Down-regulation of the α-Gal Epitope Expression in N-Glycans of Swine Endothelial Cells by Transfection with the N-Acetylglucosaminyltransferase III Gene

MODULATION OF THE BIOSYNTHESIS OF TERMINAL STRUCTURES BY A BISECTING GlcNAc*

The down-regulation of the α-Gal epitope (Galα1,3Galβ1-R) in swine tissues would be highly desirable, in terms of preventing hyperacute rejection in pig-to-human xenotransplantation. In an earlier study, we reported that the introduction of the β1,4-N-acetylglucosaminyltransferase (GnT) III gene into swine endothelial cells resulted in a substantial reduction in the expression of the α-Gal epitope. In this study, we report on the mechanism for this down-regulation of the α-Gal epitope by means of structural and kinetic analyses. The structural analyses revealed that the amount of N-linked oligosaccharides bearing the α-Gal epitopes in the GnT-III-transfected cells was less than 10% that in parental cells, due to the alteration of the terminal structures as well as a decrease in branch formation. In addition, it appeared that the addition of a bisecting GlcNAc, which is catalyzed by GnT-III, leads to a more efficient sialylation rather than α-galactosylation. In vitro kinetic analyses showed that the bisecting GlcNAc has an inhibitory effect on α-galactosylation, but does not significantly affect the sialylation. These results suggest that the bisecting GlcNAc in the core is capable of modifying the biosynthesis of the terminal structures via its differential effects on the capping glycosyltransferase reactions. The findings may contribute to the development of a novel strategy to eliminate carbohydrate xenoantigens.

Galα1,3Galβ1-R (α-Gal epitope)† is a major xenoantigen that causes the hyperacute rejection of pig organs in xenotransplantation to humans. The elimination of this antigen from pig tissues is highly desirable because it would prevent this type of rejection (1–3). Since the α-Gal epitope is synthesized by UDP-Galβ1,4Galα1,3-galactosyltransferase (α1,3-GT), inactivation of the α1,3-GT gene represents the most promising strategy for the elimination of α-Gal epitopes. However, pigs that lack the α-Gal epitope have not yet been established. Alternatively, an alternate strategy is the overexpression of a glycosyltransferase such as GDP-Fucβ1,2Galα1,2-fucosyltransferase (α1,2-FT), CMP-sialic acid β1,2-3-sialyltransferase (α2,3-ST), and CMP-sialic acid β1,2,6-sialyltransferase (α1,2,6-ST), all of which would be expected to compete with α1,3-GT for a common substrate, N-acetyllactosamine structure. In fact, such an enzymatic competition was successful in reducing the α-Gal epitope, to some extent, as reported previously (4–10).

On the other hand, we have established a unique strategy involving the N-acetylglucosaminyltransferase (GnT) III gene, as evidenced by the substantial reduction of the α-Gal epitope in transfected swine endothelial cells (SEC) and transgenic mice (7, 10–13). In the SEC transfected with the GnT-III gene, the antigenicity to normal human serum and cell lysis mediated by normal human serum was reduced considerably. Although this strategy is distinct from enzymatic competition with α1,3-GT, these levels of reduction were found to be comparable in the case of transfection with a competitive glycosyltransferase, α1,2-FT gene (11). Since GnT-III catalyzes the transfer of GlcNAc to a core β-mannose, producing a bisecting GlcNAc, and is one of the glycosyltransferases that act on the core region of N-glycans, this reduction by GnT-III is not dependent on competition with α1,3-GT. It is well known that the prior addition of a bisecting GlcNAc by GnT-III inhibits further branch formation by UDP-GlcNAcα1,3-d-Man β1,4-N-acetylglucosaminyltransferase IV and UDP-GlcNAcα1,6-d-Man β1,6-N-acetylglucosaminyltransferase V (GnT-V) and leads to...
a decrease in branching structure (14–16). Since the α-galactosyl residues can be potentially transferred to all branches in N-glycans, the decrease in branching, as the result of the introduction of the GnT-III gene, might result in a decreased amount of N-glycans from various glycoproteins (25–34). However, an actual role(s) of the bisecting GlcNAc in the regulation of N-glycan biosynthesis has not fully been demonstrated in 
vitro because of the lack of a comprehensive structural analysis of the cellular oligosaccharides. Such a study in which total oligosaccharides from the GnT-III transfected cells are analyzed and compared with those from the parental cells would allow us to elucidate clearly the regulation of N-glycan biosynthetic pathway by GnT-III in the cells. This would be useful in terms of understanding the mechanism for the GnT-III-conducted down-regulation of the α-Gal epitope more clearly and, thereby, would contribute to the development of an enzymatic remodeling based strategy, aimed at attempting to overcome the α-Gal epitope-mediated hyperacute rejection.

In this study, we prepared free oligosaccharides that were labeled with a fluorescent reagent, 2-aminoypyridine, from the transfected SEC overexpressing GnT-III, and we analyzed their structures. A comparison of these structures with those from normal SEC permitted us to estimate quantitatively the magnitude of the reduction of the α-Gal epitopes and to analyze alterations in the terminal sequences, as well as the core structures. Furthermore, detailed kinetic studies involving α1,3-GT and ST were carried out in order to examine the effect of the bisecting GlcNAc on the susceptibilities of the substrates to these capping reactions. The results suggest that GnT-III also regulates the biosynthesis of the terminal structure via the addition of the bisecting GlcNAc even though the enzyme is involved in the formation of only the core structure.

A decrease in branching structure (14–16). Since the α-galactosyl residues can be potentially transferred to all branches in N-glycans, the decrease in branching, as the result of the introduction of the GnT-III gene, might result in a decreased number of α-Gal epitopes. Nevertheless, the reduction of xenogeneic antigenicity, as was found in the GnT-III-transfected SEC used in our previous study (7, 10, 11), was much more than expected, only from a decrease in branch formation, and, therefore, also appears to involve other effects of the bisecting GlcNAc. This has prompted us to investigate the structural basis of the reduction of α-Gal epitopes in GnT-III-transfected SEC.

The effects of the bisecting GlcNAc on the actions of several glycosyltransferases and α-mannosidase have been studied on a case by case basis using 
vitro activity assays that are based on substrate specificity (17–24). These reports seem to be generally consistent with the previous structural analyses of N-glycans from various glycoproteins (25–34). However, an actual role(s) of the bisecting GlcNAc in the regulation of N-glycan biosynthesis has not fully been demonstrated in 
vitro because of the lack of a comprehensive structural analysis of the cellular oligosaccharides. Such a study in which total oligosaccharides from the GnT-III transfected cells are analyzed and compared with those from the parental cells would allow us to elucidate clearly the regulation of N-glycan biosynthetic pathway by GnT-III in the cells. This would be useful in terms of understanding the mechanism for the GnT-III-conducted down-regulation of the α-Gal epitope more clearly and, thereby, would contribute to the development of an enzymatic remodeling based strategy, aimed at attempting to overcome the α-Gal epitope-mediated hyperacute rejection.

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**EXPERIMENTAL PROCEDURES**

**Materials**—α2,3-Sialidase (Salmonella typhimurium LT2) and End-β-galactosidase (Escherichia freundii) were purchased from Takara and Seikagaku Corp., respectively. All other glycosidases were purchased from Glyco, Nacalai Tesque, Seikagaku Corp., Sigma, and Takara. All glycosyltransferases were purchased from Calbiochem. The standard pyridylaminated (PA-) GG, GS, SG, SS, and galacto-tri-, -tri-, and -tetraantennary oligosaccharides were purchased from Takara. PA-galacto biantennary oligosaccharide was prepared from a glycopeptide of a casein by protease and glycosidase digestions and by mass spectrometric analysis (38). These PA-oligosaccharides were also used as the standards in structural analyses involving HPLC. All other chemicals used were of the highest grade available.

**Cell Cultures**—An SEC line from porcine aorta, MYP-30 (39), and COS-1 cells was maintained at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 0.1 mg/ml streptomycin under a humidified atmosphere of 95% air and 5% CO2. Collagen-coated dishes were used for culture of MYP-30 cells.

**Establishment of Stable Transfectant for GnT-III**—A cDNA for human GnT-III was subcloned into an expression plasmid vector, pCXN2 (40), in which the inserted cDNA is expressed under the control of the SV40 promoter and cytomegalovirus enhancer. The plasmid was transfected into COS-1 cells by a LipofectAMINE™ Reagent (Life Technologies, Inc.). The transfected cells were maintained for several days and were then subjected to selection with 0.4 mg/ml G418 (Life Technologies, Inc.). The resulting resistant cells were then incubated using cloning cylinders.

**Transient Expression of α1,3-GT in COS-1 Cells**—An expression plasmid in which a cDNA for swine α1,3-GT (42) was inserted into the pCXN2 was used to express transiently the glycosyltransferase in COS-1 cells. The plasmid was purified by CsCl gradient ultracentrifugation and used in the transfection experiment. Transfection of the cDNA (20 μg) into COS-1 cells was performed by electroporation using a Gene Pulser (Bio-Rad) under conditions of 300 V/cm and 960 microfarads. The cells were harvested after an appropriate period for use in lectin blot analysis.

**Lectin Blot Analysis**—A lectin blot analysis using a Griffonia simplicifolia IB4 lectin that binds the α-galactosyl epitope was carried out in order to examine and compare the levels of α-Gal epitopes. Cell homogenates (5 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% gel under reducing conditions, according to the methods of Laemmlli (43). The separated proteins were transferred electrophoretically onto a nitrocellulose membrane (Schleicher & Schuell). The blots were blocked in phosphate-buffered saline containing 0.05% Tween 20 and 2% bovine serum albumin and then incubated with biotinylated 5 μg/ml G. simplicifolia IB4 (Vector) for 2 h. After washing with phosphate-buffered saline, the blots were incubated with a horseradish peroxidase-avidin complex (Vector) and developed using an ECL detection system (Amersham Pharmacia Biotech).

**Preparation of PA-oligosaccharides from Cells**—Cells were harvested from 20 dishes (10 cm) of subconfluent cultures and were resuspended in 10 mM Tris-HCl buffer (pH 7). These cells were lysed by sonication and then lyophilized. Lipids were removed by two successive extractions with CH3Cl/methanol: first at a ratio of 2:1 and second at a ratio of 1:2. The residue was dried to give the glycoprotein fractions. N-Linked oligosaccharides in the glycoprotein fraction were liberated by mild acid hydrolysis and were analyzed at 100 °C for 1 h and were re-N-acetylated, as described by Hase et al. (36). The reducing ends of the oligosaccharides were labeled with 2-aminoypyridine, according to the methods of Kondo et al. (37) with minor modifications. In brief, the oligosaccharides obtained by hydrazinolysis were dried and mixed with 200 μl of a coupling reagent, which was prepared by dissolving 552 mg of 2-aminoypyridine (Nacalai Tesque) in 200 μl of glacial acetic acid and a small volume of methanol. The mixture was heated at 90 °C for 60 min. To this solution, 200 μl of a reducing reagent, 39 μl of borane-dimethylamine complex (Wako Pure Chemicals) in 200 μl of glacial acetic acid, which was prepared just prior to use, was added, and the resulting solution was heated at 80 °C for 50 min. Excess reagents were removed by benzene extraction and gel filtration, as described previously (44, 45). The resulting pyridylaminated (PA) oligosaccharides were further purified using a Cellulose Cartridge Glycan preparation kit (Takara) according to the procedures recommended by the supplier.

**HPLC for Oligosaccharide Structural Analyses**—In the structural analysis of cellular N-glycans, the PA-oligosaccharides prepared from whole cells were subjected to an HPLC system (Shimazu) equipped with a TSK-gel 80TM column (4.6 × 150 mm, Tosoh). Elution was performed at a flow rate of 1.0 ml/min at 55 °C using 20 mM ammonium acetate buffer (pH 4.0) as solvent A and the same buffer containing 1% 1-butanol as solvent B. The column was pre-equilibrated with 10% solvents B and, after injection of a sample, the PA-oligosaccharides were separated by a linear gradient of 0–25% of solvent B for 60 min. The eluted PA-oligosaccharides were monitored by a fluorescence detector (model RF-10AXL, Shimazu) at excitation and emission wavelengths of 220 and 400 nm, respectively. Separation of saialyl, nanaosialo, and disialo forms of biantennary oligosaccharides was carried out by anion exchange HPLC using a DEAE-5PW (7.5 × 75 mm, Tosoh), as described previously (46).

**Glycosidase Digestion**—For α-galactosidase digestion, PA-oligosaccharides were incubated with 250 milliunits of 
oglyco-β-galactosidase (Glyco) in the presence of 0.1% of α-galactonic acid γ-lactone
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(Agalsaccharides) in 50 μl of 0.1 x citrate-phosphate buffer (pH 6.0). To digest the PA-agalsaccharides into their corresponding core structures, a complete glycosidase mixture that consists of α-galactosidase, sialidase, α-fucosidase, and β-galactosidase was used. The agalsaccharides were incubated in 60 μl of 0.1 x citrate-phosphate buffer (pH 5.0) with 100 milliunits of jack bean α-galactosidase, 50 milliunits of A. ureafaciens sialidase (Nacalai Tesque), 50 milliunits of bovine kidney α-fucosidase (Sigma), and either 50 milliunits of jack bean β-galactosidase (Seikagaku Corp.) or 10 milliunits of Streptococcus 6646K β-galactosidase (Seikagaku Corp.). In the estimation of the contents of the α-galactosylated oligosaccharides, a mixture in which the α-galactosidase was omitted was used. In this mixture, jack bean β-galactosidase was also replaced with Streptococcus 6646K β-galactosidase. When a fraction of sialylated oligosaccharide was determined on the basis of the difference between the presence and absence of the sialidase, the glycosidase mixture from which the sialidase had been omitted was used, along with the complete mixture. All the above glycosidase digestions of PA-agalsaccharides were performed at 37 °C for 24 h.

Glycosyltransferase Activity Assays—Glycosyltransferase activities were assayed using PA-agalsaccharide substrates, as described previously (47, 48). The cell homogenates were incubated in the appropriate reaction mixture for the determination of each enzyme activity. The reaction mixtures used for GnT-III and GnT-V have been described previously (47), and the concentration of the PA-agalactobiantennary oligosaccharide as an acceptor substrate was 10 μM. The activity of α1,3GT was assayed using a reaction mixture that contains 20 mM MES, 5 mM MnCl₂, 0.5% (v/v) Triton X-100, 2 mM UDP-Gal, 3 μM PA-lacto-N-neotetraose as the acceptor substrate (pH 6.2). The activity of β1,4GT was determined using 2 mM UDP-Gal and 10 μM PA-agalactobiantennary oligosaccharide was used as a donor and an acceptor substrate, respectively. The enzyme and the substrates were incubated in the presence of 0.1% (v/v) Triton X-100 in 50 mM sodium cacodylate buffer (pH 6.0). The enzyme reactions were conducted at 37 °C for 1–4 h and terminated by boiling for 5 min, followed by centrifugation at 12,000 × g for 5 min. The supernatants were subjected to HPLC using a TSK-gel was assayed, 5 mM CMP-sialic acid and 1 mM UDP-sialic acid, and a column temperature of 40 °C. The resulting supernatants were analyzed by reversed or high pressure liquid chromatography (HPLC) in which the 1-butanol concentration was 0.02%, for the non-biected biantennary oligosaccharides were used as a donor and an acceptor substrate, respectively. Each of the reaction mixtures and immediately boiled to terminate the reactions. The samples were appropriately diluted with H₂O and centrifuged at 12,000 × g for 5 min. The resulted supernatants were analyzed by reversed or normal phase HPLC, as described below.

HPLC Analyses of the Products in the Kinetic Study—In the analysis of the α-galactosylated PA-agalsaccharides by reversed phase HPLC, the chromatographic conditions were the same as those used in the activity assay, except 1-butanol was added at a concentration of 0.25 μl/m. When the products were analyzed by normal phase HPLC, an Amide 80 column (4.6 × 150 mm, Tosoh) was used with a flow rate of 1.0 ml/min and a column temperature of 40 °C. The eluents were monitored by the fluorescence detector at excitation and emission wavelengths of 310 and 380 nm, respectively. Analysis of sialylated products was carried out by the reversed phase HPLC in which the 1-butanol concentration was 0.02%, for the non-biected oligosaccharides, and 0.08%, for the bisected types.

Identification and Separation of Four Possible Oligosaccharide Species in the Reactions of Biantennary Oligosaccharides with α1,3GT or α2,6-ST—When digalacto non-biected biantennary oligosaccharide was used as a substrate for α1,3-GT and α2,6-ST, all three possible reaction products and the substrates were simultaneously separated by the reversed phase HPLC. The peaks were identified via conversion into identifiable forms by successive digestion with β-galactosidase and α-galactosidase. This treatment converted αGalG, αGG, GaG, and GG to GG, GGN, GgN, and GgN, respectively, all of which could successfully be identified. Substitution of sialidase for α-galactosidase allowed the identification of peaks corresponding to sialylated products of both non-biected and bisected oligosaccharides. In the α-galactosylation of the bisected biantennary, because the α-galactosyl oligosaccharides could not be cleanly separated by a single chromatographic run, the mono-α-galactosyl oligosaccharide fraction was obtained by normal phase HPLC, followed by β-galactosidase/α-galactosidase digestion and reversed phase HPLC analyses. These chromatographic separations allowed all fractions of the oligosaccharide species, which were produced during the reactions to be determined. In glycosidase digestions in this analysis, oligosaccharides were incubated at 37 °C for 2 h with 0.5 milliunits of Streptococcus 6646K β-galactosidase in 100 mM citrate-phosphate buffer (pH 6.0) in a total volume of 10 μl and, after boiling for 5 min, 50 μg/ml β-galactosidase y-galactosidase (1 μl) and either 20 milliunits of α-galactosidase (1 μl) and 2.5 units of sialidase (1 μl) were then added, followed by further incubation at 37 °C for 5 h.

Protein Determination—Protein concentrations were determined with a BCA protein assay kit (Pierce) using bovine serum albumin as a standard.

RESULTS

A lectin blot analysis, using G. simplicifolia IB4 isolectin (IB4), was carried out in order to compare the levels of an α-Gal epitope in normal SEC and GnT-III-transfected SEC. As shown in Fig. 1, normal SEC express a high level of the α-Gal epitope, and this level is comparable to that in the α1,3-GT-transfected COS cells (αGal-T-COS) were separated by electrophoresis on a 10% SDS-polyacrylamide gel and analyzed by lectin blotting using IB4 lectin. Details of the experimental conditions are described under “Experimental Procedures.”

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As a result, two of these three peaks were identified as core followed by reversed phase HPLC and mass spectrometry, and peaks were collected and analyzed by glycosidase digestion.

Prepared by hydrazinolysis of whole cell lysates, followed by re-N-acetylation and fluorescence labeling with 2-aminopyridine. These PA-oligosaccharides were analyzed by reversed phase HPLC (Fig. 2). The elution profile of the transfected SEC was different from the profile of the normal SEC, indicating that the oligosaccharide structures in SEC had been totally altered as the result of GnT-III transfection.

When the PA-oligosaccharides were digested by α-galactosidase, three major peaks were observed in the profile of the normal SEC, all of which were α-galactosylated (Fig. 2). These peaks were collected and analyzed by glycosidase digestion followed by reversed phase HPLC and mass spectrometry, and as a result, two of these three peaks were identified as core α,1,6-fucosylated and non-fucosylated digalacto-biantennary oligosaccharides. In the case of the transfected SEC, on the other hand, α-galactosidase digestion resulted in essentially no change in the elution profile. Consistent with the results from the lectin blot analysis, this suggests that a substantial fraction of N-glycans in the normal cells contain α-Gal epitopes, whereas most of N-glycans in the transfected cells do not. Thus, the results further confirm that a dramatic reduction in the α-Gal epitope by the expression of GnT-III had occurred.

To assess the changes in the core structure by GnT-III, PA-oligosaccharides from normal and GnT-III-transfected SEC were exhaustively digested with a complete mixture of glycosidases, which contained sialidase, α-galactosidase, β-galactosidase, and α-fucosidase, followed by analysis using reversed phase HPLC (Fig. 3). The elution profile for the normal SEC showed four major peaks that corresponded to PA-agalacto-bi-, -tri-, -tri‘-, and -tetraantennary oligosaccharides, indicating that the SEC are capable of producing N-glycans with a variety of branches. In contrast, only one major peak was found in the transfected cells, which was identified as the PA-agalacto-bisected biantennary oligosaccharide. This indicates that the biosynthesis of multiantennary oligosaccharides is inhibited, in the case of the GnT-III-transfected SEC. This inhibition by GnT-III appears to constitute a mechanism for reducing the α-Gal epitopes in the cells, since it leads to a decrease in number of potential sites for α-galactosylation.

Since polygalactosamines are absent in N-glycans from both normal and transfected SEC, as indicated by the analysis with endo-β-galactosidase (data not shown), most of the complex type N-glycans were digested into the corresponding core structures by the complete glycosidase mixture. When α-galactosidase was omitted from the mixture, α-galactosylated oligosaccharides were not converted to the core structures that had already been identified. Therefore, a variation in the peak area for each core structure between the cases in the absence and presence of α-galactosidase should represent the oligosaccharide fraction that contained at least one α-galactosyl residue. This permitted the content of the oligosaccharide that contained one or more α-galactosyl residue to be estimated separately for each of bi-, tri-, tri‘-, and tetraantennae. As shown by a comparison of the profiles obtained in the absence and presence of α-galactosidase, it was found that a large portion of any of the bi-, tri-, tri‘-, and tetraantennary oligosaccharides contains α-galactosyl residues in normal SEC (Fig. 3A and C). On the other hand, α-galactosyl residues are not found in most oligosaccharides from the transfected SEC, which predominately consist of the bisected biantennary type (Fig. 3B and D). The total amount of α-galactosylated oligosaccharides in the GnT-III-transfected SEC was estimated to be less than 10% that of the normal cells.

A similar analysis was also carried out to examine sialylation at the non-reducing terminus. As shown for the analysis of
α-galactosylation, a difference in a peak area between the absence and presence of sialidase in the glycosidase mixture was attributed to the fractions of oligosaccharides associated with sialylation. In contrast to the case of α-galactosylation, it was found that the GnT-III-transfected SEC produce sialylated N-glycans at levels comparable to normal cells (Fig. 3, B and F). Furthermore, comparison of sialylated fractions in the biantennary species that were non-bisected in the normal SEC and bisected in the transfected SEC indicates that sialylation is much more prevalent in the transfected cells than in the normal cells. It appears that the sialylation is predominantly an α2,6-linked type rather than an α2,3-linkage, as evidenced by the combined use of sialidases with different preferences toward α2,3- and α2,6-linkages (data not shown).

The non-reducing terminal structures of the biantennary oligosaccharides in normal and GnT-III-transfected SEC are summarized in Table I. Such structural differences caused by the transfection with GnT-III cannot be explained by variations in the activities of glycosyltransferases involved in the biosyntheses of the terminal structures because the activities of these glycosyltransferases in the transfected cells were similar to those in the normal cells (Table II). Thus, it can reasonably be assumed from the results obtained from the structural analyses that the non-bisected and bisected oligosaccharides otherwise prefer either of two competitive terminal modifications, α-galactosylation and sialylation, in the cells.

Modulation of biosynthetic pathway of the terminal structure by the addition of the bisecting GlcNAc appeared to be based on an alteration in the susceptibilities of the oligosaccharide toward α1,3-GT and α2,6-ST. Thus, a relatively detailed time course study was carried out to examine the mechanism of this modulation in terms of a kinetic basis. Reactions involving the conversion as the digalacto-biantennary sugar chain to the di-α-galactosylated or di-sialylated form are illustrated in Fig. 4. The reaction rate, v, for each step follows Michaelis-Menten kinetics. Under conditions where the concentrations of glycosyl donors and acceptor oligosaccharides are saturating and sufficiently lower than the $K_v$ values, respectively, the velocities can be approximated as $v = \frac{k_{cat}[G]}{K_{m}^{acceptor}}[E]_{0}][S]$, where $E_0$ and S represent the total enzyme concentration and oligosaccharide acceptor concentration, respectively, and the rate equation may then be treated as a simple second-order reaction. Since $[E_0]$ is constant during the reaction, the equation can further be converted to $v = k \cdot [S]$, where k equals $k_{cat}[E_0]/K_{m}^{acceptor}$ and thus can be regarded as a pseudo first-order rate constant. Therefore, the time course for possible oligosaccharide species that appear during the

![Fig. 4. Reaction scheme for the formation of the di-α-galactosylated or the di-sialylated biantennary oligosaccharide.](image)

**Table I**

| Terminal glycosylation       | Normal cells $^a$ | GnT-III transfected cells $^b$ |
|-----------------------------|------------------|-------------------------------|
| α-Galactosylated            | 41               | 4 $^c$                        |
| Sialylated                  | 6 $^d$           | 53 $^e$                       |
| α-Galactosylated/sialylated | 7                | 4                             |
| β-Galactosylated or         | 46               | 39                            |
| N-acetylglucosaminylated    |                  |                               |

$^a$ Non-bisected oligosaccharides, as the result of the absence of the bisected sugar chains in the cells.

$^b$ Represents bisected oligosaccharides, since the transfected cells produce nearly no detectable non-bisected biantennary oligosaccharides.

$^c$ Di-α-galactosylated bisected biantennary oligosaccharide was not detected.

$^d$ Mono-sialylated, 4%; di-sialylated, 2%.

$^e$ Mono-sialylated, 31%; di-sialylated, 22%.

**Table II**

| Cells          | GnT-III | GnT-V | β1,4-GT | α1,3-GT | α2,6-ST |
|----------------|---------|-------|---------|---------|---------|
| Normal         | 27      | 350   | 26      | 14      | 32      |
| GnT-III-transfected | 1900   | 13    | 430     | 12      | 14      |

$^a$ ND, not detected.

α-galactosylation and the sialylation of the biantennae may be defined by Equations 1–4.

$$[GG] = [GG_0]e^{-(k_1+k_3)t}$$ (Eq. 1)

$$[XG] = \frac{k_1[GG_0]}{k_3} \left( e^{-(k_1+k_3)t} - e^{-k_3t} \right)$$ (Eq. 2)

$$[GX] = \frac{k_3[GG_0]}{k_2} \left( e^{-(k_1+k_3)t} - e^{-k_3t} \right)$$ (Eq. 3)

$$[XX] = \frac{k_3k_4[GG_0]}{k_3 - (k_1 + k_2)} \left[ \frac{1}{k_2} \left( e^{-(k_1+k_3)t} + \frac{1}{k_2} \left( e^{-(k_1+k_3)t} \right) \right) \right] + \left[ \frac{1}{k_4} \left( e^{-(k_1+k_3)t} \right) \right]$$ (Eq. 4)

where $[GG_0]$ indicates initial concentrations of unmodified substrate oligosaccharides, GG and GG(Gn). The conditions used for the kinetic analysis might not be physiological in terms of the substrate concentrations. In general, however, the difference in the reactivities of the substrates can be estimated by comparing $k_{cat}/K_m$. It would be useful to treat these enzyme reactions as combination of pseudo first-order reactions, even though the model may not directly be applicable to other conditions.

The reactions with α1,3-GT and α2,6-ST were carried out under the aforementioned conditions that allow for pseudo first-order kinetics, and the products were analyzed at various intervals by HPLC to determine the concentrations of the four possible oligosaccharide species. Time courses for the α-galactosylation and sialylation of the non-bisected and bisected biantennary oligosaccharides are shown in Fig. 5. To obtain the rate constants shown in Fig. 4, all plot sets were fit to Equations 1–4 by a non-linear regression analysis (Table III). Although α1,3-GT preferred the Mana1,6-branched to the Mana1,3-
The importance of the values of the constants of the non-bisected oligosaccharide. This simulation emphasizes a factor of 3 results in curves that begin to approach those for the extent of modification. The curve for the extent of α-galactosylation was compared between the non-bisected and the bisected oligosaccharides on the basis of the rate constants obtained from the kinetic analysis (Fig. 5). Since the presence of the bisecting GlcNAc appears to inhibit preferentially the values obtained for the non-bisected sugar chain. On the other hand, the curve for extent of sialylation of the non-bisected sugar chain resembles that for the bisected oligosaccharides. A theoretical curve for each plot set was drawn by fitting data to the equation (see text). Lower panels show a comparison of the time courses of the extent of α-galactosylation (C) and sialylation (F) between non-bisected (solid curves) and bisected oligosaccharides (dashed curves).

FIG. 5. Time courses of α-galactosylation and sialylation of the non-bisected and the bisected biantennary oligosaccharides. Left (A—C) and right (D—F) show comparisons of α-galactosylation and sialylation, respectively. Upper panels (A and D) and middle panels (B and E) indicate data for non-bisected and bisected oligosaccharides, respectively. Open circles denote the substrates, closed circles the mono-α-galactosylated at Man1,6-branched, open triangles the mono-α-galactosylated at Man1,3-branched, closed triangles the di-α-galactosylated products, closed squares the monosialylated (at Man1,3, branch), and open squares the disialylated products. A theoretical curve for each plot set was drawn by fitting data to the equation (see text). Lower panels show a comparison of the time courses of the extent of α-galactosylation (C) and sialylation (F) between non-bisected (solid curves) and bisected oligosaccharides (dashed curves).

### Table III

| Reaction steps | Pseudo first-order rate constants (min⁻¹) |
|---------------|------------------------------------------|
|               | Non-bisected | Bisected | Bisected/Non-bisected |
| α-Galactosylation |            |          |                      |
| \( k_1 \)     | 0.071       | 0.023    | 0.32                 |
| \( k_2 \)     | 0.041       | 0.0029   | 0.07                 |
| \( k_3 \)     | 0.024       | 0.0024   | 0.10                 |
| \( k_4 \)     | 0.062       | 0.049    | 0.79                 |
| Sialylation   |            |          |                      |
| \( k_5 \)     | 0.11        | 0.037    | 0.34                 |
| \( k_6 \)     | 0.013       | 0.020    | 1.5                  |

\( ^a \) Defined in Fig. 4.

\( ^b \) Ratio of the rate constants for bisected and non-bisected oligosaccharides.

branch regardless of the presence of the bisecting GlcNAc residue, the presence of this residue lowered the rates of all steps in the α-galactosylation. In particular, the step in which the di-α-galactosylated oligosaccharide was formed from the oligosaccharide with mono-α-galactosyl residue linked to the Man1,6-branched was affected to a greater extent, thus leading to a marked suppression of the formation of the di-α-galactosyl oligosaccharide (Fig. 5, A and B). The time course for the extent of α-galactosylation was compared between the non-bisected and the bisected oligosaccharides. This simulation emphasizes the importance of the values of the constants \( k_2 \) and \( k_3 \) in reducing formation of the di-α-galactosylated sugar chain. In Fig. 6B, the estimated values of the constants for the α-galactosylation of the non-bisected and bisected oligosaccharides have been switched to illustrate further the effect of the values of the four constants on extent of modification. The curve for the extent of α-galactosylation of the non-bisected sugar chain is only minimally affected if the values of \( k_2 \) and \( k_3 \) are replaced with the corresponding constants obtained for the bisected sugar chain. On the other hand, the curve for extent of α-galactosylation of the non-bisected sugar chain resembles that for the bisected sugar chain if the values of \( k_2 \) and \( k_3 \) for the bisected sugar chain are substituted for the corresponding values obtained for the non-bisected sugar chain. Since the presence of the bisecting GlcNAc appears to inhibit preferentially α-galactosylation of the Man1,3-branch, due to its greater effects on \( k_2 \) and \( k_3 \) (Fig. 4, Table III), the first step in the synthetic pathway through \( k_4 \) would become predominant, and, because of the diminution of \( k_3 \), formation of the di-α-galactosylated product would be reduced. These inferences reasonably account for the results from the kinetic analysis (Fig. 5, A and B).

In contrast, although the rate of the first sialylation was modestly decreased by the presence of the bisecting GlcNAc, the rate of formation of the di-sialylated oligosaccharide was not altered by the bisecting GlcNAc because of the enhancement of the second sialylation step (Fig. 5, D and E). In both non-bisected and bisected sugar chains, the first sialylation occurred on the Man1,3-branches, as evidenced by the conversion into identifiable forms, GGn and GGnGn, by β-galactosidase/sialidase digestion (data not shown). As a result, the time course for the extent of the sialylation of the non-bisected vis à vis the bisected oligosaccharides was not significantly different (Fig. 5F). These results suggest that the presence of the bisecting GlcNAc residue markedly inhibits α-galactosylation but does not greatly affect the sialylation. These differential effects of the bisecting GlcNAc on these two competitive modifications of non-reducing terminal residues appear to constitute the mechanism by which the GnT-III-conducted down-regulation of the formation of α-galactosyl epitopes occurs.

**DISCUSSION**

The purpose of this study was to investigate the mechanism by which GnT-III overexpression causes a dramatic decrease in the expression of the α-galactosyl epitope in terms of structural and enzymatic bases. In the case of GnT-III-transfected SEC, it was found that the substantial fraction of N-glycans actually remains in the biantennary form, as the result of the inhibitory effect of the bisecting GlcNAc residue on the actions of other GnTs, which are involved in branch formation (14–16). Furthermore, it appears that α-galactosyl residues are “replaced” by sialyl residues in the transfected cells, although the enzyme activities involved in these modifications are not significantly affected by the transfection. These structural alterations with
Respect to non-reducing end groups are based on the differential effects of the bisecting GlcNAc on αL,3-GT and αL,6-ST, as shown by the kinetic analysis. It seems certain that the down-regulating mechanism consists, at least, of both a reduction in the extent of branching and an alteration in the susceptibility of the non-reducing terminal residues to capping by glycosyltransferases. These findings provide an enzymatic basis for the down-regulation of the αL-galactosylation steps (Fig. 5 and Table III) are inhibited. In contrast, the action of αL,2,6-ST may be sensitive only to steric hindrance in the proximity of the Manα3 branch. It seems likely that such a difference in the susceptibilities of the capping enzymes constitutes the molecular basis for the regulation of the biosynthesis of the terminal structures on GnT-III.

This study proposes a possible mechanism of the down-regulation of the α-gal epitope in the GnT-III-transfected cells and, furthermore, first demonstrates the roles of GnT-III and the bisecting GlcNAc in the regulation of N-glycan biosynthesis within cells by a structural analyses of whole cellular oligosaccharides. The present findings clearly show that GnT-III actually regulates branch formation in the core of the oligosaccharides in vivo, further supporting a previous suggestion obtained, based on an in vitro analysis (21, 23, 24). Moreover, the analysis of the cellular oligosaccharides and the related kinetic analysis suggest the “distant” effect of the bisecting GlcNAc which confers the regulation of the biosynthesis of the non-reducing terminal structures. Thus, the bisecting GlcNAc would have dramatic effects on a variety of steps in the biosynthesis of the complex and hybrid types of N-glycans, leading to dramatic alterations in the ultimate oligosaccharides on the cell surface. Therefore, it is conceivable that GnT-III has the potential to regulate a variety of biological processes in cells via altering critical oligosaccharide structures that are involved in glycoprotein functions. GnT-III might thus be regarded as a distinctive key glycosyltransferase in the control of cellular functions via its effects on N-glycan biosynthesis.

REFERENCES
1. Joziassie, D. H., and Oriol, R. (1999) Biochim. Biophys. Acta 1455, 403–418
2. Platt, J. L. (1998) Nature 392, 11–17
3. Sandrin, M. S., and McKenzie, I. F. (1994) Immunol. Rev. 141, 169–190
4. Sandrin, M. S., Fodor, W. L., Mouhtouris, E., Osman, N., Cohney, S., Rollins, S. A., Guilmette, E. R., Setter, E., Squinto, S. P., and McKenzie, I. F. (1995) J. Biol. Chem. 270, 13611–13627
5. Cohney, S., McKenzie, I. F., Patton, K., Prenzonsa, J., Ostenried, K., Fodor, W. L., and Sandrin, M. S. (1997) Transplantation 64, 495–500
6. Costa, C., Zhao, L., Burton, W. V., Bondioli, K. R., Williams, B. L., Hoagland, T. A., Ditsiou, P. A., Ebert, K. M., and Fodor, W. L. (1999) FASEB J. 13, 1762–1773
7. Miyagawa, S., Tanemura, M., Koyota, S., Koma, M., Ikeda, Y., Shirakura, R., and Taniguchi, N. (1999) Biochem. Biophys. Res. Commun. 264, 611–614
8. Sharma, A., Okabe, J., Birch, P., McClellan, S. B., Martin, M. J., Platt, J. L., and Logan, J. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7190–7195
9. Tanemura, M., Miyagawa, S., Koyota, S., Koma, M., Matsuha, H., Tsuji, S., Shirakura, R., and Taniguchi, N. (1998) J. Biol. Chem. 273, 16421–16425
10. Koma, M., Miyagawa, S., Honke, K., Ikeda, Y., Koyota, S., Miyoshi, S., Matsuha, H., Tsuji, S., Shirakura, R., and Taniguchi, N. (2000) Glycobiology 10, 745–751
Down-regulation of the α-Gal Epitope by GnT-III

11. Tanemura, M., Miyagawa, S., Ibara, Y., Matsuda, H., Shirakura, R., and Taniguchi, N. (1997) Biochem. Biophys. Res. Commun. 235, 359–364
12. Tanemura, M., Miyagawa, S., Ibara, Y., Mikata, S., Matsuda, H., Shirakura, R., and Taniguchi, N. (1997) Transplant. Proc. 29, 891–892
13. Miyagawa, S., Nakai, R., Yamada, M., Tanemura, M., Ikeda, Y., Taniguchi, N., and Shirakura, R. (1999) J. Biochem. (Tokyo) 126, 1067–1073
14. Schachter, H., Narasimhan, S., Gleenon, P., and Vella, G. (1983) Can. J. Biochem. Cell Biol. 61, 1049–1066
15. Schachter, H. (1986) Biochem. Cell Biol. 64, 163–181
16. Gu, J., Nishikawa, A., Tsuruoka, N., Ohno, M., Yamaguchi, N., Kangawa, K., and Taniguchi, N. (1993) J. Biochem. (Tokyo) 113, 614–619
17. Harpaz, N., and Schachter, H. (1980) J. Biol. Chem. 255, 4885–4893
18. Longmore, G. D., and Schachter, H. (1982) Carbohydr. Res. 100, 365–392
19. Allen, S. D., Tsai, D., and Schachter, H. (1984) J. Biol. Chem. 259, 694–6990
20. Gleenon, P. A., and Schachter, H. (1983) J. Biol. Chem. 258, 6162–6173
21. Blanken, W. M., Van Vliet, A., and Van den Eijnden, D. H. (1984) J. Biol. Chem. 259, 15131–15135
22. Paquet, M. R., Narasimhan, S., Schachter, H., and Moscarello, M. A. (1984) J. Biol. Chem. 259, 4716–4721
23. Narasimhan, S., Freed, J. C., and Schachter, H. (1985) Biochemistry 24, 1694–1700
24. Easton, E. W., Boccher, J. M., and Van den Eijnden, D. H. (1991) J. Biol. Chem. 266, 21674–21680
25. Yamashita, K., Hitoi, A., Matsuda, Y., Tsuji, A., Katunuma, N., and Kobata, A. (1983) J. Biol. Chem. 258, 1098–1107
26. Yamashita, K., Hitoi, A., Tsuji, A., Katunuma, N., and Kobata, A. (1983) Cancer Res. 43, 4601–4605
27. Tsuji, T., Irimura, T., and Osawa, T. (1981) J. Biol. Chem. 256, 10497–10502
28. Savvidou, G., Klein, M., Grey, A. A., Derrington, K. J., and Carver, J. P. (1984) Anal. Biochem. 135, 372–379
29. Mizochi, T., Taniguchi, T., Shimizu, A., and Kobata, A. (1982) J. Immunol. 129, 4020–4026
30. Mellis, S. J., and Baenziger, J. U. (1983) J. Biol. Chem. 258, 11546–11556
31. Bayard, B., Kerkauert, J. P., Streeker, G., Dorland, L., Van Halbeck, H., and Vliegenthart, J. F. G. (1983) Eur. J. Biochem. 137, 319–323
32. Baenziger, J. U., and Fiete, D. (1979) J. Biol. Chem. 254, 2400–2407
33. Baenziger, J., and Kornfeld, S. (1974) J. Biol. Chem. 249, 7270–7281
34. Baenziger, J., and Kornfeld, S. (1974) J. Biol. Chem. 249, 7260–7269
35. Seko, A., Kobetsu, M., Nishizono, M., Enoki, Y., Ibrahim, H. R., Juneja, L. R., Kim, M., and Yamamoto, T. (1997) Biochem. Biophys. Acta 1335, 23–32
36. Hase, S., Ibuki, T., and Ikenaka, T. (1984) J. Biochem. (Tokyo) 95, 197–203
37. Kondo, A., Suzuki, J., Kuraya, N., Hase, S., Kato, I., and Ikenaka, T. (1990) Agric. Biol. Chem. 54, 2169–2170
38. Ikeda, Y., Koyota, S., Tohma, T., Soejima, H., Niikawa, N., and Taniguchi, N. (1997) Biochem. Biophys. Res. Commun. 235, 359–364
39. Miyagawa, S., Shirakura, R., Iwata, K., Nakata, S., Matsumiya, G., Izutani, H., Matsuda, H., Terada, A., Matsumoto, M., Nagasawa, S., and Seya, T. (1994) Transplantation 58, 834–846
40. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Gene (Amst.) 108, 193–199
41. Ibara, Y., Nishikawa, A., Tohma, T., Soejima, H., Niikawa, N., and Taniguchi, N. (1995) J. Biochem. (Tokyo) 113, 692–698
42. Strahan, K. M., Gu, F., Preece, A. F., Gustavsson, L., Andersson, L., and Gustafson, K. (1995) Immunogenetics 41, 101–105
43. Laemmli, U. K. (1970) Nature 227, 680–685
44. Iwase, H., Ishii, K. I., Urata, T., Saijo, T., and Hotta, K. (1990) Anal. Biochem. 188, 200–202
45. Hase, S., Koyama, S., Daiyansu, H., Takezono, H., Hara, S., Kobayashi, Y., Kyogoku, Y., and Ikenaka, T. (1990) J. Biochem. (Tokyo) 100, 1–10
46. Yamamoto, S., Hase, S., Fukuda, S., Sano, O., and Ikenaka, T. (1989) J. Biochem. (Tokyo) 105, 547–555
47. Taniguchi, N., Nishikawa, A., Fujii, S., and Gu, J. G. (1989) Methods Enzymol. 179, 397–408
48. Morita, N., Hase, S., Ikenaka, K., Mikoshii, K., and Ikenaka, T. (1988) J. Biochem. (Tokyo) 103, 332–335
49. Hironaka, T., Furukawa, K., Esmon, P. C., Yokota, T., Brown, J. E., Sawada, S., Fournel, M. A., Kato, M., Minaga, T., and Kobata, A. (1993) Arch. Biochem. Biophys. 307, 316–330
50. Langeveld, P. M., Noolken, M. E., Hard, K., Todd, P., Vliegenthart, J. F., Reuse, J., and Hudon, B. G. (1991) J. Biol. Chem. 266, 2622–2631
51. Galili, U., Shohet, S. B., Kobrin, E., Stults, C. L., and Macher, B. A. (1988) J. Biol. Chem. 263, 17755–17762
52. Platt, J. L., Lindman, B. J., Chen, H., Spitalnik, S. L., and Bach, F. H. (1990) Transplantation 50, 817–822
53. Sepp, A., Skuce, P., Lindstedt, R., and Lechner, R. I. (1997) J. Biol. Chem. 272, 2304–2310
54. Brissos, J. R., and Carver, J. P. (1983) Biochemistry 22, 3671–3680
55. Brissos, J. R., and Carver, J. P. (1983) Can. J. Biochem. Cell Biol. 61, 1067–1078
56. Brissos, J. R., and Carver, J. P. (1983) Biochemistry 22, 3680–3686
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