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Production of Therapeutic Glycoproteins through the Engineering of Glycosylation Pathway in Yeast

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Abstract The application of recombinant DNA technology to restructure metabolic networks can change metabolite and protein products by altering the biosynthetic pathways in an organism. Although some success has been achieved, a more detailed and thorough investigation of this approach is certainly warranted since it is clear that such methods hold great potential based on the encouraging results obtained so far. In last decade, there have been tremendous advances in the field of glycobiology and the stage has been set for the biotechnological production of glycoproteins for therapeutic use. Today glycoproteins are one of the most important groups of pharmaceutical products. In this study the attempt was made to focus on identifying technologies that may have general application for modifying glycosylation pathway of the yeast cells in order to produce glycoproteins of therapeutic use. The carbohydrates of therapeutic recombinant glycoproteins play very important roles in determining their pharmacokinetic properties. A number of biological interactions and biological functions mediated by glycans are also being targeted for therapeutic manipulation *in vivo*. For a commercially viable production of therapeutic glycoproteins a metabolic engineering of a host cell is yet to be established.

Keywords: Metabolic engineering, *Saccharomyces cerevisiae*, glycoprotein, sugar nucleotide transporter, glycosyltransferase, glycosidase

INTRODUCTION

To create arrays of enzymatic activities that can synthesize a novel structure by the expression of heterologous genes is a relatively new and challenging area in biotechnology. The cloning and expression of heterologous genes enable the post-translational protein processing to be altered. Currently, the engineering of an expression host cell for the purpose of the enhanced production of recombinant proteins is a very novel and growing area. The overall notion is to genetically modify the metabolic pathways of an organism in order to synthesize a particular metabolite of interest. As a matter of fact, there are only a few cases to date in which such a metabolic engineering cycle has been implemented with success. The expression of biosynthetic genes for a secondary metabolite in a heterologous host can result in the construction of an array of enzymatic activities that yield novel products. The expression of a heterologous protein does not necessarily guarantee the appearance of the desired activity. It also provides a new idea of a "minimum genome factory", where cells, like microorganisms, can be modified their genome order or structure minimized by using the total genome information in advance to be suitable for the production of particular target components.

A protein, which carries one or more oliggosaccharide chains covalently attached to the polypeptide backbone, is called glycoprotein [1]. Among all different types of covalent modifications that nascent proteins undergo in living organisms, the glycosylation is the most common and conserved one [2]. The assembly of oligosaccharides is a quite complex process. In an individual glycoprotein more than one carbohydrate unit is often present, attached at different positions either by an *N*-linkage, an *O*-linkage or both. The *N*-linked (Asn-linked) oligosaccharides are sugar chains covalently linked to asparagine residues of a polypeptide chain. *N*-Glycans share a common pentasaccharide core region and can be divided into three main classes: high-mannose-type, complex-type, and hybrid-type. *O*-Linked (*O*-(Ser/Thr)-linked) oligosaccharides, are typically linked to the polypeptide via *N*-acetylgalactosamine (GalNAc) to a serine or threonine residue and can be extended in a variety of different classes [3]. *N*-Glycosylation is the broadest base of structural information about glycans in living organisms. Most well-characterized pathways for different classes of glycans occur within the secretory pathway along the protein movement through ER-Golgi-plasmamembrane pathway [4].

In yeast *Saccharomyces cerevisiae*, like all other eu-

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karyotic cells, the assembly of *N*-linked precursor oligosaccarides begins in the ER with the synthesis of the precursor $GlcNAc_2Man_9Glc_3$ on the lipid carrier dolichol-pyrophosphate. This glycan is transferred to nascent secretory proteins when they enter into the ER lumen and is then extensively modified as glycoproteins along the movement through the secretory pathway. This modification involves the removal of some sugar residues and the addition of others in both the ER and Golgi apparatus [2]. This processing occurs in a stepwise manner catalyzed by a number of glycosyltransferases. The initial steps of *N*-glycosylation in the ER are nearly identical between yeast and mammals. However, in the latter stage, yeast *S. cerevisiae* elongates oligosaccharides to poly-mannose strucrures without adding the peripheral *N*-acetylglucosamine, galactose, and sialic acid residues that are characteristics of complex oligosacchrides found in mammalian cells. Thus, it is a challenge to manipulate and reconstitute the glycosylation pathway in yeast to produce complex glycoforms *in vivo*.

THERAPEUTIC GLYCOPROTEINS

Therapeutic glycoproteins are the most important class of biotechnology products today. Erythropoietin (EPO), the granulocyte-macrophage-colony stimulating factor, and tissue plasminogen activator are the most important products among them. Additionally numbers of new recombinant glycoproteins are currently under development as potential therapeutic agents. In most of the cases, glycosylation must be optimized to ensure a prolonged circulatory half-life in the blood. The manipulation of glycans to promote the targeting of specific cell types has also been an essential element for drug design of several successful therapeutic glycoproteins. EPO is the most well-studied and successful product to date. EPO is a haemopoietic glycoprotein hormone, produced mainly by the kidney in humans, which stimulates the self-renewal and differentiation of late erythroid progenitor cells toward mature red blood cells through highly specific receptors. It has proved to be of great value in treating anemia caused by bone marrow suppression. Natural and recombinant forms of EPO carry four-sialylated complex type *N*-glycans. The *in vitro* activity of deglycosylated EPO is equal to that of fully glycosylated EPO, however, the *in vivo* activity of the deglycosylated form is less than 10% of glycosylated EPO, because poorly glycosylated forms of EPO are rapidly cleared by filtration in the kidney. Furthermore, undersialylated EPO is rapidly cleared by Gal/GlcNAc/Man receptors in hepatocytes and macrophages [5]. Both processes can be reduced by having fully sialylated sugar chains and by increasing the number of tetra-antennary sugar chains [7]. Decrease in the clearance rate of EPO, in turn, increases its *in vivo* activity nearly by 10-fold [6,7]. EPO is perhaps unusual, since it is small enough to be cleared by the kidneys if it is underglycosylated. For most glycoprotein therapeutics, minimizing clearance by the Gal/GlcNAc/Man receptors through the maintenance of fully sialylated sugar chains is the most important consideration. In contrast to other blood hormones such as interleukin-3, a granulocyte and macrophage colony stimulating factor, and granulocyte colony stimulating factor, all of which believed to be both synthesized and active in bone marrow, EPO is first produced in the human adult kidney and thereafter delivered to the bone marrow by circulation [7]. Human erythropoietin (HuEPO) was purified to homogeneity from the urine of aplastic anemia patients [8], which opened the door for further studies on the molecular biology and glycobiology of HuEPO. HuEPO consists of 165 or 166 amino acids and is heavily glycosylated [9,10]. These sugar chains contribute about 40% of the mol.wt. of HuEPO.

The carbohydrates of therapeutic recombinant glycoproteins play important roles in determining the phermacokinetic properties. The biological interactions and biological functions mediated by glycans are also being targeted for therapeutic manipulation *in vivo*. Examples of carbohydrate-based therapeutics currently being developed include the inhibitors of microbial pathogens and their toxins, cancer vaccines, and drugs designed to suppress the immune system for the treatment of inflammation and transplant rejection. The most important class of biotechnology products to date is therapeutic glycoproteins, which are typically produced as recombinant products in cell culture systems or in transgenic animals. The control of glycosylation takes on major importance during the development of these drugs, since glycan chains have a dramatic effect on the stability, action, and pharmacodynamics in intact organisms. In most cases, glycosylation must be optimized to ensure prolonged circulatory half-life in the blood. The manipulation of glycans to promote the targeting of specific tissues and cell types has also been an essential element of drug design for several successful therapeutic glycoproteins.

Recombinant glycoprotein therapeutics has proven to be invaluable pharmaceuticals for the treatment of chronic and life-threatening diseases. Although these molecules are extraordinary efficacious, many diseases have high dosage requirements of several hundred milli grams of protein for each administration. Multiple doses at this level are often required for treatment. One of the major challenges currently facing the biotechnology industry is the development of a large-scale, cost-effective production and manufacturing process for these biologically synthesized molecules. The metabolic engineering of animal cell hosts promises to address this challenge by substantially enhancing the recombinant protein quality, productivity, and biological activity.

The high cost and complexity of production is the main limiting factor to develop carbohydrate-based therapeutic compounds for clinical application in adequate quantities. The development of cost effective technologies for production appears to be a promising solution to this problem.

DEVELOPMENT OF NOVEL THERAPEUTIC GLYCOPROTEIN USING YEAST AS HOST

In recent years, recombinant proteins have been produced by a wide variety of microorganisms. Among them *Escherichia coli*, the yeast *Saccharomyces cerevisiae*, and various strains of Bacilli are the most important [11], although, as the genetics of other types of microorganisms becoming more and more clear, reports are also appearing on the use of alternate hosts, *e.g.*, the yeast *Pichia pastoris* [12]. The yeast *Saccharomyces cerevisiae* is probably the second most widely used host organism for the production of heterologous proteins. Although the maximum specific growth rate of this yeast is much slower than that of *E. coli*, yeast still grows faster than most mammalian cell lines. Any microorganisms can be used to produce biotechnology products for industrial use, however, for products intended for human use, the host organism must be chosen very carefully.

In this study on glycotechnology for the production of therapeutic glycoproteins, *S. cerevisiae* was chosen as a host, because this yeast possesses the ability to glycosylate proteins and appears to be one of the safest organisms to produce therapeutic glycoproteins for human use. The initial steps of *N*-linked glycoprotein biosynthesis, which involves the synthesis of $Glc_3Man_9GlcNAc_2$-PP-dol, the transfer of the oligosaccharide from lipid to protein, and the subsequent trimming to $Man_9GlcNAc_2$ in the ER, are nearly identical in yeast (*S. cerevisiae*) and mammals. However, in the latter stage of glycosylation, *S. cerevisiae* elongates the oligosaccharides to poly-mannose structures (Fig. 1) without adding the peripheral *N*-acetylglucosamine, galactose, and sialic acid residues which are the characteristics of the complex oligosaccharides found in mammalian cells. Thus, it is a challenge to manipulate and reconstitute the glycosylation pathways in yeast to produce complex glycoforms *in vivo* (Fig. 2).

The first aim of this research was to convert the sugar chain of *S. cerevisiae* to a mammalian-type $Man_5GlcNAc_2$ sugar chain, because it is an intermediate where mammalian-type sugar chains and *S. cerevisiae* sugar chains are identical. Therefore, accordingly to the overall strategy, an attempt was made first to trim down the sugar chains to $Man_5GlcNAc_2$ and then to express all the required glycosyltransferases, glycosidases, and sugar nucleotide transporters with the appropriate localization and topology, to produce mammalian-type sugar chains (Fig. 2).

The mutant yeast *Δoch1Δmnn1Δmnn4* was selected as the starting strain [13], within which three genes involved in mannose or mannosylphosphate transfer were disrupted in order to produce a $Man_8GlcNAc_2$ structure in *S. cerevisiae* [14,15]. In a previous study by the current authors, an *och1*mutant was isolated, which exhibited a deficiency in the outer chain elongation in *S. cerevisiae* [16], then the corresponding (*OCH1*) gene was cloned that encoded an α-1,6-mannosyltransferase. The *OCH1* gene sequence predicts a 55 kDa protein consisting of 480 amino acids and an Och1 protein (Och1p) is an integral type II membrane protein, in which the C-terminal major region resides in the luminal side and contains four *N*-linked oligosaccharide chains. In yeast cells, the Och1p adds an α-1,6-mannose to the inner core ($Man_8GlcNAc_2$) to create $Man_9GlcNAc_2$ [14]. The disruption of the *OCH1* gene results in the accumulation of $Man_8GlcNAc_2$ and a slightly larger sugar chain. The glycoproteins from *S. cerevisiae* are highly immunogenic, however, the proteins secreted from the *mnn1* mutant are less immunogenic than those from wild type cells. Early works by Ballou and co-workers showed that the *N*-linked and *O*-linked oligosaccharides isolated from the *mnn1* mutant lack an immunogenic terminal α1,3-mannose linkage [17]. The extracts from the mutant do not exhibit the same α-1,3-mannosyltransferase activity detectable in wild-type extracts [18]. The availability of the cloned *MNN1* gene facilitates the straightforward construction of yeast strains with a null mutant [19] for the large-scale production of non-immunogenic oligosaccharides.

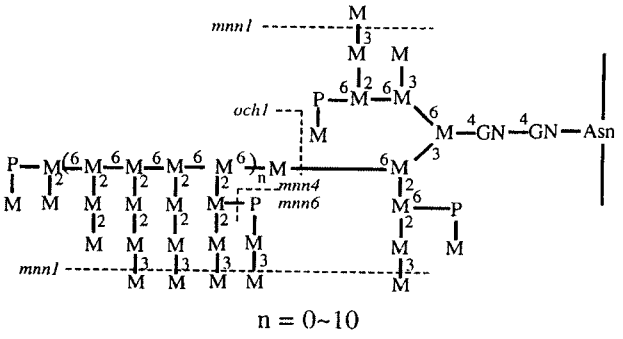

Fig. 1. Structure of *N*-linked oligosaccharides in *S. cerevisiae*. M, mannose; GN, N-acetylglucosamine; P, phosphate ; Asn, asparagine (for *mnn1*, *och1*, *mnn4*, and *mnn6* mutants, *see* text).

In *S. cerevisiae*, the glycan portion is not only composed of neutral oligosaccharides containing mannose residues and *N*-acetylglucosamine, but also of acidic oligosaccharides containing mannosylphosphate residues. In *S. cerevisiae*, very little is known about the function of oligosaccharide phosphorylation, however, it does not serve as a vacuolar targeting signal as in mammalian cells [20]. The mannosylphosphate residues confer a net negative charge on the cell wall of *S. cerevisiae*. The transfer of mannosylphosphate requires two genes, *MNN4* and *MNN6*, [15,21] and their functional analysis suggests a mechanism for mannosylphosphate transfer. It has been shown that there is a mannosylphosphate transfer complex, which contains at least Mnn4p and Mnn6p, and is localized in the Golgi apparatus (Shimma *et al.*, manuscript in preparation). The current authors believe that Mnn6p is a transferase, whereas Mnn4p serves as a positive regulator of the mannosylphosphate transfer [15,22]. The

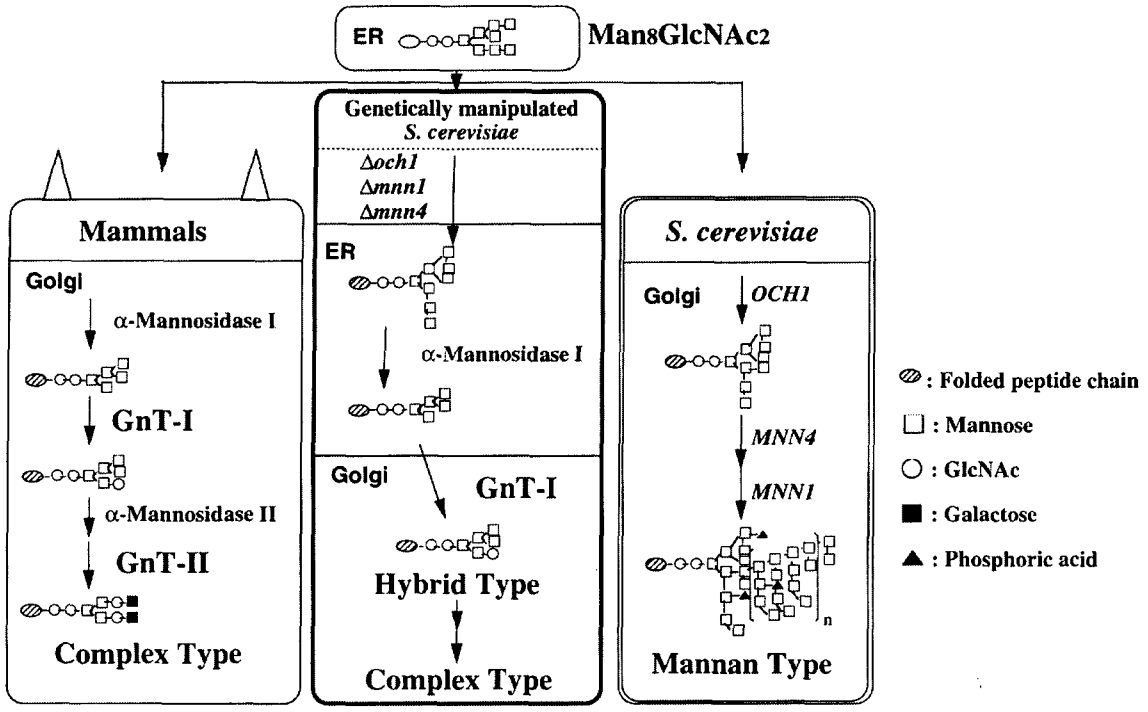

Fig. 2. Overall outline and strategy for the glycan engineering of *S. cerevisiae*, and comparison of *N*-glycan biosynthetic pathways in mammalian cells and *S. cerevisiae*.

disruption of the *MNN4* gene leads to a dramatic decrease in the mannosylphosphate content in the cell wall mannans compared to that of wild type cells [15]. However, no differences were found in the *N*-linked oligosaccharides of the mannoprotein between the *Δoch1Δmnn1* cells and *Δoch1Δmnn1Δmnn6*, where *MNN6* encodes the mannosylphosphotransferase [21]. These results suggest the existence of redundant genes for the oligosaccharide phosphorylation in this yeast. An HPLC analysis of the *N*-linked oligosaccharides from the mannoproteins was performed to examine the effect of the *Δmnn4* mutation on the *N*-linked core oligosaccharides *in vivo*. A comparison of the oligosaccharide profile of the mannoproteins prepared from *Δoch1 Δmnn1 Δkre2* cells with that of the mannoproteins prepared from *Δoch1 Δmnn1 Δkre2 Δmnn4* cells indicated a significant decrease in acidic oligosaccharides caused by the *Δmnn4* mutation [22].

As described above, *Δoch1Δmnn1Δmnn4* triple mutant cells were selected to produce less yeast specific mannosylphosphorylated oligosaccharides ($Man_8GlcNAc_2$) (Figs. 1 and 2). To convert toward a mammalian hybrid-type sugar chain, it is necessary to further trim the mannose residues of $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ using α-1,2-mannosidase. Several genes for α-1,2-mannosidase have been isolated from mammals, yeast, and fungi [23,24], and certain mammalian α-1,2-mannosidase genes have also been cloned [25,26]. A truncated soluble form of human α-1,2-mannosidase IB has been produced as a secreted protein in *Pichia pastris* [27]. The *S. cerevisiae* α-1,2-mannosidase gene (*MNS1*) has also been cloned and expressed in *S. cerevisiae* [28]. The *Aspergillus* α-1,2-mannosidase gene (*msdS*) has been cloned and expressed successfully in yeast cells as a chimeric gene with a signal sequence derived from of the aspergillopepsin I gene from *Aspergillus saitoi* [29, 30]. Recombinant α-1,2-mannosidase activity was detected in the culture medium, indicating that the products of the *msdS* gene passed through the yeast secretory pathway. Therefore, this enzyme will be useful to produce mammalian-type sugar chains in yeast, if it can be retained at the endoplasmic reticulum (ER) or Golgi apparatus of yeast. In yeast *S. cerevisiae* the His-Asp-Glu-Leu (HDEL) C-terminal sequence of proteins acts as a retention/retrieval signal for the ER [31]. Proteins with an HDEL sequence will be trapped by a membrane-bound receptor (Erd2p) [32,33].

In this project, *A. saitoi* α-1,2-mannosidase was expressed in yeast by adding "HDEL" to the C-terminus of the α-1,2-mannosidase open reading frame. The cloned α-1,2-mannosidase was also shown to convert Asn-linked $Man_8GlcNAc_2$ oligosaccharides into $Man_5GlcNAc_2$, the intermediate form for hybrid- and complex type sugar chains, in yeast cells with three disrupted genes involved in mannose and mannosylphosphate transfer (*OCH1, MNN1, and MNN4*) [13].

The next step of *N*-linked oligosaccharide modification is to add GlcNAc residues in the Golgi apparatus. To make this possible the *N*-acetylglucosaminyltransferase and UDP-GlcNAc transporter are necessary to be

introduced in the yeast, apart from the availability of $Man_5GlcNAc_2$ acceptor, which resides in the proper localization. It has already been reported that rat UDP-*N*-acetylglucosamine:α-3-D-mannoside β-1,2-*N*-acerylglucosaminyltransferase I (GnT-I) was successfully expressed in *S. cerevisiae* [34]. After the expression and careful characterization of expressed GnT-I in *S. cerevisiae*, it was revealed that the expressed enzyme was localized in various organelles, but mostly in the Golgi apparatus. In this case, the Golgi localization of GnT-I is important because α-1,2-mannosidase is localized in the ER, thereby making $Man_5GlcNAc_2$, the acceptor for GnT-I. As previously mentioned, another requirement is a UDP-GlcNAc transporter. The donor substrate for GnT-I, UDP-GlcNAc, is synthesized in the cytosol and needs to be transported from the cytosol to the lumen of the Golgi apparatus in order to provide a donor substrate for the sugar transfer. Two choices of UDP-GlcNAc transporters were identified, from which one can be selected. In this search for a homologue of the *K. lactis MNN2-2* UDP-GlcNAc transporter in *S. cerevisiae*, the gene *YEA4*, which encoded a protein with 40% identity and 70% similarity to the amino acid sequences of the Mnn2-2 protein, was identified. Therefore, the possibility that its product is a UDP-GlcNAc transporter was tested and demonstrated that UDP-GlcNAc was transported into the ER lumen. This transport was saturable and showed apparent K_m values comparable to those measured for the *K. lactis* Golgi-localized UDP-GlcNAc transporter [35,36] and rat liver ER-localized UDP-GlcNAc transporter [37]. To assess the possible biological role of this UDP-GlcNAc transporter in the ER membranes, a yeast strain deleted for *YEA4* was constructed and analyzed. The null mutant cells showed less UDP-GlcNAc transport activity compared to the wild type cells. Therefore, the *S. cerevisiae* Yea4 protein, which exhibited a high homology with the *Kluyveromyce lactis* UDP-GlcNAc transporter, is indeed a UDP-GlcNAc transporter as expected [38].

An alternative choice is a human UDP-GlcNAc transporter. A human UDP-GlcNAc transporter was identified through a homology search in the expressed sequence tags database based on its similarity to the human UDP-galactose transporter. The human transporter gene was expressed in *S. cerevisiae* and the membrane fractions prepared from the yeast cells showed UDP-GlcNAc specific transporting activity [39]. Its Golgi localization and topology were also confirmed by using Golgi and ER marker enzymes (unpublished data).

For the current purpose the latter transporter was selected for use, because the *S. cerevisiae* Yea4p is localized in the ER, whereas the human counterpart is localized in the Golgi, plus it seems to be more advantageous to have the UDP-GlcNAc transporter in the Golgi as rat GnT-I is mostly produced in the Golgi compartment of yeast.

The remaining part of the research (Fig. 2) involves the expression of mannosidase II in the Golgi of *S. cerevisiae* with an appropriate topology, which will trim the $GlcNAcMan_5GlcNAc_2$ structure to $GlcNAcMan_3GlcNAc_2$, plus a simultaneous attempt to express human UDP-*N*-acetylglucosamine: the alpha-6-D-mannoside-beta-1, 2- *N*- acetylglucosaminyltransferase II gene (MGAT2) [40] in *S. cerevisiae*.

The successful addition of GlcNAc to $GlcNAcMan_3GlcNAc_2$ serves as an acceptor for the galactose addition. Similar to the GlcNAc addition, the *in vivo* galactose addition requires the expression of β-1,4-galactosyltranferase in the Golgi lumen and a UDP-galactose (UDP-Gal) transporter to provide the substrate for the enzyme. The mammalian β-1,4-galactosyltranferase is one of the most well studied glycosyltransferases. The expression and characterization of human β-1,4 -galactosyltranferase in *S. cerevisiae* [41], and the Golgi localization and *in vivo* activity have all been reported [42]. Thus it has been established that human β-1,4-galactosyltranferase is functional in the Golgi lumen of *S. cerevisiae*. Regarding the UDP-Gal transporter, the study was already reported on the UDP-Gal transport activity in *S. cerevisiae* and its localization in the Golgi apparatus, however, the gene has yet to be identified [43]. Accordingly, *Schizosaccharomyces pombe* α-1,2-galactosyltrans-ferase was expressed in *S. cerevisiae* to make a tool for an assay of the UDP-Gal transporter, because the expression of galactosyltransferase provides an efficient antiporter (UMP) generation system in *S. cerevisiae* (Fig. 3). The transport of UDP-Gal in *S. cerevisiae* is time and protein dependent and saturable with an increased substrate concentration [43,44].

While the gene responsible for the UDP-Gal transport in the Golgi lumen is yet to be identified in *S. cerevisiae*, the cDNAs for the human and *S. pombe* UDP-Gal transporters have already been cloned and characterized [45-48]. The human UDP-Gal transporter was expressed in *S. cerevisiae* in an active form, thus demonstrating by *in vitro* transport activity that the cDNA encodes the transporter, and not a regulatory protein [49,50]. However, there are no *in vivo* studies to demonstrate whether or the expressed foreign sugar nucleotide transporter actually functions in *S. cerevisiae*. Therefore, to assess the power of the human UDP-Gal transporter in *in vivo* galactosylation, the α-1,2-galactosyltransferase gene of *S. pombe* and a UDP-Gal transporter gene of human origin were co-expressed in *S. cerevisiae*, then *in vivo* galactosylation was observed, thereby demonstrating the first *in vivo* neo-galactosylation system in *S. cerevisiae* [51]. In addition, various factor(s) involved in this *in vivo* galactosylation were also addressed. A detailed analysis indicated that this α-1,2-galactosyltransferase adds galactose to both *N*- and *O*-linked oligosaccharides [51]. The expressed α-1,2-galactosyltransferase is mainly localized in the Golgi lumen [43] and results of *in vivo* galactosylation indicate that they are working in the same environment as the endogenous one in *S. cerevisiae*. The human β-1,4-galactosyltranferase was also introduced into *S. cerevisiae* and its Golgi localization was confirmed (unpublished data). In the present engineering project

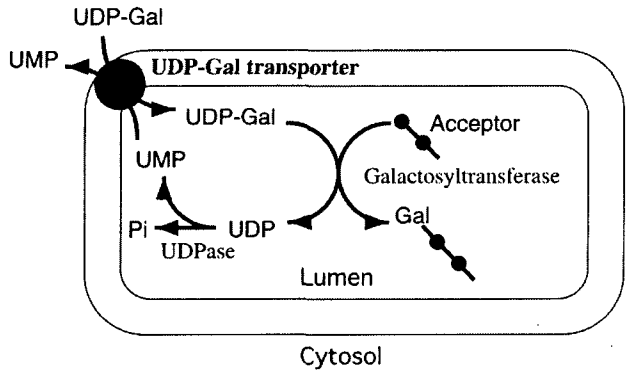

Fig. 3. Transporters for nucleotide sugars (UDP-Gal as an example) localized in Golgi. These proteins are antiporters, and the corresponding nucleoside monophosphate is carried into the cytosol with nucleotide sugar transport.

of glycosylation pathway, machinery for the addition of sialic acid was not included, because the *in vivo* synthesis of CMP-sialic acid (donor substrate) is very difficult, if not impossible at this point. Therefore, sialic acid can be added by an *in vitro* enzymatic reaction after the successful synthesis of asialoglycoproteins.

If the alteration of the biosynthetic pathway of yeast toward the production of a mammalian-type glycan is successful using this approach, it will provide a great potential as an attractive tool for the design and economic production of future therapeutic glycoproteins.

CONCLUSION

The application of glycobiology in medicine is mainly focused on the role of carbohydrates known to have important physiological functions.

It is likely that additional therapeutic opportunities will emerge as the roles of newly described or yet uncovered carbohydrate-binding proteins are elucidated. In recent years, accumulation of evidence on the role of the carbohydrates as recognition molecules is the most rewarding. Different novel approaches to evaluate the structural and biological functions of carbohydrates will set the stage for developing new glycoprotein therapeutics. In fact, the number of therapeutics in which the action is mediated by carbohydrates is currently increasing. Advances in technology will soon facilitate the production and commercialization of such compounds. One can expect that the knowledge thus accrued will lead to novel approaches for treating various diseases, such as microbial infections, inflammation, and cancer.

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