% Man_5 and thus provided the basis for the further humanization of glycosylation pathways in *P. pastoris*. After the successful generation of Man_5, we showed that by introducing an active *GnTI* gene and a UDP-GlcNAc transporter, we were able to add a terminal GlcNAc residue to the 1,3 arm of the Man_5 glycan, thereby generating the first genetically engineered yeast that efficiently produces hybrid N-glycans. As in the mannosidase case, active GnTI was identified by introducing a GnTI catalytic domain/Golgi leader library into the yeast and screening for transformants that acquired the ability to produce $GlcNAcMan_5$ at high efficiency. After knocking out the initiating α-1,6-mannosyltransferase activity, and introducing α-1,2-mannosidase, GnT1 and the UDP-GlcNAc transporter into *P. pastoris* we were able to show the secretion of a reporter protein with over 90% of the N-glycans have the desired GlcNAcMan5 structure (**Fig. 3b**)[31]. The remaining glycans contain mostly mannosylphosphate, which can be eliminated in *P. pastoris* by the elimination of two genes associated with the transfer of this sugar[32]. We have expressed several recombinant proteins in these strains and have found expression levels to be similar to wild-type yeast. Recent work by Contreras' group[33] at Ghent University (Belgium) has also shown the *in vivo* production of nonsialylated hybrid N-linked glycans in a glycoengineered strain of *P. pastoris*.

After the *in vivo* production of hybrid N-glycans, we have gone on to further humanize the glycosylation of *P. pastoris* to perform glycosylation of the complex type[34]. By successfully introducing α-mannosidase II and GlcNAc-transferase II (GnTII) activities, following the combinatorial library approach discussed above, we were able to generate yeast strains that perform essentially homogenous glycosylation containing $GlcNAc_2Man_3$ (**Fig. 3c**).

More recently, we have demonstrated the proper localization of active β-1,4-galactosyltransferase (GalT) and UDP-galactose-4-epimerase in *P. pastoris* conferring the transfer of galactose onto both terminal GlcNAc residues of a $GlcNAc_2Man_3$ glycan, resulting in the production of $Gal_2GlcNAc_2Man_3$ *in vivo*[35] (**Fig. 3d**). This has rendered the humanization of *P. pastoris* one step away from completion. Unfortunately, the final transfer of sialic acid will be most challenging, as unlike GlcNAc and galactose, a source of endogenous sialic acid is not known to exist in yeasts or other fungal organisms. Thus, in addition to the requirement for a sialyltransferase enzyme, several other genes involved in the synthesis of CMP-sialic acid must also be genetically engineered into the host. We have recently shown that the precursor CMP-sialic acid, can be produced in *P. pastoris* and used to transfer sialic acid onto a secreted reporter protein (Gerngross, T.U., unpublished data).

Unlike proteins obtained from mammalian cell culture, which are heterogenous, we have found that yeast cells can be engineered to secrete glycoproteins with exceptional glycan uniformity[31,34,35]. Although yeast-based manufacturing is expected to reduce the production cost of protein-based therapeutics, we believe the main

© 2004 Nature Publishing Group http://www.nature.com/naturebiotechnology

benefit of using glycoengineered yeast lies in the ability to rigorously control glycosylation of the final product and thereby improve clinically relevant characteristics such as half-life or tissue distribution. Using different yeast strains, a protein can be expressed in its various glycoforms (**Fig. 3**) and glycosylation-mediated functions can be elucidated, provided an appropriate assay can be developed. Most importantly, once a given glycoform has been identified, scaling-up and manufacturing are done on the same platform allowing the large-scale production of specific glycoforms.

The future of humanized yeast

With the advent of humanized yeast strains and their ability to exercise direct control over glycosylation, we see the advantages of mammalian cell culture fading, and expect a significant number of biopharmaceuticals to be switched to yeast-based expression platforms. The short turn-around time to obtain gram quantities of a therapeutic protein (typically 4–6 months for a purified protein), combined with the demonstrated scalability of yeast offer an attractive alternative to current mammalian cell culture processes. Although this is expected to reduce the cost of biomanufacturing, we believe that the main benefit of using glycoengineered yeast strains lies in the ability to rigorously control glycosylation of the final product and thereby improve clinically relevant properties of the molecule. Instead of glycoform mixtures produced by mammalian cell culture processes, it is now possible to use glycoengineered yeast strains to produce a glycoprotein with a single type of N-glycosylation (**Fig. 3b–d**). Most cost-of-goods analyses focus on the cost required to produce a given amount of recombinant protein. What is often absent in this analysis is the quality of the final product, which can have an impact on dosage requirements and therapeutic effect and ultimately market share. Considering the ability of yeast systems to express full antibodies[20], cytokines, serum proteins and other therapeutically relevant proteins, we expect yeast-based therapeutic protein expression to become a serious contender in any manufacturing strategy.

© 2004 Nature Publishing Group http://www.nature.com/naturebiotechnology

COMPETING INTERESTS STATEMENT

The authors declare competeing financial interests (see the *Nature Biotechnology* website for details).

Published online at http://www.nature.com/naturebiotechnology/

1. Walsh, G. Biopharmaceutical Benchmarks—2003. *Nat. Biotechnol.* **21**, 865–870 (2003).
2. Punt, P.J., van Biezen, N. Conesa, A., Albers, A., Mangnus, J. & van den Hondel, C. Filamentous fungi as cell factories for heterologous protein production. *Trends Biotechnol.* **20**, 200–206 (2002)
3. Werten, M.W.T., van den Bosch, T. J., Wind, R.D., Mooibroek, H. & De Wolf, F.A. High-yield secretion of recombinant gelatins by *Pichia pastoris. Yeast* **15**, 1087–1096 (1999).
4. Humphreys, A. & Boersig, C. Cholesterol drugs dominate. *MedAdNews* **22**, 42–57 (2003).
5. Coloma, M.J., Clift, A., Wims, L. & Morrison, S.L. The role of carbohydrate in the assembly and function of polymeric IgG. *Mol Immunol.* **37**, 1081–1090 (2000).
6. Mistry, P.K., Wraight, E.P. & Cox, T.M. Therapeutic delivery of proteins to macrophages: implications for treatment of Gaucher's disease. *The Lancet,* **348**, 1555–1559 (1996).
7. Grabowski, G.A. *et al.* Enzyme therapy in type 1 Gaucher disease: comparative efficacy of mannose-terminated glycocerebrosidase from natural and recombinant sources. *Ann. Intern. Med.* **122**, 33–39 (1995).
8. Grabenhorst, E., Schlenke, P., Pohl, S., Nimtz, M. & Conradt, H.S. Genetic engineering of recombinant glycoproteins and the glycosylation pathway in mammalian host cells. *Glyconj. J.* **16**, 81–97 (1999).
9. Suzuki, T., Kitajima, K., Inoue, S. & Inoue, Y. *N*-glycosylation/deglycosylation as a mechanism for the post-translational modification/remodification of proteins. *Glycoconj J.* **12**, 189–193 (1995).
10. Takeuchi, M. *et al.* Relationship between sugar chain structure and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA.* **86**, 7819–7822 (1989).
11. Yuen, C.T. *et al.* Relationships between the *N*-glycan structures and biological activities of recombinant human erythropoietins produced using different culture conditions and purification procedures. *Br. J. Haematol.* **121**, 511–526 (2003).
12. Erbayraktar, S. *et al.* Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity *in vivo. Proc. Natl. Acad. Sci. USA* **100**, 6741–6746 (2003). Erratum in: *Proc. Natl. Acad. Sci. USA* **100**, 9102 (2003).
13. Friedman, B. *et al.* A comparison of the pharmacological properties of carbohydrate remodeled recombinant and placental-derived beta-glucocerebrosidase: implications for clinical efficacy in treatment of Gaucher disease. *Blood* **93**, 2807–2816 (1999).
14. Umana, P., Jean-Mairet, J., Moudry, R., Amstutz, H. & Bailey, J.E. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat. Biotechnol.* **17**, 176–180 (1999).
15. Warner, T.G. Enhancing therapeutic glycoprotein production in Chinese hamster ovary cells by metabolic engineering endogenous gene control with antisense DNA and gene targeting. *Glycobiology* **9**, 841–850 (1999).
16. Warner, T.G. Metabolic engineering glycosylation: biotechnology's challenge to the glycobiologist in the next millennium. in *Part IV Biology of Saccharides.* (eds. Ernst, B., Hart, G. & Sinay, P.) 1043–1064 (VCH-Wiley, New York, 2001).
17. Cregg, J.M., Cereghino, J.L., Shi, J. & Higgins, D.R. Recombinant protein expression in *Pichia pastoris. Mol. Biotechnol.* **16**, 23–52 (2000).
18. Mochizuki, S. *et al.* Expression and characterization of recombinant human antithrombin III in *Pichia pastoris. Protein Expr. Purif.* **23***,* 55–65 (2001).
19. Ward, M. *et al.* Characterization of humanized antibodies secreted by *Aspergillus niger. Appl. Environ. Microbiol.* **70**, 2567–2576 (2004).
20. Ogunjimi, A.A., Chandler, J.M., Gooding, C.M. Recinos, A. & Choudary, P.V. High-level secretory expression of immunologically active intact antibody from the yeast *P. pastoris. Biotechnol. Letters,* **21**, 561–567 (1999).
21. Maras, M. & Contreras, R. Methods for modifying carbohydrate moieties. US patent 5,834,251 (Dec. 30, 1994).
22. Maras, M. *et al. In vitro* conversion of the carbohydrate moiety of fungal glycoproteins to mammalian-type oligosaccharides. *Eur. J. Biochem.* **249**, 701–707 (1997).
23. Kornfeld, R. & Kornfeld, S. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54**, 631–664 (1985).
24. Brockhausen, I., Carver, J.P. & Schachter, H. Control of glycoprotein synthesis. The use of oligosaccharide substrates and HPLC to study the sequential pathway for *N*-acetylglucosaminyltransferases I, II, III, IV, V and VI in the biosynthesis of highly branched *N*-glycans by hen oviduct membranes. *Biochem. Cell Biol.* **66**, 1134–1151 (1988).
25. Silberstein, S. & Gilmore, R. Biochemistry, molecular biology and genetics of oligosaccharyltransferase. *FASEB J.* **10**, 849–858 (1996).
26. Gemmill, T.R. & Trimble, R.B. Overview of N- and O-linked oligosaccharide structures found in various yeast species. *Biochim. Biophys. Acta* **1426**, 227–237 (1999).
27. Tulsiani, D.R., Hubbard, S.C., Robbins, P.W. & Touster, O. Alpha-D-mannosidases of rat liver Golgi membranes. Mannosidase II is the $GlcNAcMan_5$-cleaving enzyme in glycoprotein biosynthesis and mannosidases IA and IB are the enzymes converting Man_9 precursors to Man_5 intermediates. *J. Biol. Chem.* **257**, 3660–3668 (1982).
28. Kalsner, I., Hintz, W., Reid, L.S. & Schachter, H. Insertion into *Aspergillus nidulans* of functional UDP-GlcNAc: α3-D-mannoside β-1,2-*N*-acetyl-glucosaminyltransferase I, the enzyme catalyzing the first committed step from oligomannose to hybrid and complex N-glycans. *Glycoconj. J.* **12**, 360–370 (1995).
29. Martinet, W., Maras, M., Saelens, X., Jou, W.M. & Contreras, R. Modification of the protein glycosylation pathway in the methylotrophic yeast, *Pichia pastoris. Biotechnol. Letters* **20**, 1171–1177 (1998).
30. Chiba, Y. *et al.* Production of human compatible high mannose-type ($Man_5GlcNAc_2$) sugar chains in *Saccharomyces cerevisiae. J. Biol. Chem.* **273**, 26298–26304 (1998).
31. Choi, B.K. *et al.* Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris. Proc. Natl. Acad. Sci. USA* **100**, 5022–5027 (2003).
32. Stadheim, T.A., Bobrowicz, P., & Wildt, S. Methods for eliminating mannosylphosphorylation of glycans in the production of glycoproteins. US Patent Appl. No. 60/532,461 (December 23, 2003).
33. Vervecken, W. *et al. In vivo* synthesis of mammalian-like, hybrid-type *N*-glycans in *Pichia pastoris. Appl Environ Microbiol.* **70**, 2639–2646 (2004).
34. Hamilton, S.R. *et al.* Production of complex human glycoproteins in yeast. *Science*, **301**, 1244–1246 (2003).
35. Bobrowicz, P. *et al.* Engineering of an artificial glycosylation pathway blocked in core oligosaccharide assembly in the yeast *Pichia pastoris*: production of complex humanized glycoproteins with terminal galactose. *Glycobiology* **14**, 757–766 (2004).