Cell-specific basolateral membrane sorting of the human liver Na\textsuperscript{+}-dependent bile acid cotransporter

AN-QIANG SUN,1 IKYORI SWABY,1 SHUHUA XU,2 AND FREDERICK J. SUCHY

1Department of Pediatrics, Mount Sinai School of Medicine, New York, New York 10029; and 2Department of Pediatrics, Yale University School of Medicine, New Haven, Connecticut 06520

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Sun, An-Qiang, I’kyori Swaby, Shuhua Xu, and Frederick J. Suchy. Cell-specific basolateral membrane sorting of the human liver Na\textsuperscript{+}-dependent bile acid cotransporter. Am J Physiol Gastrointest Liver Physiol 280: G1305–G1313, 2001.—The human Na\textsuperscript{+}-taurocholate cotransporting polypeptide (Ntcp) is located exclusively on the basolateral membrane of hepatocyte, but the mechanisms underlying its membrane sorting domain have not been fully elucidated. In the present study, a green fluorescent protein-fused human NTCP (NTCP-GFP) was constructed using the polymerase chain reaction and was stably transfected into Madin-Darby canine kidney (MDCK) cells. Taurocholate uptake studies and confocal microscopy demonstrated that the polarity of basolateral surface expression of NTCP-GFP was maintained in MDCK cells but was lost in Caco-2 cells. Nocodazole (33 μM), an agent that causes microtubular depolymerization, partially disrupted the basolateral localization of NTCP-GFP by increasing apical surface expression to 33.5% compared with untreated cells (P < 0.05). Brefeldin A (BFA; 1–2 μM) disrupted the polarized basolateral localization of NTCP, but monensin (1.4 μM) had no affect on NTCP-GFP localization. In addition, low-temperature shift (20°C) did not affect the polarized basolateral surface sorting of NTCP-GFP and repolarization of this protein after BFA interruption. In summary, these data suggest that the polarized basolateral localization of human NTCP is cell specific and is mediated by a novel sorting pathway that is BFA sensitive and monensin and low-temperature shift insensitive. The process may also involve microtubule motors.

Bile acid transporters; protein sorting mechanism; epithelial cells

MAINTENANCE OF THE BILE ACID pool in the enterohepatic circulation depends on the polarized distribution of high-affinity transport systems in the liver and intestine. The availability of cloned cDNAs for both rat and human liver Na\textsuperscript{+}-taurocholate (TC) cotransporting polypeptides (Ntcp), which mediate bile acid uptake by the hepatocyte, allows a thorough analysis of the mechanisms required for sorting of these proteins to the basolateral plasma membrane. These transporters contain seven potential transmembrane domains with an approximate molecular mass of 50 kDa, localize to basolateral surface of hepatocytes and transport conjugated bile acids in a Na\textsuperscript{+}-dependent fashion. They are expressed in differentiated mammalian hepatocytes in a developmentally regulated pattern (13). However, the molecular mechanisms underlying the sorting and targeting of these bile acid transporters to the basolateral plasmatic membrane remain unknown.

Previous studies from our laboratory demonstrated that rat liver Ntcp can be expressed on the basolateral plasma membrane of Madin-Darby canine kidney (MDCK) cells (31). Rat Ntcp has two potential Tyr-based basolateral sorting motifs in its cytoplasmic tail. Removal of the cytoplasmic tail of Ntcp resulted in the accumulation of the truncated protein intracellularly. Moreover, the truncated rat Ntcp protein could be redirected apically by the addition of a sequence containing a apical sorting signal from the rat ileal bile acid transporter (31). Thus a sorting signal in the cytoplasmic domain of Ntcp is likely to be responsible for its basolateral localization. Human NTCP has ~78% identity and 86% similarity with the rat Ntcp at the amino acid level. These two proteins are most divergent at the cytoplasmic tail, with 57% identity and 68% similarity. However, the two potential Tyr-based basolateral-sorting motifs are conserved.

In the present studies, green fluorescent protein (GFP) was fused with human NTCP. GFP presumably contains no sorting signal and has been used for cellular trafficking studies by other laboratories. GFP-fused human liver NTCP (NTCP-GFP) was constructed by PCR and expressed in COS, MDCK, and Caco-2 cells. Analysis of the transport function and cellular localization of the fused transporter protein after treatment with several cytoskeletal and transport vesicle-disrupting agents as well exposure to low temperature were used to further define the potential sorting mechanisms of this transport protein. The results demonstrated that the polarized basolateral expression of human NTCP-GFP is maintained in MDCK cells, but it is lost in Caco-2 cells. Moreover, unlike most other basolateral membrane proteins, the membrane sorting of human NTCP-GFP is brefeldin A (BFA) sensitive and is not blocked by monensin treatment and low temperature. These studies suggest that a novel BFA-
sensitive pathway mediates the basolateral membrane localization of human NTCP-GFP.

MATERIALS AND METHODS

Materials

Restriction enzymes were obtained from New England BioLabs (Beverly, MA). Cell culture media were get from GIBCO BRL (Gaithersburg, MD). [3H]TC (2.1–3.47 Ci/mmol) was purchased from DuPont NEN (Boston, MA). Unlabeled TC was purchased from Sigma Chemical (St. Louis, MO). Subcloning reagents, enzymes and competent cells were obtained from Stratagene (La Jolla, CA), GIBCO BRL, and Invitrogen (Carlsbad, CA).

Plasmid construction. The coding sequence of human hepatic NTCP was fused with Hind III site at NH2 end and BamHI site at COOH terminus and amplified by PCR using full-length cDNA as template. The PCR product was subcloned into a GFP vector, pEGFP-N2 (Clonetech, Palo Alto, CA), using standard techniques. The positive clones containing the human NTCP cDNA insert were identified by restriction enzyme mapping and sequenced on both strands using the ABI automated DNA sequencer model 377 at the DNA Core Facility, Mount Sinai School of Medicine. These positive clones were used for further study.

Cell culture and transfection. COS-7 (SV40 transformed monkey kidney fibroblast) cells were maintained in complete DMEM containing 10% (vol/vol) fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Transient DNA transfection was carried out by Lipofectin-mediated transfection (GIBCO BRL) according to manufacturer's directions. MDCK II cells were maintained in complete MEM-essential amino acid solution medium that was supplemented with 10% (vol/vol) fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Caco-2 cells (human colonic adenocarcinoma cell line) from ATCC (Rockville, MD) were maintaining in DME with 4.5 g/l glucose, 1 mM sodium pyruvate, and 0.01 mg/ml human transferrin, 10% (vol/vol) fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Stable DNA transfection was carried out by Lipofectin-mediated transfection (GIBCO BRL) according to manufacturer’s directions. Briefly, ~5–10 × 10^5 cells were plated on a 100-mm tissue culture dish. On the next day, the 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; GIBCO BRL). Ten to fifteen 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 TC uptake assay and confocal microscopy (see below). All of the cells were maintained in a humidified incubator at 37°C under 5% CO2-95% O2.

Transport studies. The Na+-dependent TC uptake assay was performed as described by Liang et al. (16). The Transwell filter system (Costar, Cambridge, MA) was used for the study of polarity of TC 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 trans-epithelial transport of [14C]mannitol, which was <10%, as described previously (31). TC uptake was performed at 37°C for 10 min. The confluent cell monolayers grown on Transwell filters were washed twice with warm uptake buffer [in mM: 116 NaCl (or choline), 5.3 KCl, 1.1 KH2PO4, 0.8 MgSO4, 1.8 CaCl2, 11 d-glucose, and 10 mM HEPES, 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 [3H]TC at the final concentrations. After a 10-min incubation, the uptake assay was 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 Optifluor (DuPont NEN). The protein was determined with the Bio-Rad protein assay kit.

Blot hybridization. Blot hybridizations were done according to standard techniques.

Confluent microscopy. Confocal microscopy was performed on a confluent monolayer of GFP fused human NTCP transfected cells. Transfected cells were cultured on glass coverslips according to the methods used previously (31). The confluent cells were fixed in 4% paraformaldehyde at room temperature for 30 min. The NTCP-GFP proteins were detected by a Leica TCS-SP (ultraviolet) four-channel confocal laser scanning microscope in the Imaging Core Facility Microscopy Center of the Mount Sinai School of Medicine.

Biological reagent treatments. The effects of biological agents on delivery of these transporters to cell surface were performed as described before (4, 18, 28). Bile acid transport assays and confocal fluorescence microscopy analysis were utilized to localize NTCP-GFP.

BFA is a fungal metabolite that disrupts the Golgi compartment and inhibits vesicular transport. In our experiments, BFA was added to a final concentration of ~1–2 μM, as described previously for MDCK II cells (4, 18).

Nocodazole causes microtubule depolymerization. Transfected cells in Transwell culture were treated with 33 μM nocodazole (equivalent to 10 μg/ml) for 15 h, as described before (28). The ability of nocodazole treatment to disrupt microtubules was confirmed by immunocytochemical analysis with an anti-tubulin monoclonal antibody.

Monensin is a cationic ionophore that exchanges Na+ for H+, thereby increasing the pH of acid intracellular vesicles. Monensin was evaluated at a final concentration of 1.4 μM, as described previously for MDCK II cells (1, 4).
Statistics Analysis

Most of the results were expressed as means ± SE and examined by Student’s t-test. When two or more tests were performed in a subject, the mean is used for the group statistics. Results of different groups or categories were compared by using the unpaired t-test.

RESULTS

Construction and Expression of a NTCP-GFP in COS-7 Cells

To follow the sorting of Ntcp in a cell culture model, a human NTCP-GFP was created. The coding sequence of human NTCP was amplified by PCR using the full-length cDNA as template. The product was subcloned into a GFP vector, pEGFP-N2, to generate a fused protein (NTCP-GFP) in which the COOH terminus of human NTCP was fused with the NH₂ terminus of GFP. The construct was then sequenced to verify the authenticity of the insert. The human NTCP-GFP construct was then transfected using Lipofectin into COS-7 cells. To demonstrate the cellular expression of the GFP-labeled transport protein, transfected COS-7 cells were then analyzed for mRNA and protein by Northern and Western blotting. As shown in Fig. 1 (left), an mRNA of ~3 kb was readily detected in the transfected cells. Western blotting demonstrated an approximate 75-kDa band as expected for the molecular mass of the fusion protein (human NTCP is a 50-kDa protein and GFP is a 27-kDa protein) (Fig. 1, right).

Next, to examine the bile acid transport function and surface expression of this chimeric protein, the transfected COS-7 cells were incubated in a transport buffer containing either 116 mM Na⁺ or an equal amount of choline. As shown in Fig. 2, an incubation buffer containing choline did not support [3H]TC transport. However, in the presence of Na⁺, TC uptake was stimulated over 30-fold in COS-7 cells transfected with the NTCP-GFP plasmid constructs or wild-type NTCP. No significant TC uptake was detected in cells transfected with the plasmid vector alone or in nontransfected COS-7 cells. These data demonstrate that the NTCP-GFP is functionally similar to the wild-type NTCP.

To confirm the surface expression of NTCP-GFP, transfected COS-7 cells were cultured on glass coverslips and examined by fluorescence confocal microscopy. When viewed enface, the GFP-fused transporter was detected on the plasma membrane of the transfected COS-7 cells, a nonpolarized cell line (Fig. 3, middle). In contrast, in cells expressing GFP protein alone, fluorescence was predominantly localized to the area of the nucleus (Fig. 3, right). These studies demonstrate that, similar to wild-type human NTCP, the GFP-fused human NTCP is targeted to the plasma membrane of transfected COS-7 cells.

Fig. 1. Northern and Western blotting analysis of human Na⁺-taurocholate cotransporting polypeptide-green fluorescent protein (NTCP-GFP) expression in transfected COS-7 cells. Left: total RNA from wild-type COS-7 cells (lane 1) and transfected with human NTCP-GFP construct (lane 2) was probed with ³²P-labeled human NTCP cDNA (exposure: ~70°C 16 h). Total RNA was purified by TRizol Reagent (GIBCO BRL), and 10 μg were loaded per lane. Right: total protein homogenates from wild-type COS-7 cells (lane 1) and COS-7 cell transfected with human NTCP-GFP construct (lane 2) or pEGFP-N2 vector only (lanes 3) were separated by 10% SDS-PAGE and analyzed by Western blot. A rabbit polyclonal anti-GFP antibody (from CloneTech) was used in this study. The migrations of protein and RNA standards in an adjacent lane are indicated.

Fig. 2. Sodium dependence of taurocholate uptake in human NTCP-GFP transfected COS-7 cells. COS-7 cells were either untransfected or transfected with pEGFP-N2 vector, human NTCP-GFP construct, or wild-type NTCP. Taurocholate influx was measured with 10 μM [³H]taurocholate in the presence of sodium- or choline (116 mM)-containing buffers at 37°C for 10 min. Values are means ± SE of 3 independent experiments performed in triplicate and are presented as pmol·mg protein⁻¹·min⁻¹.

Fig. 3. Fluorescence microscopy of transfected COS-7 cells expressing human NTCP-GFP. COS-7 cells were cultured on glass coverslips and fixed in 4% paraformaldehyde at room temperature for 30 min. Selected images show fluorescence distribution of COS-7 cells nontransfected (left), transfected with human NTCP-GFP constructs (middle), or pEGFP-N2 vector (right).
Expression of NTCP-GFP in MDCK II and Caco-2 Cells

To explore further the cell specificity of NTCP sorting to the plasma membrane, the NTCP-GFP chimera was stably expressed in MDCK II and Caco-2 cells. We have previously used these cell lines in studies of the polarity of bile acid transporters from liver and intestine (31). Northern and Western blots demonstrate that human NTCP-GFP expressed in MDCK and Caco-2 cells have a molecular mass of mRNA and protein similar to that found in transfected COS-7 cells (data not shown). Stably transfected MDCK and Caco-2 cells were grown as confluent monolayers on Transwell filters and fixed with 4% paraformaldehyde. Fluorescent images, both en face and in X-Z cross-section, were then gathered using laser scanning confocal microscopes. As can be seen in Fig. 4 (top), the steady-state distribution of NTCP-GFP is restricted to the basolateral (predominantly lateral in these experiments) membrane of transfected MDCK cells. In contrast, when this protein is stably expressed in Caco-2 cells, confocal microscopy reveals that the NTCP-GFP protein is found on both the apical and basolateral membrane surfaces (Fig. 4, bottom).

To define the steady-state distribution of human NTCP-GFP, bile acid transport was assayed across the apical and basolateral membranes of MDCK cells, which were grown until confluent on permeable Transwell filter inserts. As shown in Fig. 5, ~80% of Na\(^+\)-dependent TC uptake was detected from the basolateral surface of NTCP-GFP transfected MDCK cells. It has been reported that, in contrast to MDCK cells, Caco-2 cells may express an Na\(^+\)-dependent bile acid transport system (9). Our results demonstrated that the baseline of \(^{3}\)H/TC uptake of wild type Caco-2 cells was ~550–850 cpm/well. The \(^{3}\)H/TC uptake of NTCP-GFP stably transfected Caco-2 cells was increased about two- to threefold compared with wild-type Caco-2 cells. Therefore, Na\(^+\)-dependent TC uptake by human NTCP-GFP in transfected Caco-2 cells was calculated by subtracting the \(^{3}\)H/taurocholate uptake of nontransfected Caco-2 cells from that of transfected Caco-2 cells.

Analysis of Potential Basolateral Sorting Mechanisms for NTCP-GFP

A series of experiments were performed to determine whether NTCP-GFP is recognized and sorted by a pathway similar to that used by other basolateral membrane and secretory proteins. To this end, several cytoskeletal and transport vesicle-disrupting agents, as well as the effects of low-temperature shift, were used to determine the potential mechanisms used for sorting of NTCP-GFP to the basolateral membrane.

First, nocodazole was used to test whether the basolateral localization of NTCP-GFP involved microtubules. Nocodazole causes microtubule depolymerization and has been used to block sorting of proteins to both the apical and basolateral surfaces (28). The effects of nocodazole on NTCP-GFP delivery to the cell surface was examined by incubating transfected MDCK cells in a medium containing 33 \(\mu\)M nocodazole for 15 h at 37°C. To ensure that nocodazole disrupted microtubules, MDCK cells were grown on coverslips in
the presence or absence of nocodazole and examined by immunofluorescent microscopy using anti-tubulin antibodies. After treatment with this agent, a diffuse cytoplasmic staining was observed, rather than the characteristic network of tubulin (data not shown). The effect of nocodazole on the surface expression of NTCP-GFP was then determined. Fluorescence confocal microscopy demonstrated that nocodazole treatment increased the sorting of NTCP-GFP to the apical membrane with rare flecks of apical staining in the X-Z sections (Fig. 6A). Tc uptake studies on transfected MDCK cells grown on Transwell filters showed that nocodazole partially disrupted the polarized surface expression of NTCP-GFP by increasing the apical localization of the protein to ~33.5% (P < 0.05) compared with untreated cells (22.6%) (Fig. 6, B and C).

BFA, a fungal metabolite, causes disassembly of the Golgi apparatus and effectively interrupts vesicular transport from the Golgi to the plasma membrane in several cell lines. In MDCK cells and cultured neurons, it has been shown that BFA inhibits apical or axonal sorting of proteins but does not affect the basolateral or dendritic sorting of proteins (4, 10, 17, 18). However, results from other investigators suggest that basolateral sorting of some proteins may also be interrupted by BFA (3, 20). In the present study, fluorescence confocal microscopy demonstrates that delivery of NTCP-GFP to the basolateral surface of MDCK cells was severely disrupted by BFA after incubation of the transfected MDCK cells in a medium containing 1 μM BFA for 15 h at 37°C. Both fluorescent confocal microscopy and TC transport studies showed that NTCP-GFP proteins were randomly targeted to both the apical and basolateral domains of the plasma membrane (Fig. 6).

The effect of monensin on NTCP-GFP sorting to the basolateral membrane was then examined. Monensin reversibly raises the pH of intracellular vesicles and inhibits recycling of membrane receptors and other glycoproteins (1, 2, 5, 24, 30, 33). In this study, monensin was evaluated at a final concentration of 1.4 M, as described previously in studies involving MDCK II cells (1, 4). The results of fluorescence confocal microscopy and TC uptake experiments across the apical and basolateral compartments showed that monensin did not effect the polarized membrane targeting of NTCP-GFP (see Fig. 6).

The results of these experiments indicate that the basolateral sorting of NTCP-GFP is critically dependent on a BFA-sensitive mechanism. Moreover, microtubules also appear to have a role in the polarized membrane sorting pathway for NTCP.

To gain further insight into the possible sorting pathway for human NTCP, the effect of a low-temperature shift (20°C) on the distribution of NTCP-GFP was studied. This low-temperature block has been shown to inhibit the classic secretory pathway by preventing secreted proteins from exiting the Golgi apparatus and has been reported to block sorting of some apical and basolateral membrane proteins (19, 27). TC transport assays and confocal microscopy were used to examine the distribution of NTCP-GFP in stably transfected MDCK cells, incubated at 20°C. The results show that, after 6 h of incubation at 20°C, there was no significant change detected in the polarity of TC uptake (Fig. 7A). On confocal microscopy there was also no change in the basolateral localization or accumulation of NTCP-GFP within Golgi complex in stably transfected MDCK cells examined after 6 h of incubation at 20°C (Fig. 7B, top left and middle). Moreover, a prolonged block of 6–10 h or at lower incubation temperature of 15°C still had no significant effect on the distribution of NTCP-GFP (data not shown).

An experiment was then done to determine whether basolateral localization of NTCP-GFP in MDCK cells could be restored at low temperature (20°C) after disruption by BFA. MDCK cells stably transfected with NTCP-GFP were treated with 2 μM BFA for 3 h at 37°C. The MDCK monolayers expressing NTCP-GFP were then transferred to a medium containing 5 mM cycloheximide and incubated at 20 or 37°C in the presence or absence of 2 μM BFA for 1.5 h before the cells were fixed for microscopy. The NTCP-GFP protein was distributed to both the apical and basolateral domains in a nonpolarized fashion after 3 h of incubation at 37°C with BFA (Fig. 7B, top right). Some bright, punctate staining of NTCP-GFP was also found throughout the cytoplasm of cells treated with BFA. Figure 7B (bottom) shows that the basolateral surface distribution of NTCP-GFP was rapidly restored by removal of BFA from the incubation media at both 20 and 37°C for only 1.5 h. Because normal cell polarity was restored in the presence of cycloheximide, these findings indicate that the NTCP-GFP proteins detected at the basolateral surface were derived from a preexisting pool of the protein within the cell and not newly synthesized proteins. In cells maintained at 20°C with BFA still in the medium, the NTCP-GFP remained in a nonpolarized distribution (Fig. 7B, bottom left). These experiments indicate that NTCP-GFP is transported to the basolateral plasma membrane via a novel temperature-insensitive pathway.

DISCUSSION

Previous studies have shown that various epithelial cells have the ability to sort proteins to distinct surface membrane domains (12, 26). A Tyr-based sorting motif may be involved in basolateral sorting in many polarized epithelial cell types, but the process may be cell specific. For example, the H^+K^+-ATPase β-subunit, which contains a Tyr-based motif in its cytoplasmic tail, is restricted to the basolateral membrane of MDCK cells. However, in LLC-PK1 cells, this protein accumulates at the apical membrane domain (26). This suggests that, although different classes of signals can specify the same distribution or dynamic behavior, distinct components of the cellular sorting machinery are responsible for interpreting their message (26). The question has thus been raised as to whether the mechanisms of basolateral delivery of the liver bile acid transporter are cell specific or whether the transporter protein contains specific signals that could direct it to
this membrane compartment in any polarized cell. To answer these questions, we have established stably transfected MDCK and Caco-2 cell lines that express the human NTCP-GFP. In MDCK cells, newly synthesized proteins are sorted primarily via the exocytic route, while Caco-2 cells use both exocytic and endocytic-transcytotic pathways are used (21, 29). Caco-2 cells resemble small intestinal enterocytes both in morphology and function. These cells transport small molecules in a manner similar to isolated ileal enterocytes and therefore have been used as a model for studying the molecular basis of ileal bile acid transport (9).

Our results demonstrate that NTCP-GFP is similar to the wild-type human NTCP protein in terms of transport function and surface membrane localization in transfected COS-7 cells. As we have shown for rat Ntcp, the physiological basolateral localization of human NTCP in hepatocytes was reproduced in stably transfected MDCK cells (31). In addition TC transport by MDCK cells stably transfected with NTCP-GFP cDNA exhibits the same features of the liver bile acid transport system, such as Na\(^+\) dependence, saturation of transport at high substrate concentrations, and inhibition by other bile acids. These results support our hypothesis that sorting information is embodied in the NTCP protein itself and is functional in heterologous polarized MDCK cells. In contrast, in NTCP-GFP stably transfected Caco-2 cells, confocal microscopy and polarized transport assays revealed that the surface expression of NTCP-GFP protein is not polarized on the plasma membrane. Thus sorting signals intrinsic to NTCP are likely recognized and interpreted differently by sorting machinery present within various cells.

Between the rough endoplasmic reticulum (ER) and the Golgi apparatus, proteins traffic via vesicles that shuttle between the transitional elements and the cis-Golgi cisternae (23, 27). Such vesicles are closely associated with a tubular membrane system at the cis-face of the Golgi apparatus. The cycling between ER and cis-Golgi may determine the cellular localization of proteins (27). Most previous studies have showed that treatment with microtubule-disrupting drugs, such as nocodazole, specifically affects polarized delivery of proteins to the apical but not basolateral membrane (4, 28). However, Lafont et al. (15) reported that, in epithelial cells, microtubule motors are involved in the movement of apical and basolateral vesicles. They suggested that the differential requirement for microtubule-FIG. 6. Effects of biological drug treatment on the polarized localization of human NTCP-GFP in stably transfected MDCK cells. The stably transfected MDCK cells were treated with or without nocodazole (33 \(\mu\)M), Brefeldin A (BFA; 1 \(\mu\)M), and monensin (1.4 \(\mu\)M) (see Methods). A: cellular localization of human NTCP-GFP in the absence and presence of cytoskeletal- and transport vesicle-disrupting drugs. Stably transfected MDCK cells were grown on glass coverslips until confluent and fixed with 4% paraformaldehyde after biological drug treatments. Confocal enface and X-Z cross-section photomicrographs were performed on the confluent monolayer of MDCK cells stably transfected with human NTCP-GFP and incubated with or without the reagent to be tested as described in Methods. B: MDCK cells stably expressing human NTCP-GFP were grown on permeable Transwell filter inserts for 5–7 days to ensure a polarized phenotype. The effects of drug treatments on polarity of the Na\(^+\)-dependent taurocholate uptake were measured by incubating cells in 10 \(\mu\)M \(^{3}H\)taurocholate (with Na\(^+\) buffer) at 37°C for 10 min. Values are means value \(\pm\) SE of 3 independent experiments performed in triplicate presented as pmol-mg protein \(-1\)-min \(^{-1}\) of taurocholate uptake. C: percentage of total \(^{3}H\)taurocholate uptake from apical and basolateral surface of stably transfected MDCK cells after biological drug treatments. *Significant difference for each NTCP-GFP stably transfected MDCK cell treated with drugs compared with control cells, \(P < 0.05\) (by unpaired \(t\)-test).
Our results demonstrated that nocodazole could partially interrupt the polarized basolateral membrane targeting of human NTCP-GFP. Two possibilities may explain these findings: 1) this basolateral sorting process may involve both microtubule-dependent and -independent pathways, and interrupting microtubule-dependent pathway may stimulate the independent pathway to recover trafficking, or 2) this drug does not fully disrupt microtubules in intact cells. Despite these possibilities, our results demonstrate that polarized basolateral delivery of human NTCP-GFP protein at least partially requires intact microtubules.

BFA and monensin are both agents that interrupt vesicular transport. Because of the existence of different populations of transport vesicles and/or specific components which insert different proteins into specifically targeted vesicles, the transport mechanisms of various proteins have been shown to be differentially affected by these reagents (1, 3, 7, 18, 32). These studies suggest that multiple basolateral sorting mechanisms mediate the polarized membrane distribution of secretory and membrane proteins. For example, several groups have shown that BFA selectively inhibits the apical sorting of proteins, such as the apical membrane protein dipeptidyl peptidase IV in MDCK cells, whereas it is without effect on the polarization of various basolateral proteins, such as uromorulin and the follicle-stimulating hormone receptor (4, 6, 10, 17, 18). However, the results from other two groups indicate that BFA inhibits the delivery of the low-density-lipoprotein receptor and the polymeric immunoglobulin receptor from the trans-Golgi network to the cell basolateral surface in MDCK cells (3, 20). Our results demonstrate that BFA treatment totally disrupted the polarity of NTCP-GFP localization on the basolateral membrane. This suggests that the sorting of human NTCP-GFP involves a BFA-sensitive basolateral pathway that may be interrupted by BFA at the stage of movement from ER to the Golgi.

Monensin can block glycoprotein transport along the secretory pathway in the cis- or medial Golgi in most mammalian cells and may affect transport of apical and basolateral proteins differently (1, 4, 5, 24, 30, 33). For example, treatment with monensin inhibited the expression of the basolateral vesicular stomatitis virus glycoprotein but did not affect the polarity of influenza hemagglutinin (1, 2, 4, 25). We found that monensin had no significant effect on the sorting of human NTCP-GFP.

Low temperature has also been shown to inhibit the ER to Golgi transport of some proteins and has been used to follow the movement of transport vesicles within cells (14, 22). Low-temperature shift inhibits the classic secretory pathway by preventing apically and basolaterally targeted proteins from exiting the Golgi apparatus (19, 22). Our results demonstrated that incubation at 20°C did not block NTCP-GFP polarized membrane sorting. It may be argued that, because of the slow sorting process of NTCP in MDCK cells, incubation for 6 h at 20°C may not be sufficient to alter the membrane sorting of NTCP. This does not seem to be the case, because a prolonged incubation at 20°C (10 h) or at lower temperature (15°C) had no significant effect on the basolateral surface distribution. Previous studies indicate that BFA affects ER to
Golgi transport prior to the temperature-sensitive step and at 15–20°C newly synthesized virus membrane glycoproteins accumulate in tubulovesicular structures between the rough ER and Golgi apparatus (27). Fire et al. (11) showed that low-temperature shift (22°C) slowed the endocytosis of receptor-mediated clathrin-coated protein. Our results showed that pretreatment with BFA did not change or inhibit the repolarization of the basolateral distribution of human NTCP-GFP at 20°C. Taken together, these results suggest that, in contrast to most other basolateral proteins reported previously, human NTCP-GFP is transported to basolateral plasma membrane via a novel temperature-insensitive pathway.

In summary, our data suggested that the polarized basolateral localization of human NTCP is cell specific. In contrast to other basolateral membrane proteins reported previously, the polarized basolateral localization of human NTCP is mediated by a novel sorting pathway that is BFA-sensitive and monensin and low temperature-shift insensitive. It may also involve microtubule motors. Our results agree with the previous observations indicating that sorting mechanisms may vary among cell types. This property may provide greater flexibility for various cell types in generating and maintaining cell polarity.

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REFERENCES


