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Expression of human glycosyltransferase genes in yeast as a tool for enzymatic synthesis of sugar chain

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We planned the production of human glycosyltransferases in yeast for the enzymatic synthesis of various sugar chains. More than 160 genes encoding various glycosyltransferases were prepared as N-terminal transmembrane region truncated forms by PCR and were inserted into the entry vector of Invitrogen Ltd's Gateway system. About fifty glycosyltransferases were chosen for the synthesis of human type oligosaccharides, and expressed as two different forms in yeast. One is a soluble form, which is secreted into the culture medium by methylotrophic yeast, and the other is an immobilized form, which is displayed at the budding yeast cell wall as a fusion protein with Pir protein. To date, in both systems, some sialyltranferases and fucosyltransferases have been produced as active forms, indicating the potential usefulness of these systems for the enzymatic synthesis of various types of human sugar chains attached to proteins and lipids.
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Introduction

More than half of the mammalian proteins are glycosylated and contain various types of sugar chains, including N-linked, O-linked, and phosphatidylinositol-linked sugar chains. The lack of these sugar chains has been described to produce malfunctions of many glycoproteins *in vivo*. For instance, a proteinous hormone, erythropoietin (EPO), has three N-glycans and one O-glycan, and is known to lose its erythropoietic activity, when its branch and/or length of N-glycans is either eliminated or reduced [1]. The Notch receptor on the surface of the cells possesses many O-linked glycans with different lengths of sugar chains depending on the type of cells. The Notch is activated by different kinds of ligands, which recognize the sugar chains of the Notch receptor, leading to different fates of the cells [2].

The elucidation of the structure and function of sugar chains is indispensable to understanding many biological processes. This information can be applied to a variety of practical uses. Sugar chains attached to proteins and lipids affect various biological processes, such as cellular differentiation, adhesion, immune response and infection, and the distribution and *in vivo* half-life of proteins, in addition to the embryogenesis and development of animals. These functions are important in basic research and can be applied practically. However, the current glyco-engineering technology for manipulating sugar chains is too immature to be able to freely synthesize various kinds of sugar chains. To check the biological function of sugar chains, it is necessary to prepare a significant amount of various sugar chains, either free or attached to proteins and lipids. Although both chemical and enzymatic approaches are possible, we have selected an enzymatic method, because enzymatic reactions will be more suitable for producing homogenous sugar chains and active glycoproteins, without harming the activities of native proteins and living cells.

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Development of AIST Glyco-Gene Library

We can access any sequence information through the internet after the completion and disclosure of the human genome sequencing data in June 2000. At that time about one hundred human glycosyltransferase genes had been cloned. It was very timely to clone all the remaining and uncharacterized glycosyltransferase genes by taking advantage of the human genome information. Based on the numbers of sugar chain biosynthesis genes estimated in yeast and nematode, we predicted the number of total human glycosyltransferase genes to be around 1% of the total ORFs, and the number was later reported to be about 30,000 ORFs.

The Glyco-Gene (GG) project, supported by the New Energy and Industrial Technology Development Organization (NEDO), aims at cloning all the remaining human glycosyltransferase genes under the leadership of Dr. Narimatsu of the Research Center for Glycoscience (RCG), National Institute of Advanced Industrial Science and Technology (AIST) in Japan. As a result of the GG project, nearly two hundred glycosyltransferase genes were cloned and collected in AIST. We have tried to make the best use of this property to develop a production system for human glycosyltransferases for the enzymatic synthesis of various types of human sugar chains.

As a first step, we have selected fifty human glycosyltransferase genes that are well characterized and suitable for the synthesis of typical sugar chains attached to proteins and lipids. These genes include sialyltransferases (ST), fucosyltransferases (FucT), galactosyltransferases (GalT), N-acetyl-glucosaminyltransferases (GnT), and peptide-N-acetyl-galactosaminyltransferases (ppGalNAcT). We planned to express these genes in lower eukaryotic microorganisms, such as yeast, to develop an efficient production system of glycosyltranferases for industrial use.

Program for production of glycosyltransferases in yeast

It is a general feature of glycosyltransferases that they contain a catalytic domain in the C-terminal region and a membrane-spanning region at the N-terminus. It is also known that many glycosyltransferases are secreted into the culture medium, when the membrane-spanning region is truncated. Thus, we planned to express a truncated form of glycosyltransferases in yeast.

Truncated forms of glycosyltransferase genes were prepared by PCR using a special adaptor sequence in the primer DNA, which can be applied with the Gateway system of Invitrogen Ltd. More than 160 glycosyltransferase genes have been introduced into the entry vector of the Gateway system, which is very effective, because once a certain ORF is introduced into the entry vector, it is easy to transfer the ORF into desired expression vectors, such as for E. coli, yeast, insect cells, mammalian cells, etc.

We are now expressing several glycosyltransferase genes as soluble and secreted forms in methylotrophic yeast cells (Figure 1A and C). In collaboration with Kirin Brewery Co. Ltd., we have developed expression and secretion systems in the unique methylotropic yeast, *Ogatae minuta*, in which the genes for endogenous yeast-type sugar chain synthesis were deleted (our unpublished results). We have also succeeded in producing a significant amount of several glycosyltransferases in methylotropic yeasts to prepare crystals for three dimensional structure analyses (our unpublished results).

As an alternative approach, we are trying to produce immobilized enzymes that are convenient for industrial use, because they can be packed in columns for automated bioreactors and can be easily separated from the reaction mixture. Immobilized enzymes are produced for practical uses, but most of them are prepared by the chemical immobilization of purified soluble enzymes. The purification of soluble enzymes from culture media or cell homogenates is time consuming and requires sophisticated protocols dependent on the properties of each enzyme protein. Furthermore, the chemical immobilization of enzymes often causes partial reduction of enzymatic activities due to the chemical modification of proteins. We have tried to immobilize glycosyltransferases *in vivo* at the yeast cell surface by fusion with cell wall Pir proteins (Figure 1B and D). In this case, it is desirable to immobilize glycosyltransferases at the N-terminal ends, because endogenous glycosyltransferases reside *in vivo* at the Golgi membrane through the N-terminal transmembrane regions. Once constructed genetically engineered yeast strains, which express glycosyltransferases covalently-linked to the cell wall protein, a simple cultivation of the yeast cells and a centrifugation of the culture broth provide an enormous amount of immobilized enzymes.

Production of immobilized glycosyltransferases at the yeast cell surface

A method of immobilizing proteins at the yeast cell wall was independently developed by the Klis group in Netherlands [3] and the Ueda group in Japan [4], using glycosyl-phosphatidylinositol (GPI) as an anchor for the proteins to bind onto the β-1,6-glucan of the cell wall. Many yeast cell wall mannoproteins are known to be members of the GPI anchored proteins, which are transported from ER to the plasma membrane along the protein secretion pathway, then transferred to the cell wall. The GPI anchor signal sequence is located at the C-terminal end of the precursor proteins [5]. However, since several glycosyltransferases lose their enzymatic activities when fused with the GPI anchor signal at the C-terminus, this method is not suitable for the immobilization of glycosyltransferases.

Recently, it has been reported that the yeast *Saccharomyces cerevisiae* contains another group of cell wall mannoproteins, Pir, which are covalently bound to the β-1,3-glucan of the cell wall in a different manner from the GPI anchor [6]. The molecular mechanism of Pir protein binding to the cell wallβ-1,3-glucan is still unclear, but Pir protein sequences possess the binding potential irrespective of the fusion site, either in the middle or at the N- or C-terminus of the target proteins. This property is useful for immobilizing glycosyltransferases. For example, alpha-1,2-galactosyltransferase from fission yeast *Schizosaccharomyces pombe* maintained a high enzymatic activity when expressed as a fusion protein with Pir protein in *S. cerevisiae* . The same enzyme completely lost its enzymatic activity when fused with the GPI anchor signal sequence at the C-terminus [7].

However, the activity of human alpha-1,3-fucosyltransferase encoded by *FUT6* was not very strong when expressed at the cell surface as a fusion protein with Pir1 in *S. cerevisiae*. It is possible that the endogenous Pir1 proteins may occupy the space in the cell wall and the Pir1-Fut6 fusion protein cannot be expressed at the cell wall. Since Pir proteins form a Pir 1-4 gene family, we constructed yeast cells with disrupted chromosomal

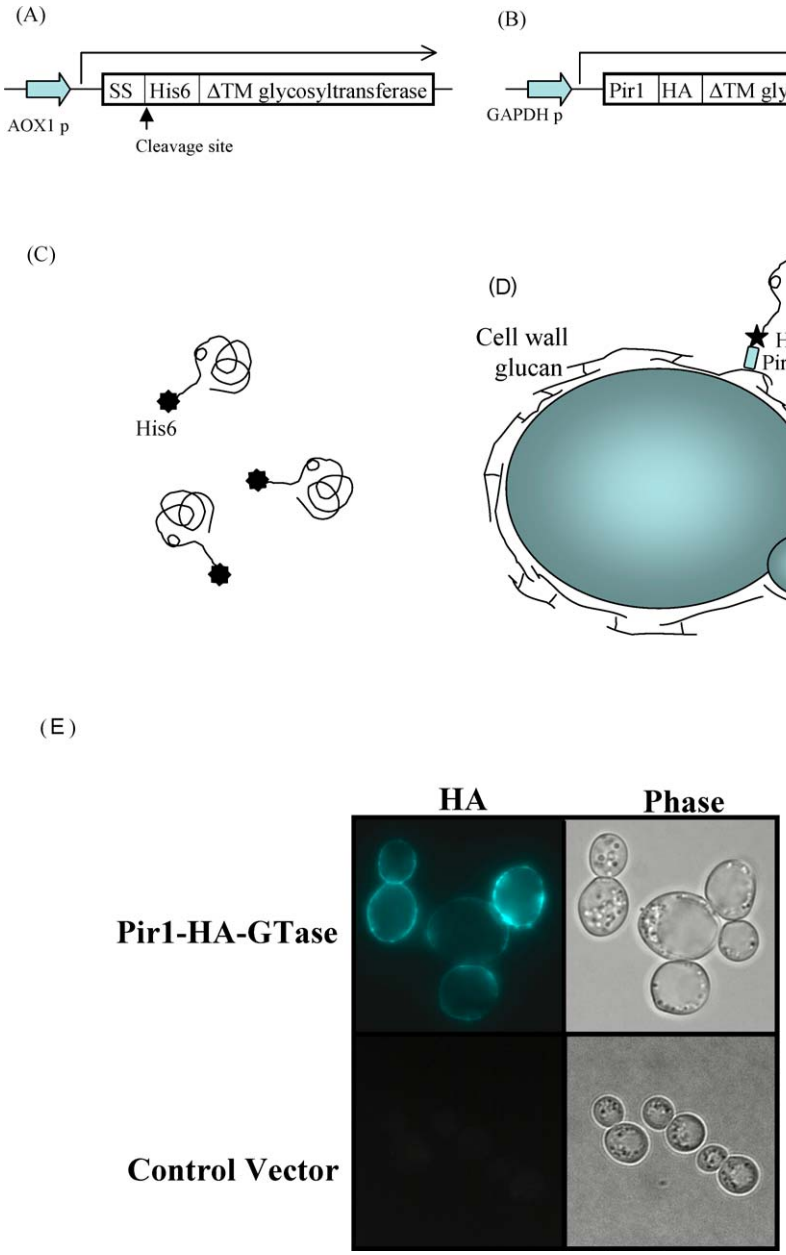

Figure 1. Expression of soluble and immobilized glycosyltransferases in yeast. (A) Construction of expression vector for soluble form in methylotrophic yeast. AOX1 is a strong promoter induced by methanol, SS and His6 mean the signal sequence of secreted protein and tandem repeats of the His tag sequence, respectively, and ΔTM glycosyltransferase means glyosyltransferase whose transmembrane region near the N-terminus is deleted. (B) Construction of an expression vector for the immobilized form through yeast cell wall protein Pir1 in budding yeast. HA means three tandem repeats of influenza hemagulutinin sequence. (C) Soluble glycosyltransferases produced by methyotrophic yeasts and secreted into the culture medium. (D) Immobilized glycosyltransferases located at the budding yeast cell surface. (E) Fluorescent microscopy of cell wall immobilized Pir1-sialyltransferase fusion protein.

PIR genes. The *PIR1* disrupted cells showed a higher fucosyltransferase activity than the wild type cells. Furthermore, an increase in numbers of *PIR* gene disruption resulted in a higher activity, except the cells disrupted for all *PIR1-4* genes, which showed a lower activity than the *PIR1-3* triple disrupted cells, probably due to the impairment of cell growth [8].

These observations prompted us to construct an expression vector in which the transmembrane truncated glycosytransferase genes were constructed as fusion proteins with Pir1 and HA tag sequences at the N-terminus to be displayed at the yeast cell wall (Figure 1A and D). We selected fifty human glycosyltransferase genes as candidates to express in this system.

The number of enzymes we selected at the initial stage will be enough to judge the usefulness of our system for the enzymatic synthesis of various sugar chains. When sialyltransferase ST3GalI was expressed in this system, the fusion protein was detected by a fluorescence-labeled anti-HA tag antibody and the fluorescent signal was observed at the yeast cell surface under fluorescence microscopy (our unpublished results) (Figure 1E). The cell wall fractions of these cells were prepared after centrifugation of cell lysates, then the enzyme activity was assayed using pyridylaminated asialoGM1 oligosaccharide as an acceptor sugar chain. The enzymatic transfer of sialic acid to the acceptor was confirmed by the HPLC analysis of the reaction product (our unpublished results).

Problems to be solved for yeast expression system

So far eight sialyltransferases and nine fucosyltransferases have been expressed in our yeast system, but only 3 and 2 genes of each enzyme group showed a significant enzyme activity. This unexpectedly small numbers of active enzyme production in yeasts is our current problem. This may be due to the use of pyridylaminated sugar chains as an acceptor for enzyme assay, because it is possible that the enzyme may require a part of protein as well for the acceptor recognition. It is also possible that the yeast high-mannose type glycosylation of the fusion proteins may inhibit the original human enzyme activity. Alternatively, it is also possible that some human glycosyltransferases may require a complex formation for their enzyme activity, as reported for *POMT1* and *POMT2* [9].

In spite of such difficulties in developing the expression and production system of human glycosyltransferases in yeast cells, our plan has a potential merit for the enzymatic synthesis of sugar chains by glycosyltransferases. The prototype of an automated sugar chain synthesizer called 'Golgi' has been developed by the Nishimura group at Hokkaido University. We think that the usefulness of this apparatus will depend on the lineup of glycosyltransferases, which will be packed in a column of the synthesizer. Therefore, it is important to evaluate the usefulness of our yeast-immobilized glycosyltransferases in the above automatic sugar chain synthesizer system. So far, when the immobilized FUT6 enzyme was applied to the synthesizer system, nearly 50% fucosylation of the acceptor was observed (personal communication by Dr. S. Nishimura). It is our dream to synthesize any desired sugar chain with a variety of yeast-immobilized glycosyltransferases prepared by a simple overnight cultivation of yeast cells within the near future.

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