Presence of A Higher Molecular Weight β -1,4-Galactosyltransferase in Mouse Liver

Masahiro Kido^{1,2}, Masahide Asano³, Yoichiro Iwakura³, Masao Ichinose², Kazumasa Miki⁴ and Kiyoshi Furukawa¹

¹Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173–0015,

²Department of Gastroenterology, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113–8655,

³Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo

108–0071 and ⁴First Department of Internal Medicine, Toho University School of Medicine, Ota-ku, Tokyo 143–8541

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Histochemical analyses of β -1,4-galactosyltransferase (β -1,4-GalT) I-knockout mouse liver using anti-bovine β -1,4-GalT I antibody and *Ricinus communis* agglutinin (RCA)-I which interacts with oligosaccharides terminated with β -1,4-linked galactose revealed that weak fluorescence derived from the lectin is present in the mutant mouse in contrast to the disappearance of the immunofluorescence. Lectin blot analysis of membrane glycoproteins showed that many protein bands react with RCA-I although their reactivities are weak in the mutant mouse liver. Upon pretreatment of blots with β -

1,4-galactosidase or N-glycanase, no lectin binding was observed, indicating that a part of N-linked oligosaccharides of β -1,4-GalT I-knockout mouse liver is galactosylated by yet unidentified β -1,4-GalT. Gel filtration column chromatography of solubilized mouse liver transferases revealed that β -1,4-GalT activities in wild-type mouse are detected in fractions 42 and 50 while those in the mutant mouse are detected only in fraction 42. The results indicate that mouse liver contains a high molecular weight β -1,4-GalT (110 K) in addition to a previously known β -1,4-GalT (46 K).

Key words: β -1,4-galactosyltransferases, High molecular weight, N-linked oligosaccharides, Mouse liver

I. Introduction

A variety of carbohydrate antigens including sialyl Lewis X, Lewis X, ABO blood group determinants, I and i antigens, polysialic acid and HNK-1 carbohydrate are expressed on the Gal β 1 $\rightarrow$ 4GlcNAc group of N-linked oligosaccharides, and some of them are involved in cellular interactions as ligands [reviewed in 9]. For many years β -1,4-galactosyltransferase (β -1,4-GalT) which forms the Gal β 1 $\rightarrow$ 4GlcNAc structure was considered to be a single gene product. However, at least two types of β -1,4-GalT differing in binding to α -lactalbumin and in affinity towards acceptor *N*-acetylglucosamine were reported to occur in porcine trachea, BHK cells and human B cells [10,

12, 28]. In addition, the occurrence of high molecular weight species of β -1,4-GalT was reported in human tumor effusion fluids [19, 30]. These reports suggest that mammalian cells contain multiple β -1,4-GalTs.

In order to isolate novel genes which encode proteins with β -1,4-GalT activities, we screened human cDNA libraries with human β -1,4-GalT cDNA as a probe. However, no clone was obtained by such a method. In 1994, the gene encoding β -1,4-*N*-acetylglucosaminyltransferase which transfers *N*-acetylglucosamine from UDP-GlcNAc to *N*-acetylglucosamine, thus forming the GlcNAc β 1 $\rightarrow$ 4GlcNAc group, was isolated from a snail prostate gland cDNA library [6]. Since this transferase

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Correspondence to: Dr. Kiyoshi Furukawa, Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173–0015, Japan.

Abbreviations: CBB, Coomassie Brilliant Blue; FITC, fluorescein isothiocyanate; Gal, galactose; β -1,4-GalT, β -1,4-galactosyltransferase; GlcNAc, *N*-acetylglucosamine; GlcNAc β -S-*p*NP, *p*-nitrophenyl-*N*-acetyl-1-thio- β -D-glucosamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; PVL, *Psathyrella velutina* lectin; RCA-I, *Ricinus communis* agglutinin-I; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

and mammalian β -1,4-GalTs share the same acceptor molecules and contain the same decapeptide, Y(W/F)GWGGEDDD, in their C-terminal regions, the peptide was considered to be a part of the acceptor recognition domain [reviewed in 9] and should be included also in novel β -1,4-GalTs if present. Using the degenerated primers as prepared based on the decapeptide sequence and mRNAs from a human breast tumor cell line, we obtained several PCR products which were not included in the existing β -1,4-GalT gene and finally isolated a novel β -1,4-GalT clone with 4.7 kb by screening the cDNA library [25, 26]. After this finding, several groups isolated five more human genes which encode proteins with β -1,4-GalT activities [1, 2, 15, 17, 18, 27]. They are now named β -1,4-GalTs II, III, IV, V, VI and VII according to the homology distances (55 to 25%) closer to the previously known β -1,4-GalT which is referred to as β -1,4-GalT I [reviewed in 3, 9]. Based on the amino acid residues composed, their molecular weights are estimated to be within a range from 30 K to 50 K [3, 9]. The present study shows that mouse liver contain two species of β -1,4-GalT with molecular weights of 46 K and 110 K, the former of which disappeared in β -1,4-GalT I-knockout mouse liver, indicating that the 110 K β -1,4-GalT is not a dimeric form of β -1,4-GalT I and could be an entirely new enzyme.

II. Materials and Methods

Chemicals

Fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (H+L) and FITC-labeled RCA-I were purchased from Jackson Immuno Research Laboratories Inc. (West Grove, PA) and Honen Oil Co. (Tokyo), respectively. Peroxidase-labeled RCA-I, diplococcal β -galactosidase, jack bean β -N-acetylhexosaminidase, recombinant N-glycanase were obtained from Seikagaku Kogyo Co. (Tokyo). Unlabeled UDP-Gal and *p*-nitrophenyl-N-acetyl-1-thio- β -D-glucosamine (GlcNAc β -S-pNP), and UDP-[³H]Gal (48.8 Ci/mmol) were purchased from Sigma Chemical Co. (St. Louis, MO) and New England Nuclear (Boston, MA), respectively. Peroxidase-labeled streptavidine, 4-chloro-1-naphthol and hydrogen peroxide were obtained from Nacalai Tesque Co. (Kyoto). Sephacryl S-200 HR and Sep-Pak C₁₈ cartridges were obtained from Amersham Pharmacia Biotech Ltd. (Uppsala, Sweden) and Waters (Milford, MA), respectively. Rabbit anti-bovine milk β -1,4-GalT (β -1,4-GalT I) polyclonal antibody and biotinylated *Psathyrella velutina* lectin (PVL) were kindly supplied by Dr. J. H. Shaper in Johns Hopkins University at Baltimore (USA) and by Dr. N. Kochibe in Gunma University (Japan), respectively.

Histochemical stainings for light microscopy

Mouse liver tissues were fixed in 4% formaldehyde in 10 mM phosphate-buffered saline (pH 7.2) and embedded in O. C. T. compound. Cryosections with 10 μ m thickness

were incubated with rabbit anti-bovine β -1,4-GalT I polyclonal antibody which was kindly provided by Dr. J. H. Shaper in Johns Hopkins University, Baltimore, and then with FITC-labeled goat anti-rabbit IgG (H+L), or with FITC-labeled RCA-I at 4°C according to the method described previously [13]. The sections were mounted in 90% glycerol-0.1 M Tris-HCl buffer (pH 8.5) and examined under a fluorescent microscope

Lectin blot analysis

Membrane glycoprotein samples were prepared from livers of wild-type and β -1,4-GalT I-knockout mice as raised previously [4]. Samples were subjected to SDS-polyacrylamide (7.5%) gel electrophoresis (SDS-PAGE) and transferred to polyvinylidenedifluoride membrane. 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. Lectin blot analysis using peroxidase-labeled RCA-I or biotinylated PVL and peroxidase-labeled streptavidine was performed, and lectin-positive bands were visualized by incubation with chromogenic peroxidase substrate, 4-chloro-1-naphthol, by the method described previously [23]. In some experiments, BSA-blocked blots were treated with 200 munits of diplococcal β -galactosidase in 150 μ l of 0.3 M citrate phosphate buffer (pH 6.0), 2.5 units of jack bean β -N-acetylhexosaminidase in 150 μ l of 0.3 M citrate phosphate buffer (pH 6.0) or 2.5 units of N-glycanase in 150 μ l of 0.1 M phosphate buffer (pH 8.2) prior to incubation with lectin.

Gel filtration column chromatography

Mouse livers were homogenized in 10 mM Tris-HCl buffer (pH 7.6) containing 0.25 M sucrose and 1 mM EDTA, and the homogenates were spun down at 8,000 $\times$ g for 20 min at 4°C. The resultant supernatant was centrifuged at 105,000 $\times$ g for 60 min, and the pellets were solubilized in 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 7.0) containing 1% Triton X-100 for 18 hr at 4°C as described previously [8]. The solution was centrifuged again at 105,000 $\times$ g for 60 min at 4°C, and the supernatant which contains solubilized β -1,4-GalTs was applied to a Sephacryl S-200 HR column (1.5 cm $\times$ 28 cm) equilibrated with 10 mM MES buffer (pH 7.0) containing 0.15 M NaCl and 0.1% Triton X-100, and each 0.5 ml was fractionated.

Assay of β -1,4-GalT activities

The transferase assay was performed by the method described previously [26]. In brief, the reaction mixture contained 10 mM MES buffer (pH 7.0) containing 4 mM 5'-AMP, 250 μ M UDP-[³H]Gal, 1 mM GlcNAc β -S-pNP, 20 mM MnCl₂ and enzyme preparation in a total volume of 50 μ l. After incubation the product was isolated by using Sep-Pak C₁₈ cartridge and the radioactivity incorporated into the product was determined.

III. Results and Discussion

In animal cells β -1,4-GalT I is present in the Golgi apparatus where it is involved in the galactosylation of glycoproteins. In order to confirm that the enzyme is not expressed in β -1,4-GalT I-knockout mouse, the cryosec-

tions prepared from the mouse livers were incubated with anti-bovine β -1,4-GalT I antibody. Immunofluorescence microscopic observation showed that FITC-labeled spots are present at supranuclear regions of wild-type mouse hepatocytes (panel a in Fig. 1) which are considered to be Golgi apparatus, while no spots are detectable in β -1,4-

Fig. 1. Staining of mouse liver cryosections with anti-bovine β -1,4-GalT I antibody (panels a and b) or with RCA-I (panels c and d). The sections were prepared from wild-type (panels a and c) and β -1,4-GalT I-knockout (panels b and d) mice. Magnifications: $\times 300$ in panels a and b; $\times 200$ in panels c and d.

GalT I-knockout mouse hepatocytes (panel b in Fig. 1), indicating that β -1,4-GalT I is not expressed in the mutant mouse. Since β -1,4-GalT I is involved in the galactosylation of N-linked oligosaccharides which are rich in cell surface glycoproteins, the cryosections from mouse livers were incubated with FITC-labeled RCA-I which preferentially interacts with oligosaccharides terminated with the Gal β 1 $\rightarrow$ 4GlcNAc group [5]. The strong fluorescence was present mainly on hepatocyte cell surfaces and inner surfaces of blood vessels in wild-type mouse liver (panel c in Fig. 1), while even in β -1,4-GalT I-knockout mouse liver the weak fluorescence remained on the cell surfaces of hepatocytes and blood vessels including vein (panel d in Fig. 1), indicating that the Gal β 1 $\rightarrow$ 4GlcNAc structure is still expressed in the mutant mouse liver.

Since cell surface glycoconjugates consist of not only glycoproteins but also glycolipids which also carry oligosaccharides with the Gal β 1 $\rightarrow$ 4GlcNAc structure, the RCA-I-positive staining detected in panels c and d of Fig. 1 could be due to glycolipids rather than glycoproteins. To address this issue, membrane glycoprotein samples were prepared from mouse livers and subjected to lectin blot analysis. CBB-staining showed that numbers and intensities in the staining of protein bands are almost the same between wild-type and mutant mice (CBB in Fig. 2), indicating that protein components are not changed significantly in the mutant mouse. Although the Gal β 1 $\rightarrow$ 4GlcNAc groups found in cell surface glycoproteins are sialylated, blots were incubated with peroxidase-conjugated RCA-I without desialylation. Many protein bands in wild-type mouse liver reacted strongly with RCA-I (lane C in Fig. 2) and those of the mutant mouse liver also reacted with the lectin though

weakly (lane D in Fig. 2). Upon treatment of blots with diplococcal β -galactosidase, which cleaves the Gal β 1 $\rightarrow$ 4GlcNAc linkage but not the Gal β 1 $\rightarrow$ 3GlcNAc linkage [11], or N-glycanase prior to incubation with lectin, no RCA-I-reactive bands were obtained in all the samples (the results from β -1,4-GalT I-knockout mouse were shown as representatives in lanes D-1 and D-2, respectively, in Fig. 2). These results indicate that RCA-I-positive bands contain N-linked oligosaccharides terminated with β -1,4-linked galactose residues and that β -1,4-GalT I-knockout mouse liver contain β -1,4-galactosylated N-linked oligosaccharides. In order to confirm that the ungalactosylated oligosaccharides are terminated with β -N-acetylglucosamine residues, lectin blot analysis was performed using PVL which interacts with oligosaccharides terminated with β -N-acetylglucosamine [7]. The results showed that strong lectin reactivity is observed towards many protein bands in β -1,4-GalT I-knockout mouse liver (lane F in Fig. 2) in contrast to several lectin-positive bands in wild-type mouse liver (lane E in Fig. 2). Upon treatment of blots with jack bean β -N-acetylhexosaminidase or N-glycanase, no lectin-reactive bands were obtained (the results from the mutant mouse were shown as representatives in lanes F-1 and F-2, respectively, in Fig. 2). These results indicate that a part or all of RCA-I-derived fluorescence in β -1,4-GalT I-knockout mouse shown in Fig. 1d is due to the presence of the Gal β 1 $\rightarrow$ 4GlcNAc structure in N-linked oligosaccharides. The presence of the residual disaccharide groups in N-linked oligosaccharides was also observed in several other tissues including the brain in the mutant mouse [14].

In order to detect β -1,4-GalT activity responsible for creating the Gal β 1 $\rightarrow$ 4GlcNAc structure on N-linked

Fig. 2. Lectin blot analysis of membrane glycoprotein samples from wild-type (lanes A, C and E) and β -1,4-GalT I-knockout (lanes B, D and F) mice. Blots were incubated with CBB for detecting proteins, RCA-I for detecting β -1,4-linked galactose-terminated oligosaccharides, or PVL for detecting β -N-acetylglucosamine-terminated oligosaccharides. Lanes D-1 and F-1 indicate that blots were treated with diplococcal β -galactosidase and jack bean β -N-acetylhexosaminidase, respectively, prior to incubation with lectin. Lanes D-2 and F-2 indicate that blots were treated with N-glycanase prior to incubation with lectin.

oligosaccharides in β -1,4-GalT I-knockout mouse liver, detergent-solubilized transferases from wild-type and mutant mouse livers were subjected to Sephacryl S-200 HR gel filtration column chromatography. β -1,4-GalT activities towards 1 mM GlcNAc β -S-*p*NP were detected in fractions 42 (K_{av} =0.17) and 50 (K_{av} =0.29) in wild-type mouse while the activity in the mutant mouse was detected only in fraction 42 (Fig. 3), indicating that the activity in fraction 50 is solely due to β -1,4-GalT I whose approximate molecular weight is 46 K which is quite consistent with those reported previously [reviewed in 20], and that mouse liver contains another β -1,4-GalT with an approximate molecular weight of 110 K.

Recently, the human genome has been shown to contain multiple genes which encode β -1,4-GalTs II, III, IV, V, VI and VII [1, 2, 15, 17, 18, 25–27] as shown in Fig. 4. Preliminary substrate-specificity studies suggested that β -1,4-GalTs III, IV and VI are involved in the biosynthesis of glycolipids [2, 17, 27] while β -1,4-GalTs II and V are involved in the biosynthesis of N-linked oligosaccharides [2, 25, 26]. In addition, β -1,4-GalT V has been shown to be able to galactosylate O-linked oligosaccharides with core 2 structure and glucosylceramide [22, 31]. The β -1,4-GalT VII cannot galactosylate *N*-acetylglucosamine but can transfer galactose to xylose attached to Ser/Thr of proteoglycan core peptides [1, 18]. In human liver, only the β -1,4-GalT V gene is expressed in addition to the β -1,4-GalT I gene [15, 26]. Similarly, the β -1,4-GalT I and V genes but not the β -1,4-GalT II gene are expressed in mouse liver [Nakamura *et al.*, manuscript submitted]. Therefore, β -1,4-GalT V is a candidate which is involved in the galactosylation of glycoproteins in β -1,4-GalT I-knockout mouse liver. Since molecular weights of all six novel β -1,4-GalTs

Fig. 3. Gel filtration column chromatography of mouse liver β -1,4-GalTs. The solubilized transferases from wild-type (open circle) and β -1,4-GalT I-knockout (closed circle) mice were applied to a Sephacryl S-200 HR column and the transferase activities in each fraction were assayed. K_{av} values were estimated by the elution positions on the column which was calibrated with glycoproteins with known molecular weights: a, IgG (160 K); b, transferrin (76 K) and c, bovine serum albumin (67 K). V_0 and V_t indicate the void and total volumes of the column, respectively.

including β -1,4-GalT V are estimated to be not more than 60 K based on the numbers of amino acid residues composed of individual proteins as shown in Fig. 4, β -1,4-GalT with 110 K could be a dimeric form of

Fig. 4. Schematic illustration of human β -1,4-GalT proteins. In each transferase, the closed box indicates a putative transmembrane domain, and other boxes indicate amino acid sequences conserved among seven h β -1,4-GalTs: a, FNRA; b, NVG; c, DVD and d, WGWGG/REDD/E, respectively. Wedges with numbers indicate the positions of Cys residues conserved among six h β -1,4-GalTs. The total number of composing amino acid residues in each transferase is shown at the right side.

β -1,4-GalT V or an entirely different protein. However, when a membrane-bound form of human β -1,4-GalT V was expressed in Sf-9 cells [24], it migrated to one with a molecular weight of 45 K as revealed by SDS-PAGE (unpublished data). Failure to detect the activity in fractions which contain 45 K protein in the mutant mouse by gel filtration column chromatography could be due to the lower concentration of acceptor GlcNAc β -S-pNP used for assay since β -1,4-GalT V has a higher K_m value towards GlcNAc β -S-pNP [21]. Higher molecular weight species of β -1,4-GalT were detected in tumor effusion fluids of human lung cancer and ovarian carcinoma [19, 30]. In the case of the ovarian carcinoma, higher molecular weight species of the transferase were considered to be oligomers of the soluble form of β -1,4-GalT I [29]. Since β -1,4-GalT with 110 K is present in β -1,4-GalT I-knockout mouse liver, it should be totally different from β -1,4-GalT I. Further studies are necessary to determine the substrate specificity of this higher molecular weight β -1,4-GalT or its contribution to the development and growth of mouse embryos since no significant histological changes were observed in most tissues including liver of β -1,4-GalT I-knockout mouse though they show growth retardation and die after birth [4, 16].

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V. References

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