Galactosylation of N-linked oligosaccharides by human β-1,4-galactosyltransferases I, II, III, IV, V, and VI expressed in SF-9 cells

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Several studies showed that SF-9 cells can synthesize the galactosylated N-linked oligosaccharides if β-1,4-galactosyltransferase (β-1,4-GalT) is supplied. The full-length human β-1,4-GalT I, II, III, IV, V, and VI cDNAs were independently transfected into SF-9 cells, and the galactosylation of endogenous membrane glycoproteins was examined by lectin blot analysis using Ricinus communis agglutinin-I (RCA-I), which preferentially interacts with oligosaccharides terminated with Galβ1→4GlcNAc group. Several RCA-I-reactive bands appeared in all of the gene-transfected cells, and disappeared on treatment of blots with β-1,4-galactosidase or N-glycanase prior to incubation with lectin. Introduction of the antisense β-1,4-GalT II and V cDNAs separately into human colorectal adenocarcinoma SW480 cells, in which β-1,4-GalT I, II, and V genes were expressed, resulted in the reduction of RCA-I binding toward N-linked oligosaccharides of the membrane glycoproteins. Differences were found in their \( K_\text{m} \) values toward UDP-Gal and GlcNAcβ-S-pNP and in their acceptor specificities toward oligosaccharides with the GlcNAcβ1→4(GlcNAcβ1→2)Man branch and with the GlcNAcβ1→6(GlcNAcβ1→2)Man branch. These results indicate that β-1,4-GalTs II, III, IV, V, and VI are involved in the N-linked oligosaccharide biosynthesis cooperatively but not in a redundant manner with β-1,4-GalT I within cells.

**Key words:** acceptor specificity/\( \text{in vivo} \) β-1,4-galactosylation/\( K_\text{m} \) value/N-glycan

**Introduction**

Several classes of glycosyltransferases have been found to consist of homologous gene families. In the case of β-1,4-galactosyltransferase (β-1,4-GalT) which transfers galactose from UDP-Gal to N-acetylglucosamine and N-acetylglucosamine-terminated oligosaccharides, seven members designated as β-1,4-GalTs I–VII form a gene family (reviewed in Amado et al., 1999; Furukawa and Sato, 1999). Studies of \( \text{in vitro} \) acceptor specificities showed that β-1,4-GalTs II and V galactosylate N-acetylglucosamine terminated on N-linked oligosaccharides (Almeida et al., 1997; Sato et al., 1998b); β-1,4-GalTs IV and V galactosylate that on O-linked oligosaccharides (Ujita et al., 1998; van Die et al., 1999); β-1,4-GalTs III, IV, V, and VI galactosylate N-acetylglucosamine or glucose on glycolipids (Almeida et al., 1997; Nomura et al., 1998; Schwientek et al., 1998, Takizawa et al., 1999; Sato et al., 2000a); and β-1,4-GalT VII, which fails to galactosylate N-acetylglucosamine but galactosylates xylose attached to proteoglycan core proteins (Almeida et al., 1999; Okajima et al., 1999). However, these studies were accomplished with using either a soluble or membrane-bound form of each β-1,4-GalT and different assay conditions, and, therefore, their properties, including acceptor specificities, may not be compared directly among them.

Because the Galβ1→4GlcNAc structure on N-linked oligosaccharides from several tissues of β-1,4-GalT I-knockout mouse was detected (Asano et al., 1997; Kido et al., 1998, 2000), some of β-1,4-GalTs II–VI could be really involved in the galactosylation of N-linked oligosaccharides \( \text{in vivo} \). To examine which of β-1,4-GalTs beside β-1,4-GalT I are involved in N-linked oligosaccharide biosynthesis \( \text{in vivo} \), individual human β-1,4-GalT cDNAs were introduced into SF-9 cells, which appeared to lack β-1,4-GalT in the Golgi apparatus and the acceptor oligosaccharides in the endogenous glycoproteins (Hollister et al., 1998; Yoshimi et al., 2000), and the galactosylation of membrane glycoproteins were examined by lectin blot analysis using RCA-I, which preferentially interacts with oligosaccharides terminated with Galβ1→4GlcNAc group (Baenziger and Fiete, 1979). To establish this further, antisense β-1,4-GalT cDNAs were introduced into human colorectal adenocarcinoma SW480 cells and the galactosylation of membrane glycoproteins was examined by lectin blot analysis. The present study showed that β-1,4-GalTs II, III, IV, V, and VI can galactosylate N-acetylglucosamine terminated on N-linked oligosaccharides.

**Results**

Expression of β-1,4-GalT proteins in SF-9 cells

Because β-1,4-GalT II, III and IV transcripts were expressed in human erythroleukemic K562 cells (Sato et al., 2000b), their full-length cDNAs were isolated from K562 cells by reverse-transcription polymerase chain reaction (PCR) using oligonucleotide primers (shown in Materials and methods) specific
to the human β-1,4-GalT II and III, and IV cDNAs already cloned by Almeida et al. (1997) and by Schwientek et al. (1998), respectively, and sequenced. The nucleotide sequences of human K562 β-1,4-GalTs III and IV match with those reported previously (Almeida et al., 1997; Schwientek et al., 1998) was confirmed. However, the nucleotide sequence of human K562 β-1,4-GalT II showed a difference in one position at 909 to that reported previously (Almeida et al., 1997) but made no change in the amino acid sequence.

To determine the nature of sugar-acceptor molecules of β-1,4-GalTs II-VI in vivo, the human β-1,4-GalT I to VI cDNAs were independently cloned into a pVL1393 vector, and the resultant plasmids were separately transfected into SF-9 cells. As described already, a very weak β-1,4-GalT activity toward p-nitrophenyl-β-N-acetyl-1-thio-β-D-glucosaminide (GlcNAcβ-S-pNP) was detected in the homogenates from untransfected SF-9 cells, which was slightly increased on transfection of a vector (less than 0.1 pmol galactose-transferred/ min-mg protein) as described previously (Sato et al., 1997), indicating that galactose is transferred to the acceptor recovered in the pass-through fraction of the column (data not shown), which was expected that Sf-9 cells provide an useful cell line to investigate the in vivo acceptor specificities of β-1,4-GalTs II, III, IV, V, and VI.

Table I. Kinetic parameters of recombinant human β-1,4-GalTs

<table>
<thead>
<tr>
<th>Transferrases</th>
<th>UDP-Gal K_m (μM)</th>
<th>GlcNAcβ-S-pNP</th>
<th>K_m (μM)</th>
<th>V_max (nmol/min-mg protein)</th>
<th>Kinetic efficiency (V_max/K_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-1,4-GalT I</td>
<td>47</td>
<td>81</td>
<td>8.3</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>β-1,4-GalT II</td>
<td>58</td>
<td>71</td>
<td>8.2</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>β-1,4-GalT III</td>
<td>95</td>
<td>63</td>
<td>3.5</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>β-1,4-GalT IV</td>
<td>105</td>
<td>238</td>
<td>6.5</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>β-1,4-GalT V</td>
<td>42</td>
<td>128</td>
<td>4.0</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>β-1,4-GalT VI</td>
<td>79</td>
<td>152</td>
<td>1.7</td>
<td>0.01</td>
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</table>

Values are means of three independent experiments. Specific activities of individual β-1,4-GalTs expressed in SF-9 cells by the experiments were 0.43–1.11 nmol galactose transferred/ min-mg protein.

It was shown that N-linked oligosaccharides from lepidopteran SF-9 cells are mainly of high mannose-type (Kubelka et al., 1994; Marchal et al., 1999). However, our lectin blot analysis of SF-9 cell membrane glycoprotein samples revealed the presence of several protein bands reacted to *Psathyrella velatina* lectin (PVL) (Yoshimi et al., 2000), which interacts with oligosaccharides terminated with β-N-acetylgalosaminosylamine (Endo et al., 1992). Because PVL-positive bands disappeared on treatment of blots with N-glycanase, it was expected that SF-9 cells contain complex- and/or hybrid-type oligosaccharides terminated with N-acetylgalosaminosylamine in addition to high mannose-type ones. Detailed structural analysis of N-linked oligosaccharides released by hydrazinolysis from SF-9 cell membrane glycoprotein samples revealed that about 7% of the total oligosaccharides are of hybrid-type with the (Manβ1→3)Manβ1→6(3)GlcNAcβ1→Manβ1→3(6)Manβ1→GlcNAcβ1→GlcNAc structure and monoaenomannose complex-type with the [Manβ1→6]GlcNAcβ1→Manβ1→3(6)Manβ1→GlcNAcβ1→GlcNAc structure (Yoshimi et al., 2000). Because co-transfection of bovine β-1,4-GalT I cDNA with human tissue plasminogen activator cDNA into SF-9 cells resulted in the β-1,4-galactosylation of N-linked oligosaccharides on vacuovirus virion glycoproteins and recombinant human tissue plasminogen activator (Hollister et al., 1998), it appeared that SF-9 cells contain a galactosylation machinery except for β-1,4-GalT. Therefore, it was expected that SF-9 cells provide an useful cell line to investigate the in vivo acceptor specificities of β-1,4-GalTs II, III, IV, V, and VI.

Because β-1,4-GalT activities were detected in SF-9 cells transfected with individual β-1,4-GalTs cDNAs, whether or not each β-1,4-GalT is involved in the galactosylation of N-linked oligosaccharides was examined in the gene-transfected cells by lectin blot analysis of endogenous membrane glycoprotein samples that contained acceptor-substrates for β-1,4-GalT. No significant differences were observed in Coomassie Brilliant Blue (CBB) staining patterns among the samples except for 50 kDa and 53 kDa bands, which appeared in those from the
Functions of \(\beta\)-1,4-galactosyltransferases I–VI

Cells transfected with the \(\beta\)-1,4-GalT II cDNA and with \(\beta\)-1,4-GalT III cDNA (lanes C and D, respectively, in Figure 1 CBB). When blots were incubated with peroxidase-conjugated RCA-I and the lectin reactivity was determined by color development within 1 min in the presence of 4-chloro-1-naphthol, a band with an apparent molecular weight of 60 kDa reacted strongly with the lectin in the samples from those transfected with \(\beta\)-1,4-GalT I, II, III, and IV cDNAs (lanes B, C, D, and E, respectively, in Figure 1 RCA-I [a]) but weakly with the lectin in the samples from those transfected with \(\beta\)-1,4-GalT V and VI cDNAs (lanes F and G, respectively, in Figure 1 RCA-I [a]). The molecular weights of RCA-I-positive bands appeared to be identical to those of PVL-positive bands as reported previously (Yoshimi et al., 2000). The results indicate that \(\beta\)-1,4-GalTs I, II, III, and IV can galactosylate the endogenous glycoprotein acceptors, most probably hybrid- and/or monoantennary complex-type acceptor oligosaccharides more effectively than \(\beta\)-1,4-GalTs V and VI. Prolonged incubation (10 min) of blots with peroxidase-conjugated RCA-I resulted in the strong color development on several protein bands whose color intensities were not discriminated among the samples (Figure 1 RCA-I [b]). However, differences in the number of lectin-positive bands and their reactivities with the lectin became more clearly (lanes B to G in Figure 1 RCA-I [b]). Very weak lectin-reactive bands were also detected in mock-transfected Sf-9 cells (lane A in Figure 1 RCA-I [a]), indicating that Sf-9 cells may contain a \(\beta\)-1,4-GalT gene that could be activated on viral infection. No lectin binding was observed when blots were treated with diplococcal \(\beta\)-1,4-galactosidase or N-glycanase prior to incubation with lectin (the results from \(\beta\)-1,4-GalT III cDNA-transfected cells are shown as representatives in lanes D1 and D2 of Figure 1 RCA-I [b], respectively). These results indicate that human \(\beta\)-1,4-GalTs I, II, III, IV, V, and VI expressed as membrane-bound forms in Sf-9 cells can galactosylate endogenous N-linked oligosaccharides.

To confirm further that \(\beta\)-1,4-GalTs are involved in N-linked oligosaccharide biosynthesis in mammalian cells which contain some or all of \(\beta\)-1,4-GalTs, human antisense \(\beta\)-1,4-GalT II and V cDNAs were independently transfected into human colorectal adenocarcinoma cell line, SW480, in which the gene expression levels of \(\beta\)-1,4-GalTs II and V were significant in addition to \(\beta\)-1,4-GalT I (Sato et al., 2000b). Lectin blot analysis of membrane glycoprotein samples from the SW480 cells revealed that the binding to RCA-I is reduced in the samples from both antisense cDNA-transfected cells when compared with that of the control sample (Figure 2). Because all the lectin-positive bands disappeared on treatment of blots with diplococcal \(\beta\)-1,4-galactosidase or N-glycanase, these results indicate that human \(\beta\)-1,4-GalTs I, II, III, IV, V, and VI expressed as membrane-bound forms in Sf-9 cells can galactosylate endogenous N-linked oligosaccharides.

![Fig. 1. Lectin blot analysis of membrane glycoproteins from Sf-9 cells transfected with human \(\beta\)-1,4-GalT I–VI cDNAs.](image1.png)

![Fig. 2. Lectin blot analysis of membrane glycoproteins from SW480 cells transfected with human anti-sense \(\beta\)-1,4-GalT cDNAs.](image2.png)
idase or N-glycanase (data not shown), at least β-1,4-GalTs II and V are involved in the galactosylation of N-linked oligosaccharides even in mammalian cells.

**Acceptor specificities of β-1,4-GalTs toward oligosaccharides with branched structures**

Because all six human β-1,4-GalTs were shown to be able to galactosylate N-linked oligosaccharides, whether or not there are differences in acceptor specificities among β-1,4-GalTs was investigated using GlcNAcβ-S-pNP, GlcNAc, oligosaccharides with the GlcNAcβ1→2Manβ1→3Man structure (oligosaccharide 3), with the GlcNAcβ1→4(GlcNAcβ1→2)Man branch (oligosaccharide 4) and with the GlcNAcβ1→6(GlcNAcβ1→2)Man branch (oligosaccharide 5) as acceptor substrates shown in Table II, and the homogenates of SF-9 cells transfected with the individual β-1,4-GalT cDNAs as enzyme sources. Because specific activities of β-1,4-GalTs expressed in SF-9 cells varied partly due to different titration levels of viruses transfected, the activities toward individual acceptor substrates were expressed as relative values to that toward GlcNAcβ-S-pNP. β-1,4-GalTs I and II galactosylated GlcNAcβ-S-pNP, oligosaccharides 4 and 5 quite effectively, but β-1,4-GaTI galactosylated GlcNAc more effectively than other acceptors while β-1,4-GaTI galactosylated GlcNAc less effectively than others (Figure 3A). β-1,4-GaTI III galactosylated GlcNAcβ-S-pNP and oligosaccharides 4 and 5 effectively, oligosaccharide 3 less effectively, but not GlcNAc. In contrast, β-1,4-GaTI, IV, V, and VI galactosylated GlcNAcβ-S-pNP and oligosaccharide 5 effectively but did so poorly for oligosaccharides 3 and 4. These transferases failed to galactosylate 1 mM GlcNAc. Because all six β-1,4-GalTs could galactosylate oligosaccharide 5 effectively, which of the N-acetylglucosamine residue of the branched oligosaccharide is galactosylated was determined by analyzing the mono-[3H]galactosylated product by the method as described previously (Shirane et al., 1999). β-1,4-GaTI preferentially galactosylated the GlcNAcβ1→2Man branch, and β-1,4-GaTI IV, V, and VI showed preferential galactosylation of the GlcNAcβ1→6Man branch (Figure 3B). No branch specificity was observed for β-1,4-GaTI and III, which galactosylated both branches almost equally. In the case of oligosaccharide 4, branch specificities among six β-1,4-GalTs were not determined because some transferases showed lower activities. Although no oligosaccharides with biantennary structures were used for assays, β-1,4-GaTI I, II, III, IV, and V cDNAs, respectively. In each case β-1,4-GaTI activities toward acceptors 2–5 were expressed by taking the activity toward GlcNAcβ-S-pNP (acceptor 1) as 100%.

Table II. Structures of acceptor oligosaccharides used

<table>
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<th>Structures</th>
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<tr>
<td>1. GlcNAcβ-S-pNP</td>
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<tr>
<td>2. GlcNAc</td>
</tr>
<tr>
<td>3. GlcNAcβ1→2Manβ1→3Manβ1→O(CH2)3COOCH2-mNP</td>
</tr>
<tr>
<td>4. GlcNAcβ1→4Manβ1→3Manβ1→O(CH2)3COOCH2-mNP</td>
</tr>
<tr>
<td>5. GlcNAcβ1→6Manβ1→3Manβ1→O(CH2)3COOCH2-mNP</td>
</tr>
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</table>

![Fig. 3. Acceptor (A) and branch (B) specificities of recombinant human β-1,4-GalTs. Numerical figures indicate acceptor oligosaccharides whose structures are listed on Table II. I–VI at the top of each panel in (A) indicate the enzyme sources prepared from SF-9 cells transfected with the β-1,4-GaTI I, II, III, IV, and V cDNAs, respectively. In each case β-1,4-GaTI activities toward acceptors 2–5 were expressed by taking the activity toward GlcNAcβ-S-pNP (acceptor 1) as 100%.](image-url)
Discussion

In the present study, human β-1,4-GalTs I, II, III, IV, V, and VI were expressed as membrane-bound forms in SF-9 cells and the galactosylation of endogenous membrane glycoproteins was examined by lectin blot analysis. Hardly any β-1,4-linked galactose residues on N-linked oligosaccharides were detected in SF-9 cells by structural analysis (Yoshimi et al., 2000) and in mock-transfected cells, although very weak, β-1,4-GalT activities toward GlcNAcβ-S-pNP were detected in the transfected cells. In contrast, significantly higher levels of β-1,4-GalT activities toward GlcNAcβ-S-pNP and of β-1,4-linked galactose residues on N-linked oligosaccharides as revealed with binding to RCA-I were detected in the cells transfected with individual human β-1,4-GalT cDNAs. Because membrane glycoproteins of SF-9 cells contain oligosaccharides with the Manα1→6(3)GlcNAcβ1→Manα1→3(6)Manβ1→GlcNAcβ1→Fucε1→1GlcNAc and (Man→1→2Manα1→6(3)GlcNAcβ1→Manα1→3(6)Manβ1→GlcNAcβ1→GlcNAc structures (Yoshimi et al., 2000), the N-acetylgalactosamine residues of the oligosaccharides should have been galactosylated by all six β-1,4-GalTs. In fact, protein bands with apparent molecular weights of 35 kDa and 60 kDa that reacted strongly with RCA-I were detected in all the samples. However, differences in the initial color development on these protein bands as shown in Figure 1 RCA-I (a) indicate that β-1,4-GalTs I, II, III, and IV can galactosylate monoantennary- and hybrid-type acceptor oligosaccharides more effectively than β-1,4-GalTs V and VI. Other differences in the reactivity and number of the lectin-positive bands among the samples could be reflected by differences in the kinetic parameters and acceptor specificities of individual β-1,4-GalTs. In this regard, our preliminary studies suggested that N-linked oligosaccharides of SF-9 cells contain yet unidentified structures having N-acetylgalactosamine residue(s) at their nonreducing termini. Therefore, it is of importance to elucidate structures of such oligosaccharides to confirm the fine acceptor specificities of individual β-1,4-GalTs.

Analysis of acceptor-specificity of β-1,4-GalT IV expressed in a soluble form showed no capability of galactosylating glycoprotein acceptors (Schwientek et al., 1998). Similarly, in a purified chimeric protein of human β-1,4-GalT V with protein A, the transferase also failed to galactosylate glycoprotein acceptors (unpublished data). However, the present study clearly demonstrated that β-1,4-GalTs IV and V expressed as membrane-bound forms in SF-9 cells can galactosylate the endogenous N-linked oligosaccharides. Because some lipid-rich environment stimulated β-1,4-GalT I activities (Mitrani and Moscarello, 1980; Mitrani et al., 1983), most of these β-1,4-GalTs may require such an environment for maintaining proper tectal structures that affect their acceptor specificities. It is of interest to investigate whether or not the purified recombinant human β-1,4-GalTs in membrane-bound forms can galactosylate glycoprotein acceptors effectively in the presence of some types of lipids. Similarly, several studies showed that β-1,4-GalTs III, IV, and VI are involved in the biosynthesis of glycolipids such as lactosylceramide and lacto-N-neotetraosylceramide in vitro (Almeida et al., 1997; Nomura et al., 1998; Schwientek et al., 1998; Takizawa et al., 1999), but the present study showed that they can also galactosylate N-linked oligosaccharides in vivo. Because they are supposed to be in the trans-Golgi (Roth and Berger, 1982) in which the galactosylation of N-linked oligosaccharides but not of glucosylceramide takes place, β-1,4-GalTs III, IV, and VI may not be involved in the biosynthesis of lactosylceramide in vivo. Concerning to this issue, it is important to elucidate the fine localization of individual β-1,4-GalTs within the Golgi apparatus once antibodies specific to individual β-1,4-GalTs are available, and it is of interest to investigate whether the galactosylation of glycolipids is also enhanced in SF-9 cells on transfection of individual β-1,4-GalT cDNAs if appropriate endogenous precursor substrates such as glucosylceramide and lacto-N-triacylglycerol are accumulated in SF-9 cells.

The present study also demonstrated that β-1,4-GalT I prefers to galactosylate the GlcNAcβ1→2Man branch and β-1,4-GalTs IV, V, and VI galactosylate the GlcNAcβ1→6Man branch predominantly, whereas no preference is observed for β-1,4-GalTs II and III. In the case of β-1,4-GalT V, prolonged incubation showed no galactosylation of another N-acetylgalactosamine residue of oligosaccharide 5, indicating that other β-1,4-GalTs could galactosylate it. Using oligosaccharides with the Galβ1→4GlcNAcβ1→2GlcNAcβ1→6Man and Galβ1→4GlcNAcβ1→6GlcNAc→2Man structures, β-1,4-GalT I was shown to galactosylate the GlcNAcβ1→6Man branch more effectively than the GlcNAcβ1→2Man branch of the monogalactosylated oligosaccharides (Ujita et al., 1999), indicating that the initial galactosylation affects the subsequent galactosylation of the branched oligosaccharides. Therefore, it is worthy of determining the order of β-1,4-GalTs for the galactosylation of all of N-acetylgalactosamine residues of highly branched oligosaccharides. In the case of β-1,4-GalT I-knockout mouse, polysialic acid, which is supposed to be expressed on highly branched N-linked oligosaccharides (Kudo et al., 1996), was expressed without reduction when compared to that of wild-type mouse (Kido et al., 1998), suggesting that β-1,4-GalTs II to VI are involved in the galactosylation of the highly branched oligosaccharides. In support of our findings in the present study, some or all of β-1,4-GalTs II, III, IV, V, and VI have been recently shown to be involved in the galactosylation of highly branched N-linked oligosaccharides using Chinese hamster ovary mutant cell lines (Lee et al., 2001).

The tissue-specific expression of β-1,4-GalTs II, III and IV in human tissues (Almeida et al., 1997; Lo et al., 1998; Sato et al., 1998b; Schwientek et al., 1998; Takizawa et al., 1999) suggests that they are contributing to tissue-specific galactosylation of N-linked oligosaccharides. Interestingly, the gene expression level of β-1,4-GalT V but not β-1,4-GalTs IV and VI, whose transcripts were under detection levels was increased on malignant transformation of NIH3T3 cells (Shirane et al., 1999; Sato et al., 2000b), indicating that β-1,4-GalT V is mainly involved in the galactosylation of the GlcNAcβ1→6Man branch, on which a variety of tumor antigens are expressed and involved in metastasis (Dennis et al., 1989; Dennis and Laferte, 1989; Ohyama et al., 1999). Further investigation is necessary as to whether suppression of the β-1,4-GalT V gene expression will result in the reduction of the tumor formation and/or tumor metastatic processes to show functions of the carbohydrates. Finally, to raise and to analyze phenotypes of individual β-1,4-GalT-knockout mice will contribute to our real understandings how individual β-1,4-GalTs are important for our lives as already shown in β-1,4-GalT I-knockout mouse (Asano et al., 1997; Lu et al., 1997).
Materials and methods

Chemicals

UDP-[3H]Gal (48.8 Ci/mmol) and NaB[1H]4 (490 mCi/mmol) were purchased from NEN-Life Science Products (Boston, MA). GlcNAcβ1→2Manβ1→3Manβ1→O(CH2)8COOCH3-m-nitrophenol (mNP), GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3Manβ1→O(CH2)8COOCH3-α-nitrophenol (α-NP), and GlcNAcβ1→6(GlcNAcβ1→2)Manβ1→3Manβ1→O(CH2)8COOCH3-mNP, which were chemically synthesized and donated by Dr. T. Kitajima, Towa Kasei Co. (Japan), and GlcNAcβ-S-pNP and UDP-Gal were purchased from Sigma Chemical Co. (St. Louis, MO). Diplococcal β-1,4-galactosidase was obtained from Roche Molecular Biochemicals (Mannheim, Germany), and N-glycanase was a gift from Dr. T. Kaizu formerly in Genzyme Japan (Tokyo). Peroxidase-conjugated R-I was purchased from Honen Oil Co. (Tokyo).

Isolation of human β-1,4-GalT II, III, and IV cDNAs

The total RNA preparation was obtained from human erythro-leukemic K562 cells with RNeasy Total RNA System (QIAGEN) from which single-strand cDNAs were prepared using a hexadeoxyribonucleotide mixture [pd (N)6, TAKARA] as a primer and Ready-To-Go™ You-Prime First Strand Beads (Pharmacia Biotech). The cDNAs encoding the full length of human β-1,4-GalTs II, III, and IV were amplified by polymerase chain reaction (PCR) (95°C, 10 min [94°C, 0.5 min; 60°C, 0.5 min; and 72°C, 1.5 min] × 40, and 72°C, 15 min) using AmpliTaq Gold™ (Perkin Elmer), the single-strand cDNAs as a template and 5’- and 3’-primers specific for each β-1,4-GalT. The following 5’- and 3’-primer pairs were used (the newly synthesized BamH I, EcoR I and Xba I sites in the primers are underlined):

- TS49 (5’-CGGATCTTGGGATGAGCAGACTG-3’) and TS50 (5’-CGGAATTCATATGTCAGCCCGAG-3’) for β-1,4-GalT II
- TS51 (5’-CGGATCCAGGATGTGCGGAGGTG-3’) and TS52 (5’-GGCTTAGAGGATGCTGGAACCTC-3’) for β-1,4-GalT III
- TS53 (5’-CGGATTCACATGCGTTCACCTACG-3’) and TS54 (5’-CGGAATTCCAGGTCATCCAGCAACCAG-3’) for β-1,4-GalT IV

PCR products were cloned into pGEM-T Easy vector (Promega) and sequenced. The nucleotide sequences of human β-1,4-GalT II, III, and IV cDNAs isolated in the present study were deposited in the DDBJ/GenBank/EMBL data base (accession numbers: AB024434, AB024435, and AB024436, respectively).

Plasmid construction and expression of β-1,4-GalT cDNA in SF-9 and SW480 cells

Plasmid containing each β-1,4-GalT cDNA was digested with BamH I and EcoR I or BamH I and Xba I, and the fragment was ligated into the BamH I–EcoR I or BamH I–Xba I site of baculovirus transfer vector pVL1393 (Pharmingen). The resultant plasmids, pVL1393/β-1,4-GalT II, pVL1393/β-1,4-GalT III, and pVL1393/β-1,4-GalT IV, were transfected independently with BaculoGold™ Linearized Baculovirus DNA into SF-9 cells by the method described in BaculoGold™ system’s protocol (Pharmingen). Recombinant baculoviruses were obtained after two successive amplification in SF-9 cells.

Similarly, plasmids, pVL1393/β-1,4-GalT V, pVL1393/β-1,4-GalT V and pVL1393/β-1,4-GalT VI were also transfected independently into the cells as described previously (Sato et al., 1998a, 2000a; Sato and Furukawa, 1999). The titers of the viruses to be used for infection to SF-9 cells (9 × 10⁶ cells/10 cm φ) were determined with monitoring the enzyme activities of individual β-1,4-GalTs.

Human colorectal adenocarcinoma cell line, SW480, was obtained from the Institute of Development, Aging and Cancer, Tohoku University, Sendai, and grown at 37°C in RPMI1640 containing 10% fetal calf serum, 50 units/ml penicillin, and 50 mg/ml streptomycin. Fragments containing a partial coding sequence of human β-1,4-GalT II (–5 to 500) and a partial coding sequence of human β-1,4-GalT V (–5 to 500) were subcloned in an antisense orientation into a mammalian expression vector, pcDNA3.1 (Invitrogen), and the expression vectors were named pcDNA3.1/GTII AS and pcDNA3.1/GTV AS, respectively. For transfection, 1 × 10⁵ SW480 cells were seeded in tissue culture dishes with a diameter of 35 mm and cultured 37°C for 24 h in a CO2 incubator. Two micrograms of pcDNA3.1, pcDNA3.1/GTII AS, or pcDNA3.1/GTV AS formulated with a FuGENE reagent (Boehringer Mannheim, Mannheim, Germany) was added to the cells grown at the 60–70% confluent stage and cultured for 72 h. The plasmid-transfected cells were selected with a medium containing genetin (700 µg/ml G418 sulfate, Sigma) for 2 weeks.

Determination of β-1,4-GalT activities

The harvested cells were washed with phosphate buffered saline (pH 7.2) three times, suspended in 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 7.0) containing 1.25% Triton X-100, and then sonicated. The cell homogenates were used for transferase assays as an enzyme source. β-1,4-GalT activities were determined by the method described previously (Sato et al., 1998b). In all instances the reaction mixture contained 100 mM MES buffer (pH 7.0) containing 4 mM 5’-AMP, 250 mM UDP-β-[3H]Gal, 1 mM appropriate acceptor, 20 mM MnCl₂, and enzyme preparation in a total volume of 50 µl. After incubation the product was isolated by using a Sep-Pak C₁₈ cartridge or an AG X₁ column (Cl⁻ form), and radioactivity incorporated into the product was determined. To diminish or inhibit β-N-acetylgalosaminidase activity possibly present in the cell homogenates (Kubelka et al., 1994), pH of the assay mixture was adjusted to 7.0 rather than 6.0, at which maximal activities of β-1,4-GalT (Furukawa and Roth, 1985) and β-N-acetylgalactosaminidase were obtained (Altman et al., 1995), and, in some assays, 2 mM N-acetyl-glucosamine was included in the reaction mixture (Bendiak and Schachter, 1987).

Lectin blot analysis of membrane glycoproteins

Lectin blot analysis of membrane glycoprotein samples from SF-9 cells and gene-transfected cells was performed as described previously (Sato et al., 1993). Membrane glycoproteins were separated by 7.5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to the method of Laemmli (1970), and separated proteins were electrophoretically transferred to polyvinylidenedifluoride membranes by the method described previously (Towbin et al., 1979). Blotted membranes were blocked with 1% bovine serum albumin and incubated with peroxidase-conjugated

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RCA-I. In some experiments, blotted membrane blocked with bovine serum albumin was treated with 3.5 U N-glycanase in 150 μl of 0.1 M phosphate buffer (pH 8.2), 1.0 U of jackbeak β-N-acetylhexosaminidase in 150 μl of 0.3 M citrate-phosphate buffer (pH 5.0), or 40 μl of diplococcal β-1, 4-galactosidase in 150 μl of 0.3 M citrate-phosphate buffer (pH 6.0) per one lane at 37°C for 24 h before incubation with lectin.

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Abbreviations

β-1,4-GalT, β-1,4-galactosyltransferase; CBB, Coomassie Brilliant Blue; GlcNAcβ-S-pNP, p-nitrophenyl-N-acetyl-1-thio-β-β-glucosaminidase; MES, 2-(N-morpholino)ethanesulfonic acid; m-NP, m-nitrophenol; PVL, Psathyrella velutina lectin; RCA-I, Ricinus communis agglutinin-I; PCR, reverse-transcription polymerase chain reaction.

References


