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Association of the 16 kDa subunit c of vacuolar proton pump with the ileal Na+-dependent bile acid transporter: protein-protein interaction and intracellular trafficking

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Abstract:

The rat ileal apical sodium-dependent bile acid transporter (Asbt) transports conjugated bile acids in a Na⁺-dependent fashion and localizes specifically to the apical surface of ileal enterocytes. The mechanisms that target organic anion transporters to different domains of the ileal enterocyte plasma membrane have not been well defined. Previous studies from our laboratory demonstrated that rat Asbt follows an apical sorting pathway that is brefeldin A-sensitive and insensitive to protein glycosylation, monensin treatment, and low temperature shift. Furthermore, a 14-mer signal sequence which adopts a beta-turn conformation is required for apical localization of rat Asbt. In this study, a vacuolar proton pump subunit (VPP-c, 16 kDa subunit c of vacuolar H⁺-ATPase) has been identified as an interacting partner of Asbt by a bacteria two-hybrid screen. A direct protein-protein interaction between Asbt and VPP-c was confirmed in an in vitro pull-down assay and in an in vivo mammalian two-hybrid analysis. Indirect immunofluorescence confocal microscopy demonstrated that the Asbt and VPP-c colocalizated in transfected COS 7 and MDCK cells. Moreover, bafilomycin A1 (a specific inhibitor of VPP) interrupted the colocalization of Asbt and VPP-c. A taurocholate influx assay and membrane biotinylation analysis showed that treatment of bafilomycin A1 resulted in a significant decrease in bile acid transport activity and the apical membrane localization of Asbt in transfected cells. Thus, these results suggest that the apical membrane localization of Asbt is mediated in part by the vacuolar proton pump associated apical sorting machinery.
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Introduction:

Bile acids are essential for the solubilization and transport of dietary lipids and lipid-soluble nutrients and are the major products of cholesterol catabolism. Maintenance of a sufficient concentration of bile salt molecules in the enterohepatic circulation depends upon high affinity transport systems in the liver, intestine, and kidney. Bile acid transporters belong to large gene families and have been found to mediate uptake and secretion of cholephilic compounds, contribute to cholesterol homeostasis, and are involved in bile formation. In the past decade, many of these proteins have been cloned and characterized from the liver, blood-brain barrier, placenta, kidneys, and intestine. Interruption of the enterohepatic circulation of bile acids or malfunctioning of these transporters may result in impaired bile formation and fat malabsorption.

During the past decade, the regulation of bile acid transporters has been extensively studied at transcriptional level [1]. By contrast, the mechanisms underlying posttranslational regulation and polarized expression of bile acid transporters on the plasma membranes of the intestine and kidney cells have not been fully defined. The Asbt is located on the apical surface of ileal enterocytes and kidney cells and plays a major role in the recovery of bile acids in a Na⁺-dependent fashion [2]. Mutations in the Asbt gene are associated with bile acid malabsorption in human [3]. Little is known about proteins that interact with and regulate trafficking of bile acid transporters.

In this study, we provide the first evidence that the 16 kDa subunit c of the vacuolar proton pump (VPP-c, 16 kDa subunit c of vacuolar H⁺-ATPase, NM_009729) directly interacts with rat ileal Na⁺-dependent bile acid transporter (Asbt) and contributes to Asbt apical membrane localization. The subunit c is a highly hydrophobic proteolipid with four putative
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transmembrane helices and assembles into a 260- kDa Vo complex of the vacuolar proton pump
[4]. Our result suggests an important new role for the subunit c of VPP in protein trafficking.
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**Materials and Methods:**

**Plasmid construction and clones:**

BacterioMatch two-hybrid (B2H) vector- The BacterioMatch$^M$T Two-Hybrid System (Stratagene) was used for cDNA library screening to search for protein-protein interactions. Fragments encoding full coding sequence of rat Asbt were cloned into the pBT vector of B2H System (Stratagene). The full coding sequence of the identified binding proteins (i.e. 16 kDa subunit c of vacuolar proton pump, VPP-c) was cloned in-frame into the SalI/NotI sites of pTRG vector to generate the plasmid pTRG-Binding protein (pTRG-VPP-c).

Mammalian cell expression vectors- Wild type rat Asbt cDNAs was subcloned into the mammalian expression vector pCMV2 at the Hind III/Sal I sites as described previously [5, 6]. For the Mammalian two-hybrid (M2H) system, pBIND and pACT (Promega) are fusion vectors for the linkage of proteins to the Gal4 DNA binding domain and to the VP16 transactivation domain, respectively. The segment encoding the C terminus of rat Asbt (aa #308-348) or full coding sequence of rat Asbt were amplified by PCR and cloned in-frame into the SalI/NotI sites of pBIND fusion vector to produce plasmid pBIND-Asbt tail and pBIND-Asbt. A complete coding sequence of the identified binding protein (VPP-c) cDNA was inserted in-frame into the SalI/NotI sites of pACT fusion vector to produce the plasmid construct pACT-VPP-c. A cDNA fragment encoding VPP-c was also inserted in-frame into the BamHI/EcoRI sites of pcDNA3.1/Myc-His (+)A vector to produce Myc-His-epitope tagged construct, VPP-c-MH.

The bacterial expression vector pGEX-3X (Life Technologies) was used to produce GST fused recombinant proteins in *E coli*. The full coding sequence of VPP-c was subcloned in-frame into the BamHI/EcoRI sites of pGEX-3X to produce the GST-fused plasmid constructs.
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All of the positive clones containing cDNA inserts were identified by restriction enzyme mapping and sequenced using the ABI automated DNA sequencer model 377 at the DNA Core Facility, Mount Sinai School of Medicine.

**BacterioMatch two-hybrid system XR plasmid cDNA library screen and protein-protein interaction assay:**

A library screen based on B2H system (Stratagene) was performed to identify proteins that interact with rat Asbt. In this study, the mouse kidney BacterioMatch\textsuperscript{MT} two-hybrid system XR cDNA library in the pTRG plasmid (Stratagene) was screened with the constructed pBT-Asbt using the protocol provided by the manufacturer with some modification. Briefly, pBT-Asbt and pTRG-cDNA library plasmids were first introduced into B2H reporter strain, and transformants were selected on LB-CTCK plates (LB-agar plates supplemented with 250 µg/ml carbenicillin, 15 µg/ml tetracycline, 34 µg/ml chloramphenicol, and 50 µg/ml kanamycin). Then, the selected colonies were screened for β-galactosidase activity by growth on X-gal indicator plates (LB-agar plates supplemented with 15 µg/ml tetracycline, 34 µg/ml chloramphenicol, 50 µg/ml kanamycin, 80 µg/ml X-gal, and 0.2 mM β-galactosidase inhibitor (phenylethyl β-D-thio galactoside)) for 17-30 hr at 30°C. To confirm the detected protein-protein interactions, we then retransformed the reporter strain with the isolated target plasmid plus bait plasmid as described by the manufacturer (Stratagene).

**In vitro binding assay (GST pull-down):** For expression of the glutathione S-transferase (GST) fusion proteins, the appropriate plasmids (GST-VPP-c, see above for coordinates) were transformed into *Escherichia coli* (\textit{E. coli}) DH5α (Life Technologies). The fusion proteins were
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affinity-purified on glutathione-agarose beads as described by Liu et al. [7]. Briefly, GST fusion proteins were expressed in E Coli DH5α cells and induced by the addition of 0.5~2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The harvested bacteria were homogenized in extraction buffer [phosphate buffered saline (PBS) containing 1% Triton X-100, 5 µg/ml leupeptin, 5 µg/ml pepstatin], and centrifuged to remove the insoluble proteins and cell debris right after the extraction process. The extracts were mixed with 200 µl of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) for 5 min. The Sepharose beads were washed with extraction buffer and used for the GST pull-down assay or GST affinity chromatography. The purity of fusion proteins was verified by Coomassie Blue staining of SDS-PAGE and the concentration was determined by a protein assay kit (Bio-Rad). To examine the binding of VPP-c with rat Asbt in vitro, the GST pull-down experiments were performed by using ProFound™ Pull-Down GST Protein:Protein Interaction Kit (PIERCE Biotechnology) as described by manufacturer. The bound proteins were denatured in sample buffer and separated by 12% SDS-PAGE, and were detected by immunoblotting with anti-Asbt antibodies as described previously [5].

Mammalian two-hybrid (M2H) assay for proteins interaction in vivo: When cell cultures reached about 50% confluence, COS-7 cells were co-transfected with 1 µg each of the pBIND-Asbt and pACT-VPP plasmids using Lipofectin (Life Technologies) according to the manufacturer’s recommendations. Plasmid pUC19 were used as a carrier to bring the total amount of DNA in the transfection solution to 3 µg. The cultures were harvested 48 h after transfection and lysed. The firefly luciferase activity was determined according to manufacturer's recommendations.
Cell culture and chemical treatments: COS-7 (SV40 transformed monkey kidney fibroblast) and MDCK II (Madin-Darby canine kidney) cells were used in this study. The plasmid pCMV2-Asbt was used to transiently transfect COS-7 for the GST pull-down assay. pBIND-Asbt and pACT-VPP-c were used to transiently co-transfect COS-7 for M2H. The MDCK II cells were stably co-transfected with pCMV2-Asbt and VPP-c-MH plasmids for confocal microscopy analysis. Cell culture and DNA transfection of cells were performed as described previously [5]. For bafilomycin A1 treatment, the cotransfected cells were incubated with 50 nM bafilomycin A1 (BA1)(Sigma) in culture medium for 16 hr at 37 °C.

Indirect immunofluorescence microscopy was carried out as described previously [5]. Briefly, indirect immunofluorescence microscopy was performed on a confluent monolayer of transfected cells cultured on glass coverslips. The cells were fixed and permeabilized for 7 min in methanol at -20°C, followed by rehydration in PBS. Nonspecific sites were blocked with normal goat serum for 60 min at room temperature (RT). The primary antibody was diluted in the blocking buffer (1% BSA in PBS-0.2% Triton X-100) and incubated for 2 h at RT in a humid chamber. After washing with PBS for 15–30 min, the cells were incubated with secondary antibodies conjugated to FITC or Texas red for 1 h. After being washed with PBS, the cells on coverslips were inverted onto a drop of VectaShield. Fluorescence was examined with a Leica TCS-SP (UV) 4-channel confocal laser scanning microscope in the Imaging Core Facility Microscopy Center, Mount Sinai School of Medicine. A rabbit polyclonal antibody against COOH-terminal 14 amino acid (aa) of rat Asbt and mouse anti-Myc antibodies were used for this study as described previously [5, 6].
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**Bile Acid influx assay:** Na⁺-dependent taurocholate (TC) influx assays were performed as described previously using a transwell filter culture system [5].

**Domain selected biotinylation** was performed essentially as described by Lisanti et al. [8] and Altin et al. [9]. Briefly, polarized monolayers of transfected cells grown on 24 mm trans-well filters were washed with ice-cold PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂ (PBS-C/M). Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL)(0.5 mg/ml in PBS-C/M, pH 8.5) was added either to the apical or basolateral compartment of the filter chamber. Compartments not receiving sulfo-NHS-Biotin were filled with an equivalent volume of PBS-C/M alone. After 30 min of incubation at 37 °C, filter chambers were washed with ice-cold PBS-C/M. Three filter chambers were used per experimental condition. After extraction with 1% SDS, the biotinylated transporters were analyzed by SDS-PAGE.
Results:

Identification of Asbt associated proteins by BacterioMatch two-hybrid library screening:

A cDNA library screening based on the BacterioMatch two-hybrid (B2H) system was used to identify proteins that interact with rat Asbt. For this purpose, the rat Asbt coding sequence was subcloned into plasmid pBT to serve as bait (pBT-Asbt). A mouse kidney BacterioMatch two-hybrid system XR cDNA library in the pTRG plasmid (Stratagene) was screened with the constructed pBT-Asbt. We screened about 2.5 million clones and identified more than 50 positive clones grown on LB-CTCK agar plates. These positive clones were then tested on β-galactosidase plates, 18 of them showed increased β-galactosidase activity (data not show), indicating a possible protein-protein interaction. After isolation and purification of the selected clones, the plasma DNAs were sequenced. The DNA sequence analysis revealed that the clone #13 (pTRG-13) matched the full sequence encoding the mouse 16 kDa subunit c of vacuolar proton pump (VPP-c, 16 kDa subunit c of vacuolar H+-ATPase, NM_009729). The amino acid sequence of mouse VPP subunit c is identical to the sequence of the rat protein (NM_130823)[10].

To further verify the interaction between Asbt and VPP-c from the library screening, the mouse/rat VPP subunit c coding sequence was subcloned into pTRG plasmid (pTRG-VPP-c). The purified pBT-Asbt and pTRG-VPP-c constructs were cotransformed into the bacterial reporter strain and tested on LB-CTCK and X-Gel indicator plates. All transformants (coexpression of VPP-c and Asbt) grew well on the LB-CTCK plate (Figure 1 left panel) and had strong β-galactosidase activity (blue color) on the X-Gel indicator plates (Figure 1 right panel). The negative control (pBT-GF2/pTRG-vector) showed no β-galactosidase activity (white color).
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Interaction between Asbt and VPP-c in vitro (GST Pull-down assay).

An in vitro interaction between Asbt and VPP-c was verified using the GST pull-down assay. First, the cDNA coding sequence of VPP-c was subcloned into pGEX3X vector to generate a GST fused VPP-c (GST-VPP-c), and the fusion protein was expressed in the DH5α bacterial strain. Affinity-purified GST and GST-VPP-c immobilized to the glutathione-Sepharose beads were incubated with lysates of Asbt expressing COS-7 cells. The bound proteins eluted from the beads were separated by SDS-PAGE, and electroblotted onto nitrocellulose membrane. The proteins were then detected using a specific rat Asbt C-terminal antipeptide antibody. Figure 2 shows that purified GST did not pull down Asbt in this assay (Panel A, lane 4), whereas GST-VPP-c efficiently pulled down a non-glycosylated Asbt protein (~34 kDa, Panel A, lane 3). To further verify the possibility of direct-interaction of Asbt with VPP-c, the bound proteins from GST pull-down experiments were denatured in sample buffer, separated by 12% SDS-PAGE, and detected by Coomassie blue staining. The results show that a single protein (a ~34 kDa band) was pull-downed by GST-VPP-c (Figure 2, panel B, lane 2), but no protein was bound by GST-Sepharose (Figure 2, panel B, lane 3). This suggests that Asbt interacts with VPP-c directly.

Examination of protein-protein interaction by in vivo mammalian two-hybrid-luciferase assay.

To further confirm the interaction of Asbt and VPP-c in mammalian cells, we performed a mammalian two-hybrid assay (Promega) in which the expression of the firefly luciferase gene was driven by Gal4-specific enhancer elements. The complete coding sequence of rat Asbt cDNA was inserted in-frame into the SalI/Not I sites of pBIND fusion vector (pBIND-Asbt). A
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cDNA fragment encoding the complete coding region of VPP-c was generated by PCR and was inserted in-frame into the Sal I/Not I sites of pACT fusion vector (pACT-VPP-c). The two constructs were co-transfected into COS-7 cells. To examine the importance of cytoplasmic-tail of Asbt in protein binding to VPP-c, a cDNA encoding the 40 amino acid cytoplasmic tail fragment of Asbt was also inserted in-frame into the SalI/Not I sites of pBIND fusion vector (pBIND-Asbt tail) and co-transfected with pACT-VPP-c into the cells. By analogy to the B2H system, the interaction between Asbt and VPP-c brings together the Gal4D binding domain and the VP16 transactivation domain of the fusion proteins and activates the luciferase-reporter gene in COS-7 cells (Figure 3). Figure 3 shows that a more than 10-fold increase of luciferase activity was observed in Asbt and VPP-c co-transfected cells compared with non-transfected cells. Therefore, any post-translational modifications that may occur in mammalian cells, but are absent in bacteria, do not interfere with the interaction of Asbt and VPP-c. However, only a slight increase of luciferase activity was observed in cells cotransfected with Asbt-tail and VPP-c cDNAs (Figure 3).

Colocalization of Asbt and VPP-c in transfected mammalian cells.

Next, we examined the subcellular localization of Asbt and VPP-c. A Myc-epitope-fused VPP-c (VPP-c-MH) and rat Asbt were cotransfected into COS-7 or MDCK cells. In transfected COS 7 cells, co-localization of Asbt and VPP-c-MH was detected in a punctate cytoplasmic and perinuclear pattern (Figure 4, Panel I, C and D, yellow color). Since flat cells of non-polarized MDCK cells would be better for the visualization of protein co-localization, we selected the flat non-polarized MDCK cells for this study. In transfected MDCK cells, the co-localization of Asbt and VPP-c was observed near the nuclear region (yellow color, Figure 4, Panel II, C and
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D). This is in agreement with the physical interactions detected in the two hybrid and pull-down assays, and suggests that in transfected MDCK cells, the membrane sorting of Asbt may be mediated in part by the VPP-c and through other unknown sorting mechanisms.

The effect of bafilomycin A1 on membrane localization of Asbt.

To examine a possible role of VPP-c in the cellular trafficking of Asbt, we pretreated the cotransfected cells with 50 nM bafilomycin A1 (BA1), a specific inhibitor of the vacuolar proton pump, for 16 h at 37 °C. The confocal image shows that BA1 treatment significantly interrupted Asbt and VPP-c-MH subcellular distribution and colocalization in both COS-7 (Figure 4. Panel III) and MDCK cells (Figure 4. Panel IV). In contrast to the colocalization of Asbt with VPP-c-MH shown as yellow, the distinct localization of VPP-c-MH and Asbt is seen as separate green and red labels, respectively (Figure 4, Panel III and IV, D). After treatment with BA1, a diffuse cytoplasmic staining was observed for VPP-c-MH in both COS-7 and MDCK cells (Figure 4, Panel III and IV, A). In both COS-7 and MDCK cells, a significantly increase in the amount of Asbt protein accumulated intracellularly at ER and Golgi regions after BA1 treatment (Figure 4, Panel III and IV, B).

To further verify the effect of BA1 treatment on the membrane distribution of Asbt, the polarized taurocholate (TC) influx assay and domain specific biotinylation experiments were performed in transfected MDCK cells. Figure 5 (left panel) shows that the initial TC influx activity decreased more than 30% after BA1 treatment comparing with that of BA1 untreated MDCK cells. Similarly, biotinylation analysis demonstrated that the apical membrane distribution of Asbt proteins was significantly decreased by about 26.2±4.1% after BA1 treatment compared with that of BA1 untreated MDCK cells (Figure 5. Central and Right panels). Thus, these results indicate further that the subunit c of vacuolar proton pump (VPP-c)
Protein interaction of Asbt interacts with Asbt and may contribute, at least partially, to the apical membrane localization of Asbt in epithelia cells.
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Discussion

The physiologic functions of ion transporters are determined in part by their subcellular distributions and a complex interaction of protein molecules modulating their activities and intracellular trafficking [11]. Sorting signal motifs for apical membrane targeting, such as N- and O-glycosylation, the GPI anchor, and PDZ binding sequence, have been reported [8, 12, 13]. Most recent studies demonstrated that a Tyr-based sorting signal motif and beta-turn structure are also involved in the apical targeting of Megalin [14] and Asbt [15], respectively. This suggests that the more precise structural features and possibly a tissue-specific regulator may be important as determinants for apical membrane sorting. Over the past decades, studies of various membrane trafficking steps have indicated that specific proteins (e.g., clathrin-coated complex Munc18-2, syntaxin 3, dynein, and PDZ domain containing proteins) play a role in regulation of apical protein localization [16-20]. These factors may be directly or indirectly associated with the sorted proteins and cooperate with each other to generate targeting specificity in the secretory pathway [21]. Some membrane proteins (e.g., Matrix metalloproteinases) contain multiple sorting signal motifs and are regulated by several sorting factors and sorting mechanisms [22, 23].

The vacuolar H⁺-ATPases (V-ATPases) are a family of ATP-dependent proton pumps (VPP) which reside predominantly within intracellular membrane compartments including endosomes, lysosomes, cation-coated vesicles, and the Golgi complex [24, 25]. The acidic luminal pH within these organelles and vesicles of the secretory pathway is established by V-ATPases and is vital for processes such as intracellular membrane transport, receptor-mediated endocytosis, protein processing and degradation, and intracellular targeting of lysosomal enzymes [24-33]. They are multisubunit, heteromeric proteins composed of two structural
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domains, a peripheral, catalytic $V_1$ domain and a membrane-spanning $V_0$ domain. The $V_0$ domain is responsible for proton translocation and the $V_1$ domain is responsible for ATP hydrolysis. All of the subunits of the H$^+\cdot$V-ATPase (VPP) have been now identified and many of their structural and molecular properties characterized [24]. The integral $V_0$ domain of the V-ATPase complex composed of five different subunits (a, c, c', c'', and d) mediates proton translocation. Six copies of the c subunit are found in the $V_0$ complex. The 16 kDa subunit c is highly hydrophobic, contains four putative transmembrane helices [4, 33]. These subunits appear to remain tightly associated with the $V_0$ domain through protein-protein interaction. Previous studies have demonstrated that VPP is required for proper vesicular trafficking through the trans-Golgi network (TGN) [34] and/or Golgi to plasma membrane delivery of proteins [35]. In both kidney and reproductive tract, VPP-rich cells have a high rate of apical membrane recycling. VPP molecules are transported between the cell surface and the cytoplasm in vesicles that have a well-defined 'coat' structure [36]. van Weert et al. [34] demonstrated that inhibition of the VPP by bafilomycin A1 induces retrograde transport of proteins from the trans-Golgi network into the Golgi. Palokangas et al. [37] indicated that VPP might be involved with the early secretory pathway and retrograde transport from the pre-Golgi intermediate compartment and the Golgi complex. Interactions between VPP and the microtubule or microfilament-based cytoskeletons have been found in osteoclasia and intercalated cells [33]. The $V_0$ domain of V-ATPase appears to have direct role independent of proton pumping in membrane fusion essential for intracellular membrane trafficking along biosynthetic and endocytic routes [33].

Recent studies demonstrate that VPP may directly and/or indirectly interact with other proteins [38]. In HepG2 cells, the function of VPP is critical to achieving timely secretion and correct N-linked glycan modifications of proteins that follow the constitutive secretory pathway.
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[35]. Geyer et al. [39] demonstrated that existence of a shared domain in three different families of proteins, Subunit H of the VPP, beta-adaptins, and beta-subunit of COPI. Moreover, Geyer et al. [40] suggest that the H subunit of VPP can function as an adaptor for interactions between Nef and AP-2. Mandic et al. [31] showed that the negative factor from SIV binds to the catalytic subunit H of VPP to internalize CD4 and to increase viral infectivity. Miura et al. [41] have shown that the E subunit of VPP by direct interaction with mSos1 may participate in the regulation of the mSos1-dependent Rac1 signaling pathway involved in growth factor receptor-mediated cell growth control. The 16 kDa subunit c of VPP has been reported to interact with several proteins, such as p12I protein [42]. The 16 kd subunit c is also capable of forming gap junctions and binding with β1 integrin independently of ATPase activity [28, 33]. The subunit c of VPP may also interact with F-actin, and thus may function as an anchor protein regulating the linkage between VPP and the actin-based cytoskeleton [43]. Goldstein et al. [27] demonstrated that hydrophobic, intramembrane interactions govern the association of E5, 16kDa subunit c of VPP and the PDGF receptor, suggesting a ligand-independent mechanism for receptor activation and a potential link between receptor signal transduction pathways and membrane pore activity. An association of V-ATPase with a calcium-releasing channel and the CIC-5 chloride channel have also been defined [33].

The mechanism of Asbt apical membrane targeting has not been fully established. Previous studies from our laboratory demonstrated that rat Asbt follows an apical sorting pathway that is brefeldin A-sensitive and insensitive to protein glycosylation, monensin treatment, and low temperature shift [15]. Furthermore, a 14-mer signal sequence which adopts a beta-turn conformation is required for apical localization of rat Asbt [15]. In this study, the
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VPP-c (16 kDa subunit c of vacuolar H+ -ATPase) has been identified as an interacting partner of Asbt. The results shown that the VPP-C interacts with non-glycosylated Asbt.

Bafilomycin treatment caused the accumulation of the Asbt protein largely at perinuclear ER-Golgi regions and a decrease in the initial apical taurocholate transport activity in Asbt transfected cells. Recent studies have identified subunit c as a key part of the binding site for the highly specific V-ATPase inhibitor bafilomycin [33, 44]. Zhang et al. [4] reported that bafilomycin also binds to a 100 kDa subunit of Vo complexes. There are several possible explanations for these effects of bafilomycin on the Asbt membrane sorting in transfected cells: (1) The 16 kDa subunit c of V-ATPase containing the binding site for the inhibitor bafilomycin may contribute to the directional sorting of Asbt from the Golgi complex to the plasma membrane. (2) It is possible that as a result of bafilomycin binding to 100 kDa subunit of Vo, the conformations of other Vo subunits including subunit c have been altered. These conformational changes of VPP-c may interfer with the interaction of VPP-c with Asbt, and thereby partially interrupt Asbt membrane sorting. (3) Previous studies demonstrated that the Vo domain remains assembled as a 260-kDa complex even after dissociation of the V1 subunits and that the clathrin-coated vesicles contain a significant population of Vo domains not complexed with V1 subunits [4]. The bafilomycin treatment may alter vesicle movement and inhibit trafficking of the transporters to the cell surface. (4) The process of vesicle acidification is also likely to play a role in proper targeting of Asbt to the apical membrane, particularly in the Golgi since the 16 kDa subunit c is associated with the non-glycosylated form of Asbt. However, the effects of V-ATPase on trafficking of Asbt may transcend its properties as a proton pump.

There are a number of yet to be defined steps in which V-ATPase and/or its subunit c of the Vo domain may influence the continuous flow of membranes leading to the correct targeting
Protein interaction of Asbt of Asbt to the apical membrane. In transfected MDCK cells, the Asbt protein partially overlaps with VPP-c and bafilomycin treatment results only in partial interruption of apical membrane localization and initial TC transport activity of Asbt. These results suggest that Asbt apical membrane sorting may involve a VPP-c mediated pathway and other unknown mechanism(s) in epithelial cells. Multiple apical targeting mechanisms for G-protein-coupled receptors in polarized renal epithelial cells have been reported by Saunders and Limbird [23]. Our previous studies show that the cytoplasmic tail of rat Asbt is important for its apical membrane localization. However, in M2H system, only a slight increase of luciferase activity was observed in the Asbt-tail and VPP-c co-transfected cells. This suggests that the tail of Asbt may only weakly interact with VPP-c and/or the cytoplasmic tail of Asbt alone may not sufficient for binding VPP-c. To understand how the Asbt and VPP-c proteins interact with each other requires further investigation.
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Abbreviations footnote:

Asbt = rat apical sodium-dependent bile acid transporter;

VPP = vacuolar proton pump;

VPP-c = 16 kDa subunit c of vacuolar proton pump (subunit c of vacuolar H\(^+\)-ATPase, NM 130823)
Protein interaction of Asbt

**Figure Legends**

**Figure 1.** Protein-protein interaction of rat Asbt and subunit c of vacuolar proton pump (VPP-c).

Each pair of plasmids, as indicated, in the vector pBT (i.e. pBT-Asbt and pBT-GF2) and the vector pTRG (i.e. pTRG-VPP-c, pTRG-Gal11P, and pTRG vector alone) was co-transformed into bacterial reporter strains. The known interaction between GF2 and Gal11P is used as a positive control, whereas the lack of interaction of GF2 and pTRG vector serves as a negative control. **Left panel:** Retransformation of the reporter strain with isolated pBT-Asbt and pTRG-VPP-c: Bacterial transformants were grown on LB-CTCK plates (see Materials and Methods). **Right panel:** The specificity of protein-protein interactions was confirmed using the β-galactosidase reporter: Bacterial transformants were selected on X-Gal indicator plate.

**Figure 2.** Direct interaction between Asbt and VPP-c in vitro (GST Pull-down assay).

(A) Purified GST (lane 4) or GST-VPP-c (lane 3) immobilized onto glutathione-Sepharose beads was incubated with cell extracts prepared from COS 7 cells transfected with a plasmid encoding Asbt, and was examined by western blotting with a specific rat Asbt C-terminal antipeptide antibody. The cell extracts from Asbt transfected COS-7 cells that were treated with tunicamycin to abolish the glycosylation of Asbt or without tunicamycin are shown on lane 2 and lane 1, respectively. Asbt (upper) as well as its non-glycosylated form (lower) are indicated by arrows.

(B) The bound proteins by GST pull-down experiments were denatured in sample buffer, separated by 12% SDS-PAGE, and detected by Coomassie blue staining. The results show that a single protein band (~34 kDa) was pull-downed by GST-VPP-c (lane 2), but no protein was found by GST pull-down (lane3). Lane 1 shows the protein molecular mass standards.
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**Figure 3. Examination of the interaction between Asbt and VPP-c by a Mammalian two-hybrid-luciferase assay.** The Gal4 DNA recognition sequence in the reporter plasmid drives the expression of the firefly luciferase. A cDNA fragment encoding the 40 amino acid cytoplasmic tail of rat Asbt (pBIND-tail) or the full open reading frame of rat Asbt (pBIND-Asbt) is linked to a sequence encoding Gal4DBD in the vector pBIND. A cDNA fragment encoding the full open reading frame of VPP-c is linked to a sequence encoding the VP16AD in the vector pACT (pACT-VPP). The plasmids, as indicated, were co-transfected into COS-7 cells. After incubation, the firefly luciferase activity was determined in cell lysates. All experiments were performed at least twice with triplicate samples and depicted as means ± S.E..

**Figure 4. Cellular co-localization of Asbt and VPP-c in transfected cells by confocal microscopy.**

(Panel I and II) Interaction of Asbt and subunit c of vacuolar proton pump (VPP-c) in situ: COS-7 or MDCK cells were cotransfected with rat Asbt and Myc-His-epitope tagged VPP-c plasmids (VPP-c-MH). VPP-c-MH was detected by mouse anti-Myc antibodies and visualized by fluorescein-labeled anti-mouse IgG (A, green). Asbt was detected by a specific rat Asbt C-terminal antipeptide antibody and visualized by Texas-Red-labeled anti-rabbit IgG (B, red). (C) shows a merged picture and (D) shows a enlarged merged picture. The Arrows (in panel D) indicate the colocalization of Asbt and VPP-c (yellow color).

(Panel III and IV) Effects of bafilomycin A1 (BA 1) treatment on the cellular distribution of Asbt and VPP-c-MH in transfected COS-7 and MDCK cells. The transfected cells were treated with 50 nM BA 1, over night, at 37 °C. The cells were grown on glass coverslips and fixed with cold 100% MtOH after BA 1 treatment. (Bar = 5 µm)
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**Figure 5. Effects of bafilomycin A1 (BA 1) treatment on initial transport activity and polarized membrane distribution of rat Asbt in stably transfected MDCK cells.**

The co-transfected cells were pretreated with (+) or without (-) 50 nM bafilomycin A1 for 16 h at 37 °C.

(Left panel). MDCK cells cotransfected with Asbt and VPP-c-MH plasmid DNA were grown on 6 mm permeable transwell filter inserts for 5 days to ensure a polarized phenotype. The effects of BA1 treatment on polarity of the Na+-dependent taurocholate uptake were measured by incubating cells in 10 µM [3H]TC at 37 °C for 10 min.

(Central panel). Domain specific biotinylation: Transfected MDCK cells were grown on 24 mm permeable transwell filter inserts for 5-7 days. Sulfo-NHS-LC-Biotin was added either to the apical or basolateral compartment of the filter chamber. Compartments not receiving sulfo-NHS-Biotin will be filled with an equivalent volume of PBS-C/M alone. After extraction with 1% SDS, the biotinylated Asbt was analyzed by Western blotting. The Asbt was detected by a specific rat Asbt C-terminal antipeptide antibody (upper panel.). The protein concentration was normalized to the amount of actin protein detected by an anti-actin antibody (lower panel).

(Right panel) Densitometric analyses of membrane distribution of rat Asbt with or without BA 1 treatment. (n=2)

All experiments were performed at least twice with triplicate samples and depicted as means ± S.E..
Protein interaction of Asbt

Figure 1
Protein interaction of Asbt

Figure 2
Protein interaction of Asbt

Figure 3

|            | PBIND | PACT | PACT-VPP-C | PBIND-tail | PBIND-Asbt |
|------------|-------|------|------------|------------|------------|
| Relative Luciferase Activity | -     | +    | +          | -          | -          |
Protein interaction of Asbt

Figure 4
Protein interaction of Asbt

Figure 5
Association of the 16 kDa subunit c of vacuolar proton pump with the ileal Na+-dependent bile acid transporter: Protein-protein interaction and intracellular trafficking

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