Expression System for Human Glycosyltransferases and Its Application

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Yasunori Chiba,1,* Hiromi Ito,1 Takashi Sato,1 Yoshie Takahashi,1 Yoshifumi Jigami1 and Hisashi Narimatsu1

1Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST)
(Tsukuba Central 6, 1−1−1, Higashi, Tsukuba 305−8566, Japan)

Abstract: Glycosyltransferases are of growing importance in in vitro synthesis of oligosaccharides and modification of glycoproteins, and several expression systems for recombinant glycosyltransferases have been investigated. We have created gene libraries of human glycosyltransferases using the Gateway® system, and each gene encoding the catalytic domain of the glycosyltransferase was expressed as a soluble enzyme using human embryonic kidney (HEK) 293T cells or yeast expression systems. In the case of the mammalian expression system, HEK293T transfectant cells successfully expressed most of the human glycosyltransferases. On the other hand, 23 glycosyltransferases were secreted into the culture media as active forms among 53 genes tested in the methylotrophic yeast Ogataea minuta. In a further study involving the optimization of the yeast expression system, we found that several factors, such as cultural conditions, chaperone activity in the host cells and truncation of the glycosyltransferase gene to be expressed were critical for high-level production. In the case of ST3Gal-I, optimization of each parameter caused a greater than 300-fold increase in the activity in the culture supernatant. Finally, the library of glycans or glycopeptides having various structures was synthesized by combination with glycosyltransferases from HEK293T cells. We also demonstrated the modification of various pyridylaminated N-glycan structures by sequential reactions with the recombinant enzymes from the yeast system. Our expression system for human glycosyltransferases may be applicable to the preparation of glycan arrays and the production of therapeutic glycoproteins with homogeneous glycans.

Key words: glycosyltransferases, glycan synthesis, glycogens, glycan array, yeast

Glycosylation of proteins and lipids is the most complex of their various co-translational and post-translational modifications. Glycan modification contributes to the folding and conformational stability of many proteins,9 and modified glycoproteins and glycolipids on the cell surface are known to act as ligands for host-pathogen interactions,9 cell adhesion and cell signaling.10 These phenomena are influenced by changes in glycan structures. Since it is known that glycans on proteins and lipids are synthesized by sequential reactions by glycosyltransferases, the alteration of the glycan structure is dependent on the expression of glycan synthesis-related genes encoding glycosyltransferases, sulfotransferases, glycosidases, sugar-nucleotide synthetases, and sugar-nucleotide transporters. Glycan structures are defined by not only the expression level of the genes but also several other factors, such as substrate specificity, competition of more than two glycosyltransferases for the same substrate, localization of glycosyltransferases, and concentration of donor substrate as sugar-nucleotides in the Golgi apparatus. Therefore, the expression of glycosyltransferases and the determination of their enzymatic characterizations in detail are significant for the elucidation of the biosynthetic machinery of glycans.

In addition to basic study of glycosyltransferases, recombinant enzymes are available for the synthesis of glycans and glycopeptides and for the modification of glycoproteins in vitro. In recent years, mass spectrometry,5,6 high-performance liquid chromatography (HPLC),5,6 and lectin microarray systems3,4 have been developed for the structural analysis of glycans. A glycan library composed of a variety of structure-defined glycans is indispensable for quantitative analyses using these technologies. Since human glycosyltransferases have a strict specificity toward donor and acceptor substrates, it is reasonable to employ in vitro synthesis of glycans by glycosyltransferases for producing glycan standards. Glycan array technology, and the ease with which the glycans can be customized for display, may enhance our ability to detect unknown lectins in mammals and to probe pathogen-specific glycan interactions.10,11 Application of glycosyltransferases in other areas of interest includes the modification of glycans attached to glycoproteins, which has also become an important topic in recent years. Because biologics and biosimilar drugs such as therapeutic antibodies and cytokines are the largest class of new drug candidates being developed by pharmaceutical companies, there are strong concerns that the heterogeneity of the glycan structure may sometimes affect their in vivo activity. Therefore, the development of technology to produce glycoproteins with homogeneous glycans is essential.

In our research center, the Glycogene (GG) Project was started in 2001, in which as many novel genes as possible, which were the candidates for glycogenes including glycosyltransferases and sugar nucleotide transporters, were searched using bioinformatics technology, and then cloned and expressed in various expression systems.22 At
Table 1. A list of the glycosyltransferase genes cloned into the entry vectors.

<table>
<thead>
<tr>
<th>Akt-T(11):</th>
<th>Fuc-T1, T2, T3, T4, T5, T6, T7, T8, T9, POFUT1, POFUT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal-T(16):</td>
<td>β3Gal-T1, T2, T3, T4, T5, T6, β4Gal-T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T13, T14, T15, β3GalNac-T1, T2, GALGT, GALGT2, β4GalNac-T3, T4, A (ABO)</td>
</tr>
<tr>
<td>GlcNac-T(23):</td>
<td>β3Gn-T2, T3, T4, T5, T6, T7, T8, AMPGT1, 2, 3, 4, 4b, 5, MFNG, RFNG, core2GnT1, 2, 3, 4GnT1, 2, 3, α4Gn-T</td>
</tr>
<tr>
<td>GalNac-T(21):</td>
<td>ppGalNac-T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T13, T14, T15, β3GalNac-T1, T2, GALGT, GALGT2, β4GalNac-T3, T4, A (ABO)</td>
</tr>
<tr>
<td>Glc-T(3):</td>
<td>GlcAT-S, GlcAT-P, GlcAT-I (OG linkage)</td>
</tr>
<tr>
<td>Glc-T(1):</td>
<td>β3Glc-T</td>
</tr>
<tr>
<td>Heparin/Chondroitin(1):</td>
<td>EXT1, 2, EXT1, 2, 3, ChSy, ChPF/CSS2, CSS3, CSSGlcA-T, CSGalNac-T1, T2</td>
</tr>
<tr>
<td>Xyl-T(2):</td>
<td>Xyl-T1, TII</td>
</tr>
<tr>
<td>Sulfo-T(34):</td>
<td>HS3ST1, 2, 3A1, 3B1, 4, 5, NDST1, 2, 3, 4, 4H2ST1, H5ST1, 2, 3, CHST1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, UST, D4ST, GAL3ST1, 2, 3, 4, 4GALNAC4S-6ST</td>
</tr>
</tbody>
</table>

Expression of glycosyltransferases in HEK293T cells.

Combined with the data of the GG project, 186 genes have been so far identified as glycosyltransferases that encode glycosyltransferases and sugar-nucleotide transporters. [12] These genes have been cloned into the entry vector pENTR/D-TOPO (Invitrogen Japan KK., Tokyo, Japan). Certain glycosyltransferases encode the enzymes involved in the synthesis of lipid-linked oligosaccharide intermediates in the lumen side of the endoplasmic reticulum (ER). Since these enzymes are generally multiple membrane-spanning proteins and use dolichyl phosphate glucose and dolichyl phosphate mannos as a donor substrate, we removed these genes from the list of expression of glycosyltransferases for glycan synthesis in vitro. Most of the other genes encode Golgi-resident glycosyltransferases that are transmembrane proteins with type-II topology. [12] In order to express them in a soluble form into the medium, we created a truncated gene that encoded a catalytic domain of glycosyltransferase. Eventually, 141 truncated glycosyltransferase genes were cloned into the entry vector (Table 1).

Next, all truncated genes in the entry clones were re-cloned into the mammalian destination vector, pFLAG-CMV-1 or -3, by use of the LR recombination reaction. Each destination vector was transfected into HEK293T cells and the culture medium was collected after incubation at 37°C for 72 h. Anti-FLAG antibody resin was then added to the culture medium and the resin was recovered and washed with buffer. The enzyme immobilized in the resin was used for the synthesis of glycans and glycopeptides. [14]

To produce a glycan library simultaneously in a short time, a new synthetic approach has been developed (Fig. 1), in which various glycan and glycopeptide structures are prepared by sequential and incomplete glycosyltransferase reactions in a single tube. [15] In the developed synthesis method, the initial substrate (glycan, peptide or glycopeptide) and donor substrate were incubated with a glycosyltransferase, and the reaction was stopped at ~50% yield of the product. After stopping the reaction by heat, the next enzyme and donor substrate were added to the reaction mixture, in which both the 50% yield of the product and the resultant initial substrate became the acceptor substrate of the second glycosyltransferase reaction. Again, the second reaction was interrupted at the halfway point, and thus four different products were obtained. When the reaction was continued repeatedly, the number of glycans (or glycopeptides) was essentially doubled during each succeeding process. Of course, this strategy is not applicable for every glycosyltransferase. Since the variety of glycans (glycopeptides) in the library depends on the substrate specificity of the glycosyltransferase, the selection of enzymes, the reaction sequence, and the structure of the starting material are significant factors for the systematic construction of the libraries.

Combination of the incomplete reactions of glycosyl-
transf erase s allowed us to synthesize ~300 kinds of O-linked glycans on the peptides and 50 kinds of both N- and O-glycans and glycan chains of glycolipids to date. These structures contain sialylated and sulfated glycans and glycan epitopes, such as branching structures, Lewis structures, and antigens of the ABO blood group (Table 2).

These synthesized glycans and glycopeptides have been used for several applications. One application is as a standard for the structural analyses by nuclear magnetic resonance, HPLC, lectin array, mass spectral analysis and so on. For example, Kameyama et al. reported a new mass technology based on the comparison of the signal intensity profiles of an observational multistage tandem mass (MS’) between the analyte and a library of observational mass spectra built up by acquiring MS’ spectra of structurally defined oligosaccharides prepared by glycosyltransferases. Next, molecular-weight-tagged glycopeptide libraries have been applied for the screening of ligand specificity for lectins by mass spectrometry. In fact, the specificity of jacalin to immobilized lectin with an affinity for them; thus, the screening system, specific glycopeptides are captured by the type 2 glycans. Interestingly, GII/4 VLP, which is known as a global epidemic genotype, binds to all 6 monovalent glycans (A-, B- and H-antigens on type 1 or 2 glycan), suggesting that this characteristic may be linked with the worldwide transmission of GII/4 strains.

In conclusion, we have succeeded in expressing many glycosyltransferases in mammalian cells; these enzymes are useful for the synthesis of a variety of glycans and glycopeptides available for the evaluation of glycan structures and glycan functions.

Expression of glycosyltransferases in methylotrophic yeast cells.

As described above, the use of human glycosyltransferases as tools for glycan synthesis is a very attractive option because of their strict regioselectivity and stereospecificity; however, their application as industrial catalysts still awaits their availability in mass quantities. There appear to be several reasons for this. First, the stability of glycosyltransferases is believed to be lower than that of other proteins. In fact, the glycosyltransferases have a tendency to become inactivated by incubation at 37°C. The second reason is the cost of the reaction, in which the glycosyltransferase requires both donor and acceptor substrates. It is expensive to synthesize sugar-nucleotides and glycosyltransferases, and to modify glycans from a cost-effectiveness perspective, although most sugar-nucleotides are commercially available from several companies. Another reason is that the industrial demand for glycosyltransferases has been very low until recently.

Several successful efforts at heterologous expression in alternative hosts, such as Escherichia coli,[21–25] fungi[24,25] and insect cells,[26,27] have been reported. However, only a few enzymes have been expressed on an industrial scale. In order to solve such problems, we attempted to establish a yeast expression system for the large-scale production of a variety of human glycosyltransferases.

Methylotrophic yeasts have the ability to utilize methanol as the sole carbon source for growth; therefore, they can produce heterologous proteins with high productivity using methanol-inducible promoters. Protein expression systems have been established in four species of methylotrophic yeasts, Candida boidinii, Hansenula polymorpha, Pichia pastoris and P. methanolica; however these are already patented.[26] Thus, we have chosen a new methylotrophic yeast strain, O. minuta,[20] to create a good host to produce heterologous proteins with high productivity, and have reported the construction of the host—vector system and successful expression of several human glycoproteins in O. minuta.[20–22] In the first attempt, 53 among 141 truncated glycosyltransferase genes cloned into the entry vector were chosen for expression. The genes were re-cloned...
into the *O. minuta* destination vector, pOMEA1-HisFLAG, by LR clonase. Each destination vector was used for the transformation of *O. minuta* TK5-3 strains, and the transformants were selected by adenine auxotrophy. As the gene was integrated into the genome DNA, introduction of the genes was confirmed by genomic PCR with specific primers for each glycosyltransferase gene. After each transformant was cultured in methanol-inducing medium, the expression of glycosyltransferase was checked by western blotting and measurement of the activity. Among the 53 genes tested, 23 glycosyltransferases were secreted into the culture media as active forms although the activities did not reach an industrial scale (Table 3). One characteristic of the expression pattern is that fucosyltransferases were expressed satisfactorily. On the other hand, galactosyltransferases and N-acetylgalcosaminyltransferases are not well established in *O. minuta*, although the reason for the same remains unclear.

In a further study involving the optimization of glycosyltransferase expression, we investigated several factors affecting the expression level of glycosyltransferases, and alpha-2,3-sialyltransferase (ST3Gal) 1\(^{12}\) was chosen as an expression model of glycosyltransferase. First, we found that cultural conditions were significant for expression of ST3GalI. For example, addition of casamino acid to the medium caused a 2-fold increase in the activity (Fig. 2). Reducing the temperature from 30°C to 20°C was also very effective in increasing the expression in *O. minuta*.

**Table 3. Glycosyltransferases expressed in *O. minuta.***

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuc-T</td>
<td>Fuc-T1, T3, T5, T6, T8, T9</td>
</tr>
<tr>
<td>Sia-T</td>
<td>ST3Gall, II, III, VI, ST6Gall, ST6GalNAcI</td>
</tr>
<tr>
<td>Gal-T</td>
<td>B (ABO), β4Gal-T1</td>
</tr>
<tr>
<td>GalNAc-T</td>
<td>A (ABO), ppGalNAc-T1, T2, T4, T6, T10, ppGalNAc-T13</td>
</tr>
<tr>
<td>GlcNAc-T</td>
<td>IgT1, 2, MGAT1, 2, 4a, 5, core2GnT1</td>
</tr>
</tbody>
</table>

Bold indicates the glycosyltransferases expressed after optimization of several factors for high-level production (see text).

The use of a protease-deficient host led to ~3-fold increased production compared to that by the TK5-3 strain. The introduction of protein disulfide isomerase (PDI), known as one of the chaperones that catalyze the formation and breakage of disulfide bonds between cysteine residues within proteins in the endoplasmic reticulum, into the host cells also increased ST3GalI activity, although the expression level of the protein was not changed according to western blotting data (manuscript in preparation). These results indicated that a part of the expressed ST3GalI is misfolded or all ST3GalI proteins are partly misfolded, which causes a decrease in the specific activity of ST3GalI expressed by *O. minuta* cells without the overexpression of PDI. Overall, the optimization of each parameter caused a greater than 300-fold increase in the activity in the culture supernatant in the case of ST3GalI.

Truncation of the stem region of glycosyltransferases was critical for high-level production. Most glycosyltransferases are composed of a short cytoplasmic tail in the N-terminus and transmembrane domain, followed by the stem region and catalytic domain containing a DXD or DXH motif responsible for binding of the divalent cation\(^{20}\). The stem region is flexible and appears to be unstable during expression in *O. minuta*. We designed two expression vectors for the β1,6-N-acetylgalcosaminyltransferase-V (MGAT5) gene\(^{25}\) in *O. minuta*; one gene encodes both the stem region and the catalytic region (Δ32) while the other encodes only a catalytic region (Δ188).\(^{13}\) Western blotting showed higher expression of MGAT5Δ188 than MGAT5Δ96, and the obvious activity of MGAT5 was confirmed in the culture medium of MGAT5Δ188, whereas MGAT5Δ32 culture medium gave no activity. The results indicate that truncation of the stem region is effective for both secretion of the protein and stability against proteases.

Since a certain amount of glycosyltransferases could be obtained, we demonstrated the synthesis of asialotetraantennary type N-glycan by sequential reactions using the recombinant enzymes. We prepared three kinds of glycosyltransferases as follows: \(\beta\)-1,4-N-acetylgalcosaminyltransferase-Iva (MGAT4a),\(^{8,75}\) MGAT5 and \(\beta\)-1,4-galactosyltransferase I (β4Gal-T1)\(^{70}\) by culturing in a jar fermenter. First, MGAT4a and/or MGAT5 were incubated with 2-aminopyridine (PA)-labeled agalactobiantennary type glycan (PA-Sugar Chain 012; Takara Bio Inc.) and UDP-GlcNAc at 37°C, and each reaction condition was determined by monitoring the product on HPLC. The structure of each product was confirmed by comparison with authentic samples and by MALDI/TOF-MS analysis. Next, the product of MGAT4a and MGAT5 was incubated with β4Gal-T1 and UDP-Gal. The position of the product peak corresponded to that of the authentic sample, i.e., asialo-tetraantennary type glycans (PA-Sugar Chain 004; Takara Bio Inc.). Another example is the sialylation of the glycopeptide, PLAG/T-I, in which the PLAG domain of human podoplanin with core 1 type O-glycan was modified by recombinant ST3GalI\(^{79}\). HPLC and MS analysis data indicated that sialyl core 1 structure was created on the PLAG domain without degradation of the PLAG peptide. Taken together, our data demonstrate that the yeast system provides valuable tools for the pro-
duction of novel glycans and the modification of glyco-

peptides.

Conclusion.

Applications of glycans, glycopeptides and glycopro-
teins are now extremely diverse. Previously, chemical
synthesis was the first choice for the large scale pro-
duction of novel glycans. However, the chemical synthesis of various glycans requires great effort and appears to be beyond any reasonable cost-benefit ratio. We have succeeded in produ-
ing a variety of human glycosyltransferases by both mammalian and yeast systems; the former is useful for the preparation of glycan libraries, while the latter helps in the large-scale production of glycans and modification of glycoconjugates. We have studied another approach, i.e., the in vivo creation of human-type glycoco-
jugates in yeast cells by disruption of yeast-specific glycosyltrans-
ferase genes and the introduction of glycosyltransferase genes required for production of mammalian-type glycans. The combination of our technologies may provide us a wider variety of glycans and glycoconjugates, which would lead to further progress in glycoscience and glyco-
techology.

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ヒト糖転移酵素の生産系の開発と応用

千葉靖典1, 伊藤浩美1, 佐藤隆1, 高橋佳江1
地崎芳文2, 成松久1

1産業技術総合研究所酵素開発研究センター
(305-8566 つくば市栄1-1 つくば中央第6)

糖鎖の機能解明や糖鎖構造分析のための標準品を合成するための一つの手段として、糖転移酵素の利用が考えられる。糖転移酵素は基質特異性が明確である一方、酵素自体は安定で大量に生産することが難しいため、糖転移酵素を利用した糖鎖合成の産業的な利用は難しいと考えられてきた。一方、安価な生産のためには大量生産技術が確立している酵素等の代替宿主を用いることが期待されているが、ヒトの糖転移酵素を多量に発現させた例はあまりない。われわれは動物細胞(HEK293T細胞)とメノール処理酵母(Ogataea minuta)を宿主としてヒト糖転移酵素の生産法の開発と応用を検討した。既知の情報ならびに当センターで新規にクローニングした遺伝子を含め、糖鎖合成関連遺伝子をライブラリーユー化した。糖転移酵素のほとんどはHEK293T細胞で可溶型酵素として発現が可能であった。ビーズ上に固定した糖転移酵素を利用し、さまざまな糖鎖、糖ペプチドの合成を行った。また合成した糖鎖の一部は膜上に固定し、糖鎖チップの生産を行った。今後はさらに糖鎖の種類を増やすことで、糖鎖と結合するタンパク質の特異性をより厳密に決定に利用できると考えている。一方、酵母の発現系については、導入した糖転移酵素の半量多様性が発現が確認されなかったため、種々の条件の最適化を検討した。その結果、従来の条件では活性がほとんどみられなかった糖転移酵素も活性が確認できるようになり、ある酵素では数百倍の生産性の向上に成功した。次に、天然からは大量調製が困難なN型多共岐糖鎖の調製を行った。アガラクト型複合型2分岐鎖を主材として、糖転移酵素を逐次作用させることにより、シアリオイ型3分岐、4分岐型糖鎖の生産に成功した。今後、酵素法による糖鎖の大量調製が可能となり、糖鎖チップへの応用や糖タンパク質化酵薬品の原料への活用が期待できる。本研究はNEDO「糖鎖機能活用技術開発」プロジェクトにおいて実施したものである。

【質問】
京大・生命科学 山本
シャペロンの導入。コドンの最適化、膜結合領域の削除によって、酵素活性が上昇するのは、酵素を宿主とし
た際の特異的にみられることでしょうか。
【答】
酵素の小胞体内は動物細胞と比べると比較的還元環境であるといわれているため、シャペロンの導入は酵素特
異的な可能性があります。コドンの最適化や膜結合領域の削除は他の宿主でも酵素活性の上昇に有効であると思
われます。

【質問】
大阪市大院・理 伊藤
実際にアスパラシン型糖鎖を、このような転移酵
素群で合成する場合、最初の糖受容体はどのようなものを利用する予定でしょうか。
【答】
アスパラシン型糖鎖を生成次第が複雑なため、根元から合成していくことは非常に難しいです。したがって、鶏卵卵黄等から抽出可能な二本鎖複合型のアス
パラシン型糖鎖を糖受容体とすることが現実的かと考えます。