Degradation of the Apical Sodium-dependent Bile Acid Transporter by the Ubiquitin-Proteasome Pathway in Cholangiocytes*

Received for publication, January 28, 2004, and in revised form, August 9, 2004
Published, JBC Papers in Press, August 10, 2004, DOI 10.1074/jbc.M400969200

Xuefeng Xia, Marlon Roundtree, Afkhamoressadat Merikhi, Xiaohui Lu, Shujun Shentu, and Gene LeSage‡
From the Division of Gastroenterology, Hepatology and Nutrition, Department of Internal Medicine, University of Texas at Houston Medical School, Houston, Texas 77030

To attenuate injury during cholestasis, adaptive changes in bile acid transporter expression in the liver provide alternative bile acid excretory pathways. Apical sodium-dependent bile acid transporter (ASBT) (SLC10A2), only expressed in the liver on the cholangiocyte apical membrane, is rapidly regulated in response to inflammation and bile acids. Here, we studied the mechanisms controlling ASBT protein levels in cholangiocytes to determine whether ASBT expression is regulated by ubiquitination and disposal through the proteasome. Protein turnover assays demonstrated that ASBT is an unstable and short-lived protein. Treatment with MG-132, a proteasome inhibitor, causes time-dependent increased ASBT levels and increased intracellular accumulation of ASBT. In cells cotransfected with green fluorescent protein-tagged ASBT and hemagglutinin-tagged ubiquitin, we demonstrated coimmunoprecipitation and colocalization of ASBT and ubiquitin. Interleukin-1β (IL-1β) induced down-regulation of ASBT is abrogated by a JNK inhibitor and is accompanied by an increase in ASBT polyubiquitin conjugates and a reduced ASBT half-life. In phosphorylation-deficient S335A and T339A mutants, the ASBT half-life is markedly prolonged, IL-1β-induced ASBT ubiquitination is significantly reduced, and IL-1β fails to increase ASBT turnover. These results indicate that ASBT undergoes ubiquitin-proteasome degradation under basal conditions and that ASBT proteasome disposal is increased by IL-1β due to JNK-regulated serine/threonine phosphorylation of ASBT protein at both Ser-335 and Thr-339. These studies are the first report of regulation of a bile acid transporter expression by the ubiquitin-proteasome pathway.

ASBT mediates bile acid absorption from the lumen of the terminal ileum, renal tubules, and bile ducts (1–3). Bile acids are the major products of cholesterol catabolism and function in the processes of bile secretion, intestinal absorption of lipids and lipid-soluble nutrients, and cholesterol elimination from the body (4). ASBT plays a key role in the recovery of bile acids from the intestinal lumen or renal tubules to prevent loss of bile acids via stool and urine, respectively (2, 5). The role of ASBT in bile ducts is less well known. ASBT may function primarily following liver injury since the recently described ASBT knockout mouse (6) is grossly indistinguishable from wild-type mice, yet ASBT is up-regulated in liver injury due to bile duct ligation (7). ASBT, expressed on the apical membrane of cholangiocytes, is poised to absorb bile acids from ductal bile. Absorbed bile acid molecules are returned via the peribiliary plexus to the hepatic sinusoids, again removed by hepatocytes to be secreted into bile (8). We have proposed that the exchange of bile acids between cholangiocytes and hepatocytes (termed cholehepatic shunting) potentially prevents bile acid-induced liver injury due to extrahepatic biliary obstruction by maintaining bile acid flux and preventing intracellular bile acid accumulation in the liver (9). Recently, the inflammatory cytokine IL-1β has been shown to down-regulate ASBT in the terminal ileum (10). Dysregulation of the ASBT adaptation to cholestasis (due to increased expression of IL-1β) could blunt the compensatory up-regulation of ASBT in response to cholestasis and promote bile acid-induced liver damage (9).

Recent data demonstrate that the ubiquitin-proteasome degradation system affects the activity of some membrane transporters (11–16). The system is responsible for the disposal of many of the short-lived proteins in eukaryotic cells (17–19). The ubiquitin-proteasome pathway targets proteins for degradation via covalent tagging of the substrate protein with a polyubiquitin chain (20). This degradation pathway is implicated in the regulation of many short-lived proteins involved in essential cellular functions, including cell cycle control, transcription regulation, signal transduction, and protein translocation (17, 21–24). The proteins degraded by this pathway are covalently modified on lysine residues by fixation of a 8-kDa polypeptide, called ubiquitin, in a three-step process (18). In the first step, ubiquitin is activated by an ubiquitin-activating enzyme. The activated ubiquitin is subsequently transferred to an ubiquitin carrier protein. Finally, ubiquitin-protein ligase catalyzes the covalent binding of ubiquitin to the target protein. Following this process, multiubiquitinated proteins are rapidly degraded by the 26 S proteasome.

We speculated that the initial ASBT down-regulation due to ileal inflammation or due to IL-1β in vitro (10) is caused by enhanced ASBT disposal by the ubiquitin-proteasome pathway. Here we present results demonstrating that ASBT is an unstable protein that is rapidly degraded. Moreover, we show that the rapid IL-1β-dependent reduction of ASBT in chol-
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giocytes is due to increased ASBT disposal through the ubiquitin-proteasome pathway. IL-1β-mediated down-regulation of ASBT expression requires phosphorylation of ASBT since mutation of two ASBT phosphorylation sites motifs reduces the rate of ASBT disposal under basal conditions and markedly reduces IL-1β-dependent ubiquitination and disposal of ASBT. These results indicate that the proteasome plays an important role in the regulation of ASBT protein level.

EXPERIMENTAL PROCEDURES

Materials and Plasmids—Dulbecco’s modified Eagle’s medium was purchased from Invitrogen. Fetal bovine serum was obtained from Sigma. IL-1β was purchased from R&D Systems Inc. (Minneapolis, MN), and ubiquitin antibody, MG-132, phenylmethylsulfonyl fluoride, N-acetyl-leu-leu-norleucine (ALLN), lactacystin, TPCK, TLCK, aprotinin, SP600125, SB203580, and U0126 were purchased from Calbiochem. β-actin monoclonal antibodies, cycloheximide (CHX), and routine research reagents were purchased from Sigma. The green fluorescent protein (GFP) and hemagglutinin (HA) mouse monoclonal antibodies were from Santa Cruz Biotechnology Inc. ASBT antisera was a gift from Dr. Paul Dawson. Wild-type rat ASBT cDNA was subcloned into a GFP vector, pEGFPN2 (gift from Dr. An-Qiang Sun, Mount Sinai School of Medicine, New York, NY). The pME18S-FLAG-tagged ASBT was constructed as follows. pME18S-FLAG-ASBT was generated by subcloning the ASBT cDNA into the EcoRI/XhoI sites of pME18s-FLAG plasmid. ASBT construct was assembled by PCR using the following synthetic oligonucleotides as primers: 5′-ATCTGGAGATCCGATACT-3′, 5′-CAATACGTTGAAATCCCTTGTTTG-3′, 5′-CCTCCGTCTGTTCCCCAAATGC-3′, and 5′-TTCTCATCTGGTTGAAATCCCTTGTTTG-3′ for pME18s-FLAG-ASBT. The PCR products were inserted into the EcoRI/XhoI sites of pME18S-FLAG plasmid.

Site-directed Mutagenesis—ASBT-S335A, ASBT-T339A, and double mutant (STIA/AA) were made using rat ASBT-GFP as template. The QuikChange™site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturer’s directions for potential changes in alanine residues according to the manufacturer’s directions with minor modifications as described previously (25). The positive clones were verified by DNA sequencing.

Cell Culture and Transfection—Mz-ChA-1 cholangiocarcinoma cell line (human gall bladder in origin) was a gift from Dr. Fitz (University of Amsterdam). Cells were cultured in complete Dulbecco’smodified Eagle’s medium (containing 10% (v/v) fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mm l-glutamine). Transient DNA transfection was carried out by LipofectAMINE 2000-mediated transfection (Invitrogen) according to the manufacturer’s directions. Briefly, 1 × 10⁶ cells were plated on a 6-well plate. The next day, cells were ~70% confluent and were transfected with 2 μg of DNA. On the following day, transfected Mz-ChA-1 cells were recovered in complete medium, and the transfected cell lines were selected by growth in the antibiotic G418 (1 mg/ml) (Invitrogen). 10–15 days after transfection, stable cell lines were obtained by subcloning individual colonies. Positive clones were selected by immunofluorescence microscopy, and expression was subsequently tested by Western blotting. All of the cells were maintained in a humidified incubator at 37 °C under 5% CO₂ atmosphere.

The HEK 293 cells, a human embryonic kidney cell line, were maintained in Dulbecco’s modified Eagle’s medium/nutrient mixture F12 (supplemented with 10% fetal calf serum), 1% penicillin/streptomycin, and 1% glucose and incubated at 37 °C under 5% CO₂. The HEK 293 cells were plated in 6-well culture dishes for Western blotting. 24 h after plating, HEK 293 cells were cotransfected with expression plasmids by the calcium phosphate coprecipitation method. Briefly, 200 μl of 300 mM CaCl2, 200 μl of 2× Hepes-buffered saline and a total 4 μg of plasmid were mixed gently and then added to the culture medium in 6-well dishes. Transfection efficiency into HEK 293 cells with calcium phosphate coprecipitation was very high, and up to 50–80% of cells were successfully transfected. Normal rat cholangiocytes (NRC) were isolated and cultured as we described previously (26).

Western Blot Analysis—Cells were rinsed with ice-cold PBS and resuspended into the lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and protease inhibitor mixture (Roche Applied Science). Following a brief sonication, cell extracts were centrifuged to remove cell debris. Protein concentration was measured by the method of Bradford with bovine serum albumin as a standard. Cell lysates (20 μg) were then separated through 10% SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was incubated for 1 h at room temperature in Tris-buffered saline/Tween 20 (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) with 5% nonfat milk. The membrane was then incubated with anti-GFP antibody (1:500) for 1 h in Tris-buffered saline/Tween 20. The detection system was horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). Specific binding of the antibody was visualized by the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) according to the manufacturer’s instructions. The intensity of the bands was determined by scanning video densitometry using the ChemiImager™ or 5500 low light imaging system (Alpha Innotech Corp., San Leandro, CA).

Immunoprecipitation—Cells were lysed at intervals using Nonidet P-40 lysis buffer containing 50 mM Tris-Cl, pH 7.8, 150 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, protease inhibitor mixture, 1 mM benzamidine, 5 mM sodium orthovanadate, and 5 mM sodium fluoride. HA and GFP monoclonal antibodies were added to 500 μg of lysates and incubated at 4 °C for 3 h. Protein G plus agarose beads (Santa Cruz Biotechnology) were added to the antigen-antibody mixture and further incubated overnight at 4 °C. Beads were washed thoroughly (four times) with phosphate-buffered saline containing 0.1% Nonidet P-40 and 0.1% SDS and subjected to Western blot analysis using anti-HA or anti-GFP monoclonal antibody.

ASBT Turnover Assays—For half-life analysis, Mz-ChA-1 cells stably expressing ASBT-GFP were treated with CHX. Mz-ChA-1 cells expressing ASBT-GFP were plated in 6-well culture dishes. Orid inde cultured near confluence (90–100%) were treated with 100 μg/ml CHX to block protein synthesis. Cells were harvested in lysis buffer at the indicated time points. Protein levels were then examined by Western blotting for GFP significance as described above.

Immunofluorescence—ASBT-GFP and HA-ubiquitin coexpression was assessed via indirect immunofluorescence. Mz-ChA-1 cells were seeded in 5% PBS and Dulbecco’s modified Eagle’s medium:Hams F-12 on Lab-Tek Chamber slides (Nalge Nunc International). Slides were then washed with PBS, fixed in −20 °C methanol for 10 min, air-dried, and washed in 0.3% Tween/PBS for 5 min. Slides were blocked with 1% normal sheep serum in 1× antibody dilution buffer (1× bovine serum albumin in 0.3%Tween/PBS) for 1 h and incubated with 1:200 HA antibody overnight at 4 °C in a humid chamber to prevent evaporation of the antibody solution. After washing in 0.3% Tween/PBS for 10 min (3×), slides were reprobed with goat anti-mouse Alexa 594 (1:500; Molecular Probes, Eugene, OR) for 2 h at room temperature in a dark humid chamber and then washed in 0.3% Tween/PBS in 1× PBS for subsequent washing steps. Slides were stained with 4,6-diamidino-2-phenylindole, mounted with Gel/Mount (Biomeda, Foster City, CA) and visualized. Samples without primary antibody were used as control. Images were acquired with a Nikon TE2000-U microscope outfitted with epifluorescence and a Cascade digital camera (Roper Scientific, Inc., Tucson, AZ), and images were acquired and processed using Metamorph software version 6.0 (Universal Imaging, Downingtown, PA).

Statistical Analysis—Data are presented as means ± S.D. Statistical significance was determined by Student’s t test. We assigned significance at p < 0.05.

RESULTS

ASBT Is a Short-lived Protein—To study the stability of ASBT protein, we determined the ASBT protein half-life in Mz-ChA-1 cells stably expressing ASBT-GFP. Whole cell extracts were isolated from cells at various times following the addition of 100 μg/ml CHX. As shown in Fig. 1A, the inhibition of protein synthesis resulted in the loss of ASBT by 12 h with over half of the protein decayed by 6 h. The mean half-life for ASBT-GFP was found to be 5.7 ± 1.2 h in three experiments. ASBT-GFP in Mz-ChA-1 cells exhibited a similar half-life as compared with ASBT-GFP (6.3 ± 1.2 versus 5.7 ± 1.2 h, respectively). Since expressed GFP protein is stable and FLAG-tagged and GFP-tagged ASBT are similarly unstable, it is unlikely that the GFP tag alters ASBT stability.

Proteasome Inhibitors Cause a Time- and Dose-dependent Accumulation of ASBT—To demonstrate the role of the proteasome in the degradation of ASBT, the effect of protease inhibitors on the steady-state levels of ASBT was assessed by immunoblot analysis of Mz-ChA-1 cells stably expressing wild-type ASBT-GFP. Although the protease inhibitors aprotinin, TLCK, TPCK,
and phenylmethylsulfonyl fluoride did not significantly affect ASBT expression, the selective proteasome inhibitors, lactacystin, ALLN, and MG-132, led to a marked increase in steady-state levels of ASBT (Fig. 2A). Treatment with MG-132 induced a time-dependent increase in ASBT (Fig. 2B). Increased ASBT was evident as early as 4 h following treatment and progressively accumulated during the 24 h following the application of MG-132. In the presence of this inhibitor for 8 h, an upregulating of ASBT-GFP to higher molecular weight forms was observed, consistent with accumulation of ubiquitinated ASBT (27). Concentrations of MG-132 as low as 5 μM induced significant increases in ASBT within 6 h of application (Fig. 2C), with higher concentrations of MG-132 resulting in higher levels of ASBT immunoreactivity. Also, fluorescence microscopy showed that exposure to MG-132 increased the total cellular GFP fluorescence signal (Fig. 2D). A similar increase in cellular GFP fluorescence was obtained using the highly specific proteasome inhibitors lactacystin and ALLN (data not shown). From these results, it is concluded that accumulation of post-translational modified ASBT is induced only by proteasome-specific protease inhibitors.

Since NRC cells express significant amounts of endogenous ASBT protein, the influence of MG-132 on endogenous ASBT level was analyzed. Western blot analysis using a specific ASBT antiserum demonstrated that treatment of NRC cells with MG-132 (Fig. 2E) resulted in a 2-fold increase of endogenous ASBT protein levels. These results indicate that both endogenously and exogenously expressed ASBT proteins are degraded by the ubiquitin-proteasome pathway.

ASBT Protein Is Ubiquitinated—To determine the intracellular distribution of ASBT-GFP, fluorescence microscopy was used to monitor the expression of GFP-tagged ASBT after exposure to a proteasome inhibitor (Fig. 3A). Control experiments indicate that the presence of an N-terminal GFP tag does not alter the kinetics of ASBT degradation (data not shown). In untreated cells, ASBT-GFP fluorescence was detected in the plasma membrane and intracellularly in a reticular distribution. Exposure to the proteasome inhibitor, MG-132, increased total cellular ASBT-GFP fluorescence and increased the fluorescence signal at a distinct site adjacent to the nucleus. After 16 h of exposure to the inhibitor, nearly all of the ASBT-GFP fluorescence was present in a single large, juxtanuclear structure that appears to impinge upon and to distort the contour of the nuclear envelope. These findings are consistent with previous studies that show accumulation of ubiquitinated conjugates in a vesicle as a consequence of overwhelming the proteasome (28). In both the presence and the absence of MG-132, the green fluorescent signal was observed throughout the cytoplasm when only GFP was transfected into Mz-ChA-1 cells (data not shown).

We investigated whether or not ubiquitin was present in the GFP-tagged ASBT-containing structure that is formed in response to proteasome inhibition. Mz-ChA-1 cells were transiently transfected with GFP-tagged ASBT together with excess plasmid encoding HA-tagged ubiquitin and analyzed by immunofluorescence microscopy using antibody to HA. The majority of HA-tagged ubiquitin protein colocalized with GFP-ASBT (Fig. 3B). We next determined whether GFP-tagged ASBT and HA-tagged ubiquitin coimmunoprecipitate. GFP-tagged ASBT and HA-tagged ubiquitin were transiently expressed in HEK293 cells, and immunoprecipitates were then collected with anti-GFP or anti-HA antibodies and subjected to immunoblot analysis with antibodies directed against the HA epitope or GFP (Fig. 3C). In cells cotransfected with GFP-tagged ASBT and HA-tagged ubiquitin, GFP-ASBT and larger molecular weight forms of GFP-ASBT (consistent with ubiquitin-conjugated ASBT and labeled ubiquitin-ASBT) were detected in HA and GFP immunoprecipitates (Fig. 3C, upper panels). In cells transfected with only GFP-ASBT, GFP-ASBT and ubiquitin-ASBT were detected in GFP but not HA immunoprecipitates (Fig. 3C, upper panels). Ubiquitin-HA was detected in HA immunoprecipitates only in cells expressing HA-tagged ubiquitin (Fig. 3C, lower left panel). Ubiquitin-HA was detected in GFP immunoprecipitates only in cells cotransfected with GFP-tagged ASBT and HA-tagged ubiquitin but not in single transfections (Fig. 3C, lower right panel). The data show that GFP-tagged ASBT and HA-tagged ubiquitin coimmunoprecipitate. Similar GFP immunoblots of GFP immunoprecipitates from cells cotransfected with GFP-tagged ASBT and HA-tagged ubiquitin as compared with cells transfected with GFP-ASBT alone (Fig. 3C, upper right panel) show that endogenous and expressed ubiquitin modifies ASBT in a similar way.

IL-1β Induces JNK-dependent Ubiquitination and Disposal of ASBT—We first sought to determine whether the IL-1β down-regulation of ASBT is due to increased proteasome-mediated degradation. Treatment with 1 ng/ml IL-1β for 4 h reduced ASBT protein levels by 70% as compared with saline control (Fig. 4A, first and second lane). IL-1β regulates the activity of a variety of target genes and transcription factors through several signal transduction cascades, including the three main mitogen-activated protein kinases (MAPK), which are JNK, MEK, and p38 MAPK (29). To investigate which pathway is primarily involved, stably expressing ASBT-GFP cells were preincubated with known inhibitors of these three signaling pathways immediately prior to exposure to IL-1β (Fig. 4A). Preincubation with a known MEK (25 μM U0126) or p38 MAPK (25 μM SB203580) inhibitor had no significant effect on IL-1β-mediated down-regulation of ASBT protein levels, whereas JNK inhibitor (25 μM SP600125) completely blocked the effects of IL-1β.

We next determined whether IL-1β alters the rate of ASBT disposal and increases ASBT ubiquitination. Consistent with IL-1β shortening the half-life of ASBT in the presence of CHX, the loss of the ASBT was faster in the presence of IL-1β (half-life of ASBT-GFP and GFP proteins. The half-life of ASBT-GFP (A), GFP protein (B), and ASBT-FLAG (C) was determined by Western blot analysis in cells treated with CHX at the time points shown. Mz-ChA-1 cells were transfected with the GFP-tagged ASBT (A), GFP (B), or ASBT-FLAG (C) expression vectors. The cells were harvested after treatment, and 50 μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis using anti-GFP (A and B) or anti-FLAG (C) antibody. Actin remaining demonstrates equal loading conditions. The detection system was horseradish peroxidase-conjugated secondary antibody and ECL. These panels are representative of at least three independent experiments.
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The Role of Phosphorylation in the Disposal of ASBT—Because IL-1β targets ASBT for degradation, we hypothesized that ASBT phosphorylation is the initial trigger for ASBT ubiquitination. In our search for a potential phosphorylation site, we focused on serine/threonine residues in the cytoplasmic tail of the protein. To assess the contribution of these residues to phosphorylation and turnover of ASBT, mutants were constructed by site-directed mutagenesis. Serine 335 was replaced by alanine (S335A), and threonine 339 was replaced by alanine (T339A). Furthermore, a double mutant was constructed (S335A/T339A). Turnover of ASBT was measured in the presence of CHX. As can be seen in Fig. 5A, the turnover of ASBT was prolonged in both the S335A mutation and the T339A mutation, and the double mutation further reduced turnover of ASBT as compared with single mutations. Therefore, the mutations appear to specifically affect the normal degradation pathway of ASBT.

If ASBT phosphorylation is the initial trigger for ASBT ubiquitination, we anticipated that the S335A and T339A ASBT mutants would be resistant to IL-1β-induced proteasome-dependent ubiquitination and disposal of ASBT. Fig. 5B shows that IL-1β-induced ubiquitination of ASBT was reduced in the S335A, T339A, and S335A/T339A mutants. Furthermore, S335A, T339A, and S335A/T339A mutants are resistant to IL-1β enhanced disposal of ASBT (Fig. 5C). Taken together, the data show that IL-1β-induced phosphorylation of serine 335 and threonine 339 is required for IL-1β triggered ubiquitination and disposal of ASBT.

DISCUSSION

Our results demonstrate for the first time that the expression of a bile acid transporter is regulated by the ubiquitin-proteasome pathway. Protein turnover assays demonstrated that ASBT is a short-lived protein (6 h). MG-132, a proteasome inhibitor, induces accumulation of endogenous ASBT in NRC and accumulation of GFP-tagged ASBT expressed in Mz-ChA-1 cells. In cells cotransfected with green fluorescent protein-tagged ASBT and HA-tagged ubiquitin, we demonstrated co-immunoprecipitation and colocalization of ASBT and ubiquitin. IL-1β-induced down-regulation of ASBT is abrogated by a JNK inhibitor (but not with inhibitors of p38 MAPK or MEK). IL-1β-induced down-regulation of ASBT is accompanied by an increase in ASBT ubiquitin conjugates and a reduced ASBT half-life. Finally, in phosphorylation-deficient S335A and T339A mutants, the ASBT half-life is markedly prolonged,
IL-1β-induced ASBT ubiquitination is reduced, and IL-1β fails to increase ASBT turnover. Taken together, these results indicate that ASBT is targeted to the ubiquitin-proteasome pathway for degradation under basal conditions and that IL-1β leads to an increased targeting of ASBT to the ubiquitin-proteasome system and disposal in a process that is affected by JNK-regulated serine/threonine phosphorylation of ASBT protein at both Ser-335 and Thr-339.

The ubiquitin-proteasome system plays a critical role in the regulation of the levels of many proteins. The first step in this process, the ubiquitination of proteins, which are subsequently degraded by the 26 S proteasome complex, is a highly regulated process (30). Several studies with mutated ABC transporters, such as cystic fibrosis transmembrane conductance regulator, copper-transporting P-type adenosine triphosphatase (ATP7B), multidrug resistance protein 2 (MRP2), and bile salt export pump (BSEP), have shown that the intracellular sequestering of membrane transporter proteins that results from mutation-induced misfolding is subsequently degraded by the ubiquitin-proteasome system (12, 14, 31, 32). Immature forms of a number of membrane transporters (e.g. cystic fibrosis transmembrane conductance regulator, epithelial sodium channel (ENaC), and aquaporin-1 (33–35) and membrane receptors (36–39) also enter the proteasome for degradation, which appears to be the abnormal folding of otherwise normal proteins. Recent studies suggest that up to 30% of newly synthesized proteins never attain native structure because of errors in translation or post-translational processes necessary for proper protein folding (40), and these defective ribosomal products are quickly disposed through the proteasome. ASBT polyubiquitination and degradation described in this study is unlikely solely due to protein misfolding since we found the ASBT disposal to be regulated by extracellular signals and ASBT phosphorylation. Previous studies have shown that not only the normal protein aging process but also processes that lead to stress-induced degradation or denaturation of proteins (e.g. high detergent conditions) will target proteins to the proteasome (41). Postsynthetic damage of ASBT (or other hepatic transporters), the extracellular domain of which is exposed to detergent environment of bile, may also potentially trigger degradation through the proteasome pathway.

Bile flow is rapidly and markedly reduced in hepatic inflammation, correlating with suppression of critical hepatic bile acid transporter gene expression (42). Recent findings indicate that inflammation-mediated down-regulation of bile flow is due to a complex and coordinated reduction in the expression and function of critical hepatic membrane transporters at both transcriptional and post-transcriptional levels. Little is known of the underlying cellular and molecular mechanisms, but several groups have focused their efforts by trying to link transporter gene down-regulation to the various arms of the intracellular signaling mechanisms invoked during the hepatic inflammatory response. This is relevant to the current study since these signaling arms involve pathways in which the ASBT disposal is regulated and thus may potentially explain aspects of the proteasome pathway.

Fig. 3. Ubiquitinated ASBT molecules accumulate. A, the time course of ASBT accumulation. Mz-ChA-1 cells stably expressing low levels of ASBT-GFP were incubated in the presence of 10 μM MG-132 for the times indicated. Note the redistribution of ASBT from the plasma membrane to intracellular structures after 4 h of MG-132 treatment (×60 objective). B, colocalization of ASBT-GFP with HA-tagged ubiquitin (HA-Ub). Mz-ChA-1 cells were transiently cotransfected with ASBT-GFP and HA-tagged ubiquitin cDNA and incubated in the presence of 10 μM MG-132 for 12 h. Cells were imaged by fluorescence microscopy for GFP and immunofluorescence for HA. ASBT-GFP and HA-ubiquitin fluorescence are primarily intracellular, and the merged image of ASBT-GFP and HA-ubiquitin fluorescence reveals strong colocalization (×40 objective). C, ASBT-GFP and HA-tagged ubiquitin coimmunoprecipitate. GFP-tagged ASBT or HA-tagged ubiquitin was transfected, or both were cotransfected in HEK 293 cells. After 24 h, cells were treated with 10 μM MG-132 for 6 h. Immunoprecipitates were collected with antibodies against HA (left panel) or GFP (right panel) and blotted with antibodies against the HA (lower panels) or GFP (upper panels). Initial immunoblots were performed in GFP immunoprecipitates blotted for HA and HA immunoprecipitates blotted for GFP. Membranes were then stripped and then rebotted for the other antibody.
response to inflammation or intracellular accumulation of bile acids (42–46). Previous studies have shown that the stress-induced alteration of ASBT genetic expression due to inflammation and bile acids is a consequence of trans-activation of the ASBT promoter by c-Jun/c-Fos and liver receptor homologue-1 (LRH-1), respectively (10, 47) and that hepatocyte nuclear factor 2 (ATF2) by phosphorylation (49, 50). Phosphorylation plays an important role in targeting many substrates for ubiquitination and can either inhibit or increase the targeting of substrates to the ubiquitin-proteasome system (51).

The novel observation in this study is the evidence that a cytokine-dependent immediate cellular response also markedly alters ASBT expression by enhanced ASBT entry into the proteasome disposal pathway. Thus, IL-1β has the dual effect of suppressing ASBT transcription and increasing ASBT disposal in cholangiocytes. Further studies are needed to determine the respective roles of transcriptional and post-transcriptional mechanisms for ASBT regulation in relevant animal models for inflammatory liver and small bowel disease.

The ubiquitination and targeting of ASBT to the proteasome are dependent on JNK-regulated serine/threonine phosphorylation of ASBT protein at both Ser-335 and Thr-339. These are the only potential phosphorylation sites in the ASBT C-terminal cytoplasmic tail, which overlaps with Ser-335 and Thr-339, respectively. The only two potential protein kinase C and casein kinase II phosphorylation sites in the ASBT C-terminal cytoplasmic tail, Thr-339 and Ser-335, are potential protein kinase C and casein kinase II phosphorylation residues, respectively. Similar to these findings, JNK has been shown to control ubiquitination of other stress-modulated factors including c-Jun and activating transcription factor 2 (ATF2) by phosphorylation (49, 50). Phosphorylation plays an important role in targeting many substrates for ubiquitination and can either inhibit or increase the targeting of substrates to the ubiquitin-proteasome system (51).
no simple correlation between the phosphorylation state of ASBT and its ubiquitination.

New therapies are needed to reverse the cholestasis that is associated with inflammatory liver disease. Inhibition of degradation may be an attractive method to maintain the levels of ASBT in the face of inflammation. These studies suggest that a novel method to accomplish this is through pharmacological inhibition of proteasome disposal of ASBT.

Acknowledgments—We thank Dr. An-Qiang Sun for providing the rat ASBT-GFP expression vector, Dr. Paul Dawson for the ASBT antibody, Dr. Fitz for Mz-ChA-1 cholangiocarcinoma cells, and Dr. Wen- zheng Zhang for discussing the manuscript.

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J. Biol. Chem. 2004, 279:44931-44937.
doi: 10.1074/jbc.M400969200 originally published online August 10, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400969200

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