# Normal levels of serum glycoproteins maintained in β-1,4-galactosyltransferase I-knockout mice

Masahiro Kido[a,b], Masahide Asano[c], Yoichiro Iwakura[c], Masao Ichinose[b], Kazumasa Miki[d], Kiyoshi Furukawa[a,*]

[a] *Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173-0015, Japan*
[b] *Department of Gastroenterology, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-8655, Japan*
[c] *Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-0071, Japan*
[d] *The First Department of Internal Medicine, Toho University School of Medicine, Ota-ku, Tokyo 143-8541, Japan*

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**Abstract The galactose-mediated clearance of serum glycoproteins from the circulation was evaluated using β-1,4-galactosyltransferase (β-1,4-GalT) I-knockout mice. Partial structural study of the oligosaccharides released from mouse serum glycoproteins revealed that 77.4% of the oligosaccharides from β-1,4-GalT $I^{+/+}$ mouse contain galactose, while 7.7% of those from β-1,4-GalT $I^{-/-}$ mouse were galactosylated. Under the conditions, no significant change in serum protein concentrations was observed between the normal and mutant mice. The results indicate that the hepatic asialoglycoprotein receptor-mediated system is not functioning in the clearance of endogenous serum glycoproteins.**



*Key words:* Serum glycoprotein; Undergalactosylation; Clearance

## 1. Introduction

One of the important functions of the carbohydrate moiety of glycoproteins was brought about by the fortuitous finding that administration of desialylated ceruloplasmin into rabbits resulted in rapid clearance from the circulation when compared to the fully sialylated protein which survived for days [1]. The subsequent studies dealing with a variety of serum glycoproteins which were treated with sialidase and/or β-galactosidase or galactose-oxidase and then injected into animals established that the rapid uptake of these serum glycoproteins is solely due to the exposure of the penultimate galactose residues (reviewed in [2]). Accumulation of radiolabeled asialoglycoproteins in liver showed the presence of a unique receptor on the sinusoidal surface of hepatocytes which functions in the binding, internalization and degradation of galactose-terminated serum glycoproteins [3]. The asialoglycoprotein receptor (ASGP-R) has been isolated from several mammalian liver tissues [4–7] and found to be an oligomer of two types of similar polypeptides, each of which binds a galactose residue with a low affinity [8–13]. However, oligosaccharides with specific geometric arrangement of galactose residues as in the Galβ1→4GlcNAcβ1→2Manα1→6-[Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)-Manα1→3]Man structure bind the receptor with higher affinities ([14,15] and reviewed in [16]). Furthermore, since the presence of the Galβ1→3GlcNAc group on the non-reducing termini of oligosaccharides diminishes a binding affinity [17], the presence of the Galβ1→4GlcNAc groups on the non-reducing termini of oligosaccharides is important for the clearance of serum glycoproteins.

In the present study serum protein concentrations and the galactose contents of serum glycoproteins were determined in order to evaluate the biological significance of the galactose-mediated clearance system of serum glycoproteins from the circulation in β-1,4-galactosyltransferase (β-1,4-GalT) I-knockout mice [18].

## 2. Materials and methods

### 2.1. Lectin blot analysis of serum glycoproteins and determination of serum protein concentrations

The serum samples were obtained by the conventional method from β-1,4-GalT $I^{+/+}$ and β-1,4-GalT $I^{-/-}$ mice of 1, 6 and 8 weeks old which were raised as described previously [18]. A portion of the samples was defatted with acetone and then with chloroform and methanol mixtures (2:1 and 1:2, v/v), and the remaining portion was diluted with water by 100-fold and used for determination of protein concentrations with a BCA protein assay kit [19]. The defatted samples were lyophilized and subjected to SDS-polyacrylamide (7.5%) gel electrophoresis (SDS-PAGE) and transferred to polyvinylidenedifluoride (PVDF) membranes. Western blot analysis using biotinylated *Psathyrella velutina* lectin (PVL) and peroxidase-conjugated *Ricinus communis* agglutinin (RCA)-I was performed after treatment of blots with *Arthrobacter ureafaciens* sialidase as described previously [18,20]. The blots were incubated with Coomassie Brilliant Blue (CBB) for detecting protein bands or blocked with 1% bovine serum albumin (BSA) prior to incubation with lectin. In some experiments, the blots blocked with BSA were treated with 200 mU of diplococcal β-galactosidase in 150 μl of 0.3 M citrate phosphate buffer (pH 6.0) or 2.5 U of N-glycanase in 150 μl of 0.1 M phosphate buffer (pH 8.2) prior to incubation with lectin.

### 2.2. Lectin column chromatography of N-linked oligosaccharides released from serum glycoproteins

The serum glycoprotein samples from 6 weeks old mice (10 mg each), which were dried thoroughly over $P_2O_5$ in vacuo, were subjected to hydrazinolysis for 10 h as described previously [21]. After *N*-acetylation, the liberated oligosaccharides were reduced with $NaB^3H_4$ to obtain tritium-labeled oligosaccharides for partial structural analysis. The radioactive oligosaccharides were directly digested with sialidase in 50 μl of 0.5 M acetate buffer (pH 5.0), and then applied to a DEAE-Sepharose column. The oligosaccharides obtained in the pass-through fraction of the column, which were supposed to be a mixture of neutral and desialylated neutral ones, were fractionated by RCA-I-agarose column chromatography. In some experiments, oligosaccha-

*Corresponding author. Fax: (81)-3-3579 4776.
E-mail: furukawa@tmig.or.jp


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rides were further separated by sequential column chromatography using concanavalin A (Con A)-Sepharose and *Aleuria aurantia* lectin (AAL)-Sepharose, and partial structures of oligosaccharides fractionated were determined by combination of Bio-Gel P-4 column (50 cm long) chromatography and exoglycosidase digestion according to the methods described previously [22].

*2.3. Chemicals, enzymes and lectin*

RCA-I-agarose, peroxidase-conjugated RCA-I, diplococcal β-galactosidase, jack bean β-*N*-acetylhexosaminidase and jack bean α-mannosidase were obtained from Seikagaku Kogyo Co. (Tokyo). Con A-Sepharose and BCA protein assay kit were purchased from Pharmacia Fine Chemical Co. (Tokyo) and Pierce (Rockford, IL), respectively. *A. ureafaciens* sialidase, avidine-peroxidase, 4-chloro-1-naphthol and hydrogen peroxide were obtained from Nacalai Tesque Co. (Kyoto). $NaB^3H_4$ (490 mCi/mmol) was obtained from New England Nuclear (Boston, MA). AAL-Sepharose, biotinylated *P. velutina* lectin (PVL), and recombinant N-glycanase were gifts from Dr. N. Kochibe in Gunma University, Nichirei Co. (Tokyo), and Dr. T. Kaizu previously in Genzyme Japan Co. (Tokyo), respectively.

*2.4. Oligosaccharides*

Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ ($Gal_2$·$GlcNAc_2$·$Man_3$·GlcNAc·GlcNAc$_{OT}$) was prepared from recombinant human factor VIII, and its digestion products, $GlcNAc_2$·$Man_3$·GlcNAc·GlcNAc$_{OT}$, $Man_3$·GlcNAc·GlcNAc$_{OT}$ and Man·GlcNAc·GlcNAc$_{OT}$, were prepared by treatments with respective glycosidases [23]. Subscript OT indicates an $NaB^3H_4$-reduced oligosaccharide.

## 3. Results

*3.1. Lectin blot analysis of mouse serum glycoproteins*

In order to examine the expression of the Galβ1→4GlcNAc group on individual serum glycoproteins, the samples from β-1,4-GalT $I^{+/+}$ and β-1,4-GalT $I^{-/-}$ mice of 1, 6 and 8 weeks old were subjected to lectin blot analysis. Coomassie Brilliant Blue (CBB)-staining showed that numbers and intensities in the staining of protein bands are almost the same among the samples (Fig. 1, CBB), indicating that the serum components are not changed between normal and mutant mice and among their ages. After *A. ureafaciens* sialidase treatment of blots, they were incubated with RCA-I, which preferentially interacts with oligosaccharides terminated with the Galβ1→4GlcNAc group [24]. Many protein bands of β-1,4-GalT $I^{+/+}$ mouse samples reacted strongly with RCA-I (lanes A, B and C in Fig. 1, RCA-I), while only several protein bands had week reactivities towards the lectin in β-1,4-GalT $I^{-/-}$ mouse samples (lanes A′, B′ and C′ in Fig. 1, RCA-I). Upon treatment of blots with diplococcal β-galactosidase, which cleaves the Galβ1→4GlcNAc linkage but not the Galβ1→3GlcNAc linkage [25], prior to incubation with lectin, no RCA-I-reactive bands were obtained in all samples (the result from 8 weeks old β-1,4-GalT $I^{-/-}$ mouse sample is shown as a representative in lane C′-1 of Fig. 1, RCA-I). Blots were also treated with N-glycanase and then incubated with the lectin. No RCA-I-positive bands were also detected in all samples (the result from 8 weeks old β-1,4-GalT $I^{-/-}$ mouse sample is shown as a representative in lane C′-2 of Fig. 1, RCA-I). These results indicate that RCA-I-positive oligosaccharides contain terminal β-1,4-linked galactose residue(s) and that serum glycoproteins from β-1,4-GalT $I^{-/-}$ mice also contain N-linked oligosaccharides with β-1,4-linked galactose residues whose amounts are small. In order to confirm that ungalactosylated oligosaccharides are terminated with β-*N*-acetylglucosamine residues, lectin blot analysis was performed using PVL which interacts oligosaccharides terminated with β-*N*-acetylglucosamine [26]. The re-

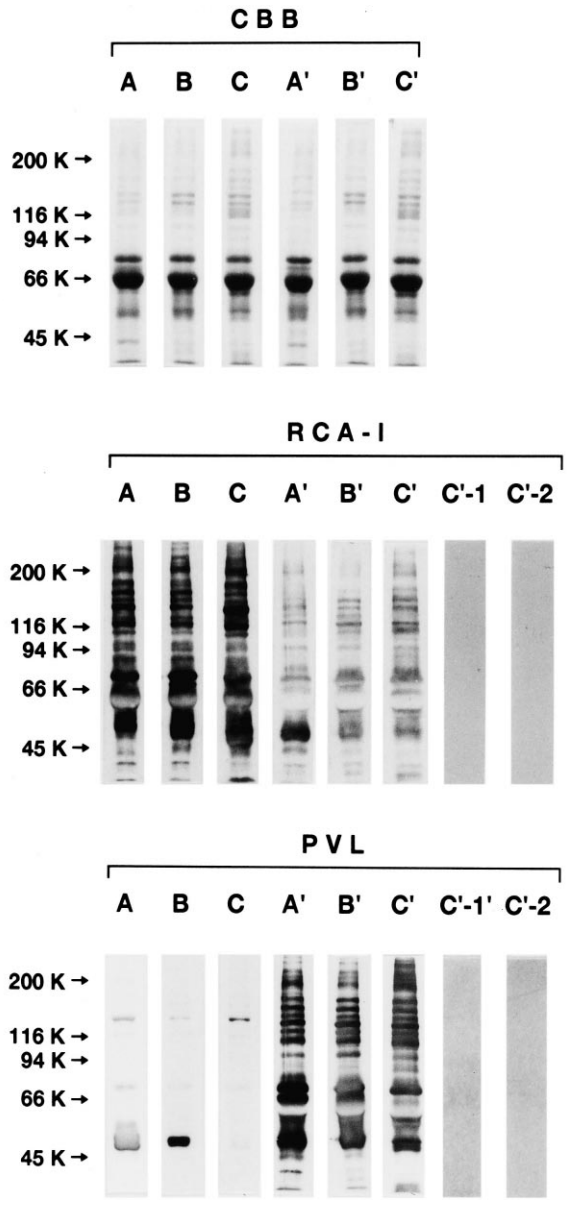


Fig. 1. Western blot analysis of mouse serum glycoproteins. The serum samples were from β-1,4-GalT $I^{+/+}$ (lanes A, B and C) and β-1,4-GalT $I^{-/-}$ (lanes A′, B′ and C′) mice of 1 week old (lanes A and A′), 6 weeks old (lanes B and B′) and 8 weeks old (lanes C and C′), respectively. CBB: filters were stained with Coomassie Brilliant Blue. RCA-I: filters were incubated with RCA-I before (lanes A, B, C, A′, B′ and C′) and after treatment with diplococcal β-galactosidase (lane C′-1) or N-glycanase (lane C′-2). PVL: filters were incubated with PVL before (lanes A, B, C, A′, B′ and C′) and after treatment with jack bean β-*N*-acetylhexosaminidase (lane C′-1′) or N-glycanase (lane C′-2).

Table 1
Relative amounts of neutral and desialylated neutral oligosaccharides fractionated by RCA-I-agarose column chromatography

| Fractions | Relative amounts (%) | |
|---|---|---|
| | Genotype +/+ | Genotype −/− |
| RCA-$I^-$ | 22.6 | 92.3 |
| RCA-$I^r$ | 10.4 | 4.2 |
| RCA-$I^+$ | 67.0 | 3.5 |

sults showed that strong lectin reactivities are observed towards most protein bands in β-1,4-GalT $I^{-/-}$ mouse samples (lanes A′, B′ and C′ in Fig. 1, PVL) in contrast to those from β-1,4-GalT $I^{+/+}$ mouse samples (lanes A, B and C in Fig. 1, PVL). Upon treatment of blots containing the mutant mouse samples with jack bean β-*N*-acetylhexosaminidase or N-glycanase, no lectin-reactive bands were obtained (the results from 8 weeks old β-1,4-GalT $I^{-/-}$ mouse sample are shown as representatives in lanes C′-1′ and C′-2, respectively, in Fig. 1, PVL). Since most lectin-positive bands disappeared when filters were treated with N-glycanase, lectin reactivities reflect the glycosylation status of N-linked oligosaccharides. Therefore, the results indicate that most complex-type oligosaccharides of serum glycoproteins from β-1,4-GalT $I^{-/-}$ mice are terminated with β-*N*-acetylglucosamine residues which are supposed to be sugar acceptors of β-1,4-GalT I.

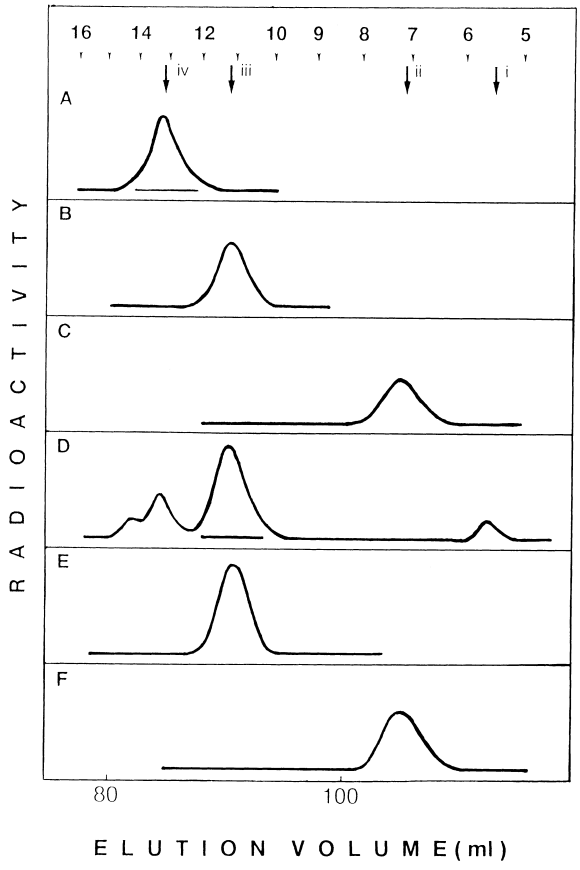


Fig. 2. Sequential exoglycosidase digestion of bi-antennary complex-type oligosaccharides. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction RCA-$I^{+}$·Con $A^{+}$·$AAL^{-}$ from β-1,4-GalT $I^{+/+}$ sample; (B) oligosaccharide in panel A digested with diplococcal β-galactosidase; (C) the peak in panel B digested with jack bean β-*N*-acetylhexosaminidase; (D) fraction RCA-$I^{-}$·$AAL^{-}$ from β-1,4-GalT $I^{-/-}$ sample after digestion with jack bean α-mannosidase; (E) oligosaccharide indicated by a bar in panel D digested with diplococcal β-galactosidase; (F) the peak in panel E digested with jack bean β-*N*-acetylhexosaminidase. The numbers at the top of the figure indicate glucose units, and the arrows i, ii, iii and iv indicate the elution positions of authentic oligosaccharides: i, Man·GlcNAc·$GlcNAc_{OT}$; ii, $Man_3$·GlcNAc·$GlcNAc_{OT}$; iii, $GlcNAc_2$·$Man_3$·GlcNAc·$GlcNAc_{OT}$; iv, $Gal_2$·$GlcNAc_2$·$Man_3$·GlcNAc·$GlcNAc_{OT}$.

Table 2
Protein concentrations in mouse sera

| Age (weeks) | Protein concentrations (mg/ml) | |
|---|---|---|
| | Genotype +/+ | Genotype −/− |
| 1 | 37.1 ± 2.18 | 33.4 ± 1.75 |
| 6 | 54.1 ± 2.87 | 50.9 ± 3.18 |
| 8 | 66.7 ± 3.19 | 59.7 ± 3.27 |

Values from three animals for each point are given as means ± standard deviations.

### 3.2. *Partial structures of N-linked oligosaccharides released from mouse serum glycoproteins*

N-linked oligosaccharides were released from serum glycoproteins of 6 weeks old β-1,4-GalT $I^{+/+}$ and β-1,4-GalT $I^{-/-}$ mice by hydrazinolysis, and the oligosaccharides were reduced with $NaB^3H_4$. Almost the same amounts of radioactivities were incorporated into two samples (data not shown), indicating that the same amounts of oligosaccharides are obtained from β-1,4-GalT $I^{+/+}$ and β-1,4-GalT $I^{-/-}$ mouse serum glycoprotein samples. After sialidase digestion, the radioactive oligosaccharides were applied to a DEAE-Sepharose column, and the pass-through fraction, in which more than 90% of the applied oligosaccharides were recovered, was subjected to RCA-I-agarose column chromatography. The pass-through fraction (RCA-$I^{-}$), the fraction that was retarded in the column (RCA-$I^{r}$) and the fraction that was bound and eluted from the column with 10 mM lactose (RCA-$I^{+}$) were obtained. The amounts of oligosaccharides in each fraction were calculated on the basis of the radioactivities in the fractions, and are summarized in Table 1. In the case of β-1,4-GalT $I^{+/+}$ mouse sample, 22.6, 10.4 and 67.0% of the oligosaccharides were separated into fractions RCA-$I^{-}$, RCA-$I^{r}$ and RCA-$I^{+}$, respectively. In contrast, 92.3, 4.2 and 3.5% of the oligosaccharides from β-1,4-GalT $I^{-/-}$ mouse sample were separated into fractions RCA-$I^{-}$, RCA-$I^{r}$ and RCA-$I^{+}$, respectively. Since oligosaccharides in fractions RCA-$I^{r}$ and RCA-$I^{+}$ are considered to have one and more than two β-1,4-linked galactose residues under the present condition respectively, about 90% of the previously galactosylated N-linked oligosaccharides of mouse serum glycoproteins were ungalactosylated by ablation of mouse β-1,4-GalT I gene. In order to confirm this further, bi-antennary complex-type oligosaccharides, most of which were recovered in fraction RCA-$I^{+}$ in the case of β-1,4-GalT $I^{+/+}$ mouse and in fraction RCA-$I^{-}$ in the case of β-1,4-GalT $I^{-/-}$ mouse were analyzed by Bio-Gel P-4 column chromatography. Oligosaccharides in fraction RCA-$I^{+}$ from β-1,4-GalT $I^{+/+}$ mouse sample were subjected to Con A-Sepharose column chromatography, and fractions Con $A^{-}$, Con $A^{+}$ and Con $A^{++}$ were obtained by elution with buffer only and buffer with 5 mM α-methyl-glucoside and 100 mM α-methyl-mannoside, respectively. Oligosaccharides in fraction RCA-$I^{+}$·Con $A^{+}$ were then separated into those with ($AAL^{+}$) and without ($AAL^{-}$) a fucose residue attached to the proximal *N*-acetylglucosamine by AAL-Sepharose column chromatography [22]. When fraction RCA-$I^{+}$·Con $A^{+}$·$AAL^{-}$ (39.6% of the total oligosaccharides) was applied to a Bio-Gel P-4 column, a single peak was eluted at the same position as authentic $Gal_2$·$GlcNAc_2$·$Man_3$·GlcNAc·$GlcNAc_{OT}$ (Fig. 2A). The peak in Fig. 2A was digested with diplococcal β-galactosidase, and the product was eluted at the same position as authentic $GlcNAc_2$·$Man_3$·GlcNAc·$GlcNAc_{OT}$, releasing two galactose residues (Fig.

2B). Upon digestion of the product in Fig. 2B with jack bean β-*N*-acetylhexosaminidase, the product was eluted at the same position as authentic $Man_3$·GlcNAc·$GlcNAc_{OT}$, releasing two *N*-acetylglucosamine residues (Fig. 2C). Oligosaccharides in fraction RCA-$I^-$ from β-1,4-GalT $I^{-/-}$ mouse sample were separated by AAL-Sepharose column chromatography, and those in fraction RCA-$I^-$·$AAL^-$ was digested with jack bean α-mannosidase to convert high mannose-type oligosaccharides into Man·GlcNAc·$GlcNAc_{OT}$ (5.5 glucose units) for separating them from complex-type oligosaccharides on a Bio-Gel P-4 column. When they were applied to the column, four peaks with effective sizes of 14.2, 13.2, 11.2 and 5.5 glucose units were obtained (Fig. 2D). The peak indicated by a bar in Fig. 2D (51.6% of the total oligosaccharides) was digested with diplococcal β-galactosidase, and the product was eluted at the same position as one in Fig. 2D and authentic $GlcNAc_2$·$Man_3$·GlcNAc·$GlcNAc_{OT}$, indicating that no galactose residues are released (Fig. 2E). Upon digestion of the product in Fig. 2E with jack bean β-*N*-acetylhexosaminidase, the product was eluted at the same position as authentic $Man_3$·GlcNAc·$GlcNAc_{OT}$, releasing two *N*-acetylglucosamine residues (Fig. 2F). When peaks with effective sizes of 14.2 and 13.2 glucose units were digested separately with jack bean β-*N*-acetylhexosaminidase, the products were eluted at the same position as authentic $Man_3$·GlcNAc·$GlcNAc_{OT}$, releasing three and four *N*-acetylglucosamine residues, respectively (data not shown). That the products with an effective size of 7.2 glucose units in Fig. 2C and F have the structure Man-$\alpha 1 \rightarrow 6(Man\alpha 1 \rightarrow 3)Man\beta 1 \rightarrow 4GlcNAc\beta 1 \rightarrow 4GlcNAc_{OT}$ was confirmed by the methods described earlier [22]. These results indicate that bi-antennary complex-type oligosaccharides in fraction RCA-$I^+$·Con $A^+$·$AAL^-$ from β-1,4-GalT $I^{+/+}$ mouse sample are fully galactosylated while those in fraction RCA-$I^-$·-$AAL^-$ from β-1,4-GalT $I^{-/-}$ mouse sample are totally ungalactosylated. Similar results were obtained by analyzing bi-antennary complex-type oligosaccharides in fraction RCA-$I^+$·Con $A^+$·$AAL^+$ from β-1,4-GalT $I^{+/+}$ mouse sample and fraction RCA-$I^-$·$AAL^+$ from β-1,4-GalT $I^{-/-}$ mouse sample (data not shown). When oligosaccharides in fraction RCA-$I^r$ from β-1,4-GalT $I^{+/+}$ mouse sample and those in fractions RCA-$I^r$ and RCA-$I^+$ from β-1,4-GalT $I^{-/-}$ mouse sample were digested with diplococcal β-galactosidase, most of the oligosaccharides were passed through an RCA-I-agarose column (data not shown), indicating that these oligosaccharides contain β-1,4-linked galactose residues and that 7.7% of the N-linked oligosaccharides of β-1,4-GalT $I^{-/-}$ mouse serum glycoproteins still contain β-1,4-linked galactose residues.

### *3.3. Determination of mouse serum protein concentrations*

The results described above showed that most serum glycoproteins of β-1,4-GalT $I^{-/-}$ mice are extremely undergalactosylated. If the galactose residues are really important for clearance of serum glycoproteins, it is expected that sera from β-1,4-GalT $I^{-/-}$ mice should contain higher concentrations of proteins due to the defect in the turnover of proteins. The serum samples obtained from β-1,4-GalT $I^{+/+}$ and β-1,4-GalT $I^{-/-}$ mice were diluted with water, and protein concentrations were determined by the BCA method [19]. As shown in Table 2, no significant difference but a slight decrease in protein concentrations was found in β-1,4-GalT $I^{-/-}$ mouse serum samples when compared to those of the age-matched β-1,4-GalT $I^{+/+}$ mice. However, there were age-related differences in the protein concentrations of both β-1,4-GalT $I^{+/+}$ and β-1,4-GalT $I^{-/-}$ mice. The protein concentrations appeared to increase with the growth of animals. The results are also quite consistent with those obtained by lectin blot analysis in which the CBB-staining of serum protein bands was almost similar between β-1,4-GalT $I^{+/+}$ and β-1,4-GalT $I^{-/-}$ mice of the same ages as shown in Fig. 1. These results indicate that no specific proteins are selectively eliminated from the circulation in β-1,4-GalT $I^{-/-}$ mice.

## 4. Discussion

It is known that elimination of serum glycoproteins from the circulation is mediated by ASGP-R which recognizes the terminal galactose residues of the glycoproteins after desialylation most probably with sialidase [27]. Since the receptor has higher affinity binding to the 2,4-branched tri-antennary complex-type oligosaccharides with β-1,4-linked galactose residues [14,15], animals without expressing the galactose residues could not survive due to higher viscosity of blood by the accumulated proteins. However, β-1,4-GalT I-knockout mice can survive up to the birth without problems in the circulation [18,28]. The present study revealed that serum glycoproteins from β-1,4-GalT I-knockout mice of three different ages lack most β-1,4-linked galactose residues. The partial structural analysis of N-linked oligosaccharides, however, revealed that 10% of the previously galactosylated oligosaccharides still contain β-1,4-linked galactose residues which are formed most probably by some of novel β-1,4-GalTs II, III, IV and V recently discovered (reviewed in [29]). Nevertheless, the serum protein concentrations in the mutant mice were shown to be in the similar levels to those of the normal mice although the age-dependent differences were found in the protein concentrations. This indicates strongly that the terminal β-1,4-linked galactose residues are not directly involved in the clearance of endogenous serum glycoproteins from the circulation. In support of this, it has been reported that serum concentrations of the galactose-terminated glycoproteins are not elevated in the ASGP-R-deficient mice [30,31]. Furthermore, no apparent charge differences were observed in human γ-glutamyltransferase with differently sialylated forms purified between rat liver and rat serum after injection, indicating that no prior desialylation is required for elimination of serum glycoproteins from the circulation [32]. Determination of serum protein concentrations showed that a slight decrease in the protein concentrations is associated with β-1,4-GalT $I^{-/-}$ mice, which could be due to the inefficient secretion of these glycoproteins by the defective glycosylation since it has been shown that inhibition of the glycosylation disturbs protein secretion [33]. These results indicate that the clearance of serum glycoproteins is regulated by some mechanism(s) other than the ASGP-R-mediated uptake. Nevertheless, specific degradation of $^{125}I$-labeled asialo-$\alpha_1$ acid glycoprotein by primary cultured mouse hepatocytes [31] indicates that the ASGP-R-mediated clearance mechanism is effective for eliminating the glycoproteins whose galactose residues are exposed under some unphysiological conditions such as virus/bacteria infection [27] probably because the exposed sugar residues could be harmful to the vascular system [34].

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