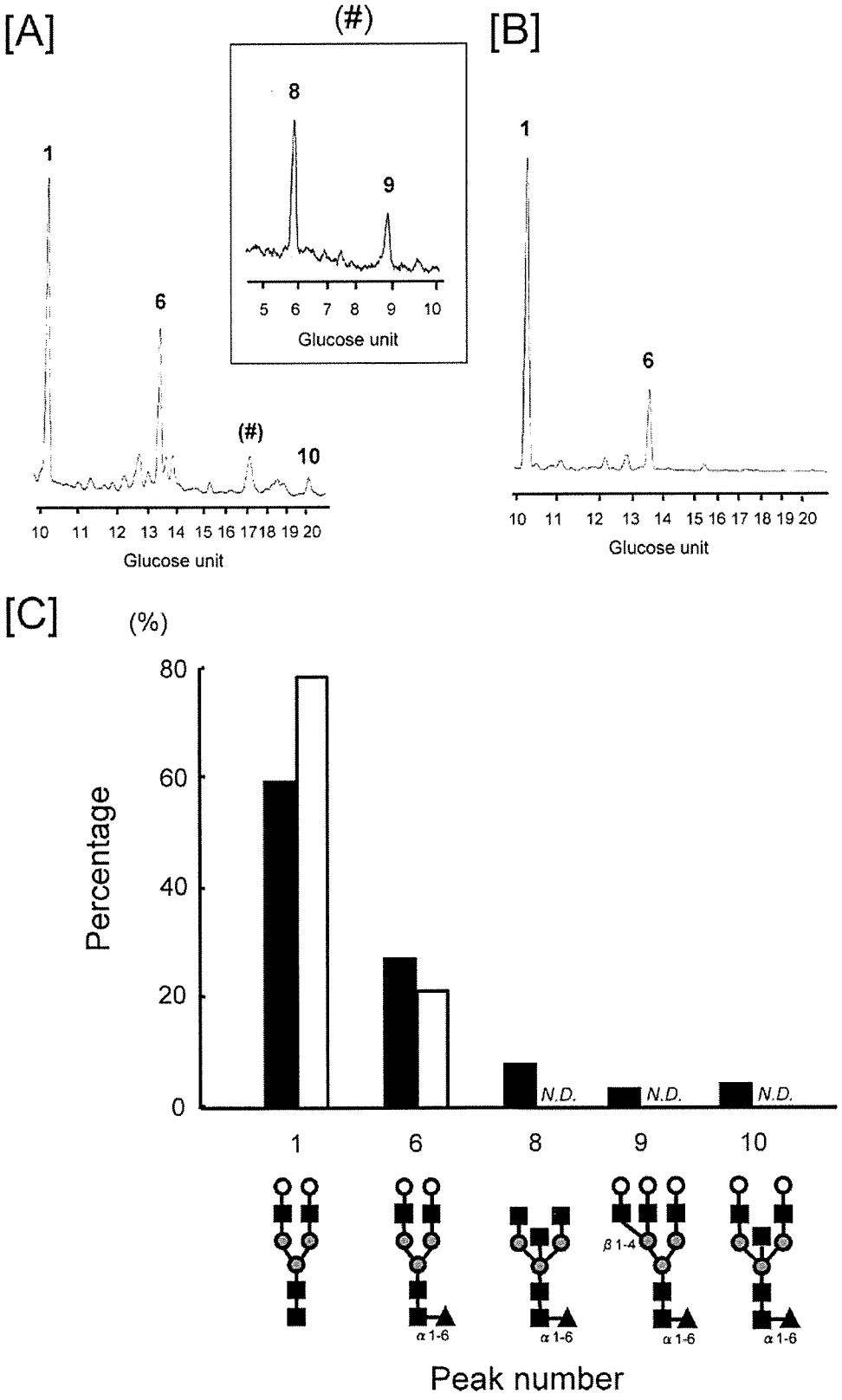

FIGURE 6. **HPLC separation of PA oligosaccharides from mouse biliary and serum glycoproteins.** Representative elution profile of PA oligosaccharides from mouse gallbladder bile (*A*) and serum (*B*) on an ODS column. The chart in the *frame* indicates the elution profile on an Amido-80 column of the 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 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.

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

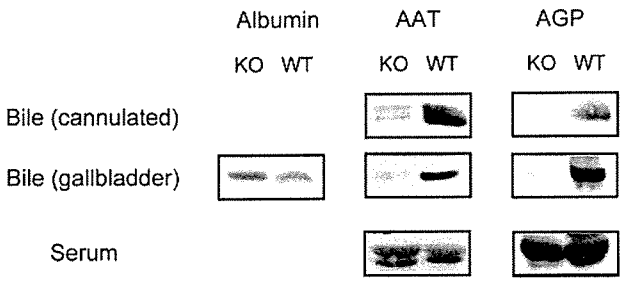

FIGURE 7. **Comparison of protein composition between wild-type and Fut8-deficient mice.** Cannulated bile (10 μl), 5 μg of protein from gallbladder bile, and serum (0.5 μl) were subjected to 12% SDS-PAGE, and Western blot analyses of albumin, AAT, and AGP were performed. *KO* and *WT* indicate Fut8-deficient and wild-type mice, respectively. Detailed procedures are described under "Experimental Procedures."

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. 1*B*). In contrast, both biliary and serum glycoproteins are weakly bound to UEA-I (*Ulex europaeus*) and Lotus (*Lotus tetragonolobus*) 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-deficinet 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- and 25-fold higher than those in the bile, respectively.

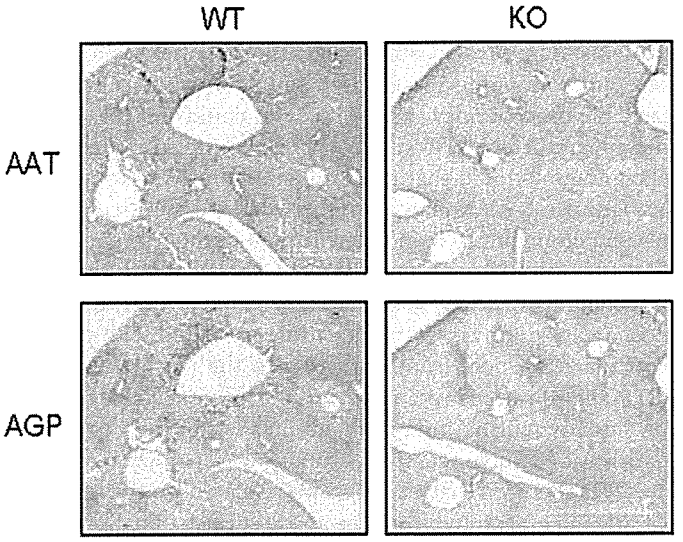

FIGURE 8. **Immunohistochemical study of Fut8-deficient mice.** Liver sections of wild-type and Fut8-deficient mice were stained with goat anti-human AAT and rabbit anti-AGP antibodies. *WT* and *KO* indicate wild-type and Fut8-deficient mice, respectively. Detailed procedures are described under "Experimental Procedures."

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-

rier 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 fucosylated oligosaccharides 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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