A 14-Amino Acid Sequence with a \( \beta \)-Turn Structure Is Required for Apical Membrane Sorting of the Rat Ileal Bile Acid Transporter

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The rat ileal sodium-dependent bile acid transporter (Asbt) is a polytopic membrane glycoprotein, which is specifically expressed on the apical domain of the ileal brush-border membrane. In the present study, an essential 14-amino acid (aa 335–348) sorting signal was defined on the cytoplasmic tail of Asbt with two potential phosphorylation sites motifs for casein kinase II (335SFQE) and protein kinase C (PKC) (338TNK). Two-dimension NMR spectra analysis demonstrated that a tetramer, 544NKGF, which overlaps with the potential PKC site within the 14-mer signal sequence, adopts a type I \( \beta \)-turn conformation. Replacement of the potential phosphorylation residue Ser335 and Thr339 with alanine or deletion of either the 4-335SFQE or 10 aa (338–348, containing 338TNKGF) from the C terminus of Asbt resulted in a significantly decreased initial bile acid transport activity and increased the basolateral distribution of the mutants by 2–3-fold compared with that of wild type Asbt. Deletion of the entire last 14 amino acids (335–348) from the C terminus of Asbt abolished the apical expression of the truncated Asbt. Moreover, replacement of the cytoplasmic tail of the liver basolateral membrane protein, Na\(^+\)/taurocholate cotransporting polypeptide, with the 14-mer peptide tail of Asbt redirected the chimeric to the apical domain. In contrast, a chimera consisting of the 14-mer peptide of Asbt fused with green fluorescent protein was expressed in an intracellular transport vesicle-like distribution in transfected Madin-Darby canine kidney and COS 7 cells. This suggests that the apical localization of the 14-mer peptide requires a membrane anchor to support proper targetting. The results from biological reagent treatment and low temperature shift (20 °C) suggests that Asbt follows a transport vesicle-mediated apical sorting pathway that is brefeldin A-sensitive and insensitive to protein glycosylation, monensin treatment, and low temperature shift.

Bile acids are the major products of cholesterol catabolism and perform an important role in bile secretion and intestinal absorption of lipids and lipid-soluble nutrients. During the past decade, considerable progress has been made in our understanding of mechanisms by which bile acids enter and exit liver and intestinal cells. By contrast, the molecular mechanisms that regulate the activity and membrane localization of these ion transporters remain poorly understood. Recent cloning of several key genes, whose protein products are integral to the enterohepatic circulation of bile acids, has provided an opportunity to investigate the cellular and molecular control of this important metabolic and physiologic pathway.

The ileal apical sodium bile acid transporter (Asbt) plays a major role in the recovery of bile acids from the intestinal lumen (1, 2). The cDNAs encoding this transporter have been cloned from several species (3, 4). Rat Asbt contains seven potential transmembrane domains, localizes to apical surface of ileal enterocytes, and transports conjugated bile acids in a Na\(^+\)-dependent fashion. Sequence analyses of Asbt revealed that there are three conserved domains in the 40-amino acid cytoplasmic tails across species. In addition, there are two potential phosphorylation sites, one for casein kinase II (CK II, 335SFQE) and another for protein kinase C (PKC, 339TNK), in the last 14 amino acids of the carboxyl terminus of rat Asbt.

The mechanisms underlying the basolateral sorting of membrane proteins have been recently defined and have been associated with sorting signals in the cytoplasm tail of these proteins. A \( \beta \)-turn structure has been found in several proteins as part of the sorting signal for the basolateral membrane targeting (6, 7). However, the apical targeting signals are less well defined and are of diverse molecular nature. Up to now, no conformational data are available for the apical sorting signals.

In the present study, we have constructed a series of trunc-
Apical Sorting Signal of Asbt

EXPERIMENTAL PROCEDURES

Materials

Cell culture media were obtained from Invitrogen. [3H]Taurocholic acid (2.1-3.47 Ci/mmol) was purchased from PerkinElmer Life Sciences. Unlabeled taurocholate was purchased from Sigma. Subcloning reagents, enzymes, and competent cells were obtained from Stratagene (La Jolla, CA), Invitrogen, and New England Biolabs (Beverly, MA).

Construction of Mutant and GFP-fused Rat Ileal Bile Acid Transporter (Asbt) cDNA

Plasmid Construction—Wild type and mutant rat Asbt cDNAs were subcloned into the mammalian expression vector pCMV2 or a green fluorescent protein (GFP) vector, pEGFPN2 (Clontech). 

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Construction of Mutant and GFP-fused Rat Ileal Bile Acid Transporter (Asbt) cDNA

Plasmid Construction—Wild type and mutant rat Asbt cDNAs were subcloned into the mammalian expression vector pCMV2 or a green fluorescent protein (GFP) vector, pEGFPN2 (Clontech) as described previously (8). A combination of restriction enzyme digestion and PCR was used to generate mutant and chimeric transporters. The PCR was done with oligonucleotide primers generated from cDNA sequencing information as described previously (5). PCR amplifications were carried out using a PTC-100™ Programmable Thermal Controller (MJ Research, Inc., Watertown, MA). After subcloning into expression vectors, the fidelity of all of the constructs was verified by DNA cycle sequencing using a PerkinElmer Life Sciences, GeneAmp 9600, ABI Prism 377 DNA Sequencer at the DNA Core Facility, Mount Sinai School of Medicine.

Truncated transporters were generated by using a PCR-based strategy to modify the coding sequence as described previously (9). All PCR-amplified products were purified by QIAquick column (Qiagen Corp.) and digested with restriction enzyme. Fragments were subcloned into the MluI and NotI sites of pcMV2 vectors. Proper orientation of constructs was analyzed by restriction enzyme digestion and DNA sequencing.

Del1—The Asbt with deletion of the potential CK II motif (aa 335–338, SPFQ) was constructed by PCR using a forward primer 5′-CCACGGTGATTGAAACTCTCCGTGTCCTCGGAGACAAAC-3′, which is designed to anneal to 5′-end coding sequence of rat Asbt cDNA with an MluI restriction site (boldface), and a reverse primer 5′-CCCGCGGCCGTC-TCTATTTCTCATCTGGTTGAAATCCCTTGT-TTGTCTCC-TGGAAATGGAACCGGAGGATAATGATGATGAGAGGCCATCTGTCGTCCTCTCGGAGACAAAC-3′, containing codons that anneal to sequences 3′-end region of rat Asbt cDNA with a NolI restriction site (underlined).

Del10—The Asbt with deletion of the carboxyl-terminal 10 amino acids (aa 329–338, containing 3′-TNKGF) was constructed by PCR using a forward primer 5′-CCACGGTGATTGAAACTCTCCGTGTCCTCGGAGACAAAC-3′, which is designed to anneal to 5′-end coding sequence of rat Asbt cDNA with an MluI restriction site (boldface), and a reverse primer 5′-CCCGCGGCCGTC-TCTATTTCTCATCTGGTTGAAATCCCTTGT-TTGTCTCC-TGGAAATGGAACCGGAGGATAATGATGATGAGAGGCCATCTGTCGTCCTCTCGGAGACAAAC-3′, containing codons that anneal to sequences 3′-end region of rat Asbt cDNA, but no SPFQ codons, with a NolI restriction site (underlined).

Del10—The Asbt with deletion of the carboxyl-terminal 10 amino acids (aa 329–338, containing 3′-TNKGF) was constructed by PCR using a forward primer 5′-CCACGGTGATTGAAACTCTCCGTGTCCTCGGAGACAAAC-3′, which is designed to anneal to 5′-end coding sequence of rat Asbt cDNA with an MluI restriction site (boldface), and a reverse primer 5′-CCCGCGGCCGTC-TCTATTTCTCATCTGGTTGAAATCCCTTGT-TTGTCTCC-TGGAAATGGAACCGGAGGATAATGATGATGAGAGGCCATCTGTCGTCCTCTCGGAGACAAAC-3′, containing codons that anneal to sequences 3′-end region of rat Asbt cDNA, but no SPFQ codons, with a NolI restriction site (underlined).

Del14—the Asbt with deletion of the potential CK II motif (aa 335–338, SPFQ) was constructed by PCR using a forward primer 5′-CCACGGTGATTGAAACTCTCCGTGTCCTCGGAGACAAAC-3′, which is designed to anneal to 5′-end coding sequence of rat Asbt cDNA with an MluI restriction site (boldface), and a reverse primer 5′-CCCGCGGCCGTC-TCTATTTCTCATCTGGTTGAAATCCCTTGT-TTGTCTCC-TGGAAATGGAACCGGAGGATAATGATGATGAGAGGCCATCTGTCGTCCTCTCGGAGACAAAC-3′, containing codons that anneal to sequences 3′-end region of rat Asbt cDNA with a NolI restriction site (underlined).

Del25—the Asbt with deletion of the carboxyl-terminal 25 amino acids (aa 304–328, containing 3′-TNKGF) was constructed by PCR using a forward primer 5′-CCACGGTGATTGAAACTCTCCGTGTCCTCGGAGACAAAC-3′, which is designed to anneal to 5′-end coding sequence of rat Asbt cDNA with an MluI restriction site (boldface), and a reverse primer 5′-CCCGCGGCCGTC-TCTATTTCTCATCTGGTTGAAATCCCTTGT-TTGTCTCC-TGGAAATGGAACCGGAGGATAATGATGATGAGAGGCCATCTGTCGTCCTCTCGGAGACAAAC-3′, containing codons that anneal to sequences 3′-end region of rat Asbt cDNA with a NolI restriction site (underlined).

Del40—the Asbt with deletion of the carboxyl-terminal 40 amino acids (aa 309–348) was constructed by PCR using a forward primer 5′-CCACGGTGATTGAAACTCTCCGTGTCCTCGGAGACAAAC-3′, which is designed to anneal to 5′-end coding sequence of rat Asbt cDNA with an MluI restriction site (boldface), and a reverse primer 5′-CCCGCGGCCGTC-TCTATTTCTCATCTGGTTGAAATCCCTTGT-TTGTCTCC-TGGAAATGGAACCGGAGGATAATGATGATGAGAGGCCATCTGTCGTCCTCTCGGAGACAAAC-3′, containing codons that anneal to sequences 3′-end region of rat Asbt cDNA with a NolI restriction site (underlined).

Del4—The Asbt with deletion of the potential CK II motif (aa 335–338, SPQF) was constructed by PCR using a forward primer 5′-CCACGGTGATTGAAACTCTCCGTGTCCTCGGAGACAAAC-3′, which is designed to anneal to 5′-end coding sequence of rat Asbt cDNA with an MluI restriction site (boldface), and a reverse primer 5′-CCCGCGGCCGTC-TCTATTTCTCATCTGGTTGAAATCCCTTGT-TTGTCTCC-TGGAAATGGAACCGGAGGATAATGATGATGAGAGGCCATCTGTCGTCCTCTCGGAGACAAAC-3′, containing codons that anneal to sequences 3′-end region of rat Asbt cDNA with a NolI restriction site (underlined).

GFP—The 14-amino acid cytoplasmic tail of rat Asbt was constructed by PCR using a forward primer 5′-GGAAAGCTTCTATTTCTCATCTGGTTGAAATCCCTTGT-TTGTCTCC-TGGAAATGGAACCGGAGGATAATGATGATGAGAGGCCATCTGTCGTCCTCTCGGAGACAAAC-3′, which is designed to anneal to 5′-end coding sequence of rat Asbt cDNA with an MluI restriction site (boldface), and a reverse primer 5′-CCCGCGGCCGTC-TCTATTTCTCATCTGGTTGAAATCCCTTGT-TTGTCTCC-TGGAAATGGAACCGGAGGATAATGATGATGAGAGGCCATCTGTCGTCCTCTCGGAGACAAAC-3′, containing codons that anneal to sequences 3′-end region of rat Asbt cDNA with a NolI restriction site (underlined).

Site-directed Mutagenesis

Potential sorting determinant residues were examined by site-specific mutagenesis. The QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to convert codons for potential sorting determinant residues to alanine residues according to manufacturer’s directions with minor modification as described previously (6). S335A—mutation at potential CK II phosphorylation residue Ser335. The rat Asbt was used as template. A S335A substitution was made using a forward primer 5′-1104TGGACCCCATGACGCAATTC-CAGGAA1129, which annealed to the coding sequence in the Ser335 region, and a compatible reverse primer, in place of the codon for Ser335 was converted to alanine residue (underlined). T339A (Mutation at Potential PK Phosphorylation Residue Thr339)—The Rat Asbt was used as template. A T339A substitution was made using a forward primer 5′-1111CATTTCAAGGCAAAAAGG-GATTTCCACC1129, which annealed to the coding sequence in the Thr339 region, and a compatible reverse primer, in place of the codon for Thr339 was converted to alanine residue (underlined).

ST/AA—the Ser335 and Thr339 double mutant was constructed by using T339A primers and Asbt-S335A mutant construct as template. GFP-fused transporters were constructed using similar PCR-based strategies. The full-length cDNA coding sequences of wild type or mutant transporters were used as templates. The PCR-generated mutant and wild type transporter cDNA products were subcloned into a GFP vector, pGFP FN2 (Clontech), using standard techniques. The coding sequences of wild type and mutant transporters were fused with HindIII site at N-end and BamHI site at C-end and amplified by PCR using full-length cDNA as template. These restriction sites were compatible with the pEFGP N2 polylinker. The PCR products were gel-purified, digested at sites incorporated at the ends of PCR products, and ligated directly to pEFGP C3 vector digested with the same restriction enzymes to produce the GFP-fused 14-amino acid tail of rat Asbt chimera.
Apical Sorting Signal of Asbt

Cell Culture and Transfection

COS 7 (SV40-transformed monkey kidney fibroblast) cells were maintaining in complete Dulbecco's modified Eagle's medium (containing 10% fetal bovine serum, 50 units/ml penicillin, 50 µg/ml streptomycin, and 2 mM L-glutamine). Transient DNA transfection was carried out by Lipofectin™-mediated transfection (Invitrogen) according to the manufacturer's directions. MDCK II cells were maintained in complete MEM-E medium that was supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin, 50 µg/ml streptomycin, and 2 mM L-glutamine. Stable DNA transfection was carried out by Lipofectin™-mediated transfection (Invitrogen) according to the manufacturer's directions. Briefly, 5×10^5 cells were plated on a 100-mm tissue culture dish. The next day, cells were ~50% confluent and were transfected with 5 µg of DNA per 100-mm tissue culture dish. On the following day, transfected MDCK cells were split at 1:3 ratios in complete medium, and the transfected cell lines were selected by growth in the antibiotic G418 (900 µg/ml) (Invitrogen). 10–15 days after transfection, large, healthy colonies were picked by cloning cylinders and transferred to 12-well plates. The expression of the transporters was assayed initially by taurocholate influx assay and confocal microscopy (see below). All of the cells were maintained in a humidified incubator at 37 °C under 5% CO_2 atmosphere.

Bile Acid Influx Transport Assay

The Na+-dependent taurocholate (TC) influx assay was done as described previously (8). The Transwell filter system (Costar, Cambridge, MA) was used for the study of polarity of taurocholate influx. Transfected and untransfected cells were grown to confluence for 5–7 days on 0.45-µm pore size Transwell filter inserts. Formation of a tight seal between the upper and lower chambers was measured by transepithelial transport of [^3]^H]mannitol, which was <10%, as described previously (5). Taurocholate uptake was performed at 37 °C for 10 min. The confluent cell monolayers grown on Transwell filters were washed twice with warm uptake buffer (116 mM NaCl (or choline), 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM MgSO_4, 1.8 mM CaCl_2, 11 mM Na-glucose, 10 mM Hepes, at pH 7.4), and each well was incubated from the apical (0.2 ml) or basolateral (0.6 ml) side with uptake buffer containing 10 µM [^3]^H]taurocholate at the final concentrations. Following 10 min of incubation, the influx assays were terminated by aspirating the medium, and the filters were successively dipped into three beakers, each of which contained 100 ml of ice-cold uptake buffer. The filters were excised from the cups, and the attached cells were solubilized in 0.2 ml of 1% SDS and transferred into scintillation vials with 4 ml of Optifluor or Aquamount (BDH). Fluorescence were examined with a Leica TCS-SP (UV) 4-channel confocal laser scanning microscope in the Imaging Core Facility, Mount Sinai School of Medicine. The 488 nm laser was used for the Live confocal protein assay kit.

In the inhibition studies, taurocholate influx in the absence of competitor was set at 100%, and all values were graphed relative to this level. The final concentration of each inhibitor was 100 µM or indicated in figure legends.

Fluorescence Confocal Microscopy

Indirect immunofluorescence microscopy for Asbt was carried out as described previously (10). Confocal microscopy of GFP-fused chimeras was performed on a convenient monolayer of transfected cells cultured on glass coverslips. Glass coverslip-grown cells were rinsed 3 times with phosphate-buffered saline, fixed for 7 min in 100% methanol at −20 °C, and rinsed 4 times with phosphate-buffered saline, and then mounted with Aquamount (BDH). Fluorescence were examined with a Leica TCS-SP (UV) 4-channel confocal laser scanning microscope in the Imaging Core Facility, Mount Sinai School of Medicine. The 488 nm wavelength line of an argon laser and the 568 nm wavelength line of a krypton laser were used. The cell monolayer was optically sectioned every 0.5 µm. Image resolution using a Leica ×63 and ×100 Neofluor objective and Leica TCS-SP software was 512 × 512 pixels.

NMR Spectroscopy

All NMR spectra were acquired at 20 and 30 °C on a Bruker DRX-600 NMR spectrometer. The NMR sample of the 20-mer peptide (2.5 mM) derived from the cytoplasmic tail of rat ileal bile acid transporter was prepared in a 10 mM phosphate buffer, pH 7.4, containing 150 mM sodium chloride in 90% H_2O and 10% D_2O. The two-dimensional homonuclear TOCSY (36 ms), NOESY (300 ms), and ROESY (300 ms) spectra were acquired with 256 and 2048 complex points in δ_ω and δ_δ, respectively (11). All NMR spectra were processed and analyzed using the NMRPipe (12) and NMRView (13) programs. A 20-mer peptide, ddmpmpsqetnkgqfpdek, corresponding to the sequence from Asp to the cytoplasmic tail of rat Asbt was synthesized by Genosys (The Woodlands, TX). The crude peptide was purified by reverse phase high pressure liquid chromatography on a YMC C-18 column. The peak fractions were subjected to matrix-assisted laser desorption ionization mass spectrometry and analytical high pressure liquid chromatography.

Biological Reagent Treatments

The effects of biological agents on delivery of these transporters to the cell surface were performed as described previously (14–16). Brefeldin A was added to a final concentration of 1–2 µM, as described previously for MDCK II cells (15, 16). Monensin was evaluated at a final concentration of 1.4 µM, as described previously for MDCK II cells (14, 15). Treatment of tunicamycin that abolishes the glycosylation of viral glycoproteins was performed as described previously (8). Inhibition of Asbt-GFP glycosylation by tunicamycin treatment was confirmed by Western blotting.

Statistics Analysis

Most of the results were expressed as mean value ± S.E. and examined by Student’s t test. When two or more tests were performed in an experiment, the mean is used for the group statistics. Results of different groups or categories were compared using the unpaired t test.

RESULTS

The Cytoplasmic Tail of Asbt Is Important for Both Membrane Localization and Substrate Binding Specificity—Previous studies (5) from our laboratory have demonstrated that a apical sorting motif is located on the cytoplasmic tail of Asbt and may transferable and capable of redirecting a protein normally sorted to the basolateral surface to the apical domain of MDCK cells.

There are three domains (amino acids 309–318, 324–328, and 338–348) that are conserved in the 40-aa carboxyl-terminal tail of ileal transporters from the rat, hamster, mouse, and human (Fig. 1A). To identify further the potential sorting motif in the cytoplasmic carboxyl terminus, a series of sequentially shorter cytoplasmic tails of Asbt protein was generated. These truncated transporters were then transfected into COS 7 and MDCK cells. The transport activity of these mutant transporters was analyzed by TC influx assay in a Transwell culture system. The results show that compared with wild type Asbt, deletions of 14 (aa 334–348), 25 (aa 322–348), and 40 (aa 309–348) amino acid residues in the carboxyl tail of Asbt did not change the Na+ dependence of taurocholate transport function but decreased the initial rates of TC transport activity by ~50, ~82, and ~95%, respectively, in transfected COS 7 and MDCK cells (Fig. 1B and Fig. 2). Moreover, in stably transfected MDCK cells, deletion of the 14 and 25 amino acid residues resulted in the mutant proteins being randomly sorted to both apical and basolateral domains (Fig. 2). These results suggested that an apical sorting determinant is present within the last 14 amino acids of Asbt cytoplasmic tail.

To examine whether the cytoplasmic tail is involved in substrate binding specificity, the effects of several bile acid analogs and organic anions on taurocholate transport activity of truncated mutants were tested. The cells were incubated in the presence or absence of 100 µM unlabeled bile acids or other organic anion competitors. Taurocholate influx in the absence of a competitor was set at 100%, and all values were graphed relative to this level of activity. Fig. 3 shows that the competitive inhibitor, cholate, inhibited to a similar degree the initial rate of TC influx in COS 7 cells expressing the wild type or truncated transporters. In contrast, the noncompetitive inhibitor bromosulfophthalein (BSP) and taurodehydrocholate demonstrated various effects on the initial rate of TC influx in COS 7 cells transfected with truncated transporters or with wild type Asbt. Compared with the wild type Asbt (BSP inhibited the TC influx ~20%), BSP had no effect or stimulated TC influx.

dnmpmpsqetnkgqfpdek, corresponding to the sequence from Asp to the cytoplasmic tail of rat Asbt was synthesized by Genosys (The Woodlands, TX). The crude peptide was purified by reverse phase high pressure liquid chromatography on a YMC C-18 column. The peak fractions were subjected to matrix-assisted laser desorption ionization mass spectrometry and analytical high pressure liquid chromatography.
Apical Sorting Signal of Asbt

Fig. 1. A. Alignment of the carboxyl-terminal cytoplasmic tail of ileal apical sodium-dependent bile acid cotransporters from rat, hamster, mouse, and human (GenBank™ accession numbers: rat, Q62633; mouse, D87059; hamster, A49876; and human, U10417). Asterisks indicate the residues shown by two-dimensional NMR to adopt a β-turn structure. The arrowheads indicate potential phosphorylation residues. B. Sodium dependence of taurocholate influx in transfected COS 7 cells. COS 7 cells were either untransfected or transfected with Asbt or deletion mutants of Asbt. TC transport was measured with 10 μM [3H]TC in the presence of Na− or choline (100 mM)-containing buffers at 37 °C for 10 min. Data are presented as pmol/mg protein/min and represent the mean values ± S.E. of three independent experiments performed in triplicate.

Fig. 2. Polarity of sodium-dependent taurocholate influx by stably transfected MDCK cells grown on Transwell filters. The rat Asbt and truncated mutants-transfected MDCK II cells were incubated in 10 μM [3H]TC (with Na− buffer) at 37 °C for 10 min. Data are presented as cpm per Transwell insert and represent the mean values ± S.E. of two to four independent experiments performed in triplicate.

by about 20% in Del14 and Del25 transfected cells, respectively. Moreover, taurodehyrocholate had no significant effect on wild type Asbt-transfected cells but stimulated the TC influx more than 40% in the Del14-transfected cells. These results suggest that the competitive and non-competitive substrates may interact with Asbt differently, and the cytoplasmic tail of Asbt may be important for the substrate binding specificity and plasma membrane delivery in the nonpolarized COS 7 cells.

Apical Sorting of Asbt Is Dependent on a 14-Amino Acid Sorting Signal on the Cytoplasmic Tail—There are two potential motifs for casein kinase II (CK II, 335SFQE) and protein kinase C (PKC, 335TNK) phosphorylation sites in the last 14 amino acids of the cytoplasmic tail of Asbt. Previous studies (17) have shown that PKC could inhibit taurocholate influx in rat hepatocytes. In addition, a potential CK II phosphorylation site at the extreme carboxy terminus of CMV-glycoprotein B has been reported (18), which is involved in internalization and basolateral membrane localization. In order to further identify the apical sorting signal and follow the sorting of a wild type and mutant Asbt in a cell culture model, GFP-fused wild type and a series of mutant rat Asbts were created.

To examine whether the potential PKC and CK II sites are involved in the apical targeting of Asbt, we first mutated the potential phosphorylation residues Ser335 and Thr339. Three mutants were constructed by site-directed mutagenesis to replace the Ser335 (S335A-GFP), Thr339 (T339A-GFP), and both Ser335 and Thr339 (ST/AA-GFP) with alanines. Then these point mutants, and wild type Asbts were fused with the amino terminus of GFP in order to follow the intracellular localization of these proteins in transfected COS 7 and MDCK II cells. Northern and Western blotting were used to verify the cellular expression of the GFP-fused proteins (data not shown). The bile acid transport activity and cell surface expression of these mutant proteins were examined in transfected COS 7 cells by a TC influx assay and fluorescence confocal microscopy. The results show that all three of the GFP-fused point mutants were functionally similar to the GFP-fused wild type Asbt in Na+ dependence of bile acid transport and surface membrane localization in transfected non-polarized COS 7 cells (data not shown). The polarized membrane distribution of the point mutants of Asbt was then examined in stably transfected MDCK cells. The stably transfected MDCK cells were grown to confluence on permeable Transwell filter inserts. The bile acid influx was measured across the apical and basolateral membrane domains of the Transwell filter inserts. The results show that replacement of Ser335 and Thr339 reduced the initial apical transport activity more than 20% and enhanced the basolateral transport activity by 2-fold (Fig. 4), but all three of the point-mutated transporters were predominantly localized to the apical surface of MDCK cells (Table I).

To identify further the apical sorting determinants of Asbt,
domain-deleted mutants were generated. In these mutated transporters, one of the potential phosphorylation sites, S{sup 335}FQE for CK II (del4) and the last 10 amino acid including the PKC site (del10) or the last 14 amino acids including both CK II and PKC sites (del14), were deleted. These constructs were then stably transfected into MDCK cells. As shown in Fig. 5, the polarized {sup 3}H{sub T}auraclocholate transport assay demonstrated that deletion of only one of the two potential phosphorylation sites significantly decreased the initial tauraclocholate influx activity and the fidelity of polarized apical sorting but did not change the dominant apical localization. In contrast, deletion of the entire last 14 amino acid residues resulted in the GFP-fused mutant proteins being randomly sorted to both apical and basolateral domains (Fig. 5). To confirm the surface expression of the GFP-fused wild type and deleted mutants, transfected MDCK cells were cultured on glass coverslips and examined by fluorescence confocal microscopy. Similar to the GFP-fused Asbt, the majority of the four amino acids deleted (del4) and the 10 amino acids deleted (del10) mutants were predominantly detected on the apical plasma membrane of the transfected MDCK cells (Fig. 5). In contrast, deletion of the entire last 14 amino acids containing both potential phosphorylation sites resulted in the loss of apical localization polarity of the GFP-fused mutant proteins (Fig. 5). As a control, the GFP-transfected MDCK cells showed mostly nuclear localization of the protein. It is notable that with all of the mutated transporters changed by increasing the basolateral distribution more than 2-fold (Table I). With regard to the mutant (del10) containing the potential PKC site, the initial apical transport activity of del10-transfected cells was reduced by 33.2% (Fig. 5), and the basolateral localization was enhanced more than 4-fold comparing with that of wild type Asbt-GFP (Fig. 5). These results suggest that the last 14 amino acids contain an apical sorting determinant. The two potential phosphorylation sites, particularly the PKC region, may be involved in the regulation of apical membrane delivery.

The 14-Mer Apical Sorting Determinant Contains a Tetrapeptide β-Turn Structure—To determine whether the cytoplasmic tail sequence of Asbt contains any structurally ordered conformation, we conducted NMR structural analysis of a synthetic peptide containing NDMDPMPFSFQETNKGFPQDEK, derived from the cytoplasmic tail protein sequence of rat Asbt. To overcome signal overlap problems for resonance assignment of the peptide, we collected NMR spectra of the peptide at 20 and 30 °C. The two-dimensional NOESY (Fig. 6A) as well as ROEST (data not shown) spectra show that the NKGF residues of the peptide exhibit a distinct pattern of backbone H{sup 5}N-H{sup 5}C and H{sup 5}N-H{sup 5}C NOE cross-peaks that is consistent with the reverse type-I β-turn conformation (Fig. 6B). The direct evidence, supporting a type I over type II β-turn conformation, is the observation of similar intensity NOE cross-peaks of G14H-H{sup 5}C and F15H-H{sup 5}C. The former NOE peak (corresponding to ~2.2 Å in a well defined β-turn) would show significantly higher intensity than the latter one (~3.2 Å) for a type II β-turn. Small peptides containing related sequences that have been reported to adopt a stable β-turn conformation in solution include the NPXpY motif, which is known as the canonical sequence for the phosphotyrosine binding domains (19, 20). Interestingly, it has been shown that the β-turn formation of the NPXY motif does not depend on phosphorylation of the tyrosine residue (21–23) or require an amino acid tyrosine (peptides with phenylalanine substituting the tyrosine could still form a β-turn) (24, 25). Nevertheless, the Asn and a small amino acid such as glycine or alanine are highly favored at the i and i + 2 positions (26), which is consistent with our observation of the β-turn formation of the NKGF segment of the Asbt peptide in this study.

A Membrane Anchorage Domain Is Required for the Polarized Apical Localization of the 14-Mer Peptide—To examine whether the apical sorting signals contained in the 14-amino acid tail of rat Asbt is autonomous and dominant, a chimera (NtA14-GFP) composed of the 14-mer peptide and truncated liver sodium-tauraclocholate cotransporting polypeptide (Ntcp) was constructed. The wild type Ntcp is also a membrane protein with seven potential transmembrane domains but is normally sorted to the basolateral domain of the hepatocytes. This chimera was transiently transfected into COS 7 and stably transfected into MDCK II cells. The bile acid transport activity and membrane localization were examined by tauraclocholate influx assay. The cellular distribution of this GFP-fused chimera was visualized by fluorescence and analyzed by confocal microscopy. A previous study (5) from our laboratory demonstrated that the cytoplasmic tail of rat Ntcp is essential for the basolateral membrane localization. Deletion of the cytoplasmic tail of Ntcp resulted in loss of the transport activity with most of the truncated Ntcp accumulating intracellularly in the transfected cells (5, 8). The results in this study show that replacement of the C-end 56-amino acid cytoplasmic tail of rat Ntcp with the 14-mer peptide of rat Asbt resulted in increased initial tauraclocholate influx activity and apical membrane localization of this chimera transporter in transfected cells (Fig. 7). This suggested that the increased initial tauraclocholate influx activity is due to enhanced apical membrane delivery and localization of this chimeric transporter.

Previous studies have shown that apical sorting signals require a supporting membrane anchor. The apical localization of rhodopsin mediated by a cytoplasmic sorting motif also required membrane anchors provided by the transmembrane domain and/or a palmitoylation signal (27). This raises the question whether the information present in the transmembrane domains of Asbt and Ntcp is involved in the apical targeting of the 14-amino acid sequence. To answer this question, a chimera (GFP-A14) was constructed, in which GFP was fused with a triplicate 14-mer peptide of rat Asbt. This chimera...
calculated from the taurocholate uptake data in stably transfected MDCK cells.

10 filters. Asbt and truncated mutant-transfected cells were incubated in a confluent monolayer of stably transfected MDCK grown on Transwell filters. The cellular localization of GFP-A14 was analyzed by confocal microscopy. The results (Fig. 8) show that in contrast to the nuclear localization of wild type GFP, fusion of the C-end 14-mer peptide of rat Asbt with GFP, resulted in the accumulation of the fused protein (GFP-A14) intracellularly in a transport vesicle-like pattern. These results suggested that the 14-mer peptide apical sorting signal is autonomous and dominant but requires a membrane anchor to support its function.

**Apical Membrane Sorting of Asbt Is Mediated by a Pathway That Is BFA-sensitive and -insensitive to the Protein Glycosylation, Monensin Treatment, and Low Temperature Shift—**

Studies involving mutation of glycosylation sites and inhibition of N-glycosylation by tunicamycin treatment indicated that the N-linked carbohydrates were able to mediate apical membrane localization of some membrane proteins (28). The function of N-linked carbohydrate on the amino terminus of Asbt-GFP was examined by tunicamycin inhibition and site-directed mutagenesis of N-linked glycosylation sites (Asn3 and Asn10) to alanine in transfected MDCK cells. The results from taurocholate influx studies and confocal microscopy indicated that the apical surface expression of Asbt was not affected by tunicamycin treatment (Fig. 9, A–C). Moreover, the transporters with N-glycosylation site (Asn3 and Asn10) mutated to alanine were also localized to the apical membrane in stably transfected MDCK cells (data not shown). These results suggest that the polarized apical surface expression of ASBT proteins is achieved by an apical sorting pathway that is independent of a carbohydrate-mediated apical sorting pathway.

In MDCK cells and cultured neurons, it has been shown that BFA, a fungal metabolite that disrupts the Golgi compartment and inhibits vesicular transport, inhibits apical or axonal sorting of proteins but does not affect the basolateral or dendritic sorting of proteins (15, 16). Previous studies (27) demonstrated that the apical localization of rhodopsin was mediated by a brefeldin A and low temperature shift-sensitive pathway. Our results show that delivery of Asbt-GFP to the apical surface of MDCK cells was significantly disrupted by BFA after incubating the transfected MDCK cells in a medium containing 1 μM BFA for 15 h at 37 °C (Fig. 9D). The taurocholate transport studies showed that the basolateral localization of Asbt-GFP proteins was enhanced more than 2-fold after BFA treatment compared with untreated cells. The effect of monensin, another transport vesicle-interrupting reagent, on Asbt-GFP sorting to the apical membrane was also examined. Monensin reversibly raises the pH of intracellular vesicles and inhibits recycling of membrane receptors and other glycoproteins (14, 15). In this study, monensin was evaluated at a final concentration of 1.4 μM, as described previously (14, 15). The results showed that monensin did not

**TABLE I**

| Constructs | Amino acid sequence of mutant rat Asbt tail | Apical | Basolateral |
|------------|---------------------------------------------|--------|------------|
| Asbt       | Asbt1–334-335SFQETNKGFQPDEK                  | 90.6 ± 3.1 | 9.4 ± 3.1 |
| Del4       | Asbt1–334–335SFQETNKGFQPDEK                  | 79.5 ± 1.8 | 20.5 ± 1.8 |
| Del10      | Asbt1–334–335SFQETNKGFQPDEK                  | 68.3 ± 2.8 | 31.7 ± 2.8 |
| Del14      | Asbt1–334–335SFQETNKGFQPDEK                  | 53.1 ± 3.5 | 46.9 ± 3.5 |
| S335A      | Asbt1–334–335AFQETNKGFQPDEK                  | 80.2 ± 3.6 | 19.8 ± 3.6 |
| T339A      | Asbt1–334–335AFQETNKGFQPDEK                  | 83.5 ± 4.1 | 16.5 ± 4.1 |
| ST/AA      | Asbt1–334–335AFQETNKGFQPDEK                  | 79.2 ± 2.4 | 20.7 ± 2.4 |
| Nt         | Nt-cp1–306                                   | 51.5 ± 2.6 | 48.5 ± 2.6 |
| NtA14      | Nt-cp1–306-335SFQETNKGFQPDEK                 | 67.8 ± 3.5 | 32.2 ± 3.5 |

a MDCK cells stably transfected with mutant transporters indicate significant difference (p < 0.05) from wild type Asbt-transfected MDCK cells (Asbt).

![Fig. 5. Expression of Asbt and truncated mutants in stably transfected MDCK cells grown on Transwell filters.](image)

(A) Taurocholate influx

(B) Confocal microscopy

(GFP-A14) was transfected into COS 7 and MDCK II cells. The cellular localization of GFP-A14 was analyzed by confocal microscopy. The results (Fig. 8) show that in contrast to the nuclear localization of wild type GFP, fusion of the C-end 14-mer peptide of rat Asbt with GFP, resulted in the accumulation of the fused protein (GFP-A14) intracellularly in a transport vesicle-like pattern. These results suggested that the 14-mer peptide apical sorting signal is autonomous and dominant but requires a membrane anchor to support its function.

**Apical Membrane Sorting of Asbt Is Mediated by a Pathway That Is BFA-sensitive and -insensitive to the Protein Glycosylation, Monensin Treatment, and Low Temperature Shift—** Studies involving mutation of glycosylation sites and inhibition of N-glycosylation by tunicamycin treatment indicated that the N-linked carbohydrates were able to mediate apical membrane localization of some membrane proteins (28). The function of N-linked carbohydrate on the amino terminus of Asbt-GFP was examined by tunicamycin inhibition and site-directed mutagenesis of N-linked glycosylation sites (Asn3 and Asn10) to alanine in transfected MDCK cells. The results from taurocholate influx studies and confocal microscopy indicated that the apical surface expression of Asbt was not affected by tunicamycin treatment (Fig. 9, A–C). Moreover, the transporters with N-glycosylation site (Asn3 and Asn10) mutated to alanine were also localized to the apical membrane in stably transfected MDCK cells (data not shown). These results suggest that the polarized apical surface expression of ASBT proteins is achieved by an apical sorting pathway that is independent of a carbohydrate-mediated apical sorting pathway.

In MDCK cells and cultured neurons, it has been shown that BFA, a fungal metabolite that disrupts the Golgi compartment and inhibits vesicular transport, inhibits apical or axonal sorting of proteins but does not affect the basolateral or dendritic sorting of proteins (15, 16). Previous studies (27) demonstrated that the apical localization of rhodopsin was mediated by a brefeldin A and low temperature shift-sensitive pathway. Our results show that delivery of Asbt-GFP to the apical surface of MDCK cells was significantly disrupted by BFA after incubating the transfected MDCK cells in a medium containing 1 μM BFA for 15 h at 37 °C (Fig. 9D). The taurocholate transport studies showed that the basolateral localization of Asbt-GFP proteins was enhanced more than 2-fold after BFA treatment compared with untreated cells. The effect of monensin, another transport vesicle-interrupting reagent, on Asbt-GFP sorting to the apical membrane was also examined. Monensin reversibly raises the pH of intracellular vesicles and inhibits recycling of membrane receptors and other glycoproteins (14, 15). In this study, monensin was evaluated at a final concentration of 1.4 μM, as described previously (14, 15). The results showed that monensin did not

![Image](image)
significantly affect the polarized membrane targeting of Asbt-GFP (Fig. 9D). To gain further insight into the possible sorting pathway for rat Asbt, the effect of a low temperature shift (20 °C) on the distribution of Asbt-GFP was examined. Low temperature shift has been shown to block the classical secretory pathway by preventing secreted and membrane proteins from exiting the Golgi apparatus (27, 29, 30). The results show that after 6 h of incubation at 20 °C, there was no significant change detected in the initial transport activity and polarity of taurocholate influx (Fig. 9E). All of these results suggest that the apical sorting of Asbt-GFP may be mediated by a transport vesicular sorting pathway that is sensitive, at least partially, to BFA and is insensitive to the monensin treatment and low temperature shift.

**DISCUSSION**

We have systematically examined the cytoplasmic tail sequence requirements for the rat Asbt apical targeting and its possible secondary structure. A novel 14-amino acid apical plasma membrane sorting signal has been identified on the cytoplasmic carboxyl terminus of Asbt. Within this 14-mer signal sequence, a tetramer (340NKGF), which overlaps with a potential PKC phosphorylation site (339TNK), adopts a type I β-turn conformation. Moreover, this 14-mer sorting signal of Asbt requires a transmembrane anchor domain to support its appropriate apical localization, and its apical sorting may be mediated, at least partially, by BFA and is insensitive to protein glycosylation, monensin treatment, and low temperature shift.
glycosylphosphatidylinositol anchor or glycans, the apical sorting signal of Asbt is located on its cytoplasmic tail. Apical sorting signals have been found on the cytoplasmic tail of several other membrane proteins. For example, the C terminus of the GABA transporter GAT-3 (31) and cystic fibrosis transmembrane conductance regulator (CFTR) (32, 33) contain a PDZ-interacting domain that is required for the apical plasma membrane localization and interaction with the PDZ domain-containing proteins, such as EBP50 (NHERF). The PDZ-interacting domain is typically comprised of a tetrapeptide sequence, (N/P)(V/G)Y, in the low density lipoprotein receptor cytoplasmic tail of the low density lipoprotein receptor were shown to be essential for clustering in clathrin-coated pits (38). Kibbe et al. (38) suggested that the receptor internalization rates depend on the affinities of the hexapeptide motif RQVD of the NPXY motif. Aroeti et al. (6) suggested that the presence and location of specific side chains might determine the ability of a particular signal to function as a basolateral signal and/or endocytosis signal. In particular, endocytosis signals contain a Tyr (or other aromatic residue) at position i or i + 3, whereas the β-turn in the basolateral signal of the polymeric immunoglobulin receptor lacks aromatic residues but contains a bulky hydrophobic Val at position i + 2 (6). However, in all of the cases above, the results suggested that the turn structure alone is necessary, but not sufficient, for a maximal response. Up to now, it is unclear whether a unique secondary conformation is involved with apical sorting.

Our results showed that the potential CK II and PKC phosphorylation sites in this 14-mer sequence might act as part of the apical sorting signal. Replacement of the nearby potential phosphorylation residues Ser2295 and Thr2309 reduced the apical transport activity more than 20% and increased the basolateral distribution about 2-fold. Moreover, deletion of the last 10 amino acid residues containing the unique tetramer β-turn structure overlapping with the PKC site region resulted in a significant reduction of the apical sorting and enhancement of basolateral localization more than 3-fold. A computer-generated secondary structure prediction analysis (www.bu.edu/jsa/ request.htm) of the 40-amino acid sequence of the rat Asbt tail showed that a potential turn structure, which has been re-

**Fig. 9.** Effect of biological reagent treatment and low temperature shift on polarized distribution of rat Asbt-GFP in stably transfected MDCK cells. A, the polarity of the Na+-dependent taurocholate uptake was performed on a confluent monolayer of stably transfected MDCK cells incubated with Na+-butyrate (10 mm, 15 h, 37 °C). After 18 h of tunicamycin treatment (2 μg/ml for 16 h + 12 μg/ml for 2 h), the MDCK cells stably expressing Asbt were incubated in 10 μM [3H]taurocholate at 37 °C for 10 min. B, Western blot of plasma membrane fractions from stably transfected MDCK cells with (+Tun) and without (−Tun) tunicamycin treatment. C, confocal microscopy of rat Asbt expressed in stably transfected MDCK cells. The stably transfected MDCK cells were treated with tunicamycin (2 μg/ml for 16 h + 12 μg/ml for 2 h) before cells were fixed. Bar, 5 μm. D, effects of vesicular transport inhibitors on polarized taurocholate uptake in stably transfected MDCK cells. The polarity of the Na+-dependent taurocholate uptake was performed on a confluent monolayer of MDCK cells stably expressing GFP-fused rat Asbt protein cultured on a Transwell culture system. The cells were incubated with Na+-butyrate (10 mm, 15 h, 37 °C). After 15 h of brefeldin A (2 μM) or monensin (1.4 μM) treatments, the Asbt-GFP transfected cells were incubated in 10 μM [3H]taurocholate (with Na+ buffer) at 37 °C for 10 min. Asterisks for each stably transfected MDCK cells treated with drugs indicates significant difference (p < 0.05) from drug-untreated cells. E, effects of low temperature shift on polarized distribution of rat Asbt-GFP in stably transfected MDCK cells. Time dependence of polarized taurocholate uptake of rat Asbt-GFP expressed in stably transfected MDCK cells at 20 °C. The cells were grown on Transwell filter inserts until confluent and incubated at 20 °C for 0, 3, and 6 h before the bile acid transport assay. The data are presented as radioactivity (cpm) of total TC uptake per Transwell filter insert.
ported as essential for protein sorting (6, 7, 9, 21), may occur between amino acids 25 and 35. These residues overlap with the potential CK II and PKC phosphorylation sites. Further computer prediction shows that replacement of the Ser335 or the residues within a tetramer 340NKGF (N, G, and F) with alanines would result in a significant loss of the turn potential of tetramer 340NGKF. This suggests that the Ser335 (a potential CK II phosphorylation residue) may be important for stabilizing the turn conformation. In contrast, computer prediction shows that replacement of the Thr339 with alanine would have no significant effects on the tetramer β-turn potential. This suggests that the effect on the initial transport activity and apical sorting by Thr339 mutation may involve a different mechanism than that of Ser335 mutation. The results from our two-dimensional NMR analysis confirmed that the tetramer sequence 340NGKF does adopt a type I β-turn structure that is similar to the type I β-turn structure in basolateral and endocytosis signals. This experimental evidence demonstrates, for the first time, that an apical sorting signal contains a unique secondary structure and further suggests that turn conformation is a common structural motif for not only basolateral and endocytosis but also apical sorting signals. A possible model is that these sorting signal motifs share a common turn structure backbone but have individual specificity conferred by side chain variation. The side chains of the amino acid residues within and/or nearby the turn structure may favor a specific chain variation. The side chains of the amino acid residues within and/or nearby the turn structure may favor a specific conformation to interact with cytosolic proteins of the sorting machinery to regulate different sorting pathways. However, it is not clear what the relationship is between the phosphorylation and β-turn structure. More studies are necessary to understand the features of turn structure within the apical, basolateral, and endocytosis signals.

In current study, our results showed that a novel 14-mer sequence with a unique turn structure acted as an apical sorting determinant, and this 14-mer peptide can be functionally transferred to a heterologous protein and act as an autonomous apical targeting signal. This makes it more plausible that the structural characteristics of the peptide determined here are biologically relevant. In contrast to the sorting signals of CFTR and rhodopsin, the apical localization of Asbt requires a transmembrane anchorage domain to support its appropriate polarized localization. Asbt is not a glycosylphosphatidylinositol-linked protein, and inhibition of N-linked glycosylation does not inhibit the polarized apical localization of this protein. Based on these differences, we surmise that the mechanism for the apical sorting of rat Asbt may be distinct from that used for the recognition of previously described apical sorting signals.

So far, several regulator and/or adapter proteins have been reported to regulate the multiple apical sorting pathways by interacting with the cytoplasmic tail of proteins in MDCK cells. The association of CFTR with a cytoskeletal complex, e.g., EBP50 and ezrin, could serve as an anchor to determine its specific location within microdomains of the apical membrane and/or its residence time at the cell surface (39). MAL is a nonglycosylated integral membrane protein found in glycolipid- or nonglycosylated integral membrane microdomains in several cell lines including MDCK cells (40, 41). In polarized epithelial MDCK cells, MAL is necessary for normal apical sorting and is thus part of the integral machinery for glycolipid-enriched membrane-mediated apical targeting (40, 41). The results from drug treatment studies demonstrated that MAL-associated apical transport vesicles were sensitive to drugs such as monensin, chloroquine, and NH4Cl but insensitive to the drug BFA (41). It was also reported that the Tctex-1, dynin light chain, could directly interact with the carboxyl-terminal cytoplasmic tail of rhodopsin (42). The apical localization of rhodopsin can be reversibly blocked at the Golgi complex by low temperature shift and altered by BFA treatment. Tugizov et al. (18) reported that CMV glycoprotein B was transported to apical membrane independently of other envelope glycoproteins and that it colocalized with proteins in transport vesicles of the biosynthetic and endocytic pathways.

In contrast to the apically sorted proteins reported previously, our results demonstrated that the apical plasma membrane localization of Asbt was partially interrupted by BFA treatment and was not blocked by monensin treatment and low temperature shift. In addition, without the support of a membrane anchor domain, a GFP-fused 14-mer cytoplasmic tail of rat Asbt was distributed intracellularly in a transport vesicle-like pattern. The differential sensitivity to drug treatment and low temperature shift suggests that Asbt apical sorting may be mediated by a different population of transport vesicles than reported previously for other apical proteins. It is still unknown how this 14-mer sequence directs Asbt apical transport and whether the apical localization of Asbt is mediated by an unidentified factor or other apical targeting regulator/adaptors reported previously, such as Rab11/Rip11 protein complex (43), Munc 18–2 (44), and Raft-associated SNAP receptor (45). Further analysis is required to address these issues and distinguish these possibilities.

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