Targeted inactivation of sister of P-glycoprotein gene (spgp) in mice results in nonprogressive but persistent intrahepatic cholestasis


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Mutations in the sister of P-glycoprotein (Spgp) or bile salt export pump (BSEP) are associated with Progressive Familial Intrahepatic Cholestasis (PFIC2). Spgp is predominantly expressed in the canalicular membranes of liver. Consistent with in vitro evidence demonstrating the involvement of Spgp in bile salt transport, PFIC2 patients secrete less than 1% of biliary bile salts compared with normal infants. The disease rapidly progresses to hepatic failure requiring liver transplantation before adolescence. In this study, we show that the knockout of spgp gene in mice results in intrahepatic cholestasis, but with significantly less severity than PFIC2 in humans. Some unexpected characteristics are observed. Notably, although the secretion of cholic acid in mutant mice is greatly reduced (6% of wild-type), total bile salt output in mutant mice is about 30% of wild-type. Also, secretion of an unexpectedly large amount of tetra-hydroxylated bile acids (not detected in wild-type) is observed. These results suggest that hydroxylation and an alternative canalicular transport mechanism for bile acids compensate for the absence of Spgp function and protect the mutant mice from severe cholestatic damage. In addition, the spgp−/− mice display a significant increase in the secretion of cholesteryl and phospholipids into the bile. This latter observation in spgp−/− mice suggests that intrahepatic, rather than intracanalicular, bile salts are the major driving force for the biliary lipid secretion. The spgp−/− mice thus provide a unique model for gaining new insights into therapeutic intervention for intrahepatic cholestasis and understanding mechanisms associated with lipid homeostasis.

Bile acids are critical as carriers for elimination of cholesterol from the body through biliary secretion and as a detergent for the ingestion of fatty acids and fat-soluble vitamins (1). They also play important roles in regulating cell apoptosis/survival (2–6) and in regulating gene expression through the farnesoid X-activated receptor (7–12) in hepatocytes. Bile acids are synthesized in hepatocytes from cholesterol, secreted into the bile after being conjugated with glycine or taurine, reabsorbed in the small intestine, and recirculated back to hepatocytes through the portal vein. Canalicular secretion of bile acids from liver into the bile is a key process in the enterohepatic circulation of bile acids and its malfunction results in different hepatic diseases (1). Canalicular bile salt transport appears to be mediated by the sister of P-glycoprotein (Spgp) (13) also known as the bile salt export pump (BSEP) (14).

Spgp is a canalicular-specific ATP binding cassette (ABC) transporter and a close relative of the multidrug-resistant P-glycoprotein (mdr) (15, 16). Expression of the rat spgp gene in Xenopus laevis oocytes results in a 1.5-fold higher [3H]taurocholate efflux and the reconstituted membrane vesicles from Spgp-transfected s9 insect cells exhibits 5-fold stimulation of ATP-dependent taurocholate transport, with a K_m of 5.3 μM, consistent with Spgp being a bile acid transporter (14). Mutations in the spgp gene have been associated with a severe human genetic disease, type 2 Progressive Familial Intrahepatic Cholestasis (PFIC2) (17). Total bile acid secretion in PFIC2 patients is only 1% of normal, and Spgp was not detected in these patients by immunostaining (18). In mice, spgp or a nearby locus may be associated with cholesterol gallstone susceptibility (19). All of the above evidence agrees with Spgp being a canalicular bile acid transporter.

To further investigate the role Spgp plays in biliary secretion of bile acids and to understand how spgp deficiency results in PFIC2 and affects bile formation and cholesterol homeostasis, we have generated a knockout mice model by using homologous recombination. This study provides insights into the role Spgp plays in regulating bile salts and lipid transport in these mutant mice.

Materials and Methods

Generation of spgp−/− Mutant Mice. Two different ES cell lines were used. TL-1 embryonic stem cells and E14K embryonic stem cells from 129Sv/Ev mice were generous gifts from Dr. Patricia Labosky (University of Pennsylvania, Philadelphia) and Dr. Tak Mak (Amgen Institute, Toronto), respectively. Genomic DNA of murine spgp gene was isolated from a λ phage library of 129/J genomic DNA by using rat spgp cDNA as probe (15). One of the obtained genomic clones, 129J-9, and an additional fragment of 1.6 kb on the 5’ end of 129J-9, which was cloned by PCR amplification, were used to generate the targeting vector (Fig. 1A). The targeting vector was linearized with SsrI and electroporated into TL-1 and E14k embryonic stem cells at 340 V and 250 μF of capacitance. ES cell clones surviving G418 selection were screened by Southern blot analysis. Four of the 11 targeted ES cell lines were subsequently used to produce chimeric mice and chimeric mice from three lines with germline transmission were used in this study. The heterozygous mutant mice from each generation were crossed into C57BL/6J. Homozygous mutant mice were produced by intercross of the heterozygous mice. Genotypes of the ES cells and mice were determined by Southern blot analysis using a 1-kb probe upstream of the 5’ end.
of the short arm of the targeting vector (Fig. 1A and B). The expression level of the spgp gene was determined by Northern and Western blot analysis using standard methods (16). Anti-serum IW is specific for Spgp, whereas monoclonal antibody C219 detects predominantly Pgp, but will also detect Spgp (15, 16). Both Pgp and Spgp migrate with a similar molecular size.

Adult mice from generation three to four (2 to 6 months of age) were used in this study.

Bile Duct Cannulation and Collection of Bile. Animal surgery was performed by using the approved protocols of the Committee on Animal Care, University of British Columbia, according to the guidelines of the Canadian Council on Animal Care. Mice were killed by i.p. injection of Ketamine (112.5 mg/kg) and Xylazine (11.3 mg/kg) after 2 hours of fasting. The abdomen was opened, and the gall bladder was cannulated after distal common bile duct ligation (20). Bile was collected in 15 min intervals for a total of 30 min, followed by injection of 3.5 μmol/100 g body weight of [14C]taurocholate as a bolus into the jugular vein. Bile was then further collected through the cannula for another 30 min.

Transmission Electron Microscopy. Mice were killed and their livers perfusion fixed in situ by using 2.5% glutaraldehyde and post-fixed in 1% OsO4. Dehydration and embedding were performed as described in ref. 21. Sections were stained with uranyl acetate and examined by using a Philips EM400T transmission electron microscope (Eindhoven, The Netherlands).

Bile Acid Determinations. Liquid chromatography–electrospray tandem mass spectrometry (LC/MS/MS) and gas chromatography–mass spectrometry (GC/MS) were used for the identification and determination of bile acids in bile, plasma, and liver tissues. Briefly, bile acids were extracted with a C18 (octadecyl) reversed-phase column. For LC/MS/MS, bile acids were analyzed by simultaneous monitoring of parent and daughter ions for the identification of glycine and taurine conjugates (22). Identification and quantification of conjugated bile acids was achieved in 5 min. The detection limit was 1 ng and the determination was linear up to 100 ng. For GC/MS, conjugated bile acids were hydrolyzed in 2.5 M NaOH at 160°C overnight. Bile acids were then extracted, methylated, and acetylated. Identification and quantification of bile acids were achieved by GC/MS using a Hewlett-Packard 5896 gas chromatograph equipped with a Hewlett-Packard 5971A mass selective detector (MSD) in selected ion-monitoring mode (SIM). Quantification was carried out by using a correction factor obtained by using 5β-cholanic acid as internal standard.

Biliary Phospholipid and Cholesterol Determinations. Biliary lipids were extracted as described by Folch et al. (23). Phospholipids were determined enzymatically with phospholipase D and cholesterol oxidase by using a kit supplied by Wako Chemicals (Rich-
Bile acids concentration in plasma, mm Biliary cholesterol, spgp changes in hepatocytes of electroporation with the targeting vector and G418 selection, 11 ATP-binding domain of Spgp (Fig. 1 containing the coding region of Walker A of the N-terminal the spgp head), and retained biliary material in the form of lamellar and more mem-
mond, VA). Cholesterol was determined by GC/MS as described above.

Results

Inactivation of the spgp Gene in Mice. A vector for inactivation of the spgp gene was constructed by deleting a 1.4-kb fragment containing the coding region of Walker A of the N-terminal ATP-binding domain of Spgp (Fig. 1A, aa454–478). After electroporation with the targeting vector and G418 selection, 11 ES colonies were isolated by Southern blot screening. Four of the 11 targeted ES cell lines were subsequently introduced into pseudopregnant foster mother to produce chimeric mice. Germ-line transmission of the targeted mutation on spgp gene was achieved in chimeric mice from three of the four injected lines. The heterozygous mutant mice were crossed into C57Black/6J for each generation to eliminate genetic diversity, and the homozygotes were obtained by back-crossing of heterozygotes. Inactivation of the spgp gene resulting in spgp−/− mice (Fig. 1A) was confirmed by the absence of detectable Spgp mRNA and protein product (Fig. 1C and D).

Analysis of spgp−/− Mice. The spgp−/− mice are viable and fertile, but displayed growth retardation. The body weight of spgp−/− mice is about 20% lower than that of the wild-type littermates at weaning (21 days after birth). They tended to have a lower body weight throughout their life, but no advanced cholestasis was observed up to 1 year of age. Indicators of liver function such as gamma-glutamyl transferase, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, 5′-nucleotidase, plasma albumin, and bilirubin showed no significant change in spgp−/− mice compared with wild-type mice (data not shown).

Ultrastructural changes of the canaliculi were observed, including dilation of canicular lumens, partial or complete loss of microvilli, and retained biliary material in the form of lamellar whorls or more membranous appearing “fingerprints” in canicular lumens (Fig. 2A). There were also hepatocyte cytoplasmic changes, including increases in peroxisomes, lysosomes, and lipid droplets, and a decrease in glycogen content (Fig. 2B). The liver of spgp−/− mice was enlarged to 10.1% of the body weight (vs. 4.7% in wild-type; Table 1). Overall, however, the spgp−/− mice did not exhibit signs of overt cholestasis (1, 24–27).

Table 1. Comparison of the spgp−/− and wild-type mice

<table>
<thead>
<tr>
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<th>Wild type</th>
<th>spgp−/−</th>
<th>Significance†</th>
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<tbody>
<tr>
<td>Body weight at weaning, g</td>
<td>10.40 ± 0.67 (5)</td>
<td>8.16 ± 0.72 (6)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Liver weight, g/100g BW</td>
<td>4.72 ± 0.47 (4)</td>
<td>10.14 ± 0.5 (3)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Bile flow rate μl/min/100g BW</td>
<td>8.33 ± 2.71 (3)</td>
<td>6.85 ± 2.58 (3)</td>
<td>P = 0.53</td>
</tr>
<tr>
<td>Bile salt output, nmol/min/100g BW</td>
<td>249.92 ± 80.90 (3)</td>
<td>84.62 ± 30.41 (5)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Bile acids concentration in bile, mM</td>
<td>32.33 ± 7.66 (3)</td>
<td>7.60 ± 4.58 (3)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>1.16 ± 0.49</td>
<td>ND</td>
<td>Significant</td>
</tr>
<tr>
<td>Chenodeoxycholate</td>
<td>0.49 ± 0.12</td>
<td>0.11 ± 0.12</td>
<td>P &lt; 0.02</td>
</tr>
<tr>
<td>Cholate</td>
<td>21.56 ± 6.31</td>
<td>1.26 ± 1.05</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Ursodeoxycholate</td>
<td>0.51 ± 0.54</td>
<td>0.06 ± 0.08</td>
<td>P = 0.23</td>
</tr>
<tr>
<td>Muricholate (alpha)</td>
<td>0.96 ± 0.37</td>
<td>0.10 ± 0.04</td>
<td>P = 0.02</td>
</tr>
<tr>
<td>Muricholate (omega)</td>
<td>2.50 ± 0.48</td>
<td>1.57 ± 1.27</td>
<td>P = 0.30</td>
</tr>
<tr>
<td>Muricholate (beta)</td>
<td>5.15 ± 4.18</td>
<td>3.16 ± 2.62</td>
<td>P = 0.52</td>
</tr>
<tr>
<td>Tetrahydroxy</td>
<td>ND</td>
<td>1.37 ± 0.84</td>
<td>Significant</td>
</tr>
<tr>
<td>Bile acids concentration in liver, μM</td>
<td>40.07 ± 10.30 (3)</td>
<td>232.06 ± 105.07 (3)</td>
<td>P &lt; 0.04</td>
</tr>
<tr>
<td>Bile acids concentration in plasma, μM</td>
<td>7.22 ± 1.93 (4)</td>
<td>29.43 ± 24.96 (5)</td>
<td>P &lt; 0.11</td>
</tr>
<tr>
<td>Biliary phospholipids, μM</td>
<td>6.17 ± 3.60 (5)</td>
<td>15.26 ± 7.06 (3)</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>nmole/min/100g BW</td>
<td>46.27 ± 26.98 (5)</td>
<td>128.16 ± 59.30 (3)</td>
<td>P &lt; 0.03</td>
</tr>
<tr>
<td>Biliary cholesterol, μM</td>
<td>0.49 ± 0.02 (5)</td>
<td>3.45 ± 1.71 (3)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>nmole/min/100g BW</td>
<td>3.69 ± 1.98 (5)</td>
<td>28.97 ± 14.4 (3)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Bile acids/phospholipids ratio*</td>
<td>8.58 ± 3.72 (3)</td>
<td>1.48 ± 0.15 (3)</td>
<td>P &lt; 0.03</td>
</tr>
<tr>
<td>Bile acids/cholesterol ratio*</td>
<td>99.96 ± 36.02 (3)</td>
<td>6.62 ± 2.01 (3)</td>
<td>P &lt; 0.01</td>
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Body weights were obtained from mice at weaning. All other numbers are from male adult mice. All numbers are expressed as mean ± SD (n). ND, not detectable; BW, body weight.
*The bile acids:lipids ratio is the average of the ratio of bile salt output to lipids output for three animals.
†The statistical significance of differences between spgp−/− and wild-type mice was assessed using Student’s two-tailed t test.
biliary bile salts in the spgp
cholestasis patients (30). Increased hydroxylation in rodents has
and transformed into tri- or tetra-hydroxylated bile acids in
nodeoxycholic, cholic, and deoxycholic acids can be hydroxylated
presence of tetra-hydroxylated bile salts in human cholestatic
lished results). These data indicate a dramatic impairment in
distributed in
the jugular vein. Thirty minutes after infusion, radioactivity was
determined. Results are expressed as percent of total radiolabeled material infused
metabolism exist in the mutant mice, we identified and quanti-
and represent means ± SD from three animals of each genotype. Similar
results were obtained with labeled glycocholate.

The secretion of cholesterol and phospholipids is not understood
promote secretion of biliary phospholipids and cholesterol (1, 37,
different from that for bile salts (14, 20, 37). However it is well
indicated.

Tetra-Hydroxylated Bile Acids. We detected an unusually large
amount of tetra-hydroxylated bile acids (18% of total biliary bile
acids) in the spgp<sup>−/−</sup> mice that were not detected in wild-type
trols. This was confirmed with tandem mass spectrometry
(LC/MS/MS), which showed that the tetra-hydroxylated bile
salt was present mainly as a taurine conjugate with a fragment
ion at m/z 530 (Fig. 4). Several investigators have reported the
presence of tetra-hydroxylated bile salts in human cholestatic
diseases (28–34). Furthermore, it was demonstrated that che-
nodeoxycholic, cholic, and deoxycholic acids can be hydroxylated
and transformed into tri- or tetra-hydroxylated bile acids in
cholestasis patients (30). Increased hydroxylation in rodents has
also been observed following administration of toxic mono-
hydroxylated bile salts (34) and after bile duct ligation (35, 36).
Thus, the hydroxylation of bile salts in spgp<sup>−/−</sup> mice is consistent with a cholestatic phenotype. However, the amount of tetra-
hydroxylated bile salts secreted into the bile was substantial, and
this may help to modulate the severity of cholestasis and may
explain why the bile flow rate was only slightly reduced in spgp<sup>−/−</sup>
mice.

Because of a lack of standards for tetrahydroxylated bile acids,
the structure of the tetrahydroxylated bile acid species observed
in spgp<sup>−/−</sup> mice can only be postulated. However, analysis using
ionization GC/MS confirms the presence of four hydroxyl
groups and suggests that the structure of the tetrahydroxylated bile
acid is predominantly 3α,6β,7α,12α-tetrahydroxy-5β-cholanic acid (unpublished observation).

Lipid Composition of Bile in spgp<sup>−/−</sup> Mice. The secretion of chole-
sterol and phospholipids into the bile is mediated by a system(s)
different from that for bile salts (14, 20, 37). However it is well
established that bile salts, especially hydrophobic bile salts,
cause secretion of biliary phospholipids and cholesterol (1, 37,
38). The mechanisms by which the bile salts induce biliary
secretion of phospholipids and cholesterol is not understood
(39). We analyzed biliary lipids in the spgp<sup>−/−</sup> mice to examine
whether the biliary secretion of cholesterol and phospholipids
was affected. The spgp<sup>−/−</sup> mice displayed a marked increase in
biliary cholesterol (7-fold) and phospholipids (2.5-fold), poss-
sessed a more hydrophilic muriatic bile acids, such as taurine and glycine-conjugated cholic
acid, increased in the
hepatic accumulation of bile salts (Table 1, Fig. 4). Furthermore,
the disruption of the spgp gene in mice
appears to greatly affect the homeostasis of cholesterol and phospholipids.

Discussion

Sppg is considered to be the major bile salt transporter in the
canalculus (14, 40); and genetic defect of spgp gene in humans
results in very severe progressive cholestasis (17, 18). This study
shows that canalicular secretion of bile salts, especially conjugated cholic acid in spgp /−/− mice, is severely impaired. The surprising finding in this study is that the spgp /−/− mice display only mild nonprogressive cholestasis. A less severe phenotype in spgp /−/− mice compared with human PFIC2 is likely because of another canalicular bile salt transport system (as yet unidentified) that allows for the secretion of significant amounts of bile salts. The spgp /−/− mice thus provide a unique model system to investigate the pathogenesis of intrahepatic cholestasis and other related liver diseases; moreover, it may be a useful system for exploring potential approaches for therapeutic intervention for these diseases.

In the spgp /−/− mice, biliary secretion of conjugated cholic acid and some di-hydroxyl bile salts was greatly reduced, but secretion of the more hydrophilic bile salts such as the muricholates were affected. Also, a significant amount of another hydrophilic bile acid, tetrahydroxylated bile acid, appeared (Table 1 and Fig. 4). These observations imply that (i) Spgp mediates the secretion of conjugated cholic acids and other hydrophobic bile acids. This is in line with previous publications demonstrating that Spgp is a transporter of taurocholates and other bile salts in transfected systems (14, 40); and (ii) there is an alternative bile salt transport system in the canaliculus for the more hydrophilic bile salts. Rodents have a more efficient hydroxylation/detoxification mechanism in the liver (36), and the bile acid pool in mice is more hydroxylated, less hydrophobic, and less toxic (32, 41, 42). Thus in mice, elevated hydroxylation of bile salts may serve not only as a detoxification mechanism of hydrophobic bile salts, but may also allow for their clearance via the predicted alternative transport system. It is yet to be discovered whether a similar system for the transport of hydrophilic bile salts exists in humans.

In addition to Spgp, other proteins, such as FIC1 (43, 44), MRP3 (45–47), and co-cto-ATPase (48, 49), have also been proposed as potential bile acid transporters in the canaliculus. Some of these may be responsible for this alternative transport of hydrophilic bile acids. The spgp /−/− mice should be a useful system for investigating this possibility.

Whereas biliary secretion of bile salts was greatly reduced in the spgp /−/− mice, the secretion of phospholipids and cholesterol into the bile was significantly elevated (Table 1). Secretion of phospholipids and cholesterol usually correlates positively with changes in the amount of bile salts secreted in vivo (37, 39), and it is commonly accepted that bile salts drive the amount of phospholipids and cholesterol secreted into the bile. Whether or not this is accomplished at the intracanalicular level or the intrahepatic level is not well defined (37–39). The spgp /−/− mice allow us to differentiate between these two possibilities. In these mice, the increased biliary lipid secretion is observed under conditions where bile salt is reduced but intrahepatic bile salt is increased. We therefore propose that the intrahepatic site is the location for the bile salt stimulation of biliary lipid secretion.

An increased accumulation of intrahepatic bile salts could also explain the greatly increased cholesterol:phospholipid ratio in the bile of spgp /−/− mice (Table 1). Bile acid synthesis from cholesterol accounts for about 50% of cholesterol eliminated from the body (50). Accumulation of hepatic bile acids resulting from a loss of Spgp would very likely inhibit bile acid biosynthesis (8, 9) and could lead to the accumulation of cholesterol in hepatocytes. This, in turn, may stimulate elevated biliary secretion of cholesterol. Hence, the observed higher cholesterol:phospholipid ratio in the bile of spgp /−/− mice may reflect the need for cholesterol clearance. It is noteworthy that the Lith1 locus of cholesterol gallstone susceptibility in mice has been colocalized with the spgp locus (19). A higher cholesterol secretion in the spgp /−/− mice would be consistent with the suggestion that the spgp gene is the Lith1 site. The spgp /−/− mice are therefore an excellent model for investigating lipid secretion and understanding lipid homeostasis.

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