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Short-chain ubiquitination is associated with the degradation rate of a cell-surface-resident bile salt export pump (BSEP/ABCB11).

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Abbreviations: BSEP/Bsep, human/rat isoforms of bile salt export pump; Cbl, Casitas B-lineage lymphoma; EH, Eps15 Homology; Eps15, epidermal-growth factor receptor pathway substrate 15; GFP, green fluorescence protein; MDCK, Madin-Darby canine kidney; MOI, multiplicity of infection; NPF, asparagine, proline, phenylalanine; PFIC, progressive familial intrahepatic cholestasis; rCMVs, rat canalicular membrane vesicles; SD, Sprague Dawley; Ub, ubiquitin; 4PBA, 4-phenylbutyrate

Abstract

The reduced expression of the bile salt export pump (BSEP/ABCB11) at the canalicular membrane is associated with cholestasis-induced hepatotoxicity due to the accumulation of bile acids in hepatocytes. We previously demonstrated that 4-phenylbutyrate (4PBA) treatment, an FDA-approved drug for urea cycle disorders, induces the cell-surface expression of BSEP by prolonging the degradation rate of cell-surface-resident BSEP. Conversely, BSEP mutations, E297G and D482G, found in progressive familial intrahepatic cholestasis type 2 (PFIC2), reduced it by shortening the degradation rate of cell-surface-resident BSEP. Accordingly, to help the development of the medical treatment of cholestasis, the present study investigated the underlying mechanism by which 4PBA and PFIC2-type mutations affect the BSEP degradation from cell surface, focusing on short-chain ubiquitination. In Madin-Darby canine kidney II (MDCK II) cells expressing BSEP and rat canalicular membrane vesicles, the molecular weight of the mature form of BSEP/Bsep shifted from 170 kDa to 190 kDa following ubiquitin modification (molecular weight: 8 kDa). Ubiquitination susceptibility of BSEP/Bsep was reduced in vitro and in vivo by 4PBA treatment and, conversely, was enhanced by BSEP mutations, E297G and D482G. Moreover, biotin-labeling studies using MDCK II cells demonstrated that the degradation of cell-surface-resident chimeric protein fusing ubiquitin to BSEP was faster than that of BSEP itself. In conclusion, BSEP/Bsep is modified with two to three ubiquitins, and its ubiquitination is modulated by 4PBA treatment and PFIC2-type mutations. Modulation of short-chain ubiquitination can regulate the change in the degradation rate of cell-surface-resident BSEP by 4PBA treatment and PFIC2-type mutations.

Introduction

The bile salt export pump (BSEP/ABCB11) is an ATP-binding cassette transmembrane transporter located in the bile canalicular membrane, playing an indispensable role in the biliary excretion of monovalent bile acids (such as taurocholic acid) (Byrne et al., 2002; Gerloff et al., 1998; Hayashi et al., 2005b; Meier and Stieger, 2002; Noe et al., 2002; Trauner and Boyer, 2003). The secretion of bile acids into bile by BSEP provides an osmotic driving force for bile formation (Meier and Stieger, 2002; Trauner and Boyer, 2003). A hereditary defect of BSEP function results in the acquisition of progressive familial intrahepatic cholestasis type 2 (PFIC2), a fatal liver disease, characterized by cholestasis and jaundice in the first year of life (Jansen et al., 1999; Strautnieks et al., 1998). BSEP function is disrupted not only in PFIC2 but also in several cholestatic models, such as endotoxin- or drug-induced cholestasis (Elferink et al., 2004; Lee et al., 2000; Vos et al., 1998), and cholestasis in pregnancy (Crocenzi et al., 2003; Lee et al., 2000). In cholestatic patients, it is likely that impaired biliary bile acid secretion causes accumulation of bile acids in hepatocytes and progressive severe hepatocellular damage due to the toxicity produced by a high concentration of bile acids. We and other groups have reported that the functional defects of BSEP are often associated with reduced BSEP expression at the canalicular membrane (Crocenzi et al., 2003; Elferink et al., 2004; Hayashi et al., 2005a; Lee et al., 2000; Plass et al., 2004; Strautnieks et al., 2008; Vos et al., 1998; Wang et al., 2002). Moreover, we found that shortening the half-life of cell-surface-resident BSEP in addition to the proteasome-mediated degradation from the endoplasmic reticulum (ER) are responsible for the reduced cell-surface expression of BSEP in PFIC2 patients with E297G and D482G mutations (Hayashi and Sugiyama, 2007), both of which are the most frequently found in PFIC2 patients (Strautnieks et al., 2008). Conversely, 4-phenylbutyrate (4PBA), a nontoxic butyrate analogue that was originally approved for clinical use as an ammonia scavenger in subjects with urea cycle disorders (Kajimura et al., 1996), induces cell-surface expression of

BSEP by prolonging the half-life of cell-surface-resident BSEP (Hayashi and Sugiyama, 2007). Its potential therapeutic effect against cholestasis by the increasing the cell-surface BSEP expression was identified by us for the first time. Considering that, to date, there are few established medical therapies for severe intrahepatic cholestasis like PFIC2, clarifying the mechanism of BSEP degradation from the canalicular membrane will play an important role in finding new medical therapies for this condition, in which the transport function of BSEP is retained. However, this mechanism is not completely understood, although it has been reported that Hax-1 is a binding partner of Bsep and participates in internalization from the apical membrane as demonstrated from an co-immunoprecipitation study using rat canalicular membrane vesicles (rCMVs) and a pulse-chase metabolic labeling study using Hax-1-depleted MDCK cells, respectively (Ortiz et al., 2004).

Ubiquitination is a regulated post-translational modification that conjugates ubiquitin (Ub) to lysine residues of targeted proteins and determines their intracellular fate. The canonical role of ubiquitination is to mediate degradation by the proteasome of the proteins that carry a single or polymeric chain of Ub on a specific lysine residue (d'Azzo et al., 2005). However, recently, it has been revealed that Ub modification has much broader and diverse functions in cellular processes. Short-chain ubiquitination, in which targeted molecules are attached to one or two Ub molecules, has been shown to be involved in the regulation of endocytosis and degradation of receptors, channels, and transporters from the cell surface in yeast (Galan and Haguenauer-Tsapis, 1997). In higher eukaryotes, this type of ubiquitination enhances endocytosis and lysosomal degradation (Kamsteeg et al., 2006; Lin et al., 2005; Sharma et al., 2004). Therefore, in the present study, we examined the possibility that 4PBA treatment and PFIC2-type mutations modulate the short-chain ubiquitination, thereby regulating the degradation rate of cell-surface-resident BSEP.

Materials and Methods

Materials

Pharmaceutical grade 4PBA was purchased from Scandinavian Formulas Inc. (Sellersville, PA). Antibodies against BSEP (N-16) and Ub (P4D1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HA antibody (3F10) was obtained from Roche (Indianapolis, IN). Antiserum for rat Bsep (rBsep) was raised in rabbits against an oligopeptide (the carboxyl terminal of rBsep; AYYKLVITGAPIS) (Akita et al., 2001). All other chemicals were of analytical grade. MDCK II cells were cultured in Dulbecco's modified eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL) at 37 °C with 5 % CO2 and 95 % humidity.

The cDNAs of BSEP harboring the HA epitope at the N-terminus (HA-BSEP) were constructed by PCR involving human BSEP cloned into the pShuttle vector (BD Biosciences, Palo Alto, CA) using the forward primer 5' –gagacccaagctggctagcgccgcccccaccatgtacccatacgatgttccagattacgctct**atgtctgactcagtaattcttcgaag**- 3' and the reverse primer 5' –**cttcgaagaattactgagtcagacat**aagagcgtaatctggaacatcgtatgggtacatggtgggggcggcgctagccagcttgggtctc- 3' [BSEP coding sequence in bold] (Geiser et al., 2001). The construct encoding HA-BSEP-Ub$^{\Delta GG}$, in which the two last Ub glycines were deleted, was obtained by fusing Ub$^{\Delta GG}$ in frame to the C-terminus of BSEP using PCR (Geiser et al., 2001). Mutant chimeras HA-BSEP-Ub$^{\Delta GG/I44A}$, incorporating Ub I44A in addition to deletion of the last two glycines, were obtained by PCR mutagenesis (Geiser et al., 2001).

Generation of recombinant adenovirus

The BD Adeno-X Adenoviral Expression System (BD Biosciences, Palo Alto, CA) was used to create

BSEP, E297G BSEP, D482G BSEP, HA-BSEP, HA-BSEP-Ub$^{\Delta GG}$ and HA-BSEP-Ub$^{\Delta GG/I44A}$ recombinant adenoviruses as previously described (Hayashi et al., 2005a). The virus titer was quantified with an Adeno-X Rapid Titer Kit (Clontech). As a control, recombinant adenoviruses containing green fluorescence protein (GFP) were used.

Animals

Male Sprague Dawley (SD) rats (6-7 weeks old) were purchased from Nippon SLC (Shizuoka, Japan). All animals were maintained under standard conditions with a reverse dark-light cycle and were treated humanely. Food and water were available ad libitum. The studies reported in this manuscript were carried out in accordance with the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan).

Preparation of canalicular membrane vesicles

Male SD rats (6-7 weeks old) were given 0.6 g/kg/day 4PBA or vehicle by gavage in three divided doses for 10 days. Rat canalicular membrane vesicles (rCMVs) were prepared from the liver of the treated rats as described previously (Akita et al., 2001). To inhibit the ubiquitination in vitro, 10 mM N-ethylmaleimide was added to the preparing buffer. Prepared rCMVs were immunoprecipitated.

Immunoprecipitation from rCMVs and MDCK II cells

Prepared rCMVs (200 μg) were solubilized for 1 h at 4 °C in 1 mL lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % Nonidet P40, 0.5 % sodium deoxycholate, 10 mM N-ethylmaleimide and a protease inhibitor

cocktail tablet (Roche)). The mixtures were centrifuged at 150,000 × g for 1 h at 4 °C. The supernatants were precleared by adding 40 μL of protein G-sepharose beads. MDCK II cells were seeded in 10 cm culture plates at a density of 1.5×10^6 cells per plate. After a 24 h culture, confluent cells were infected with recombinant adenovirus containing cDNAs for BSEP, E297G BSEP, D482G BSEP, HA-BSEP and GFP at a 200 multiplicity of infection (MOI). 48 h after the infection, MDCK II cells were solubilized in 1 mL lysis buffer and precleared by adding 40 μL of protein G-sepharose beads. When isolating the cell-surface-resident BSEP, cell-surface biotinylation was performed as described previously (Hayashi et al., 2005a) before solubilization. The biotinylated cell surface fraction was isolated with ImmunoPure® StreptAvidin (Pierce Biotechnology). The prepared specimens were diluted 10-fold with lysis buffer, and subsequently precleared by adding 40 μL of protein G-sepharose beads. Precleared lysates from rCMVs and MDCK II cells and the precleared cell surface fraction from MDCKII cells were incubated with anti-rBsep antibody (5 μg) for 2 h at 4 °C. Then 40 μL of protein G-sepharose beads was added and incubated for 3 h at 4 °C. Immune complexes were precipitated, followed by two washes with 1 mL of high salt buffer (50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 10 mM N-ethylmaleimide, 0.1 % Nonidet P40 and 0.05 % sodium deoxycholate) and one wash with 1 mL of low salt buffer (10 mM Tris-HCl (pH 7.5), 10 mM N-ethylmaleimide, 0.1 % Nonidet P40 and 0.05 % sodium deoxycholate). Immunoprecipitated proteins were eluted for 5 min at 100 °C with 50 μL of 1 × Reducing Loading Buffer (Biolabs). The specimens were separated by 6 % SDS-PAGE and subjected to western blot analysis.

Cleavage of glycosylation

To examine the extent of glycosylation of ubiquitinated BSEP, 20 μL of immunoprecipitated specimens were digested with endoglycosidase H (EndoH) or peptide N-glycosidase F (PNGaseF) (New England Biolabs) at 37 °C

for 2 h as described by the manufacturer. The deglycosylated proteins were separated by 6 % SDS-PAGE and subjected to western blot analysis.

Western blot analysis

Specimens were loaded onto a 6 % SDS-PAGE plate with a 3.75 % stacking gel, and subjected to western blot analysis with 300-fold diluted polyclonal BSEP antibody (N-16), 1,000-fold diluted monoclonal HA antibody (3F10) and 300-fold diluted monoclonal Ub antibody (P4D1) as described previously (Hayashi et al., 2005a). Immunoreactivity was detected with an ECL Advance™ Western Blotting Detection Kit (Amersham Biosciences, Piscataway, NJ). The intensity of the band indicating the short-chain ubiquitinated BSEP and mature form of BSEP was quantified by Multi Gauge software Ver 2.0 (Fujifilm, Tokyo, Japan).

Cell-surface biotinylation and determination of degradation rate of cell-surface expressing protein

MDCK II cells were seeded on 12-well plates at a density of 2.5×10^5 cells per well. After a 24 h culture, confluent cells were infected with recombinant adenovirus containing cDNAs for HA-BSEP, HA-BSEP-Ub$^{\Delta GG}$, HA-BSEP-Ub$^{\Delta GG/I44A}$ and GFP at 200 MOI. 24 h after infection, cell-surface biotinylation was performed as described previously (Hayashi et al., 2005a).

To examine the degradation rate of the cell-surface-resident protein, biotinylated MDCK II cells were incubated for various periods at 37 °C before solubilization. Then, the remaining biotinylated protein was isolated as described above, separated by 6 % SDS-PAGE and subjected to western blot analysis.

Statistical analysis

Experiments were repeated at least three times, and graphs include means ± S.E.. P values between two variables and multiple variables were calculated at the 95 % confidence level with Student's t test and ANOVA, respectively, using Prism software (GraphPad Sofrware, Inc.).

Results

Ubiquitination of BSEP/Bsep in vitro and in vivo

To investigate whether BSEP is ubiquitinated in MDCK II cells, it was immunoprecipitated from MDCK II cells expressing HA-BSEP with rBsep antibody (Figure. 1A). Subsequent western blot analysis for Ub and HA detected a ubiquitinated BSEP band at a molecular weight of 190 kDa (Figure. 1A; upper panel) and two BSEP bands of 150 kDa and 170 kDa, respectively (Figure. 1A; lower panel). Its ubiquitinated BSEP band was also detected in immunoprecipitates prepared from the cell surface fraction of MDCK II cells with rBsep antibody (Figure. 1B; upper panel). In these immunoprecipitates, BSEP was detected as only the 170 kDa band (Figure. 1B; lower panel). The ubiquitination of Bsep was confirmed in vivo. In immunoprecipitates from rCMVs with rBsep antibody, ubiquitinated Bsep and Bsep were detected as a smear band around 190 kDa (Figure. 1C; upper panel) and only the 170 kDa band (Figure. 1C; lower panel), respectively.

Ubiquitinated BSEP was sensitive to PNGaseF, which cleaves the high mannose- and complex-type sugar chains, but was insensitive to EndoH, which does not cleave these sugar chains (Figure. 1D; upper panel), indicating that the N-glycans attached to ubiquitinated BSEP were highly modified in the Golgi complex. Ubiquitinated Bsep was also sensitive to PNGaseF and insensitive to EndoH (data not shown). These results suggest that these ubiquitinated BSEP/Bsep represent the attachment of Ub to the mature form of BSEP/Bsep, and do not represent the intermediate form of the polyubiquitinated immature BSEP/Bsep in the ER. In addition, EndoH digestion of immunoprecipitated BSEP resulted in a shift of 150 kDa band, but not the 170 kDa band (Figure. 1D; lower panel), suggesting that the 150 and 170 kDa bands represent the immature ER-resident form and the mature form of BSEP, respectively. This indication is further supported from the results showing that the cell-surface-resident BSEP in MDCKII cells was

detected as only the 170 kDa band (Figure. 1B; lower panel) and Bsep in rCMVs, which are the canalicular membrane-enriched fraction, was also detected as only the 170 kDa band (Figure. 1C; lower panel). Considering that the molecular weights of the ubiquitinated BSEP/Bsep, the mature form of BSEP/Bsep and Ub are 190 kDa, 170 kDa and 8 kDa, respectively, this shows that BSEP/Bsep can be modified with two to three Ub molecules at steady-state.

Alteration of short-chain ubiquitination susceptibility of BSEP by PFIC2-type mutations and 4PBA treatment

Previously, we reported that E297G and D482G, frequent mutations in PFIC2 patients, shorten the half-life of cell-surface-resident BSEP by approximately 1.5- and 4-fold, respectively, and conversely, 4PBA treatment prolongs cell-surface-resident BSEP 2-fold (Hayashi and Sugiyama, 2007). To explore a possible correlation between the half-life of cell-surface-resident BSEP and the short-chain ubiquitination susceptibility of BSEP, mutated BSEP was immunoprecipitated from MDCK II cells expressing E297G BSEP and D482G BSEP, and the immunoprecipitates were subjected to western blot analysis for Ub and BSEP (Figure. 2A). BSEP, E297G BSEP and Bsep were also immunoprecipitated from MDCK II cells expressing BSEP and E297G BSEP after 4PBA treatment and rCMVs prepared from 4PBA-treated SD rats, and the immunoprecipitates were subjected to western blot analysis for Ub and BSEP (Figures. 3A - C). Quantitative densitometry analysis revealed that the ratio of the short-chain ubiquitinated BSEP, PFIC2-type mutated BSEPs (Figures. 2A, 3A, B: Arrow) to the mature form of BSEP, PFIC2-type mutated BSEPs (Figures. 2A, 3A, B: Filled arrowhead) was significantly greater, 6- and 30-fold by E297G and D482G mutations, respectively, than that in wild type BSEP (Figure. 2B), and was reduced in a time-dependent manner after 4PBA treatment in vitro (Figure. 3D, E). The same 4PBA effect was also observed in vivo (Figure. 3C). This ratio was reduced 5-fold following 4PBA treatment for 10 days (Figure. 3F).

Degradation rate of cell-surface-resident BSEP-Ub chimera

To directly examine the effect of short-chain ubiquitination on the degradation rate of cell-surface-resident BSEP, MDCK II cells expressing a protein fusion of $Ub^{\Delta GG}$ or $Ub^{\Delta GG/I44A}$ to the BSEP were constructed (Figure. 4A). Since the Ub chain is extended via covalent binding between the glycine residues in the C-terminus and lysine residues, the fusion protein of $Ub^{\Delta GG}$ mimics the effect of the attachment of a Ub (Haglund et al., 2003; Kamsteeg et al., 2006). An epidermal growth factor receptor (EGFR)- $Ub^{\Delta GG}$ was actually detected in only monoubiquitinated form, although EGFR-Ub chimera protein containing wild type Ub was detected as not only monoubiquitinated form but also the polyubiquitinated form (Haglund et al., 2003). The I44A mutation in Ub has been shown to inhibit the interaction of Ub with Ub-binding adaptor proteins, which recruits certain ubiquitinated cell-surface receptors for lysosomal degradation (Sharma et al., 2004; Shih et al., 2002; Stang et al., 2004). In the previous paper examining the effect of ubiquitination on the degradation of cystic fibrosis transmembrane conductance regulator (CFTR) from the cell surface, it was demonstrated that the I44A mutation inhibited the interaction of Ub with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), the primary Ub-binding adaptor that form the sorting complex involving components of the endosomal sorting complex required for transport I, and consequently, the lysosomal degradation of CFTR-$Ub^{\Delta GG/I44A}$ was prevented in comparison with CFTR-$Ub^{\Delta GG}$, and the degradation rate of CFTR-$Ub^{\Delta GG/I44A}$ was equivalent to that of CFTR itself (Sharma et al., 2004). Therefore, the fusion of $Ub^{\Delta GG/I44A}$ to BSEP has been used as a negative control to examine only the effect of covalent binding of a Ub. Biotin-labeling studies using MDCK II cells expressing HA-BSEP, HA-BSEP-$Ub^{\Delta GG}$ and HA-BSEP- $Ub^{\Delta GG/I44A}$ demonstrated that fusion of $Ub^{\Delta GG}$ shortened the half-life of cell-surface-resident BSEP 2.5-fold, while that of $Ub^{\Delta GG/I44A}$ had no effect (Figures. 4B, C).

Discussion

The reduction of BSEP expression at the canalicular membrane causes or aggravates cholestasis (Crocenzi et al., 2003; Elferink et al., 2004; Hayashi et al., 2005a; Lee et al., 2000; Plass et al., 2004; Vos et al., 1998; Wang et al., 2002). We have previously found that shortening the half-life of cell-surface resident BSEP is partly responsible for the reduced cell-surface expression of BSEP in PFIC2 patients with E297G and D482G mutations (Hayashi and Sugiyama, 2007). Moreover, 4PBA is a potential therapeutic agent to combat cholestasis, the effect of which induces cell-surface expression of BSEP by prolonging the degradation rate of cell-surface-resident BSEP (Hayashi and Sugiyama, 2007). Accordingly, it is considered that elucidating the regulatory mechanism of BSEP degradation from the cell surface will help to establish a new medical treatment for cholestasis. However, its mechanism is poorly understood. In the present study, we examined the possibility that 4PBA treatment and PFIC2-type mutations modulate the short-chain ubiquitination, thereby regulating the degradation rate of cell-surface-resident BSEP.

Initially, ubiquitination of BSEP/Bsep in vitro and in vivo was confirmed by immunoprecipitation of protein obtained from MDCK II cells expressing BSEP and rCMVs (Figures. 1A, C). The ubiquitinated BSEP was also detected in the surface fraction (Figure. 1B). The ubiquitinated BSEP was sensitive to PNGaseF and insensitive to EndoH (Figure. 1D). Considering that the molecular weights of the mature form of BSEP/Bsep and Ub are 170 kDa and 8 kDa, respectively, it was found that BSEP/Bsep can be modified with two to three Ub molecules at a steady-state. Although the bands corresponding to the ubiquitinated BSEP/Bsep (~ 190 kDa) were not detected by both HA antibody and BSEP antibody (Figure. 1A - C), it may be accounted by the low amount of the ubiquitinated BSEP/Bsep compared with non-ubiquitinated BSEP/Bsep. This interpretation is supported by recent studies showing that the amount of monoubiquitinated ROMK1 and short-chain ubiquitinated aquaporin-2 water channel (AQP2) were much lower than

that of the non-ubiquitinated form (Kamsteeg et al., 2006; Lin et al., 2005). In case of AQP2, similar to BSEP/Bsep, short-chain ubiquitinated AQP2 in immunoprecipitates produced by AQP2 antibody were not detected by AQP2 antibody (Kamsteeg et al., 2006).

Next, the alteration in short-chain ubiquitination susceptibility was investigated to analyze the correlation with the degradation rate of BSEP from the cell surface. Quantitative densitometry analysis revealed PFIC2-type mutations, which shorten the half-life of cell-surface-resident BSEP (Hayashi and Sugiyama, 2007), induce the short-chain ubiquitination of BSEP, and conversely, 4PBA treatment, which prolongs the half-life of cell-surface-resident BSEP (Hayashi and Sugiyama, 2007), reduced it in vitro and in vivo (Figures. 2, 3), indicating a correlation between the half-life of cell-surface-resident BSEP with the ubiquitination propensity of BSEP. Moreover, the effect of short-chain ubiquitination on BSEP degradation was directly investigated using MDCK II cells expressing HA-BSEP-$Ub^{\Delta GG}$ and HA-BSEP-$Ub^{\Delta GG/I44A}$. The results of biotin-labeling studies demonstrated that fusion of $Ub^{\Delta GG}$ shortened the half-life of cell-surface-resident BSEP, while that of $Ub^{\Delta GG/I44A}$, as a negative control, had no effect, suggesting that the short-chain ubiquitination promotes the degradation of BSEP from the cell surface (Figures. 4B, C). This suggestion is further supported by the finding that the ubiquitinated BSEP is actually present at the cell surface (Figure 1B).

Although it has been reported that short-chain ubiquitination of receptors and channels promotes endocytosis from the cell surface (Haglund et al., 2003; Kamsteeg et al., 2006) and/or translocation from the endosomal compartment to lysosomes (Haglund et al., 2003; Sharma et al., 2004), the regulation of transporters by short-chain ubiquitination has not been clarified in detail in higher eukaryotes. To our knowledge, this is the first reported example in an ATP-binding cassette-type transporter in higher eukaryotes that BSEP/Bsep can be attached to Ub and its ubiquitination relates to the promotion of degradation from the cell surface. It remains unclear whether the accelerated

degradation of cell-surface-resident BSEP by short-chain ubiquitination results from the promotion of the endosomal sorting from the cell surface or the delivery from the endosomal compartment to lysosomes, or both. However, a recent report suggesting that epidermal-growth factor receptor pathway substrate 15 (Eps15) is involved in endocytosis of Bsep (Ortiz et al., 2004) may provide several clues to the role of short-chain ubiquitination in BSEP sorting. Eps15 contains various functional regions, three Eps15 Homology (EH) domains that interact with proteins containing tandem asparagine, proline, phenylalanine (NPF) repeats in the N-terminal domain, binding sites to the α-subunit of the clathrin adaptor-protein complex AP-2 and two ubiquitin-interacting motifs at the C-terminal domain (Regan-Klapisz et al., 2005). Considering that Eps15 could be involved in the initial steps of clathrin-coated-pit formation through the EH domain-NPF motif interaction (Morgan et al., 2003), and recruit ubiquitinated receptors from the plasma membrane through its ubiquitin-interacting motifs (de Melker et al., 2004; Polo et al., 2002), it is possible that the short-chain ubiquitination escorts BSEP into the forming clathrin-coated pit.

Here, we demonstrated that BSEP/Bsep can be modified by two to three Ub molecules and its ubiquitination promotes the degradation of cell-surface-resident BSEP. The ubiquitination of substrate proteins are performed by covalent attachment of Ub via the sequential action of three enzymes: a Ub-activating enzyme E1, a Ub-conjugating enzyme E2 and a Ub ligase E3 (d'Azzo et al., 2005; Hershko et al., 2000). Among these three enzymes, E3s are considered to ensure the correct timing, localization and specificity of the ubiquitination reaction, because genomic information suggests that there are several hundreds of E3s in eukaryotic cells. Therefore, the E3 responsible for the short-chain ubiquitination of BSEP is an attractive target for treatment of severe cholestatic disease. Moreover, it has also been demonstrated that E3s are regulated by various mechanisms including protein-protein interactions and phosphorylation (d'Azzo et al., 2005). For example, autophosphorylation of EGFR by ligand binding promotes its

recognition by Ring-type E3, Casitas B-lineage lymphoma (Cbl) (Rubin et al., 2005), while its Cbl-EGFR interaction is negatively regulated by protein-protein interaction with Sprouty (d'Azzo et al., 2005). In future studies, the identification of the E3s, which participate in the short-chain ubiquitination of BSEP, and the elucidation of its regulatory mechanism will help in the development of new medical treatments for severe cholestasis.

In conclusion, the results of the present study demonstrate that BSEP/Bsep is modified with two to three ubiquitins, and its ubiquitination is modulated by 4PBA treatment and PFIC2-type mutations. Modulation of short-chain ubiquitination can regulate the change in the degradation rate of cell-surface-resident BSEP by 4PBA treatment and PFIC2-type mutations. These pieces of information should be useful in understanding the pathogenic mechanism of severe cholestasis related to reduced BSEP expression at the canalicular membrane and will help in the development of new medical treatments for severe cholestasis.

References

Akita H, Suzuki H, Ito K, Kinoshita S, Sato N, Takikawa H and Sugiyama Y (2001) Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. *Biochim Biophys Acta* **1511**:7-16.

Byrne JA, Strautnieks SS, Mieli-Vergani G, Higgins CF, Linton KJ and Thompson RJ (2002) The human bile salt export pump: characterization of substrate specificity and identification of inhibitors. *Gastroenterology* **123**:1649-58.

Crocenzi FA, Mottino AD, Cao J, Veggi LM, Pozzi EJ, Vore M, Coleman R and Roma MG (2003) Estradiol-17beta-D-glucuronide induces endocytic internalization of Bsep in rats. *Am J Physiol Gastrointest Liver Physiol* **285**:G449-59.

d'Azzo A, Bongiovanni A and Nastasi T (2005) E3 ubiquitin ligases as regulators of membrane protein trafficking and degradation. *Traffic* **6**:429-41.

de Melker AA, van der Horst G and Borst J (2004) c-Cbl directs EGF receptors into an endocytic pathway that involves the ubiquitin-interacting motif of Eps15. *J Cell Sci* **117**:5001-12.

Elferink MG, Olinga P, Draaisma AL, Merema MT, Faber KN, Slooff MJ, Meijer DK and Groothuis GM (2004) LPS-induced downregulation of MRP2 and BSEP in human liver is due to a posttranscriptional process. *Am J Physiol Gastrointest Liver Physiol* **287**:G1008-16.

Galan JM and Haguenauer-Tsapis R (1997) Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein. *Embo J* **16**:5847-54.

Geiser M, Cebe R, Drewello D and Schmitz R (2001) Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. *Biotechniques* **31**:88-90, 92.

Gerloff T, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, Hofmann AF and Meier PJ (1998) The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. *J Biol Chem* **273**:10046-50.

Haglund K, Sigismund S, Polo S, Szymkiewicz I, Di Fiore PP and Dikic I (2003) Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat Cell Biol* **5**:461-6.

Hayashi H and Sugiyama Y (2007) 4-phenylbutyrate enhances the cell surface expression and the transport capacity of wild-type and mutated bile salt export pumps. *Hepatology* **45**:1506-16.

Hayashi H, Takada T, Suzuki H, Akita H and Sugiyama Y (2005a) Two common PFIC2 mutations are associated with the impaired membrane trafficking of BSEP/ABCB11. *Hepatology* **41**:916-24.

Hayashi H, Takada T, Suzuki H, Onuki R, Hofmann AF and Sugiyama Y (2005b) Transport by vesicles of glycine- and taurine-conjugated bile salts and taurolithocholate 3-sulfate: a comparison of human BSEP with rat Bsep. *Biochim Biophys Acta* **1738**:54-62.

Hershko A, Ciechanover A and Varshavsky A (2000) Basic Medical Research Award. The ubiquitin system. *Nat Med* **6**:1073-81.

Jansen PL, Strautnieks SS, Jacquemin E, Hadchouel M, Sokal EM, Hooiveld GJ, Koning JH, De Jager-Krikken A, Kuipers F, Stellaard F, Bijleveld CM, Gouw A, Van Goor H, Thompson RJ and Muller M (1999) Hepatocanalicular bile salt export pump deficiency in patients with progressive familial intrahepatic cholestasis. *Gastroenterology* **117**:1370-9.

Kajimura K, Takagi Y, Ueba N, Yamasaki K, Sakagami Y, Yokoyama H and Yoneda K (1996) Protective effect of Astragali Radix by intraperitoneal injection against Japanese encephalitis virus infection in mice. *Biol Pharm Bull* **19**:855-9.

Kamsteeg EJ, Hendriks G, Boone M, Konings IB, Oorschot V, van der Sluijs P, Klumperman J and Deen PM (2006) Short-chain ubiquitination mediates the regulated endocytosis of the aquaporin-2 water channel. *Proc Natl Acad Sci U S A* **103**:18344-9.

Lee JM, Trauner M, Soroka CJ, Stieger B, Meier PJ and Boyer JL (2000) Expression of the bile salt export pump is maintained after chronic cholestasis in the rat. *Gastroenterology* **118**:163-72.

Lin DH, Sterling H, Wang Z, Babilonia E, Yang B, Dong K, Hebert SC, Giebisch G and Wang WH (2005) ROMK1 channel activity is regulated by monoubiquitination. *Proc Natl Acad Sci U S A* **102**:4306-11.

Meier PJ and Stieger B (2002) Bile salt transporters. *Annu Rev Physiol* **64**:635-61.

Morgan JR, Prasad K, Jin S, Augustine GJ and Lafer EM (2003) Eps15 homology domain-NPF motif interactions regulate clathrin coat assembly during synaptic vesicle recycling. *J Biol Chem* **278**:33583-92.

Noe J, Stieger B and Meier PJ (2002) Functional expression of the canalicular bile salt export pump of human liver. *Gastroenterology* **123**:1659-66.

Ortiz DF, Moseley J, Calderon G, Swift AL, Li S and Arias IM (2004) Identification of HAX-1 as a protein that binds bile salt export protein and regulates its abundance in the apical membrane of Madin-Darby canine kidney cells. *J Biol Chem* **279**:32761-70.

Plass JR, Mol O, Heegsma J, Geuken M, de Bruin J, Elling G, Muller M, Faber KN and Jansen PL (2004) A progressive familial intrahepatic cholestasis type 2 mutation causes an unstable, temperature-sensitive bile salt export pump. *J Hepatol* **40**:24-30.

Polo S, Sigismund S, Faretta M, Guidi M, Capua MR, Bossi G, Chen H, De Camilli P and Di Fiore PP (2002) A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature* **416**:451-5.

Regan-Klapisz E, Sorokina I, Voortman J, de Keizer P, Roovers RC, Verheesen P, Urbe S, Fallon L, Fon EA, Verkleij A, Benmerah A and van Bergen en Henegouwen PM (2005) Ubiquilin recruits Eps15 into ubiquitin-rich cytoplasmic aggregates via a UIM-UBL interaction. *J Cell Sci* **118**:4437-50.

Rubin C, Gur G and Yarden Y (2005) Negative regulation of receptor tyrosine kinases: unexpected links to c-Cbl and receptor ubiquitylation. *Cell Res* **15**:66-71.

Sharma M, Pampinella F, Nemes C, Benharouga M, So J, Du K, Bache KG, Papsin B, Zerangue N, Stenmark H and Lukacs GL (2004) Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes. *J Cell Biol* **164**:923-33.

Shih SC, Katzmann DJ, Schnell JD, Sutanto M, Emr SD and Hicke L (2002) Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis. *Nat Cell Biol* **4**:389-93.

Stang E, Blystad FD, Kazazic M, Bertelsen V, Brodahl T, Raiborg C, Stenmark H and Madshus IH (2004) Cbl-dependent ubiquitination is required for progression of EGF receptors into clathrin-coated pits. *Mol Biol Cell* **15**:3591-604.

Strautnieks SS, Bull LN, Knisely AS, Kocoshis SA, Dahl N, Arnell H, Sokal E, Dahan K, Childs S, Ling V, Tanner MS, Kagalwalla AF, Nemeth A, Pawlowska J, Baker A, Mieli-Vergani G, Freimer NB, Gardiner RM and Thompson RJ (1998) A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nat Genet* **20**:233-8.

Strautnieks SS, Byrne JA, Pawlikowska L, Cebecauerova D, Rayner A, Dutton L, Meier Y, Antoniou A, Stieger B, Arnell H, Ozcay F, Al-Hussaini HF, Bassas AF, Verkade HJ, Fischler B, Nemeth A, Kotalova R, Shneider BL, Cielecka-Kuszyk J, McClean P, Whitington PF, Sokal E, Jirsa M, Wali SH, Jankowska I, Pawlowska J,

Mieli-Vergani G, Knisely AS, Bull LN and Thompson RJ (2008) Severe bile salt export pump deficiency: 82 different ABCB11 mutations in 109 families. *Gastroenterology* **134**:1203-14.

Trauner M and Boyer JL (2003) Bile salt transporters: molecular characterization, function, and regulation. *Physiol Rev* **83**:633-71.

Vos TA, Hooiveld GJ, Koning H, Childs S, Meijer DK, Moshage H, Jansen PL and Muller M (1998) Up-regulation of the multidrug resistance genes, Mrp1 and Mdr1b, and down-regulation of the organic anion transporter, Mrp2, and the bile salt transporter, Spgp, in endotoxemic rat liver. *Hepatology* **28**:1637-44.

Wang L, Soroka CJ and Boyer JL (2002) The role of bile salt export pump mutations in progressive familial intrahepatic cholestasis type II. *J Clin Invest* **110**:965-72.

Footnotes

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Legends for figures

Figure 1 ----- Ubiquitination of BSEP/Bsep.

(A) Ubiquitination of BSEP in MDCK II cells. BSEP was immunoprecipitated from MDCK II cells expressing HA-BSEP and GFP with anti-rBsep antibody. Immunoprecipitates were separated by 6 % SDS-PAGE and subjected to western blot analysis. Rabbit IgG served as a negative control. Filled and empty arrowheads indicate the mature and immature forms of BSEP, respectively. (B) Ubiquitination of cell-surface-resident BSEP in MDCKII cells. The cell surface fraction of MDCKII cells expressing HA-BSEP and GFP was labeled with or without biotin and isolated with ImmunoPure® StreptAvidin as described in Materials and Methods. Cell-surface-resident BSEP was immunoprecipitated from these specimens with anti-rBsep antibody. Immunoprecipitates were separated by 6 % SDS-PAGE and subjected to western blot analysis. (C) Ubiquitination of Bsep in rCMVs. Bsep was immunoprecipitated from solubilized rCMVs with anti-rBsep antibody. Immunoprecipitates were separated by 6 % SDS-PAGE and subjected to western blot analysis. (D) The extent of glycosylation of ubiquitinated BSEP. Immunoprecipitated specimens from MDCK II cells expressing BSEP with anti-rBsep antibodies were digested by EndoH or PNGaseF. The deglycosylated proteins were separated by 6 % SDS-PAGE and subjected to western blot analysis. Filled and empty arrowheads indicate the core- and non-glycosylated form of BSEP, respectively.

Figure 2 ----- The effect of PFIC2-type mutations on short-chain ubiquitinatioin of BSEP.

(A) Short-chain ubiquitination susceptibility of PFIC2-type mutated BSEPs, E297G BSEP and D482G BSEP. BSEP and PFIC2-type mutated BSEPs were immunoprecipitated from MDCK II cells expressing BSEP, PFIC2-type mutated BSEPs and GFP with anti-rBsep antibody. Immunoprecipitates (15 μL and 30 μL) were

separated by 6 % SDS-PAGE and subjected to western blot analysis. Arrow, filled and empty arrowheads indicate the short-chain ubiquitinated BSEP, the mature and immature forms of BSEP, respectively. (B) Quantification of the short-chain ubiquitinated BSEP normalized with regard to the mature form of BSEP in (A). Data are derived from the intensity of the band indicating the short-chain ubiquitinated BSEP and the mature form of BSEP from 15 μL of each specimen applied. Band density was quantified by Image Gauge software. Open, gray and closed columns represent the ratio of band density indicating the short-chain ubiquitinated BSEP to that indicating the mature form of BSEP in MDCK II cells expressing BSEP, E297G BSEP and D482G BSEP, respectively. Each bar represents the mean ± S.E., n = 3 - 4. Asterisks represent statistically significant differences between BSEP and mutated BSEP, $^{*}P < 0.05$ and $^{**}P < 0.01$.

Figure 3 ----- The effect of 4PBA treatment on short-chain ubiquitination of BSEP/Bsep.

(A, B) Short-chain ubiquitination susceptibility of BSEP (A) and E297G BSEP (B) in 4PBA-treated MDCK II cells. BSEP was immunoprecipitated from MDCK II cells expressing BSEP after 4PBA (1mM) treatment for the indicated period with anti-rBsep antibody. Immunoprecipitates were separated by 6 % SDS-PAGE and subjected to western blot analysis. Arrow, filled and empty arrowheads indicate the short-chain ubiquitinated BSEP, the mature and immature forms of BSEP, respectively. (C) Short-chain ubiquitination susceptibility of Bsep in 4PBA-treated SD rats. Male SD rats (6-7 weeks old) were given 4PBA or vehicle by gavage for 10 days at 0.6 g/kg/day before the experiments. Bsep was immunoprecipitated from solubilized rCMVs prepared from 4PBA-treated SD rats with anti-rBsep antibody. Immunoprecipitates were separated by 6 % SDS-PAGE and subjected to western blot analysis. (D, E) Quantification of the short-chain ubiquitinated BSEP and E297G BSEP normalized with regard

to the mature form of BSEP and E297G BSEP in (A, B). Data are derived from the band density indicating the short-chain ubiquitinated BSEP and mature form of BSEP by Image Gauge software. Each bar represents the mean ± S.E., n = 3. (F) Quantification of the short-chain ubiquitinated Bsep normalized with regard to Bsep in (C). Data are derived from the intensity of the band indicating the short-chain ubiquitinated Bsep and Bsep by Image Gauge software. Closed and open columns represent the ratio of band density indicating the short-chain ubiquitinated Bsep to Bsep in rCMVs prepared from SD rats, with and without 4PBA, respectively. Each bar represents the mean ± S.E., n = 4. Asterisks represent statistically significant differences between vehicle-treated SD rats and 4PBA-treated SD rats, * $^{*}P < 0.01$.

Figure 4 ----- Determination of the degradation rate of cell-surface-resident BSEP-Ub chimera.

(A) Cell-surface expression of HA-BSEP, HA-BSEP-Ub$^{\Delta GG}$ and HA-BSEP-Ub$^{\Delta GG/I44A}$ in MDCK II cells. The cell-surface fractions of MDCK II cells expressing HA-BSEP, HA-BSEP-Ub$^{\Delta GG}$, HA-BSEP-Ub$^{\Delta GG/I44A}$ and GFP were isolated by cell-surface biotinylation as described in Materials and Methods. Prepared specimens were separated by 6 % SDS-PAGE and subjected to western blot analysis. (B) The degradation rate of cell-surface-resident HA-BSEP, HA-BSEP-Ub$^{\Delta GG}$ and HA-BSEP-Ub$^{\Delta GG/I44A}$. After cell-surface biotinylation, MDCK II cells expressinig HA-BSEP, HA-BSEP-Ub$^{\Delta GG}$ and HA-BSEP-Ub$^{\Delta GG/I44A}$ were incubated for the indicated time at 37 °C. Remaining biotinylated proteins isolated with streptavidin beads were separated by 6 % SDS-PAGE and subjected to western blot analysis. (C) Quantification of band density indicating HA-BSEP, HA-BSEP-Ub$^{\Delta GG}$ and HA-BSEP-Ub$^{\Delta GG/I44A}$ in (A). The intensity of the band indicating HA-BSEP, HA-BSEP-Ub$^{\Delta GG}$ and HA-BSEP-Ub$^{\Delta GG/I44A}$ was quantified by Image Gauge software and expressed as respective percentages of the BSEP present at 0 h. Closed (●), open (○) circles

and closed square (■) represent remaining cell-surface HA-BSEP, HA-BSEP-Ub$^{\Delta GG}$ and HA-BSEP-Ub$^{\Delta GG/I44A}$, respectively, in MDCK II cells. Each bar represents the mean ± S.E., n = 3 - 5. Asterisks represent statistically significant differences between HA-BSEP and HA-BSEP-Ub$^{\Delta GG}$, $^{***}P < 0.001$.

Figure. 1

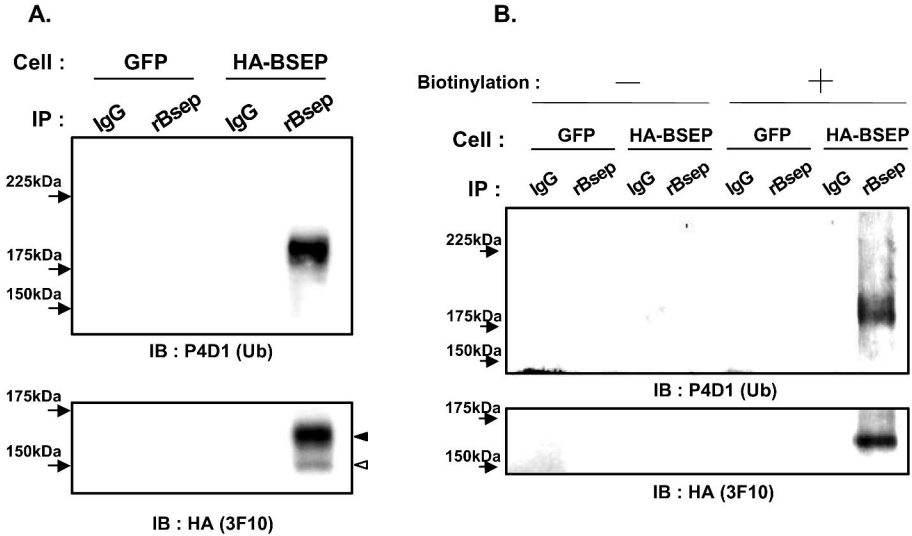

A.
Cell :
GFP
HA-BSEP
IP :
IgG
rBsep
IgG
rBsep
225kDa
175kDa
150kDa
IB : P4D1 (Ub)
175kDa
150kDa
IB : HA (3F10)
B.
Biotinylation :
—
+
Cell :
GFP
HA-BSEP
GFP
HA-BSEP
IP :
IgG
rBsep
IgG
rBsep
IgG
rBsep
IgG
rBsep
225kDa
175kDa
150kDa
IB : P4D1 (Ub)
175kDa
150kDa
IB : HA (3F10)

Figure. 1 continued

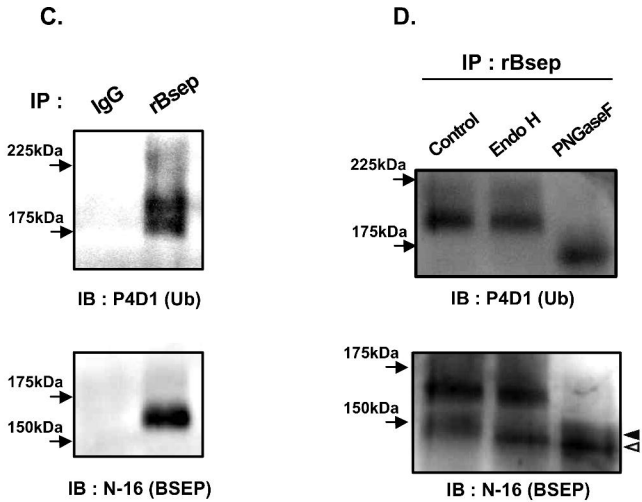


Figure. 2

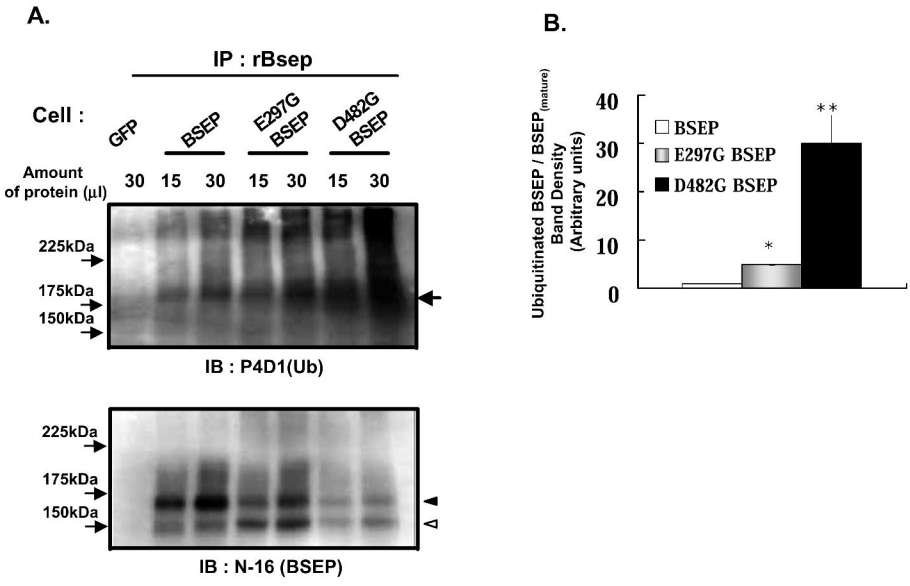

A.
IP : rBsep
Cell :
GFP
BSEP
E297G BSEP
D482G BSEP
Amount of protein (μl)
30 15 30 15 30 15 30
225kDa
175kDa
150kDa
IB : P4D1(Ub)
225kDa
175kDa
150kDa
IB : N-16 (BSEP)
B.
Ubiquitinated BSEP / BSEP(mature) Band Density (Arbitrary units)
40
30
20
10
0
BSEP
E297G BSEP
D482G BSEP
*
**

Figure. 3

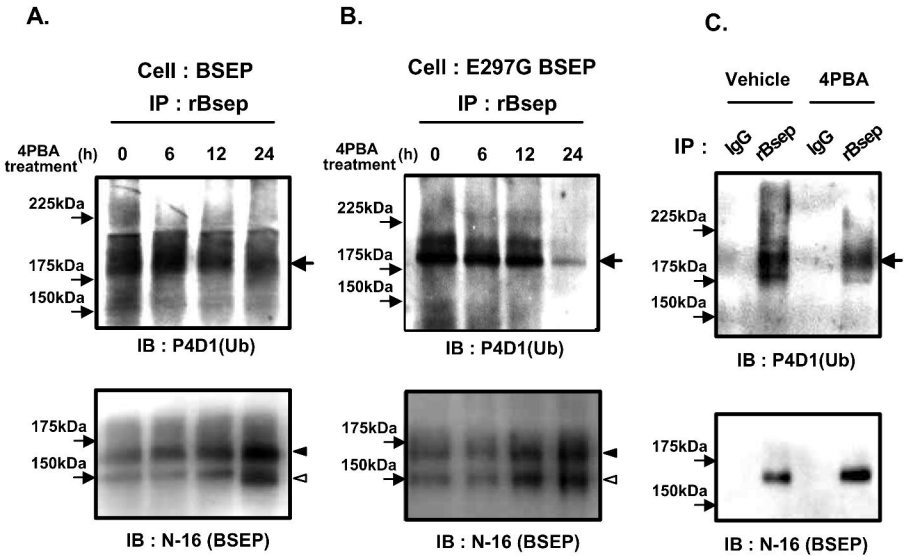

A.
Cell : BSEP
IP : rBsep
4PBA treatment (h) 0 6 12 24
225kDa
175kDa
150kDa
IB : P4D1(Ub)
175kDa
150kDa
IB : N-16 (BSEP)
B.
Cell : E297G BSEP
IP : rBsep
4PBA treatment (h) 0 6 12 24
225kDa
175kDa
150kDa
IB : P4D1(Ub)
175kDa
150kDa
IB : N-16 (BSEP)
C.
Vehicle
4PBA
IP : IgG rBsep IgG rBsep
225kDa
175kDa
150kDa
IB : P4D1(Ub)
175kDa
150kDa
IB : N-16 (BSEP)

Figure. 3 continued

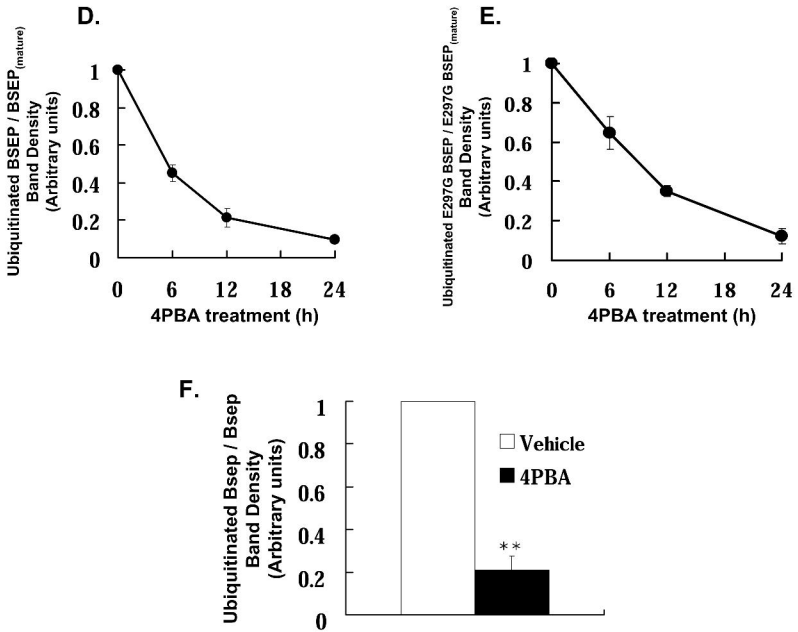
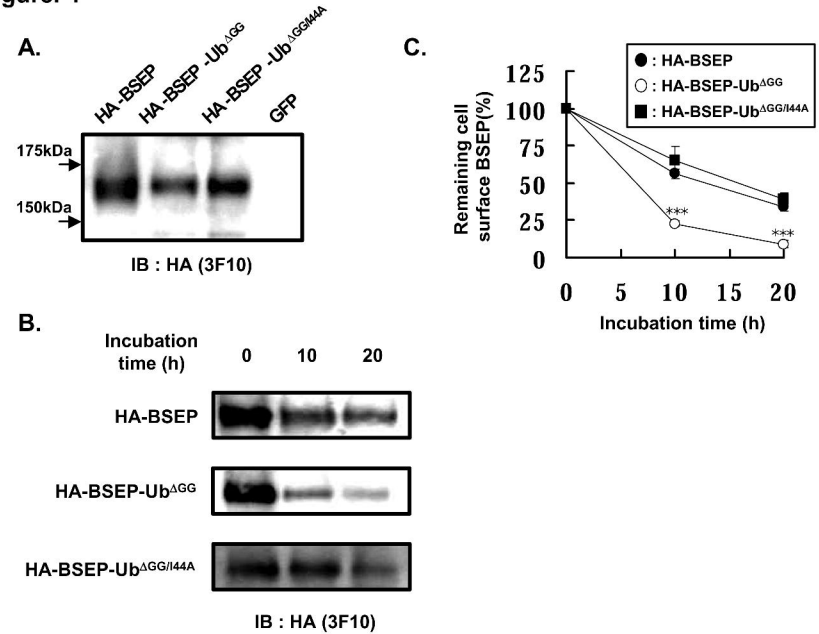


Figure. 4


A.
HA-BSEP
HA-BSEP -Ub^ΔGG
HA-BSEP -Ub^ΔGG/I44A
GFP
175kDa
150kDa
IB : HA (3F10)
B.
Incubation time (h)
0
10
20
HA-BSEP
HA-BSEP-Ub^ΔGG
HA-BSEP-Ub^ΔGG/I44A
IB : HA (3F10)
C.
● : HA-BSEP
○ : HA-BSEP-Ub^ΔGG
■ : HA-BSEP-Ub^ΔGG/I44A
Remaining cell surface BSEP(%)
125
100
75
50
25
0
***
***
0
5
10
15
20
Incubation time (h)