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Impaired Galactosylation of Core 2 O-Glycans in Erythrocytes of β1,4-Galactosyltransferase Knockout Mice

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O- and N-glycans included in erythrocyte membrane glycoproteins from β1,4-galactosyltransferase I (GalT-I) knockout mice were analyzed to examine how this enzyme deficiency affects glycosylation of proteins in erythroid cells. The results indicated that greater than 80% of core 2 O-glycans from GalT-I$^{-/-}$ mice are not galactosylated by β1,4 linkage, resulting in the expression of Neu5Acα2 → 3Galβ1 → 3(GlcNAcβ1 → 6)GalNAc, while core 2 O-glycans from GalT-I$^{+/+}$ mice are fully galactosylated and occur as Neu5Acα2 → 3Galβ1 → 3(Neu5Acα2 → 3Galβ1 → 4GlcNAcβ1 → 6)GalNAc. On the other hand, β1,4-galactosylation of N-glycans of the mutant was approximately 60% that of the wild type. Thus, it is suggested that GalT-I is predominantly responsible for β1,4-galactosylation of the core 2 O-glycan branch in erythroid cells.

A β1,4-galactosyltransferase (GalT) which is present in milk has so far been considered to be involved in the synthesis of the Galβ1 → 4GlcNAc group widely expressed as a structural moiety in N- and O-glycans. Therefore, knockout mice lacking this enzyme has been generated to examine functional roles of sugar chains by two groups (1, 2). However, these knockout mice still express low levels of β1,4-galactosylated glycans in several tissues, suggesting the presence of other compensating galactosyltransferases. Recently, several galactosyltransferases other than the milk enzyme (now called GalT-I) has been cloned, and termed GalT-II to VI (3–7). Expression of these transferases differs tissue to tissue (3–7), indicating that a distinct set of GalTs as well as other glycosyltransferases are working in each tissue or cell type. Therefore, deficiency of GalT-I would produce different galactosylation in various tissues. Actually, serum glycoproteins of GalT-I knockout mice have been shown to be markedly defective in β1,4-galactosylation of N-glycans (1), while N-glycans of brain glycoproteins from wild-type and knockout mice seem to be β1,4-galactosylated at comparable levels based on the lectin staining experiment (8).

The Galβ1 → 4GlcNAc group is expressed in O-glycans as structural units of poly-*N*-acetyllactosamine and of core 2 O-glycans. β1,4-Galactosylation of a GlcNAc residue in the core 2 branch, Galβ1 → 3(GlcNAcβ1 → 6)GalNAc, is essential for further elongation of this branch synthesizing the backbone structures for expression of functional carbohydrate structures such as selectin ligands (9–11). Quite recently, it has been shown from the *in vitro* experiments that GalT-IV is responsible for poly-*N*-acetyllactosamine synthesis in core 2 branched O-glycans (12), but there are no data on the role of GalTs in O-glycan synthesis *in vivo.* Mouse erythrocyte membrane glycoproteins have been shown to express N-glycans and core 2 O-glycans as well as core 1 O-glycans and their structures have already been elucidated (13, 14). Therefore, erythrocytes are thought as good targets especially to examine how GalT-I contributes to synthesis of the core 2 O-glycans *in vivo.* In this study, erythrocyte membrane glycoproteins are prepared from wild-type and GalT-I knockout mice, and structural analysis of their O- and N-glycans are focused on galactosylation by using accurate and sensitive methods, which have recently been developed for structural analysis of small amounts of samples (15, 16).

MATERIALS AND METHODS

Materials. Sodium borohydride, 2-aminobenzamide and dimethylamine–borane complex were purchased from Nacalai Tesque Inc., Kyoto. Anhydrous hydrazine was from Tokyo Kasei Inc. Co., Tokyo. AG-50W-X12 was obtained from Bio-Rad Lab., Hercules, CA. Ultrafree centrifugal filter units (C3LGC) were from Millipore Japan Co., Tokyo; Sep-Pak C18 cartridge from Waters Co., Milford, MA; and

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Fluorescamine from Sigma Chemical Co., St. Louis, MO. Jack bean β-*N*-Acetylhexosaminidase and endo-β-galactosidase from *Escherichia freundii* were purchased from Seikagaku Corporation, Tokyo. Concanavalin A (Con A)–Sepharose was obtained from Pharmacia Biotech Japan, Tokyo and β-galactosidase from *Diplococcus pneumoniae* was from Boehringer-Mannheim Yamanouchi, Tokyo. *Aleuria aurantia* lectin (AAL)-Sepharose was a kind gift from Dr. N. Kochibe (Gunma University).

Preparation of erythrocyte ghosts. GalT-I heterozygous (GalT-$I^{+/-}$) and homozygous mutant mice (GalT-$I^{-/-}$) and the wild-type mice (GalT-$I^{+/+}$) were generated as previously described (1). Erythrocytes from 9 weeks old mice were washed with phosphate buffered saline three times and their ghosts were prepared as previously described (15). Lyophilized ghosts were stored at −20°C.

Preparation of O-linked oligosaccharides from erythrocyte ghosts. Ghosts were delipidated by extraction twice with chloroform-methanol (2:1, v/v) and then four times with chloroform-methanol (1:2, v/v). Delipidated ghosts were suspended with 1 ml of 0.05 M NaOH containing 1 M sodium borohydride and incubated at 45°C for 16h. The released oligosaccharides were purified according to the previous method (15).

Preparation of fluorescence-labeled N-linked oligosaccharides from erythrocyte ghosts. N-linked oligosaccharides were liberated from anhydrous ghosts by hydrazinolysis (17), labeled with 2-aminobenzamide (2-AB), and purified as previously described (16). Since the previous report (14) showed that N-linked oligosaccharides of mouse erythrocyte glycoprotein are well fucosylated, the labeled oligosaccharides were further purified by AAL-Sepharose column chromatography as follows. The sample was applied to a column (1 ml) of an AAL-Sepharose and eluted with 10 ml of 10 mM ammonium acetate buffer, pH 7.0, and the fraction bound to the column was then eluted with 5 ml of the same buffer containing 1 mM fucose. The bound fraction containing N-linked oligosaccharides was evaporated, resolved in 200 μl of 1 M sodium/acetate buffer, pH 5.0 and then applied to a C18 cartridge to remove fucose and salts as previously described (16).

Analysis of O-linked and 2-AB labeled N-linked oligosaccharides by HPAEC. The O-linked oligosaccharides were analyzed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a Bio-LC system (Dionex Co., Sunnyvale, CA) equipped with a CarboPac PA-1 column (4 × 250 mm) and a pulsed amperometric detector as previously described (15). The N-linked oligosaccharides were desialylated by mild acid hydrolysis with 0.01 M HCl at 100°C for 20 min and then analyzed by HPAEC with fluorometric detection (HPAEC-FD) by adding a RF-10AXL fluorescent monitor (Shimadzu Co., Kyoto) and an ASRS-2 anion micromembrane suppressor (Dionex Co., Sunnyvale, CA) to the system as previously described (16).

HiTrap Q FPLC of 2-AB labeled N-linked oligosaccharides. Anion-exchange column chromatography with a HiTrap Q column (Pharmacia Biotech Japan, Tokyo) equipped with a RF-10AXL fluorescent monitor was carried out to separate oligosaccharides depending on their negative charges. Elution was performed with 1 mM ammonium acetate buffer, pH 7.0 from 0 to 6 min and with a linear gradient of 1 to 200 mM ammonium acetate buffer, pH 7.0, from 6 to 40 min.

Glycosidase digestion of oligosaccharides. N-Linked oligosaccharides were digested with diplococcal β-galactosidase (10 mU), or endo-β- galactosidase (5 mU) in 50 μl of 0.3 M citrate/phosphate buffer, pH 6.0 at 37°C overnight. Jack bean β-*N*-acetylhexosaminidase (1 U) was used for digestion of O-linked oligosaccharides in 50 μl of 0.3 M citrate/phosphate buffer, pH 5.0 at 37°C for 40 h. Each digestion mixture was applied to an Ultrafree centrifugal unit in order to remove the enzyme and desalted by a C18 cartridge.

Con A–Sepharose column chromatography. The labeled N-linked asialooligosaccharides applied to a column (1 ml) of Con A-Sepharose were eluted with 10 ml of 10 mM Tris–HCl, pH 7.4 containing 100 mM NaCl, 1 mM $CaCl_2$, $MgCl_2$, and $MnCl_2$. Then, the bound fraction was eluted with 15 ml of the same buffer containing 0.2 M α-methyl mannoside. All procedures were performed at room temperature.

RESULTS

Analysis of O-linked oligosaccharides. The O-linked oligosaccharides released by alkaline borohydride treatment of erythrocyte ghosts prepared from 9 weeks old mice were analyzed by HPAEC-PAD. As shown in Fig. 1A, three oligosaccharide peaks *a*, *c*, and *d* were detected from the ghost of GalT-$I^{+/+}$ mice. The sample from GalT-$I^{+/-}$ mice also gave quite similar chromatogram (Fig. 1B). Considering the previously reported structures of O-glycans of mouse erythrocyte membrane glycoproteins (13) and elution positions identical to authentic oligosaccharide standards from fetuin, the peaks *a*, *c* and *d* are identified as Neu5Acα2 → 3Galβ1 → 3GalNAc, Neu5Acα2 → 3Galβ1 → 3(Neu5Acα2 → 3Galβ1 → 4GlcNAcβ1 → 6)GalNAc, and Neu5Acα2 → 3Galβ1 → 3(Neu5Acα2 → 6)GalNAc, respectively.

On the other hand, O-linked oligosaccharides from GalT-$I^{-/-}$ mouse erythrocytes were separated into four peaks *a*, *b*, *c* and *d* (Fig. 1C). To be noted is that the peak *c* corresponding to the core 2 O-glycan dramatically decreased and the peak *b* newly appeared in the knockout sample. When the peak *b* was digested with jack bean β-*N*-acetylhexosaminidase, its elution position was shifted to that of peak *a*, Neu5Acα2 → 3Galβ1 → 3GalNAc (data not shown). Therefore, the peak *b* must be Neu5Acα2 → 3Galβ1 → 3(GlcNAcβ1 → 6)GalNAc. The proposed structures of peaks *a* to *d* of three samples and their relative molar ratios are shown in Fig. 2. The data indicate that β1,4-galactosylation of core 2 O-glycans in erythrocytes of the knockout mice decreases to approximately one-fifth of the wild-type, while synthesis of sialylated forms of the core 1 oligosaccharide (Galβ1 → 3GalNAc) does not change. The similar results were also obtained with 28 weeks old mice (data not shown). It is therefore concluded that GalT-I is predominantly responsible for β1,4-galactosylation of the core 2 branch in mouse erythroid cells.

Analysis of N-linked oligosaccharides. A mouse erythrocyte glycoprotein has been shown to express complex-type N-glycans with sialylated *N*-acetyllactosamine structures, Neu5Acα2 → 3Galβ1 → 4GlcNAc, in their outer chain moieties (14). If impaired expression of *N*-acetyllactosamine structures occurs by knockout of the GalT-I gene, it would result in reduced expression of sialylated N-glycans and accumulation of less sialylated N-glycans. Then, we tested if this is the case by charge analysis of N-linked oligosaccharides released by hydrazinolysis of erythrocyte ghosts. When the oligosaccharides from GalT-$I^{+/+}$ mice were sub-

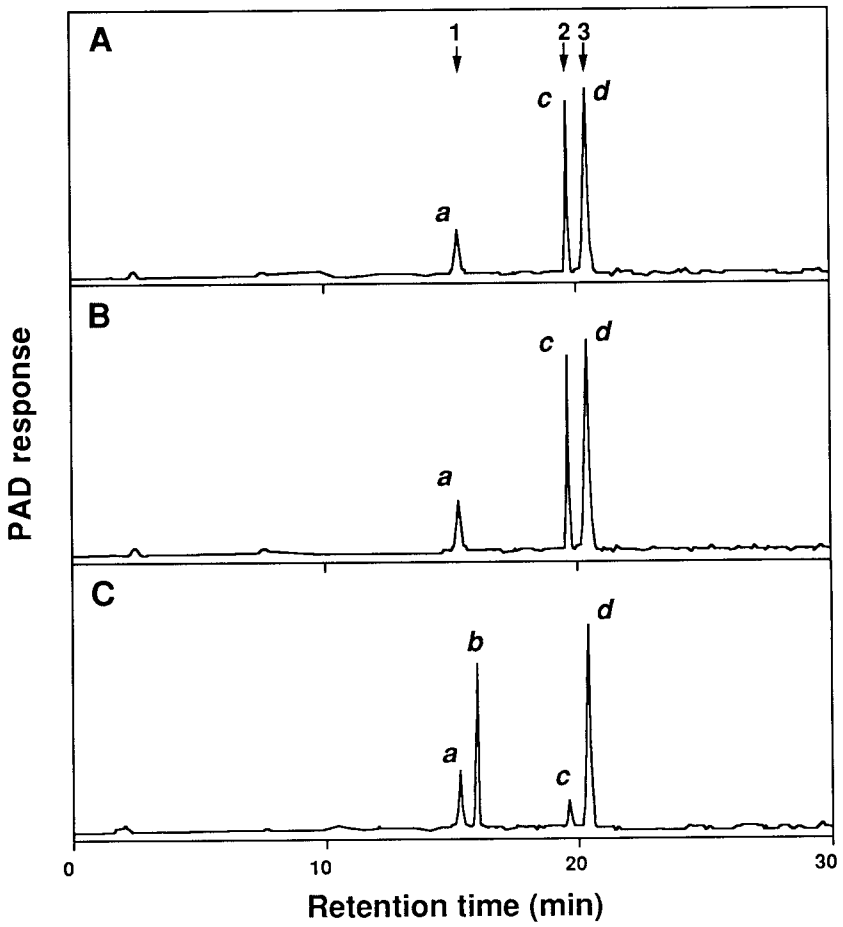

FIG. 1. Analysis of O-glycans by HPAEC-PAD. Oligosaccharides released by alkaline-borohydride treatment from delipidated erythrocyte membrane glycoproteins were analyzed by HPAEC-PAD. (A) GalT-I$^{+/+}$, (B) GalT-I$^{+/-}$, (C) GalT-I$^{-/-}$. Arrows 1, 2 and 3 in (A) indicate elution positions of standard oligosaccharide alditols prepared from fetuin: 1, Neu5Acα2 → 3Galβ1 → 3GalNAc: 2, Neu5Acα2 → 3Galβ1 → 3(Neu5Acα2 → 3Galβ1 → 4GlcNAcβ1 → 6)GalNAc; 3, Neu5Acα2 → 3Galβ1 → 3(Neu5Acα2 → 6)GalNAc.

jected to HiTrap Q FPLC, they were separated into a neutral and three acidic fractions A1 to A3 (Fig. 3A). Since all these acidic fractions were converted to neutral ones by sialidase digestion, it was evident that A1, A2, and A3 are mono-, di-, and trisialylated oligosaccharides, respectively. The oligosaccharides from GalT-I$^{+/-}$ mice were also separated in a quite similar manner as those of the wild-type (data not shown). On the other hand, the elution profile of oligosaccharides from GalT-I$^{-/-}$ mice differs from that of the wild-type (Fig. 3B). Relative molar ratios of fractions N, A1, A2 and A3 on the basis of peak areas were 15:28:40:17 (GalT-I$^{+/+}$), and 48:23:27:2 (GalT-I$^{-/-}$), respectively. An increase of fraction N in the knockout sample, from 15 to 48%, suggests less sialylation occurs possibly due to the reduced galactosylation. However, major parts of oligosaccharides are still sialylated, and degree of sialylation is not so decreased as in the case of core 2 O-glycans. These results suggest that knockout of the GalT-I gene results in a partial reduction of β1,4-galactosylation of N-glycans.

To demonstrate more directly the reduced galactosylation, the whole 2-AB labeled N-linked oligosaccharides were desialylated and analyzed by HPAEC-FD. As shown in Fig. 4A (solid line), asialooligosaccharides from GalT-I$^{+/+}$ mice gave five major peaks *a* to *e*. A quite similar profile was obtained with the sample from GalT-I$^{+/-}$ (data not shown). According to the previously elucidated structures of N-glycans of mouse erythrocytes (14), and identities of elution positions with those of authentic oligosaccharides standardized with glucose units (16), peaks *a*, *b* and *c* were estimated to be biantennary, 2,4-branched triantennary, and tetraantennary oligosaccharides with fucosylated trimannosyl cores. Glycosidase digestion analysis using endo-β-galactosidase and diplococcal β-galactosidase (data not shown) also indicated that peaks *d* and *e* are tetraantennary oligosaccharides with fucosylated trimannosyl cores containing one and two *N*-acetyllactosamine repeating units. On the other hand, many peaks were detected from the sample of the GalT-I$^{-/-}$ (Fig. 4B, solid line). When the asialo oligosaccharides from

	Proposed structure	+/+	+/-	-/-
a	Neu5Acα2→3Galβ1→3GalNAc-ol	10	11	10
b	Neu5Acα2→3Galβ1→3GalNAc-ol ↓6 GlcNAcβ1	-	-	30
c	Neu5Acα2→3Galβ1→3GalNAc-ol ↓6 Neu5Acα2→3Galβ1→4GlcNAcβ1	44	42	7
d	Neu5Acα2→3Galβ1→3GalNAc-ol ↓6 Neu5Acα2	46	47	53

FIG. 2. Proposed structures of O-glycans released from erythrocyte membrane glycoproteins of GalT-I$^{+/+}$, GalT-I$^{+/-}$ and GalT-I$^{-/-}$ mice. Values indicate percent molar ratios of peaks *a* to *d* calculated on the basis of their peak areas.

GalT-I$^{+/+}$ and GalT-I$^{-/-}$ were further digested with diplococcal β-galactosidase which specifically cleaves the Galβ1 → 4GlcNAc linkage (18), products obtained from both samples were eluted at the same positions (data not shown). Therefore, more complicated elution profile of the knockout sample is suggested to result from an incomplete β1,4-galactosylation of the GlcNAc residues in outer chain moieties of N-glycans. Since it was strongly suggested that the GalT-I$^{-/-}$ sample is composed of a mixture of completely and partially galactosylated oligosaccharides with different antennary structures, we recovered major biantennary oligosaccharides from the fraction bound to and eluted from a Con A–Sepharose column and analyzed by HPAEC-FD. The oligosaccharides from the wild-type showed a major peak of completely galactosylated biantennary oligosaccharides (Fig. 4A, dotted line), while those from GalT-I$^{-/-}$ showed two extra peaks *x* and *y* in addition to the peak *z* identical to the major peak of the wild-type (Fig. 4B, dotted line). Diplococcal β-galactosidase digestion converted these peaks to a single peak which was eluted at the same position of the peak *x* (data not shown), indicating that the peak *x* is the biantennary oligosaccharide without galactose residues and peaks *y* and *z* are those containing one and two galactose residues, respectively. On the basis of relative content of these oligosaccharides (*x*:*y*:*z* = 31:26:43), it was calculated that approximately 60% of *N*-acetylglucosamine residues in the outer chain moieties were galactosylated. This results contrasts with the fact that only one-fifth of the core 2 O-glycans from the knockout mouse are β1,4-galactosylated.

DISCUSSION

In this study, effect of the GalT-I deficiency on synthesis of core 2 O-glycans *in vivo* is presented for the first time. The results clearly show that the loss of GalT-I results in a dramatic decrease of β1,4-galactosylation of the core 2 O-glycan branch, but in a moderate decrease of that of N-glycans. It is suggested, therefore, that GalT-I is dominantly responsible for synthesis of galactosylated core 2 branch in erythroid cells. Galactosylation of small parts of core 2 O-glycans and large parts of N-glycans indicates compensation of other GalTs. Cloning data so far accumulated indicate that a β1,4GalT family is composed of at least six members called GalT-I to -VI (3–7). Since expression levels of GalTs in erythroid cells have not been so far reported, it is not clear which enzymes are compensatory in erythroid cells of GalT-I knockout mice. To be noted is, however, that residual GalTs in the knockout mice contribute to galactosylation of the core 2 branch to a less extent than that of N-glycans. Different contribution of GalT-I and other GalTs to galactosylation

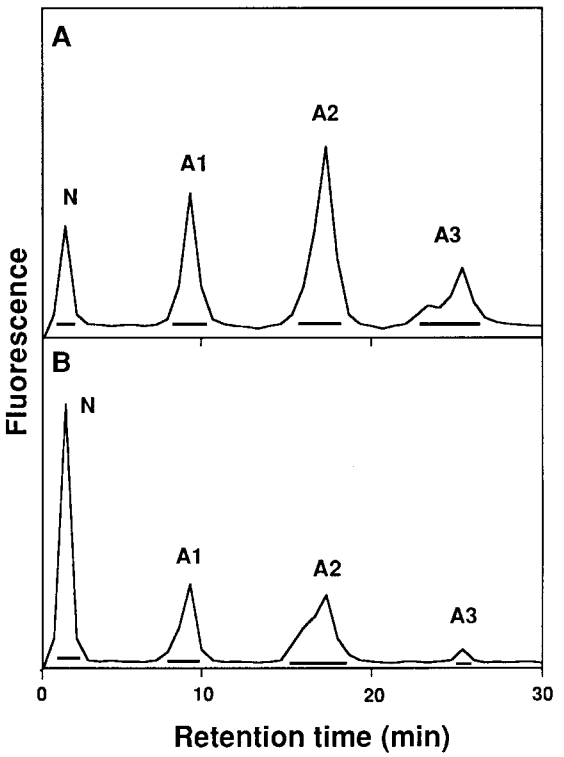

FIG. 3. Charge analysis of N-glycans by HiTrap Q FPLC. Oligosaccharides released by hydrazinolysis from erythrocyte membrane glycoproteins and labeled with 2AB were analyzed by HiTrap Q FPLC as described under Materials and Methods. (A) GalT-I$^{+/+}$, (B) GalT-I$^{-/-}$.

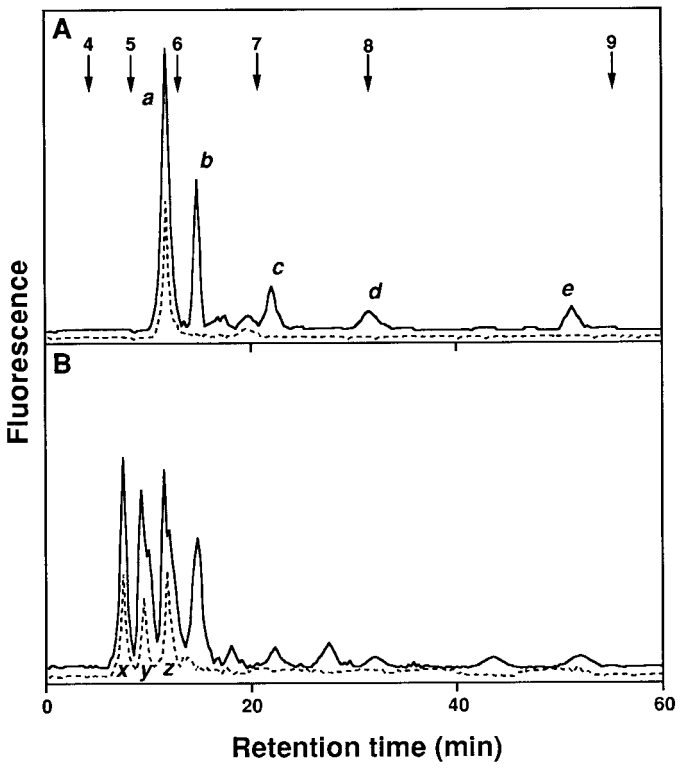

FIG. 4. Analysis of desialylated N-glycans by HPAEC-FD. The N-linked oligosaccharides from GalT-I$^{+/+}$(A, solid line) and GalT-I$^{-/-}$ (B, solid line) were desialylated and analyzed by HPAEC-FD. The Con A-bound fractions of desialylated oligosaccharides from GalT-I$^{+/+}$ (A, dotted line) and GalT-I$^{-/-}$ (B, dotted line) were also analyzed. Arrows and numbers indicate elution positions of alditols of glucose oligomers used as internal standards and glucose units, respectively.

of O- and N-glycans is suggested to partly due to distinct substrate specificities of GalTs. In connection with this respect, milk GalT-I, recombinant GalT-II and III, but not GalT-IV, have been shown to transfer Gal to asialo, agalacto-transferrin and -fetuin *in vitro* (7). GalT-V is inactive toward asialo, agalacto-transferrin (4). A recent report also shows that milk GalT-I is more active toward N-glycans related synthetic acceptors than recombinant GalT-IV (12). To be noted is also the fact that when the core 2 branched oligosaccharides, Galβ1 $\rightarrow$ 3(GlcNAcβ1 $\rightarrow$ 6)GalNAcα1 $\rightarrow$ R, were incubated with either of milk GalT-I and recombinant GalT-II, -III, -IV and -V, they were most efficiently galactosylated by GalT-IV (12). These *in vitro* data suggest that GalT-I has a preference of N-glycans to core 2 O-glycans while GalT-IV has an opposite preference. However, intrinsic enzymatic properties of these GalTs, especially GalT-I and IV, cannot explain the present *in vivo* results obtained with erythrocytes that GalT-I knockout mice have a dramatic defect in galactosylation of the core 2 O-glycan branch but a rather mild defect in galactosylation of N-glycans. We have to consider many factors controlling galactosylation; tissue (cell-type)-dependent expression levels of GalTs, physiological concentrations of donor and acceptors, structures of acceptor proteins, and so on (19). Further *in vivo* and *in vitro* investigations will have insights into the mechanism by which β-galactosylation of O- and N-glycans is regulated.

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