Fucosylation of N-Glycans Regulates the Secretion of Hepatic Glycoproteins into Bile Ducts

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Fucosylated α-fetoprotein (AFP) is a highly specific tumor marker for hepatocellular carcinoma (HCC). However, the molecular mechanism by which serum level of fucosylated AFP increases in patients with HCC remains largely unknown. Here, we report that the fucosylation of glycoproteins could be a possible signal for secretion into bile ducts in the liver. We compared oligosaccharide structures on glycoproteins in human bile with those in serum by several types of lectin blot analyses. Enhanced binding of biliary glycoproteins to lectins that recognize a fucose residue was observed over a wide range of molecular weights compared with serum glycoproteins. A structural analysis of oligosaccharides by two-dimensional mapping showed higher fucosylation in bile than in serum. To find direct evidence for fucosylation and sorting signal into bile ducts, we used α1–6 fucosyltransferase (Fut8)-deficient mice because fucosylation of glycoproteins produced in mouse liver was mainly an α1–6 linkage. Interestingly, the levels of α1-antitrypsin and α1-acid glycoprotein and haptoglobin, which are synthesized in the liver, showed higher fucosylation in bile than in serum. A disruption in this system might involve an increase in fucosylated AFP in the serum of patients with HCC.

Fucosylated α-fetoprotein (AFP), referred to as the L3 band of AFP on LCA (Lens culinaris agglutinin lectin affinity electrophoresis), is a highly specific tumor marker for hepatocellular carcinoma (HCC). Increases in AFP levels in serum are observed in patients with chronic liver disease such as liver cirrhosis, but fucosylated AFP is rarely detectable in benign liver diseases (1). α1–6 fucosyltransferase (Fut8) is involved in fucosylation of AFP, and we previously succeeded in the purification and cDNA cloning of Fut8 from porcine brain (2) and a human gastric cancer cell line (3). Although the overexpression of Fut8 in Hep3B cells increased the fucosylation rate of AFP, a high expression of Fut8 was also observed in non-cancerous liver cirrhotic tissues as well as HCC tissues (4). Therefore, other factors could be linked to specific incidences of fucosylated AFP in HCC. GDP-fucose is a donor substrate for Fut8. Recently, we developed an assay system for GDP-fucose levels in cells/tissues (5). When we determined GDP-fucose levels in liver tissues using the assay system, the levels in HCC tissues were significantly higher than those in liver cirrhosis and normal liver (6). The increases of GDP-fucose in HCC were due to the enhancement of FX (human homologue of GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase) expression, which contributed to the synthesis of GDP-fucose. Therefore, both Fut8 and FX would regulate the production of fucosylated AFP in HCC. However, increases in Fut8 and FX in HCC tissues were two or three times compared with their surrounding tissues, and another factor could be concerned in terms of increases in fucosylated AFP in serum of patients with HCC.

The sorting of glycoproteins to apical or basolateral membranes in polarized cells is a recent hot spot for discussions in cell biology. An apical sorting machinery is hardly known although polarized cells such as Madin-Darby canine kidney cells have been widely used to investigate the sorting mechanism. In experiments using Madin-Darby canine kidney cells, N-glycans are considered possible candidates of apical sorting signal based on the findings that some proteins gain...
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FIGURE 1. Comparison of human biliary and serum glycoproteins. 5 μg of proteins from human bile and serum were subjected to 12% SDS-PAGE and stained with Coomassie Brilliant Blue (A). Lectin blot analyses were performed using the same samples (B). AOL and AAL bind to fucose residue. ConA binds with high affinity to a high mannose-type, biantennary complex or hybrid type of asparagine-linked oligosaccharide. SSA and MAM bind to α2–6– and α2–3–linked sialic acid residues, respectively. 5 μg of protein from human bile and serum were subjected to 12% SDS-PAGE, and glycosylated proteins were detected according to the manufacturer’s protocol for the ECL glycoprotein detection system (Amersham Biosciences) (C). B and S indicate bile and serum, respectively. Detailed procedures are described under “Experimental Procedures.”

The ability to reach the apical membrane after the recombinant addition of an N-glycosylation site and that other proteins entirely or partially lose apical expression as a result of the removal of N-glycans by amino acid mutation or treatment with glycosylation inhibitors (7–11). It was also reported that the core region sugars, not sialic acid and galactose, were more important than the terminal sugars for apical sorting (12, 13).

In the case of the liver, major epithelial cells, hepatocytes, produce a variety of serum glycoproteins and non-glycosylated proteins, including albumin. In the system of hepatocytes, there are two kinds of secretion pathways. One pathway is to the apical surface of hepatocytes followed by secretion into bile ducts. The other is to the basolateral surface followed by secretion into blood vessels. Interestingly, the same kinds of serum proteins, including albumin, are detected in bile (14). Until now, however, few cellular or protein factors have been found to regulate these secretion pathways.

In serum glycoproteins, both α1-antitrypsin (AAT) and transferrin were reported to be fucosylated in patients with HCC (15). However, because these two glycoproteins are produced in normal hepatocytes, the rate of fucosylation of these glycoproteins that could occur in HCC tissues was quite low. The expression of Fut8 is enhanced in cirrhotic hepatocytes, leading to a slight increase in fucosylated glycoproteins in these cells even though the levels of GDP-fucose are not higher compared with HCC tissues (6). Where are these fucosylated glycoproteins produced by cirrhotic hepatocytes? To answer this question, we hypothesized that the fucosylation of glycoproteins might be a signal for sorting to the bile ducts. This hypothesis leads to the selective secretion of fucosylated glycoproteins into bile ducts. In the present study, we analyzed oligosaccharide structures on biliary glycoproteins compared with those on serum glycoproteins. Many biliary glycoproteins were strongly fucosylated compared with those in the serum as evidenced by lectin blot analyses as well as high performance liquid chromatography (HPLC) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses. Furthermore, we found that the sorting system of hepatic glycoproteins is disrupted in Fut8-deficient mice.

EXPERIMENTAL PROCEDURES

Materials—Biotinated lectins (AAL (Aleuria aurantia), ConA (Canavalia ensiformis), SSA (Sambucus sieboldiana), and MAM (Maackia amurensis)) were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Biotinated AOL (Aspergillus oryzae) and AOL-agarose were from Toyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Both AAL and AOL bind to fucose residues with the α1–2, 1–3, 1–4, and 1–6 linkage. ConA binds with high affinity to a high mannose-type, biantennary complex or hybrid type of asparagine-linked oligosaccharide. SSA and MAM bind to α2–6– and α2–3–linked sialic acid residues, respectively.

Animals—8-week-old BALB/c mice were used. The establishment of Fut8-deficient mice has been described previously (16). Body weight, liver weight/body weight, serum protein concentration, and bile protein concentration for wild-type and Fut8-deficient mice were 24.9 ± 0.57 g, 6.22 ± 0.31, 49.5 ± 3.6 mg/ml, 3.69 ± 0.42 mg/ml and 15.4 ± 1.51 g, 5.05 ± 0.28, 49.3 ± 1.8 mg/ml, 4.45 ± 2.17 mg/ml (mean ± S.D., n = 3), respectively. Although a ratio of liver weight/body weight of Fut8-deficient mice was significantly smaller than that of wild-type mice, no significant differences between the two types of mice were observed in serum and bile protein concentrations. There was no difference in the volume of bile secretion between the two types of mice. All procedures of animal experiments were performed in accordance with the Animal Ethics Committee of Osaka University.

Bile Specimens—Human bile was collected from a percutaneous transcatheater drainage tube in a patient with obstructive jaundice due to common bile duct stones. This collection was performed according to an ethical committee of Osaka University Hospital. Serum was collected from the same patient. Mice were anesthetized with ketamine and xylazine (0.8 mg and 0.08 mg/10 g body weight, respectively). Bile duct cannulation was carried out according to the method of Fischer et al. (17). Shortly, the abdomen was opened, and the gallbladder was ligated with a string. The common bile duct was cannulated with a 30-gauge needle connected to polyethylene tubing. Gallbladder bile was collected after the extraction. Protein concentrations of bile specimens were measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard after delipidation according to the method of Wessel and Flugge (18).

Purification of Human AAT, Haptoglobin (Hp), and α1-Acid Glycoprotein (AGP) from Serum and Bile—Human AAT, Hp, and AGP were purified from serum by a separate two-step
FIGURE 2. HPLC separation of PA oligosaccharides from human biliary and serum glycoproteins. Representative elution profile of PA oligosaccharides from human bile (A) and serum (B) on an ODS column. The charts in frames indicate an elution profile on an Amido-80 column of peak (I) or (II) on an ODS column. Detailed procedures are described under “Experimental Procedures.” The percentages of each peak in the total area of assigned peaks were calculated based on peak areas of ODS and Amide-80 elution profiles (C). Numbers at each peak correspond to those in Table 1. Closed and open columns indicate oligosaccharides from biliary and serum glycoproteins, respectively. N.D., not detected.
chromatography procedure. In the first step, albumin was removed from the serum by chromatography on an Econo-Pac Blue Cartridge (Bio-Rad Laboratories) using 20 mM sodium phosphate buffer, pH 7.1. The protein eluate from an Econo-Pac Blue Cartridge was concentrated on an Apollo concentrator (Orbital Sciences Corp., Topsfield, MA) and then re-equilibrated in 50 mM sodium phosphate buffer, pH 7.4, containing 0.5 M NaCl on a PD-10 column (Amersham Biosciences). The re-equilibrated eluate was then used directly in chromatography on antibody columns equilibrated with the same buffer. In the second chromatographic step on antibody columns, the re-equilibrated eluate was recirculated a fifth time. After washing with equilibration buffer, the elution of bound fraction was performed by 0.1 M glycine-HCl, pH 3.0.

To purify glycoproteins from human bile, bile was equilibrated in 50 mM sodium phosphate buffer, pH 7.4, containing

| Peak | Structure | ODS (GU) | Amide-80 (GU) | m/z |
|------|-----------|----------|---------------|-----|
| 1    | ![Structure 1](image1.png) | 10.3     | 6.9           | 1719.7 |
| 2    | ![Structure 2](image2.png) | 10.3     | 9.9           | 2450.1 |
| 3    | ![Structure 3](image3.png) | 10.3     | 10.6          | 2597.6 |
| 4    | ![Structure 4](image4.png) | 12.8     | 8.4           | 2107.3 |
| 5    | ![Structure 5](image5.png) | 12.8     | 9.1           | 2254.0 |
| 6    | ![Structure 6](image6.png) | 13.5     | 7.3           | 1888.4 |
| 7    | ![Structure 7](image7.png) | 13.5     | 10.3          | 2597.6 |
| 8    | ![Structure 8](image8.png) | 17.0     | 5.9           | 1744.8 |
| 9    | ![Structure 9](image9.png) | 17.0     | 8.6           | 2254.0 |
| 10   | ![Structure 10](image10.png) | 20.3     | 7.3           | 2068.9 |

* Numbers at each peak indicated correspond to those in Figs. 2, 4, and 6.
* Monosaccharides were denoted by: □, galactose; ■, N-acetylglucosamine; ◇, mannose; △, fucose.
* The elution positions on HPLC columns were expressed as glucose units (GU). The chromatographic conditions were described under "Experimental Procedures."
* The ions correspond to [M + H]⁺.
* The ions correspond to [M + Na]⁺.
0.5 M NaCl on PD-10 columns. These bile samples were then used directly in chromatography on antibody columns. The same methods as with serum were performed.

Lectin Blot Analysis—Lectin blot analyses were performed as described previously (19). Briefly, 5–10 μg of proteins were subjected to 12% SDS-PAGE. After the electrophoresis, the gels were blotted onto nitrocellulose membranes. The membranes were incubated with 3% bovine serum albumin in Tris-buffered saline (20 mM Tris, 0.5 M NaCl, pH 7.5; TBS) overnight and then incubated with 1.0 μg/ml of biotinlated lectins (AOL, AAL, ConA, SSA, MAM) in TBS (TBS containing 0.05% Tween 20) for 1 h. After washing with TBS, the membranes were incubated with horseradish peroxidase-conjugated avidin (VECTASTAIN ABC kit; Vector Laboratories, Burlingame, CA) for 1 h and then washed with TBS. Staining was performed with ECL Western blot detection reagents (Amersham Biosciences).

Fractionation of Human Biliary and Serum AGP by AOL Lectin Affinity Chromatography—AOL lectin affinity chromatography was performed at room temperature, using AOL-agarose column (bed volume 2 ml) and equilibrated with 10 mM Tris, 0.5 M NaCl, pH 7.4. AGP purified from human bile and serum were equilibrated in the same buffer on PD-10 column and were concentrated using an Apollo concentrator. Purified AGP sample was applied to the column. The elution of non-bound fraction was performed by five volumes of equilibration buffer, and each fraction of 0.5 ml was collected. The elution of bound fraction was performed using 50 mM fucose in equilibration buffer. Protein concentrations were assayed by spectrophotometry at 280 nm.

Western Blot Analysis—5–10 μg of proteins were subjected to 12% SDS-PAGE. After the electrophoresis, the gels were blotted onto nitrocellulose membranes. The membranes were incubated with 2% skim milk in TBS for 1 h and then incubated with 1/1000 diluted rabbit anti-human AAT, Hp (Dako Cyto- mation, Copenhagen, Denmark), goat anti-human AAT (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-mouse AGP (Life Diagnostics, Inc., West Chester, PA), 10 μg/ml of rabbit anti-human AGP (Abcam Ltd., Cambridge, UK), and horseradish peroxidase-conjugated goat anti-mouse albumin (Bethyl Laboratories, Inc., Montgomery, TX) antibodies in 2% skim milk in TBS overnight for human AAT, Hp, mouse AAT, AGP, human AGP, and mouse albumin, respectively. The membranes, except for mouse albumin, were incubated with 1/1000 diluted HRP-conjugated anti-rabbit IgG (Promega, Madison, WI) or anti-goat IgG (Dako Cytomation) antibody in 2% skim milk in TBS for 1 h after washing with TBST three times for 10 min each. Staining was performed with ECL Western blot detection reagents after washing with TBST three times for 10 min each.

Structural Analysis of Oligosaccharides from Biliary and Serum Glycoproteins—Bile and serum were equilibrated in 20 mM sodium phosphate buffer, pH 7.1, on PD-10 columns. Immunoglobulins were removed by using Protein A-Sepharose (Sigma) and Protein G-Sepharose 4 Fast Flow (Amersham Biosciences). The solutions were subjected to trichloroacetic acid precipitation, and the precipitated fraction was washed with ethanol twice. Oligosaccharides were released from the precipitated proteins and AAT, Hp, and AGP purified from bile and serum by hydrazinolysis (100 °C for 10 h) followed by N-acetylation (20).

The oligosaccharides were applied to the GlycoTAG (Takara Bio Inc., Shiga, Japan) for pyridylationammonium (21). Excess reagents were removed by chromatography on a TSK gel Amide-80 column (Tosoh Corp., Tokyo, Japan). Sialic acids of the purified pyridylaminated (PA) oligosaccharides were removed with neuraminidase treatment (from Arthrobacter ureafaciens, Nacalai Tesq, Kyoto, Japan) in 0.2 M acetate buffer, pH 7.4, at 37 °C overnight. The asialo PA oligosaccharides were separated by reverse phase HPLC on a Shim-pack CLC-ODS column (Shimadzu Corp., Kyoto, Japan) and subsequent normal phase HPLC on a TSK gel Amide-80 column. Elution and detection of PA oligosaccharides were performed as described by Tomiya et al. (22). The structures of PA oligosaccharides were determined from the elution position of each peak in combination with α-fucosidase (from bovine kidney; Sigma) and β-galactosidase (from Aspergillus oryzae; Sigma) digestion.

MALDI-TOF MS—MALDI-TOF MS of PA oligosaccharides was performed on an Ultraflex TOF/TOF mass spectrometer equipped with a reflector and controlled by the Flexcontrol 1.2 software package (Bruker Daltonics GmbH, Bremen, Germany). In MALDI-TOF MS reflector mode using positive polarity, ions generated by a pulsed UV laser beam (nitrogen laser, 1 = 337 nm, 5 Hz) were accelerated to a kinetic energy of 15 kV. A solution of 2,5-dihydroxybenzoic acid (Bruker Daltonics) in a mixture of ethanol-0.1% trifluoroacetic acid (1:2) (10 mg/ml) was used as matrix. Sample (1 μl) was added to the matrix solution (4 μl), and a part of the mixture (1 μl) was then applied to a polished stainless steel target (Bruker Daltonics).

Immunohistochemical Analysis—To detect the AAT and AGP in the mouse liver using immunohistochemical technique, whole liver tissues from wild-type or Fut8-deficient mice were fixed by 0.1 M phosphate buffer containing 4% paraformaldehyde and embedded in paraffin. For immunohistochemical analysis, the dewaxed sections were pretreated with hydrogen blocking and nonspecific staining reagent (Dako Cytomation) for 10 min at 37 °C and then incubated with 1/200 diluted goat anti-human AAT antibody (Santa Cruz Biotechnology) and 1/300 diluted rabbit anti-mouse AGP antibody (Life Diag-
FIGURE 4. HPLC separations of PA oligosaccharides from AAT, Hp, and AGP purified from human bile and serum. Representative elution profile of PA oligosaccharides from AAT (A), Hp (B), and AGP (C) purified from human bile and serum on an ODS column. The charts in frames indicate the elution profile on an Amido-80 column for peaks (I-III) on an ODS column. Detailed procedures are described under “Experimental Procedures.” The percentages of each peak in the total area of assigned peaks were calculated based on peak areas of ODS and Amide-80 elution profiles (D, AAT; E, Hp; F, AGP). Numbers at each peak correspond to those in Table 1. Closed and open columns indicate oligosaccharides from glycoproteins purified from bile and serum, respectively. N.D., not detected.
nastics) in antibody diluent buffer (Dako Cytomation) at 4 °C overnight and at room temperature for 1 h for AAT and AGP, respectively. The sections were followed by a second reaction with 1/200 diluted horseradish peroxidase-conjugated anti-goat IgG and anti-rabbit IgG antibodies (Dako Cytomation) for AAT and AGP, respectively.

The immunoreactivity was visualized using the manufacturer’s protocol for the peroxidase/3, 3’-diaminobenzidine tetrahydrochloride reagent in the DAB kit (Dako Cytomation), and sections were counterstained with Gill’s hematoxylin solution. The immunostainings were photographed using a microscope system (Microphot F-XA; Nikkon, Tokyo, Japan) and application (Photograv-250; Fuji Film, Tokyo, Japan).

RESULTS

The Comparison of Oligosaccharide Structures between Human Biliary and Serum Glycoproteins by Lectin Blot Analyses—We compared the oligosaccharide structures on biliary glycoproteins with those on serum glycoproteins by several types of lectin blot analyses. It has been reported that almost all of the serum proteins are detected in bile, but the volume of each protein might be different (14). As shown in Fig. 1A, the staining pattern with Coomassie Brilliant Blue R-250 was very similar between bile and serum proteins except for a few bands. As shown in Fig. 1B, enhanced intensities of AOL and AAL binding in biliary glycoproteins were observed over a wide range of molecular mass compared with those in serum glycoproteins. There were few changes in the SSA and MAM blot analyses, suggesting that the sialylation of biliary and serum glycoproteins was almost the same level. In the case of ConA blot analysis, the intensity of lectin binding was slightly higher in biliary glycoproteins than in serum glycoproteins. However, the differences were not prominent in comparison with AOL and AAL blot analyses. These results show that biliary glycoproteins contained many fucose residues that are recognized by AOL and AAL lectins compared with serum glycoproteins, whereas total oligosaccharide structures differed only slightly.

As shown in Fig. 1C, a slight increase in carbohydrate labeling by the ECL glycoprotein detection system (Amersham Biosciences) was observed in biliary proteins as compared with serum proteins, which was in accordance with a result of ConA blot analysis. This shows that the levels of glycoproteins were increased in bile compared with serum. Although these data might enhance the AOL and AAL lectin blots results, no dramatic changes were observed in the other lectin blots.

Structural Analysis of PA Oligosaccharides from Human Biliary and Serum Glycoproteins—As shown in Fig. 1, fucosylated glycoproteins might be selectively secreted into the bile. To determine the oligosaccharide structures on biliary and serum glycoproteins in more detail, two-dimensional mapping HPLC and MALDI-TOF MS analyses were performed. PA oligosaccharides from biliary and serum glycoproteins were separated by reverse phase HPLC (Fig. 2, A and B). Each peak was isolated and chromatographed by normal phase HPLC. The elution positions of each peak were recorded as glucose units (Table 1) and their structures were determined based on the glucose units (22). The structural assignments were supported by the MALDI-TOF MS analyses (Table 1) and digestion with α-fucosidase and β-galactosidase (data not shown). As shown in Fig. 2C, the percentages of peaks 5, 8, and 10 in fucosylated oligosaccharides on biliary glycoproteins were larger than those on serum glycoproteins. These results indicate that both α1–3 (peak 5) and α1–6 (peaks 8 and 10) linkages of fucose residues were increased in biliary glycoproteins.

The Comparison of Oligosaccharide Structures on Hepatic Glycoproteins Purified from Human Bile and Serum—To gain a better understanding of the fucosylation levels of a liver-specific protein in bile and serum, we analyzed the oligosaccharide structures on AAT, Hp, and AGP purified from bile and serum. AAT, Hp, and AGP are major serum glycoproteins that are produced mainly in the liver and possess three, four, and five N-glycans, respectively. As shown in Fig. 3, all of these hepatic glycoproteins purified from bile showed much higher reactivities to the AOL and AAL lectins than those purified from serum. These results were consistent with data for lectin blot analysis on total glycoproteins in bile and serum. The difference in AAT between bile and serum was not due to N-glycosylation but to the peptide moiety. The N-terminal sequence of biliary AAT revealed that 16 amino acid residues were removed from the N-terminals.

We next analyzed the oligosaccharide structures on hepatic glycoproteins by HPLC analysis in more detail. As shown in Fig. 4, the levels of both α1–3 and α1–6 fucosylated oligosaccharides in AAT, Hp, and AGP purified from bile were increased to a greater extent than those purified from serum.

Fractionation of Human Biliary and Serum AGP by AOL Lectin Affinity Chromatography—As shown in Figs. 1–4, glycoproteins in bile were strongly fucosylated compared with those in
Serum. However, small amounts of fucosylated proteins were present in serum. If fucosylation is one of the signals for secretion into the bile duct and a lectin(s) regulate(s) its sorting, the direct interaction of fucosylated proteins with the AOL lectin would be an important factor. To examine the interaction of biliary and serum AGPs with AOL lectin, a fractionation of biliary and serum AGP was performed using an AOL-agarose column. As shown in Fig. 5, the ratio of the fraction bound to AOL lectin in biliary AGP was much larger than that of serum AGP.

**Structural Analysis of PA Oligosaccharides from Mouse Biliary and Serum Glycoproteins**—PA oligosaccharides from biliary and serum glycoproteins in wild-type mice were separated by reverse phase HPLC (Fig. 6, A and B). Similar to the human case, oligosaccharides on biliary glycoproteins were strongly fucosylated, whereas no fucosylated oligosaccharides on serum glycoproteins were observed except for α1–6 fucosylated biantennary structure (peak 6) (Fig. 6, B and C). On the other hand, there were few numbers of α1–3 fucosylated oligosaccharides on both biliary and serum glycoproteins, different from the human case, and most of the fucose residues on mouse glycoproteins were α1–6 linkages.

**The Effect of Fucosylated Oligosaccharides on Secretion of Glycoproteins into Bile Ducts in the Mouse Liver**—To investigate the direct effect of α1–6 fucosylation of glycoproteins on sorting into bile ducts in the liver, we examined levels of hepatic glycoproteins in bile and serum using Fut8-deficient mice. Expectedly, levels of biliary AAT and AGP were markedly decreased in Fut8-deficient mice although the serum AAT and AGP levels were almost the same between wild-type and Fut8-deficient mice (Fig. 7). In contrast, the level of biliary albumin, a non-glycosylated protein, was not different in these two

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**FIGURE 6.** HPLC separation of PA oligosaccharides from mouse biliary and serum glycoproteins. Representative elution profile of PA oligosaccharides from mouse gallbladder bile (A) and serum (B) on an ODS column. The chart in the frame indicates the elution profile on an Amido-80 column of the peak (#) on an ODS column. Detailed procedures are described under “Experimental Procedures.” The percentages of each peak in the total area of assigned peaks were calculated based on peak areas of ODS and Amido-80 elution profiles (C). Numbers at each peak correspond to those in Table 1. Closed and open columns indicate oligosaccharides from biliary and serum glycoproteins, respectively. N.D., not detected.
types of mice (Fig. 7). These results suggest that α1–6 fucosylation was essential for the secretion of hepatic glycoproteins such as AAT and AGP into bile in mice.

Furthermore, we examined the localization of AAT and AGP in the liver by immunohistochemical analyses. As shown in Fig. 8, there was a marked difference in distribution patterns of these proteins. The staining of AAT and AGP in the liver from wild-type mice was detected in patches, whereas a weak staining under uniform distribution pattern was observed in the liver from Fut8-deficient mice.

**DISCUSSION**

Increases in fucosylated oligosaccharides under pathological conditions have been reported, and fucosylated AFP in HCC is one of the most representative cases. Although its mechanism might be complicated, the results of the present study demonstrate a new concept for fucosylation and protein sorting in hepatocytes.

Biliary glycoproteins contain many fucose residues that are recognized by AOL and AAL lectins compared with serum glycoproteins (Fig. 1B). In contrast, both biliary and serum glycoproteins are weakly bound to UEA-1 (*Ulex europaeus*) and Lotus (*Lotus tetratubulobulus*) lectins, which bind to α1–2 fucose residues (data not shown). These results suggest that the enhanced intensities of AOL and AAL binding in biliary glycoproteins is due to either α1–3- or α1–6-fucosylated structures on glycoproteins.

To deny the possibility that fucose residues on glycoproteins are released in blood, fucosylated AGP was incubated with serum for 72 h. Defucosylation of fucosylated biliary AGP was not observed up to 72 h (supplemental data).

Because Fut6 is not expressed in the mouse liver, most of the fucosylation of hepatic glycoproteins are α1–6 linkages (23). Therefore, the Fut8-deficient mouse is a powerful tool to show direct evidence for fucosylation and apical sorting in the liver. Low levels of AAT and AGP in the bile of Fut8-deficient mice demonstrate the importance of fucosylation on the sorting signal into bile ducts. Although the levels of AAT and AGP in bile were dramatically decreased in Fut8-deficient mice, no change in the levels of these glycoproteins in serum was observed. This is due to the difference of levels of these glycoproteins between bile and serum; physiological concentrations of AAT and AGP in the serum are 300–25-fold higher than those in the bile, respectively.

Furthermore, we found a large difference in the AAT and AGP staining pattern in the liver between wild-type and Fut8-deficient mice in immunohistochemical analyses (Fig. 8). Certain types of secretory proteins are stained with difficulty in hepatocytes because they are rapidly secreted into hepatic vessels. If there were a cargo receptor that recognized fucosylated oligosaccharides in the liver, the localization and secretion pattern might be changed. The difference in the AAT and AGP staining pattern between wild-type and Fut8-deficient mice could support this hypothesis. Identification of such types of receptors is currently under investigation in our laboratory.

Although fucosylation was dramatically increased in biliary glycoproteins, we were unable to detect other bile-specific oligosaccharide structures in the present study. It was reported that an N-glycan at a specific site played a pivotal role in apical sorting in a glycoprotein possessing plural N-glycans, such as erythropoietin (11). The reason why small amounts of fucosylated glycoproteins were detected in the serum might be that the position of the fucosylated oligosaccharide was critical for secretion into the bile. In other words, fucosylated glycoproteins might be secreted into the serum when the position of fucosylation is not readily recognized by a cargo receptor if it exists. Serum AGP was scarcely bound to the AOL lectin although the levels of fucosylation of serum AGP were relatively high compared with AAT and Hp (Fig. 5). The difference between the binding of biliary and serum AGP in AOL lectin affinity chromatography supports the hypothesis that site-specific fucosylation is also important in a sorting system. The route and regulation of intracellular trafficking of fucosylated glycoproteins are interesting issues for a subsequent study. There are few reports of vesicular transport pathways of soluble secretory proteins in hepatocytes (24). It has been reported that the intracellular traffic of vesicles is microtubule dependent. Secretory proteins in these vesicles could not interact with cytoplasmic carrier proteins on microtubules. Therefore, it is thought that secretory proteins interact with cytoplasmic car-
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Carrier proteins via cargo receptors present as transmembrane proteins. The present study suggests that there is a possible receptor containing a lectin domain that interacts with fucosylated oligosaccharides and the receptor could control sorting to the apical surface followed by secretion into bile ducts. It has been demonstrated that intracellular lectin-like proteins such as mannose-6-phosphate receptor, calnexin, calreticulin, endoplasmic reticulum–Golgi intermediate compartment-53, and vesicular integral membrane protein 36 play important roles in intracellular vesicular transport (25, 26). However, there have been few reports concerning lectins associated with polarized vesicular transport from the Golgi to the plasma membrane (27). Therefore, an investigation of the intracellular route of the receptor would be important in elucidating the intracellular pathway of soluble secretory proteins in hepatocytes.

It was reported that glycopeptides isolated from human pancreatic juice, secreted from the apical surface of pancreas cells, contained a high level of fucosylated oligosaccharides (28). This report suggested that the sorting machinery in terms of fucosylation of glycoproteins might be located in the pancreas.

The biological functions of the sorting machinery through fucosylation will need to be elucidated in larger studies. It was reported that human bile contains several cholesterol crystallization-promoting glycoproteins and that biliary Hp and AGP have highly potent cholesterol crystallization-promoting activity at physiological concentrations (29, 30). However, these promoters were identified from a ConA-bound fraction (31), and a correlation of its activity with fucosylation of glycoproteins has not been reported. Further studies will be required to determine whether fucosylated oligosaccharides on glycoproteins are associated with biological functions in bile as well as intracellular traffic in hepatocytes.

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