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Core 2 GlcNAc transferase and kidney tubular cell-specific expression

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The expression of glycan chains is precisely regulated in a time- and space-dependent manner. We summarize here our recent work on the kidney tubular cell-specific regulation of core 2 β-1,6-GlcNAc transferase. *Gsl5* gene was first identified by genetic analysis on the basis of polymorphic expression of kidney glycolipids among inbred strains of mice and turned out to be a regulatory gene controlling the level of mRNA of kidney-specific core 2 β-1,6-GlcNAc transferase. This kidney-specific core 2 GlcNAc transferase takes glycolipids having Galβ1-3GalNAc at their termini, Galβ1-3GalNAcα1- and β1-oligosaccharide derivatives, and glycoproteins having core 1 structure, as substrates. Immunohistochemistry with anti-core 2-Le^x monoclonal antibody demonstrated that vesicles located just below the microvillous membrane of proximal tubule cells were clearly stained in a *Gsl5*-wild type mouse. Western blotting with the monoclonal antibody detected a major glycoprotein with a molecular mass of 500 kDa in the microsomal fraction of the wild type mouse kidney. *In situ* hybridization with anti-sense cDNA of kidney-specific core 2 GlcNAc transferase confirmed that *Gsl5* gene controls the expression of the core 2 β-1,6-GlcNAc transferase mRNA in a proximal tubular cell-specific manner. The 5′ upstream sequences of the kidney-specific core 2 GlcNAc transferase gene in inbred and wild-derived strains of mice were analyzed, and the phylogenetic analysis of these sequences suggests that functional *Gsl5* gene might be produced by the time of subspeciation of *M. musculus*, about one million years ago.
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Introduction

Glycan chains are added to lipids and proteins, and modify biological functions of these molecules. Recognition between glycan chains and their receptor molecules plays an important role in cell-cell and cell-matrix communications [1,2]. The expression of glycoconjugates changes during embryogenesis, differentiation, and malignant transformation and is precisely regulated at the levels of transcription, translation, and post-translation [3–9]. The molecular mechanisms responsible for the time-dependent and tissue-specific regulations of carbohydrate expression are not well understood and are important research targets in glycoscience. To understand the molecular mechanisms, we focused on polymorphic differences of glycolipids and applied them to genetic analysis. This approach is able to identify genes involved in the regulation of glycan chains and can lead to studies on molecules encoded by the identified genes. Here we review the results of such a study applied to a kidney-specific and polymorphic expression of glycolipids.

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Identification of *Gsl5* gene

We found a polymorphic difference in mouse kidney glycolipids. A glycolipid identified as Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3)Gb$_4$Cer (GL-Y) is a major neutral glycolipid in most inbred strains of mice: C57BL/6, C57BL/10, BALB/c, C3H/He, WHT/Ht, and AKR, and is absent in a very limited number of strains like DBA/2 and CBA [10,11]. DBA/2 mouse kidney contains a less polar neutral glycolipid identified as Galβ1-3GalNAcβ1-4Gb$_3$Cer (GL-X). The expression of GL-Y was dominant over the expression of GL-X in F1 mice produced by the mating between BALB/c and DBA/2 mice. The backcross mice obtained with F1 and recessive DBA/2 mice segregated into two types, F1 type and DBA type, and the ratio of the

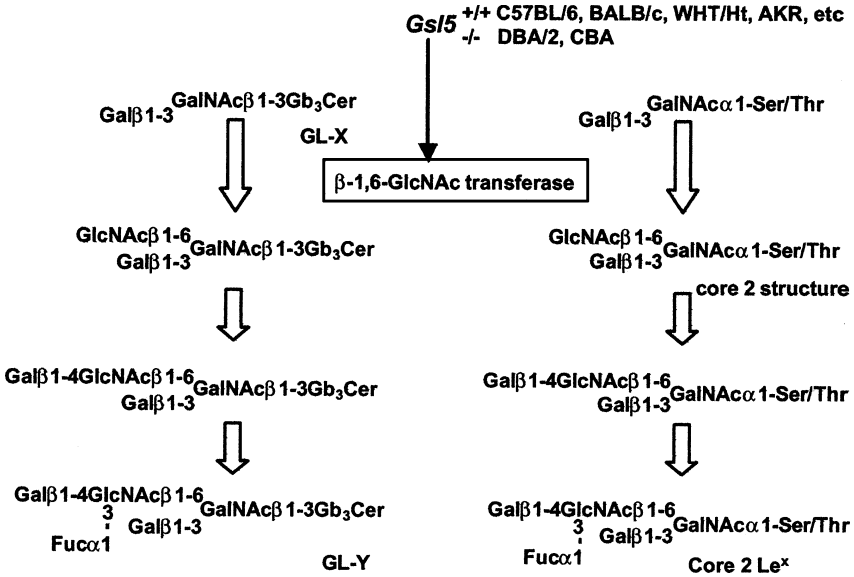

Figure 1. Biosynthetic pathways related to *Gsl5* gene. *Gsl5* regulates the level of kidney-specific β-1,6-GlcNAc transferase mRNA which transfers GlcNAc to Galβ1-3GalNAcβ1-3Gb$_3$Cer and Galβ1-3GalNAcα1-Ser/Thr of proteins. DBA/2 and CBA inbred strains of mice carry defective *Gsl5* gene and other strains like C57BL/6, BALB/c, WHT/Ht, AKR, etc. carry wild-type *Gsl5* gene. *Gsl5* acts in a mouse kidney proximal tubular epithelial cell-specific manner.

number of individuals with the F1 and DBA/2 types was almost 1:1 [11]. This result indicates that the polymorphic difference is produced by a difference of a single autosomal gene, and we named this gene *Gsl5*, glycosphingolipid regulating gene-5 [12,13]. The structures of GL-X and GL-Y suggested that the *Gsl5* gene controls the expression of GlcNAcβ1-6(Galβ1-3)Gb$_4$Cer, an immediate product of GL-X, by the regulation of β-1,6-GlcNAc transferase (GNT) which uses Galβ1-3Gb$_4$Cer (GL-X) as a substrate (Figure 1). Therefore, we set up an assay system to measure the activity of β-1,6-GlcNAc transferase using Galβ1-3Gb$_4$Cer as a substrate. The results of an assay shown in Figure 2 demonstrate that the expression of GL-Y always requires GNT activity in individual mice of the parent and backcross mice, and the lack of GL-Y expression is due to the undetectable level of the GNT activity [14].

To understand the molecular mechanism, how the expression of GNT activity is controlled by *Gsl5*, we purified GNT from wild-type mouse kidneys, characterized its substrate specificity, obtained partial amino acid sequences, and cloned its cDNA by RT-PCR with degenerate oligonucleotide primers designed on the basis of the partial amino acid sequences of the purified enzyme and kidney mRNA as a template and rapid amplification of 5′- and 3′-cDNA ends method [14,15]. The purified enzyme can use Galβ1-3GalNAcα1- or β1-*p*-nitrophenyl derivatives, asialoglycophorin, chemically modified serum albumin having Galβ1-3GalNAc structure, Gg$_4$Cer, and GL-X as good substrates, indicating that Galβ1-3GalNAc structure at the terminals is essential for the substrates. The amino acid sequence deduced from the cDNA sequence exhibited 84% identity to that of human core 2 β-1,6-GlcNAc transferase-I [16,17]. These results of substrate specificity and the sequence comparison

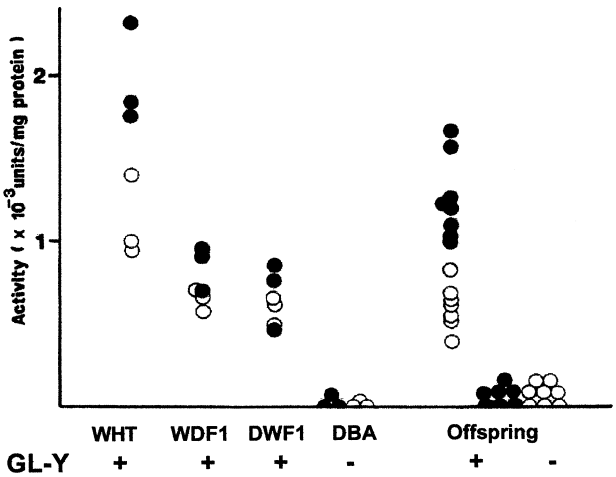

Figure 2. Relation between GL-Y expression and β-1,6-GlcNAc transferase activity in the kidneys of parent mice (WHT/Ht and DBA/2), F1 mice produced by WHT/Ht and DBA/2 (WDF1 and DWF1), and offspring mice produced by the mating with F1 and DBA/2. A circle represents single individual, and closed circles are males and open ones females.

conclude that the GNT is a mouse homologue of human core 2 β-1,6-GlcNAc transferase-I. The GNT mRNA detected by Northern blot analysis with a 494-bp probe covering a part of the *N*-terminal half of GNT is expressed abundantly in the kidney of wild type mice but not of DBA/2 type mice (Figure 3). The GNT mRNA in various tissues other than the kidney of the wild-type and DBA-type mice was not detectable at a detection sensitivity which gave clear signals with kidney RNA of the dominant-type mice. These results indicate that *Gsl5* controls

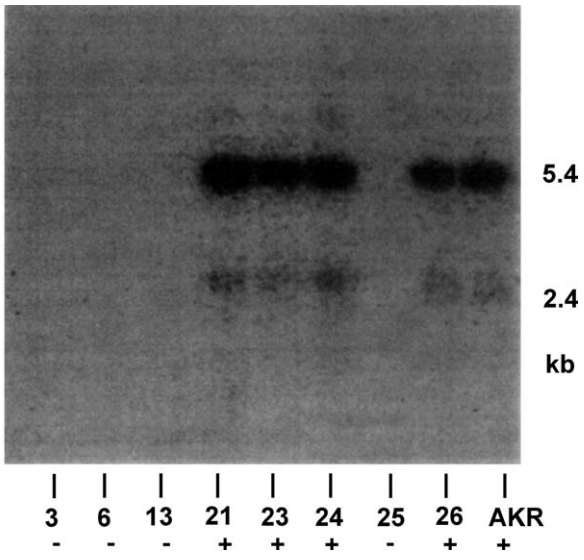

Figure 3. Northern blot of the kidney-specific β-1,6-GlcNAc transferase mRNAs from recombinant inbred strains of mice produced by AKR and DBA/2 mice. The numbers at the bottom represent the strain lines and the expression of GL-Y in the kidney of each line is indicated. Poly(A) RNAs were hybridized with a 494-bp probe. The result demonstrates that GL-Y positive individuals exhibit the signals and the negative individuals exhibit no detectable signals.

the level of mRNA encoding GNT in kidney-specific manner [16]. We also cloned a homologous cDNA from mouse submaxillary gland and found that these two cDNAs differ only in their 5′-untranslated regions and share an open reading frame (ORF) encoding for the same protein. The mRNA of the submaxillary gland type was detected in various organs of both DBA/2 and wild type mice by RT-PCR and did not exhibit a polymorphic difference. Competitive RT-PCR results indicated that the kidney-type mRNA is 20 times more abundant than the submaxillar type mRNA in the kidney of mice with the *Gsl5* wild type. Genomic composition of the GNT gene was also analyzed and the results indicate that the mouse GNT gene produces at least three transcripts which share the ORF, differ in 5′-untranslated regions, and are produced by different promoters. The *Gsl5* gene regulates only the expression of the mRNA specific to kidney but not the other two transcripts [16]. The sequence corresponding to 5′-none translated region of the third transcript reported by Warren [18] was found in the genomic sequence of GNT gene and it is interesting to note that the third transcript was cloned from T cell lymphoma derived from DBA/2 mouse.

Cell specific regulation of GNT by *Gsl5*

In situ hybridization with an antisense probe specific to the kidney-type mRNA demonstrated that the kidney-type transcript was expressed heavily in the part of the cortex adjacent to the medulla and weakly in other part of cortex in the kidney

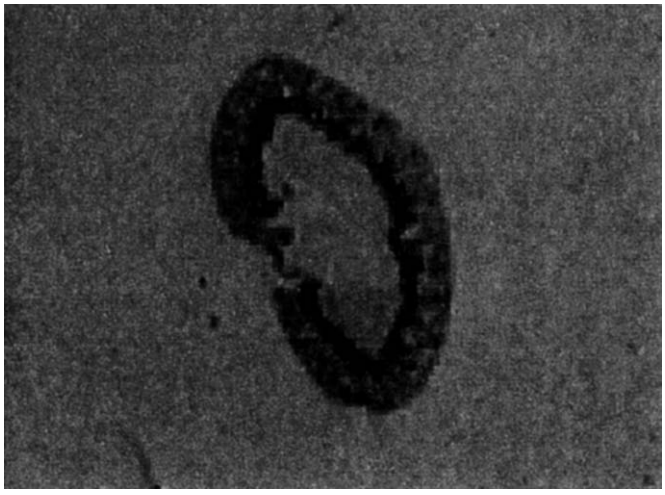

Figure 4. Detection of kidney-specific transcript of β-1,6-GlcNAc transferase by *in situ* hybridization. The mRNA was highly expressed in proximal tubular epithelial cells in the cortico-medullary region. *Gsl5* gene is active only this region.

(Figure 4). This distribution overlaps with that of proximal tubular epithelial cells. Therefore, it is concluded that the expression of GNT mRNA regulated by *Gsl5* is specific to proximal tubular epithelial cells [19].

As mentioned above, GNT regulated by *Gsl5* takes glycoproteins as well as glycolipids as substrates. Therefore, we analyzed glycoproteins carrying the core 2 structure. We used a monoclonal antibody, SA024, to detect the Lewis x antigen on the core 2 structure, Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3)GalNAcα1-. Immunohistochemistry with SA024 demonstrated that the vesicular structures just below the apical membrane of the proximal tubular epithelial cells were heavily stained in BALB/c kidney but not in DBA/2 kidney [19]. Western blotting with SA024 demonstrated that the microsomal fraction of BALB/c kidney contained a number of glycoproteins but that of DBA/2 kidney contained no corresponding bands. One major polymorphic band exhibited molecular mass of about 500 kDa. Thus, we conclude that *Gsl5* also regulates the expression of glycoproteins having core 2 structure in a proximal tubular cell specific manner [19].

Analysis of 5′-upstream sequence of GNT gene

Gsl5 controls the expression of GNT activity through the regulation of kidney-type GNT mRNA. Therefore, we analyzed 5′-upstream sequences of the GNT gene of various types of inbred and wild-derived strains of mice [20]. The sequences of 1.2 kb-long DNA fragments of 6 wild mice derived from the Asian continent or Japan with the *Gsl5*-recessive phenotype were identical to the sequence of DBA/2 mice except one strain, BLG2/Ms, which exhibited three single base substitutions and one deletion of 8 nucleotides, GTTTGTTT. These variations do not affect the function of the *Gsl5* gene and are considered to

be mutations within the recessive phenotype. The other inbred strains and two wild strains derived from the European continent with the *Gsl5*-dominant phenotype contained considerable numbers of substitutions in comparison with DBA/2 and were classified as one group. A phylogenetic tree drawn by the sequence diversity obtained by DNADIST on PHYLIP software demonstrates that mutations to differentiate the dominant and recessive types of the *Gsl5* gene occurred approximately one million years ago during subspeciation of *M. musculus* [20]. We have not been able to identify the genomic sequence of the *Gsl5* gene itself by the comparison of 1.2 kb-long 5′-upstream sequences, but we can speculate that mutations to create the functional *Gsl5* gene occurred at the same time as the subspeciation of *M. musculus*.

Discussion

The *Gsl5* gene regulates the production of the kidney-type GNT mRNA in a proximal tubular cell-specific manner, and controls the expression of the core 2-like structure of glycolipids and the core 2 structure of membrane glycoproteins in the proximal tubular cells.

Polymorphic variations of glycan chains are very well described and the best examples are histo-blood group antigens. ABO blood group and Lewis type antigens have been studied extensively and the molecular basis of the variations has been revealed to be mutations in the ORF of glycosyltrasferases responsible for the antigen biosynthesis [21–24]. We studied polymorphic variations of glycolipids in various tissues among inbred strains of mice and identified the following 5 mouse genes: *Ggm1* for liver-specific expression of GM1 [25], *Ggm2* for liver-specific expression of GM2 [26], *Gsl4* for erythrocyte-specific expression of GalCer and GM4 [27], *Gsl5* in this report, and *Gsl6* for kidney-specific expression of NeuGcα2-3Galβ1-3Gb$_4$Cer [13]. These genes were also located on mouse chromosomes: 17 for *Ggm1*, 10 for *Ggm2*, 4 for *Gsl4*, and 19 for *Gsl5* and *Gsl6*. An interesting notion is that *Ggm1*, *Ggm2*, and *Gsl5* genes do not correspond to respective glycosyltrasnferase ORF themselves, but seem to be transcription-responsible elements involved in tissue-specific expression of corresponding glycosyltransferases. Reasons for this possibility are that (1) the phenotypes caused by variations of the genes are restricted to particular tissues but not all of the tissues like ABO and Lewis antigens; (2) three genes, *Gsl5*, *Ggm1*, and *Ggm2*, regulate the level of mRNA in a tissue specific manner (unpublished results for *Ggm1* and *Ggm2*); (3) no apparent functional abnormalities are reported in the liver of mice with defective *Ggm1* and *Ggm2* genes and in the kidney of mice with a defective *Gsl5* gene. It is conceivable that these genes do not encode for tissue-specific transcription factors, because, if these factors are defective, liver and kidney malfunctions would be apparent. Therefore, these genes are possibly tissue-specific transcription-responsible elements.

However, the molecular basis of *Ggm1*, *Ggm2*, and *Gsl5* cannot be easily addressed, because culture cells maintaining tissue-specific expressions regulated by *Ggm1*, *Ggm2*, and *Gsl5* are not available at present. We experienced that primary cultures of mouse proximal tubular epithelial cells lost the GNT expression regulated by *Gsl5* within 24 h. Two possible ways to approach this subject are (1) establishment of culture cells maintaining the required phenotypes by the use of transgenic mice carrying the conditional SV-40 promoter, and (2) transgenic experiments to rescue defective phenotypes of mutant mice. We were able to rescue the defective phenotype of the *Gsl5* gene by transgenesis of a 150 kbp-long BAC clone derived from a *Gsl5*-dominant mouse into a defective DBA/2 mouse. Now, we need to dissect the clone and find responsible elements.

The phylogenetic analysis of the 5′-upstream regions of the GNT gene provided an interesting example for the process of creating functional genes. Sequence variations of the 5′-upstream regions are clearly separated into the dominant and recessive types and these two types were estimated to have separated each other one million years ago during the subspeciation of *M. musculus*. Variations found in each type are a rather good control for the accumulation of mutations within the type. Analysis of wild-derived strains demonstrated that the recessive genes distribute from Eastern Europe to Far East Asia and Central Asia. In these regions *M. musculus musculus* is an inhabitant. The dominant genes distribute in the European continent and South Asia where *M. musculus domesticus* and *M. musculus castaneus*, respectively, are habitants. Then, we ask, which gene is beneficial to support kidney function, especially proximal tubular epithelial cell function. Unfortunately, the genomic composition of the core 2 β-1,6-GlcNAc transferase-I gene in *M. musculus* is not conserved in *Rattus norvegicus* and *Homo sapiens*, and the tissue-specific promoter use including *Gsl5* is unique to *M. musculus*. However, we think that the answering of the above question can give clues and an example for considering the relation between evolution and glycan-chain diversity.

Fox *et al*. reported that immunohistochemical analysis of frozen sections of mouse kidney with SSEA-1 antibody (stage-specific embryonic anigen-1), anti-Lewis x antibody localized the antigen in proximal tubular epithelial cells [28]. The positive staining was lost by the treatment of the sections with chloroform-methanol solution, confirming that the antigens are glycolipids [28]. These results together with those of our biochemical analysis indicate that the glycolipid antigen is GL-Y, Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3)Gb$_4$Cer, because other glycolipids containing Lewis x structure are not present as major glycolipids in the kidney. The immunohistochemical staining of paraffin sections of the kidney of a *Gsl5* wild type mouse with SA024 antibody and Western blotting of the microsomal fraction with SA024 antibody indicate that the vesicles in proximal tubular epithelial cells contain a 500 kDa-glycoprotein carrying Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3)GalNAcα1-Ser/Thr. These results indicate that the proximal tubular epithelial cells express both of GL-Y and the 500 kDa

glycoprotein. The 500 kDa glycoprotein is characterized as megalin, which is a receptor molecule for various kinds of proteins exhibiting smaller molecular weight than albumin and functions to uptake these ligand proteins from glomerular filtrate urine [unpublished results and 29]. These results suggest that the *Gsl5* wild type mice have GL-Y and megalin carrying Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3)GalNAcα1-Ser/Thr or possibly other core 2 extended structures in the proximal tubular cells, and the *Gsl5* recessive mice have GL-X and megalin carrying no core 2 extended structures, but possibly NeuAcα2-3Galβ1-3GalNAcα1-Ser/Thr or NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAcα1-Ser/Thr. *N*-Glycan structures of rat megalin have been extensively analyzed [30], but *O*-glycan structures require further study. We are now asking the question whether these glycan-chain differences can affect ligand binding activity of megalin or not. If it is the case, we are able to find a clue to address the relationship between evolution and glycan-chain diversity from the functional aspect of glycan chains.

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