Vectorial transport of bile salts across MDCK cells expressing both rat Na\(^{+}\)-taurocholate cotransporting polypeptide and rat bile salt export pump

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Mita, Sachiko, Hiroshi Suzuki, Hidetaka Akita, Bruno Stieger, Peter J. Meier, Alan F. Hofmann, and Yuichi Sugiyama. Vectorial transport of bile salts across MDCK cells expressing both rat Na\(^{+}\)-taurocholate cotransporting polypeptide and rat bile salt export pump. Am J Physiol Gastrointest Liver Physiol 288: G159–G167, 2005. First published August 5, 2004; doi:10.1152/ajpgi.00360.2003.—Bile salts are predominantly taken up by hepatocytes via the basolateral Na\(^{+}\)-taurocholate cotransporting polypeptide (NTCP/SLC10A1) and secreted into the bile by the bile salt export pump (BSEP/ABC11). In the present study, we transfected rat Ntcp and rat Bsep into polarized Madin-Darby canine kidney cells and characterized the transport properties of these cells for eight bile salts. Immunohistochemical staining demonstrated that Ntcp was expressed at the basolateral domains, whereas Bsep was expressed at the apical domains. Basal-to-apical transport of taurocholate across the monolayer expressing only Ntcp and that coexpressing Ntcp/Bsep was observed, whereas the flux across the monolayer of control and Bsep-expressing cells was symmetrical. Basal-to-apical transport of taurocholate across Ntcp/Bsep-coexpressing monolayers was significantly higher than that across monolayers expressing only Ntcp. Kinetic analysis of this vectorial transport of taurocholate gave an apparent \(K_{m}\) value of 13.9 ± 4.7 \(\mu\)M for cells expressing Ntcp alone, which is comparable with 22.2 ± 4.5 \(\mu\)M for cells expressing both Ntcp and Bsep and \(V_{\text{max}}\) values of 15.8 ± 4.2 and 60.8 ± 9.0 pmol\(\cdot\)min\(^{-1}\)\(\cdot\)mg protein\(^{-1}\) for Ntcp alone and Ntcp and Bsep-coexpressing cells, respectively. Transcellular transport of cholate, glycocholate, taurochenodeoxycholate, chenodeoxycholate, glycochenodeoxycholate, tauroursodeoxycholate, ursodeoxycholate, and glycochenodeoxycholate, but not that of lithocholate was also observed across the double transfectant. This double-expressing system could be used as a model to clarify vectorial transport of bile salts across hepatocytes under physiological conditions.

bile salt transporters; hepatocyte; transcellular transport

Vectorial transport of bile salts across hepatocytes plays a vital role in their efficient enterohepatic circulation. Indeed, the highly concentrated excretion of bile salts has been demonstrated and the concentration gradient of bile salts is as steep as 100- to 1,000-fold between the portal plasma and bile. This vectorial transport is supported by the transporters located on the basolateral and bile canicular membranes (18, 33).

On the basolateral membrane of hepatocytes, bile salts are taken up from the portal vein by \(Na^{+}\)-dependent \(Na^{+}\)-taurocholate (TC) cotransporting polypeptide (Ntcp; rat Ntcp/Slc10a1 and human Ntcp/SLC10A1) (10, 11) and \(Na^{+}\)-independent organic anion-transporting polypeptides (rat Oatps/Slc21a and human OATPs/SLC21A). On the canalicular membrane, unipolar bile salts are secreted into bile by the apical bile salt export pump (rat Bsep/Abcb11 and human Bsep/ABC11) (4, 7, 23), whereas dipolar bile salts, which account for only 0.5% of total biliary bile salts in humans, are excreted by multidrug-resistance protein 2 (rat Mrp2/Abcc2 and human MRp2/ABCC2) (13, 14, 32). Thus NTcp/Ntcp and BSEP/Bsep play key roles in the transport of bile salts across hepatocytes.

The molecular properties of these two transporters have been characterized recently. For Ntcp, the transport characteristics were investigated using cRNA-injected oocytes and cDNA-transfected cell lines such as COS-7 (3, 15), Chinese hamster ovary (CHO) (31), HeLa (28), V79, and HPCT-1E3 cells (24). The transport properties of BSEP have been characterized exclusively using cRNA-injected oocytes and isolated membrane vesicles prepared from cDNA-transfected/injected SF9 cells (1, 4, 7, 9, 23, 30) or mammalian BALB-3T3 fibroblasts (9). Although the uptake and efflux transporters act synergistically to produce the vectorial transport of bile salts, previous analyses have only focused on the function of a single transporter. Therefore, it seemed desirable to establish an in vitro experimental system to allow quantitative analysis of the transcellular transport of bile salts across hepatocytes from blood to bile. Such a synergistic role of transporters may be quantitatively examined by examining the transcellular transport of substrates across the cell monolayer after transfection/infection of the respective cDNAs. Indeed, recent studies using a double transfectant of OATP8 and MRP2 (6) and that of OATP2 and MRP2 (26) have demonstrated that this experimental approach is feasible.

In the present study, we have stably transfected rat Ntcp into polarized Madin-Darby canine kidney (MDCK) cells, which were subsequently infected with a recombinant adenovirus containing rat Bsep cDNA. After it was confirmed that Ntcp and Bsep were expressed on the basolateral and apical membranes, respectively, we characterized the transport of a series of bile salts. Kinetic analysis was performed for TC, because this bile salt accounts for >80% of the bile salt pool in rats and is transported by multidrug-resistance protein 2 (rat Mrp2/Abcc2 and human MRp2/ABCC2) (13, 14, 32). Although the uptake and efflux transporters act synergistically to produce the vectorial transport of bile salts, previous analyses have only focused on the function of a single transporter. Therefore, it seemed desirable to establish an in vitro experimental system to allow quantitative analysis of the transcellular transport of bile salts across hepatocytes from blood to bile. Such a synergistic role of transporters may be quantitatively analyzed by examining the transcellular transport of substrates across the cell monolayer after transfection/infection of the respective cDNAs. Indeed, recent studies using a double transfectant of OATP8 and MRP2 (6) and that of OATP2 and MRP2 (26) have demonstrated that this experimental approach is feasible.

MATERIALS AND METHODS

Chemicals. \([^{3}H]\) cholic acid (24.5 Ci/mmol), \([^{3}H]\) taurocholic acid (2 Ci/mmol), and \([^{14}C]\) Chenodeoxycholic acid (48.6 mCi/mmol) were purchased from Amersham Biosciences Corp., Little Chalfont, United Kingdom. 

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purchased from PerkinElmer Life Sciences (Boston, MA). [3H]Glycocholic acid (57.3 mCi/mmol) and [3H]Tauroursodeoxycholic acid (57.3 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). [3H]Tauroursodeoxycholic acid (20 Ci/mmol) and unlabeled tauroursodeoxycholic acid were obtained from SibTech (Newington, CT) by customized synthesis. [3H]Tauroursodeoxycholic acid (10 Ci/mmol), [3H]Glycocholic acid (11 Ci/mmol), and [3H]Tauroursodeoxycholic acid (10 Ci/mmol), and [3H]Glycoursodeoxycholic acid (11 Ci/mmol) were synthesized in the laboratory of Alan F. Hofmann as described (29). Unlabeled tauroursodeoxycholic acid, glycoursodeoxycholic acid, and glycocholic acid were purchased from Sigma (St. Louis, MO). Unlabeled cholic acid was purchased from Wako Pure Chemicals Industries (Osaka, Japan). Unlabeled tauroursodeoxycholic acid, tauroursodeoxycholic acid, and glycoursodeoxycholic acid were kindly provided by Mitsubishi Pharma (Osaka, Japan). All other chemicals used were commercially available and of reagent grade.

Antiserum for rat Bsep was raised in rabbits against an oligopeptide (the COOH-terminus of rat Bsep; AYYKLVITGAPIS) coupled with keyhole limpet hemocyanin via m-maleimidobenzoyl-n-hydroxysuccinimide ester (1). Antiserum for rat Ntcp was raised in rabbits against a COOH-terminal fusion protein of rat Ntcp (31). Antiserum against Ntcp and Bsep were used at a dilution of 1:5,000 and 1:1,000 for immunoblotting and 1:250 and 1:50 for immunofluorescence, respectively.

Cell culture and transfection. Parental MDCK cells were cultured in DMEM with 10% fetal bovine serum and 1% antibiotic-antimycotic (GIBCO; 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) at 37°C under 5% CO2. Full-length Ntcp cDNA cloned previously in our laboratory (15) was inserted into a mammalian expression vector (pcXN2) (22) and transfected into MDCK cells using LipofectAMINE (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s instructions. Transfectants expressing Ntcp were selected with G418 (600 μg/ml). The clone with the highest Ntcp expression was screened by immunoblot analysis.

Construction of recombinant adenovirus containing rat Bsep. BD Adeno-X Adenoviral Expression System (BD Biosciences, Palo Alto, CA) was used to establish the recombinant adenovirus. Full-length rat Bsep cDNA cloned previously in our laboratory (1) was inserted into pShuttle vector resulting in the production of pShuttle-Bsep, which has an I-CeuI and a PI-SceI site upstream and downstream of the Bsep insert and cultured at confluence for 2 days and infected by recombinant adenovirus containing cDNAs for Bsep or GFP (250 MOI). Cells were harvested 48 h after infection, and expression of Ntcp was induced with 10 mM sodium butyrate. Twenty-four hours after induction, cells were fixed with ice-cold methanol for 10 min, permeabilized with 1% Triton X-100 in PBS for 10 min, and incubated for 1 h with primary antibodies at room temperature. After this, cells were washed three times with PBS and incubated with goat anti-rabbit IgG Alexa Fluor 488 (Molecular Probes, Eugene, OR), diluted 250-fold in PBS for 1 h at room temperature, and mounted in VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA). Confocal laser-scanning microscopy was performed with an LSM 510 microscope from Zeiss (Oberkochen, Germany).

Transport assays. Ntcp- or vector-transfected MDCK cells were seeded on transwell membrane inserts ( pore size of 3 μm; Falcon, Bedford, MA) in 24-well plates at a density of 1.4 × 10^6 cells per insert and cultured at confluence for 2 days and infected by recombinant adenovirus containing cDNAs for Bsep or GFP (250 MOI). Cells were harvested 48 h after infection, and expression of Ntcp was induced with 10 mM sodium butyrate (5). Then, 24 h after induction, cells were washed with transport buffer (mM): 118 NaCl, 23.8 NaHCO3, 4.83 KCl, 0.96 KH2PO4, 1.20 MgSO4, 12.5 HEPES, 5 glucose, and 1.53 CaCl2 adjusted to pH 7.4). Subsequently, 3H- or 14C-labeled substrates were added to the transport buffer in either the apical (250 μl) or basal compartment (950 μl). After the times indicated, the radioactivity in the opposite compartment was measured. The intracellular accumulation of radioactivity was determined at the end of the experiments by lysing the cells with 500 μl 0.2 N NaOH in distilled water and measuring the radioactivity in the cell lysates. Aliquots (50 μl) of cell lysate were used to determine protein concentrations by the method of Lowry et al. (16) with bovine serum albumin as a standard. The apparent intracellular concentration of substrates was determined by assuming that the cellular volume per milligram cellular protein was 4 μl.

Data analysis. For the kinetic analysis, the transcellular transport of TC determined over 2 h was used. The transcellular transport at 30 and 60 min was also determined to confirm that the transcellular transport determined over 2 h represents the initial rate of flux (data not shown). The apparent kinetic parameters for transcellular transport of TC were estimated according to the Michaelis-Menten equation by assuming one saturable and one nonsaturable component: 

\[
v_0 = \left(\frac{V_{\text{max}} \times S}{K_m + S} + PS_{\text{arr}} \times S\right)
\]

where \(V_{\text{max}}\) is the maximum uptake rate (pmol·min\(^{-1}\)·mg protein\(^{-1}\)), \(K_m\) is the Michaelis constant (μM), \(v_0\) is the initial transport velocity of substrates (pmol·min\(^{-1}\)·mg protein\(^{-1}\)), \(PS_{\text{arr}}\) is the nonsaturable permeability surface area (PS) product expressed as clearance (μl·min\(^{-1}\)·mg protein\(^{-1}\)). The uptake data were fitted to this equation by a nonlinear least-squares method with a MULTI program (34) to obtain estimates of the kinetic parameters. The input data were weighted as the reciprocals of the squares of the observed values.

KINETIC ANALYSIS OF TRANSPORTER EXPRESSION

Kinetic analysis of the transcellular transport was performed according to the following procedure. The rate of appearance of ligands in the apical compartment is described by

\[
dx_{\text{apical}}/dt = PS_{\text{net}} \times C_{\text{med}}
\]

where \(x_{\text{apical}}\) (pmol/mg protein) is the amount of ligand in the apical compartment, and \(PS_{\text{net}}\) (μl·min\(^{-1}\)·mg protein\(^{-1}\)) is the PS product defined for the ligand concentration in the medium \(C_{\text{med}}\) (pmol/μl).
The rate of accumulation of ligands in the apical compartment is also described by

\[
dx_{\text{apical}}/dt = PS_{\text{apical}} \times C_{\text{cell}}
\]

where \(PS_{\text{apical}} (\mu l \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1})\) is the clearance for the influx of ligand across the apical membrane, which is defined for the ligand concentration in the cells \(C_{\text{cell}} (\text{pmol/µL})\). In addition, the mass-balance of ligand in the cells can be described by

\[
dx_{\text{cell}}/dt = PS_{\text{basal}} \times C_{\text{med}} - (PS_{\text{apical}} + PS_{\text{basal,eff}}) \times C_{\text{cell}}
\]

where \(x_{\text{cell}} (\text{pmol/mg protein})\) is the amount of ligand in the cells, \(PS_{\text{basal}} (\mu l \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1})\) is the clearance for the influx of ligand across the basal membrane, which is defined for \(C_{\text{med}}\), and \(PS_{\text{basal,eff}} (\mu l \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1})\) is the clearance for the efflux of ligand across the basal membrane from the cell to the basal compartment, which is defined for \(C_{\text{cell}}\), respectively.

At steady-state \((dx_{\text{cell}}/dt = 0)\), Eq. 3 is simplified to Eq. 4, which gives the cell-to-medium concentration ratio at steady state \((C_{\text{cell,ss}} / C_{\text{med,ss}})\).

\[
C_{\text{cell,ss}} / C_{\text{med,ss}} = PS_{\text{basal}} / (PS_{\text{apical}} + PS_{\text{basal,eff}})
\]

Under conditions where \(PS_{\text{apical}} \gg PS_{\text{basal,eff}}\), \(PS_{\text{net}}\) can be approximated

\[
PS_{\text{net}} \approx PS_{\text{basal}}
\]

In the present study, \(PS_{\text{net}}\) and \(PS_{\text{apical}}\) were calculated by dividing the rate for the transcellular transport of ligands determined over 2 h by the medium concentration of ligands and by the apparent cellular concentration of ligands determined at the end of the experiments (2 h), respectively. When \(PS_{\text{apical}} \gg PS_{\text{basal,eff}}\) for Ntcp and Bsep coexpressing MDCK (MDCK-Ntcp/Bsep) and Bsep expressing MDCK monolayers (MDCK-Bsep), the difference between \(PS_{\text{apical}}\) for MDCK-Ntcp/Bsep and \(PS_{\text{net}}\) for MDCK-Bsep represents the clearance for the uptake mediated by Ntcp \((PS_{\text{Ntcp}})\). Consequently, \(PS_{\text{Ntcp}}\) was calculated according to

\[
PS_{\text{Ntcp}} = PS_{\text{net}} \text{ for MDCK-Ntcp/Bsep} - PS_{\text{net}} \text{ for MDCK-Bsep}
\]

RESULTS

Expression and localization of Ntcp and Bsep in MDCK cells. The expression of Ntcp and Bsep in the transfected MDCK cells was analyzed by immunoblotting (Fig. 1). As shown in Fig. 1A, Ntcp expression was detectable as a band of 55 kDa in MDCK-Ntcp and MDCK-Ntcp/Bsep. The expression of Bsep was also detectable at 160 kDa in MDCK cells transfected with Bsep cDNA (MDCK-Bsep) and in MDCK-Ntcp/Bsep cells (Fig. 1B). In the control MDCK cells, no expression of Ntcp or Bsep could be detected (Fig. 1).

The cellular localization of the recombinant transporters in the transfectants was assessed using confocal laser-scanning microscopy. In MDCK-Ntcp and MDCK-Ntcp/Bsep cells, Ntcp was localized on the basolateral membrane (Fig. 2, A and C-1). In MDCK-Bsep and MDCK-Ntcp/Bsep cells, Bsep was localized on the apical membrane (Fig. 2, B and C-2). In the present study, we infected the MDCK cells stably expressing Ntcp with recombinant adenoviruses containing Bsep cDNA. Because the Ntcp expressing MDCK cells were prepared by incubating the cells in the presence of G418 after transfection of plasmid vector containing Ntcp cDNA and neomycin resistance gene, all the cells used in the present study express Ntcp as shown in Fig. 2. In contrast, immunohistochemical studies indicated that the expression of Bsep was detectable in 5–10% of the infected cells (Fig. 2). However, the expression rate of Bsep may be higher because it is possible that there were Bsep-expressing cells at a low level that was under the detection limit of the immunohistochemical staining. Although we infected the cells with the recombinant adenoviruses at more than 250 MOI, infection of the viruses at this higher MOI resulted in the death of the cells presumably due to the toxicity of the viruses.

Fig. 1. Western blot analysis of Na\(^+\)-taurocholate-(TC) cotransporting polypeptide (Ntcp) and bile salt export pump (Bsep). The expression level of Ntcp and Bsep was determined by Western blot analysis. Crude membrane fractions (30 µg) from the control, Madin-Darby canine kidney (MDCK)-Ntcp, MDCK-Bsep, and MDCK-Ntcp/Bsep cells were separated on 12.5 and 8.5% SDS-PAGE for Ntcp (A) and Bsep (B), respectively.
MDCK-Ntcp/Bsep cells. The analysis gave $K_m$ values of $22.2 \pm 4.5 \mu M$ for doubly transfected cells and $13.9 \pm 4.7 \mu M$ for Ntcp transfected cells. The $V_{max}$ values were $60.8 \pm 9.0$ and $15.8 \pm 4.2$ pmol·min$^{-1}$·mg protein$^{-1}$ for doubly transfected and Ntcp-transfected cells, respectively. The $PS_{diff}$ was unaffected by Bsep transfection. The $PS_{diff}$ values were $0.10 \pm 0.04$ μl·min$^{-1}$·mg protein$^{-1}$ for doubly transfected cells and $0.12 \pm 0.03$ μl·min$^{-1}$·mg protein$^{-1}$ for Ntcp transfected cells.

Further evidence that the transfection of Bsep had a powerful influence was shown by the saturation of the $PS_{apical}$ (Fig. 5). As the medium TC concentration was increased to $300 \mu M$, the $PS_{apical}$ in the MDCK-Ntcp/Bsep monolayer became saturated to the same level as the control and MDCK-Ntcp monolayers (Fig. 5). $PS_{apical}$ was almost the same for the control and MDCK-Ntcp monolayers, and no saturation was observed in these monolayers.

**Transcellular transport of a series of bile salts across MDCK monolayers.** In addition to TC, the transcellular transport of a variety of natural conjugated and unconjugated bile salts was characterized. For conjugated bile salts, fluxes across the parental MDCK monolayer were symmetrical and extremely low (Fig. 6). Insertion of Ntcp (Fig. 6B) caused a marked increase in vectorial transport of taurocholate (TCDC), glycochenodeoxycholate (GCDC), tauroursodeoxycholate (TUDC), glycoursodeoxycholate (GUDC), and TC (shown previously in Fig. 3), indicating the presence of non-Bsep apical transporters (Fig. 6). Glycocholate (GC) transport increased to a much lower degree. Insertion of both transporters caused a marked increase in vectorial transport of all conjugated bile salts except TCDC and TUDC, the vectorial transport of which in the absence of Bsep was already far higher than that of any other bile salt (Fig. 6).

For unconjugated bile salts (Fig. 7), there was symmetrical transport in the parental MDCK monolayers. The magnitude of transport varied in direct proportion to the passive membrane permeability (hydrophobicity) of individual bile salts [lithocholate (LCA) > chenodeoxycholate (CDCA) = ursodeoxycholate (UDCA) > cholate (CA); Fig. 7]. Addition of Ntcp increased the transport of CDCA but not that of other unconjugated bile salt (Fig. 7). In the doubly transfected cells, vectorial transport of CA, CDCA, and UDCA but not that of LCA was observed (Fig. 7).

Finally, the $PS_{Ntcp}$ of these bile salts was calculated to quantitatively evaluate the role of basolateral transport in their transcellular transport. Figure 8 shows the $PS_{Ntcp}$ of these bile salts normalized with respect to that of TC. The rank order for
PS<sub>Ntcp</sub> was TUDC > TCDC > GCDC > GUDC, TC, CDCA > UDCA > GC > CA.

**DISCUSSION**

Hepatic vectorial transport of bile salts is supported by the uptake and efflux transporters located on the sinusoidal and bile canalicular membrane, respectively. Although the molecular properties of these two transporters have been separately characterized, there has been no description of the functional coexpression system of these transporters. In the present study, we have established MDCK cells expressing both rat bile salt uptake transporter (Ntcp) and rat bile salt efflux transporter (Bsep).

Basolateral and apical localization of Ntcp and Bsep, respectively, was confirmed by immunohistochemical analysis (Fig. 2), which is consistent with the localization in rat hepatocytes (7, 31). Although the transport of TC across the control and MDCK-Bsep was symmetrical, this bile salt was transported from the basal side to the apical side across MDCK-Ntcp and MDCK-Ntcp/Bsep monolayers (Fig. 3, B and D). Furthermore, the transcellular transport across MDCK-Ntcp/Bsep monolayers was significantly higher than that across MDCK-Ntcp monolayers (Fig. 3, B and D). These results indicate that Ntcp and Bsep exhibit a coupled transport function in this expression system. TC molecules in the basal compartment are taken up by Ntcp into the cells and then exported to the apical compartment by Bsep. The function of Bsep was further confirmed by the fact that the cellular accumulation of TC, which was determined at the end of the experiments, was significantly lower in MDCK-Ntcp/Bsep cells compared with MDCK-Ntcp cells (see RESULTS). The significant basal-to-apical flux of TC across MDCK-Ntcp indicates the presence of endogenous transporter(s) on the apical membrane, capable of extruding this bile salt from the cells (Fig. 3B). At the present moment, we cannot identify this endogenous transporter. Although it has been suggested that MDR1, MRPs, and OATPs are expressed endogenously in MDCK cells (8, 21), they cannot be candidates, if we consider their cellular localization and substrate specificity. However, we were able to analyze the transport mediated by Bsep, because basal-to-apical flux was significantly enhanced by the additional expression of Bsep (MDCK-Ntcp/Bsep monolayer), compared with the flux mediated by

![Figure 3](image3.png)

**Fig. 3.** Time profiles of the transcellular transport of [3H]TC across MDCK monolayers. Transcellular transport of [3H]TC (1 μM) across MDCK monolayers was examined as a function of time. A-D represent the data for the control, MDCK-Ntcp, MDCK-Bsep, and MDCK-Ntcp/Bsep monolayers, respectively. ○ and ● represent the transcellular transport in the apical-to-basal and basal-to-apical directions, respectively. Each point and vertical bar represents the mean ± SE of 3 determinations. Where vertical bars are not shown, the SE was contained within the limits of the symbol.

![Figure 4](image4.png)

**Fig. 4.** Concentration dependence of the transcellular transport of [3H]TC across Ntcp- and Bsep-expressing MDCK monolayers. The saturation of the basal-to-apical flux of [3H]TC (1 μM) across MDCK-Ntcp (▲), MDCK-Ntcp/Bsep (double transfectant; ●), and control MDCK monolayers (○) was studied for 2 h in the presence and absence of unlabeled TC at 37°C (A). Each symbol and bar represent the mean ± SE of 3 determinations. The solid lines represent the fitted line. B represents the saturation of transporter-mediated transport of TC, which was obtained by subtracting the transport across the control cells [nonsaturable permeability surface (PS) area (PS<sub>diff</sub> × S)] from the transport across MDCK-Ntcp and MDCK-Ntcp/Bsep cells (▲ and ●, respectively). v<sub>i</sub>, Initial transport velocity of substrates.
Ntcp and endogenous transporters in the MDCK monolayer (MDCK-Ntcp monolayer; Fig. 3, B and D).

The saturation of transport was examined using MDCK-Ntcp and MDCK-Ntcp/Bsep monolayers, which exhibit significant basal-to-apical transport of TC. The basal-to-apical flux of TC across MDCK-Ntcp and MDCK-Ntcp/Bsep monolayers was saturated with apparent \( K_m \) values of 13.9 ± 4.7 and 22.2 ± 4.5 \( \mu M \), respectively (Fig. 4). These \( K_m \) values are similar to the reported \( K_m \) value of TC for Ntcp [34 \( \mu M \); (27)], which indicates that the basal-to-apical flux of TC across MDCK-Ntcp/Bsep is dominated by the influx clearance of Ntcp. This in vitro result is consistent with our previous studies performed in situ. Previously, we performed liver perfusion studies and found that the canalicular efflux clearance (PSapical) of TC is much larger than the basolateral efflux clearance (PSbasal,eff): 69.2 ± 6.3 vs. 8.4 ± 0.6 \( \mu l\cdot min^{-1}\cdot g^{-1}\cdot liver^{-1} \) (2). From this result, we can conclude that the basaloapical flux of TC across hepatocytes is dominated by influx clearance under physiological conditions (see Eq. 6). However, to compare in vitro and in vivo results, the expression level of Ntcp and Bsep should be compared. Moreover, we have to consider the presence of other bile salt transport systems in the hepatocytes, such as uptake transporters (Oatps) and Mrp4, an efflux transporter, located on the basolateral membrane of hepatocytes (25).

The saturation of PSapical was also examined. At lower and presumably physiological medium concentrations of TC (less than ~20 \( \mu M \)), PSapical in the MDCK-Ntcp/Bsep monolayer was significantly higher than that in the MDCK-Ntcp monolayer, the latter being comparable with that in the control MDCK monolayer (Fig. 5). As the medium TC concentration was increased to 300 \( \mu M \), the PSapical in the MDCK-Ntcp/Bsep monolayer became saturated to the same level as the control and MDCK-Ntcp monolayers (Fig. 5). Although the \( K_m \) value of Bsep-mediated TC transport is reported to be 5.3 \( \mu M \) (7), we cannot determine this \( K_m \) value from the results of the present experiment because of the fact that it is difficult to determine the intracellular unbound concentration of TC in MDCK-Ntcp/Bsep cells.

Transcellular transport of other kinds of bile salts was also examined. GC, TCDC, GCD, TUDC, GUDC (Fig. 6D), CA, CDCA, and UDCA (Fig. 7D) were transported by the MDCK-Ntcp/Bsep monolayer in a vectorial manner. The transcellular transport across the MDCK-Ntcp/Bsep monolayer (Figs. 6D and 7D) was significantly higher than the control (Figs. 6A and 7A), MDCK-Ntcp (Figs. 6B and 7B), and MDCK-Bsep (Figs. 6C and 7C) monolayers except for TCDC and TUDC. This result suggests that these bile salts, except for TCDC and TUDC, are substrates of Ntcp and Bsep. Among these bile salts, TCDC, TUDC, TC, CA, and GC have been reported to be transported by Ntcp expressing oocytes and CHO9–6 cells (17, 27) and TCDC, GCD, TUDC, TC, and GC have been shown to be transported by rat Bsep expressing s9 vesicles (7, 30). In the present study, we also detected the Ntcp-mediated transport of CDCA, UDCA, GDC, and GUDC and the Bsep-mediated transport of UDCA, GUDC, CDCA, and CA. Identification of some new substrates of Ntcp and Bsep shows that this model is available for the characterization of these transporters. The transcellular transport of TCDC and TUDC across MDCK-Ntcp (Fig. 6B) was not enhanced by the additional expression of Bsep [MDCK-Ntcp/Bsep (Fig. 6D)], although this bile salt is reported to be transported by Bsep (7, 30). This difference may be accounted for by assuming that the transport capacity of endogenous (non-Bsep) transporter(s) in MDCK cells is high enough for TCDC and TUDC and, therefore, the rate-determining process for the transcellular transport of these bile salts is the uptake mediated by Ntcp. For LCA, no vectorial transport across the MDCK-Ntcp/Bsep was detectable (Fig. 7), which suggests that this bile salt is a poor substrate of Ntcp and/or Bsep.

Furthermore, to quantitatively evaluate the transcellular transport of these bile salts, the calculated PSNtcp values were compared (Fig. 8). PSNtcp was calculated using Eq. 7, which is applicable under steady-state conditions (\( dx_{cell}/dt = 0 \)). Although the PSNtcp values were determined from the results of transcellular transport experiments for 2 h, the fact that the \( x_{cell} \) of TC at 2 h (130 ± 9 pmol/mg protein) is the same as that at 10 min (133 ± 30 pmol/mg protein) suggests that the \( dx_{cell}/dt \) at \( t = 0 \) holds true for up to 2 h. On the basis of this consideration, the analysis method was validated. The rank order for PSNtcp was TUDC > TCDC > GCD > GUDC, TC, CDCA > UDCA > GC > CA. This order is the same as that reported previously using recombinant Ntcp; Meier et al. (17) have reported the following rank order: TUDC > TCDC > TC > GC > CA. These data are consistent with the hypothesis that the rate-determining process for the transcellular transport across MDCK-Ntcp/Bsep cells is the uptake process mediated by Ntcp, due to the efficient efflux mediated by Bsep. The fact that taurine-conjugated bile salts were transported to a greater extent than their corresponding glycine or unconjugated derivatives (TC > GC > CA, TCDC > GCD > CDCA and TUDC > GUDC > UDCA) may be reasonable considering the bile salt composition of rats in which most of the bile salts are taurine conjugates. We were thus able to establish an in vitro model to quantitatively evaluate the vectorial transport of bile salts. However, in the calculation of PSNtcp, it is assumed that the rate-determining process for the transcellular transport of a series of bile salts is the uptake mediated by Ntcp, as suggested for TC. If this assumption does not hold true for other bile salts,
the calculated PS_{Ntcp} values cannot be compared among bile salts.

This expression system may also be useful for the detecting the transport of bile salts mediated by Bsep. Until now, it has been difficult to study Bsep function in intact mammalian cells because most Bsep substrates are negatively charged under physiological conditions and thus cannot penetrate the cell membrane without the aid of uptake transporters. Therefore, Bsep has been studied using inside-out membrane vesicles prepared from Bsep-expressing cells. With the aid of MDCK-Ntcp/Bsep monolayers, Bsep function can be studied more effectively and sensitively compared with the current in vitro methods using isolated membrane vesicles. Indeed, using MDCK/Ntcp-Bsep monolayers, we could detect the Bsep-mediated transport of CA (Fig. 7), which has not been detectable previously using isolated membrane vesicles. Because the
transported compound is accumulated in the aqueous fluid of the apical compartment, it is easy to detect the transport mediated by Ntcp and/or Bsep due to the low background level; if the uptake of ligands into the cells and/or isolated membrane vesicles is examined, the extent of adsorption to the surface of the cells/membrane vesicles is not negligible. Detection of unlabeled bile salts from the aqueous specimens may be possible with the aid of LC-MS.

In conclusion, we have established MDCK cells expressing both basolateral Ntcp and apical Bsep that transport conjugated and unconjugated bile salts vectorially. Kinetic analysis of the transcellular transport of bile salts suggests that the Ntcp/Bsep coexpressing MDCK monolayer may be useful in analyzing the vectorial transport of individual bile salts. Our system may also be useful for analyzing the inhibitory effects of some compounds on Ntcp and/or Bsep function. Such analysis will give us suggestions for understanding of human cholestatic liver disease induced by the inhibition of NTCP and/or BSEP by drugs.

Fig. 7. Time profiles for the transcellular transport of unconjugated bile salts across MDCK monolayers. Transcellular transport of [3H]cholate (CA), [3H]chenodeoxycholate (CDCA), [3H]lithocholate (LCA), and [3H]ursodeoxycholate (UDCA; 1 μM) across the control (A), MDCK-Ntcp (B), MDCK-Bsep (C), and MDCK-Ntcp/Bsep monolayers (D) was determined as a function of time. ○ and ● represent the transcellular transport in the apical-to-basal and basal-to-apical directions, respectively. Each point and vertical bar represent the mean ± SE of 3 determinations. Where vertical bars are not shown, the SE was contained within the limits of the symbol.

Fig. 8. Comparison of the transcellular transport of bile salts across MDCK monolayer expressing both Ntcp and Bsep. On the basis of the results shown in Fig. 6, the PS_{Ntcp} was calculated for CA, GC, TCDC, GCDC, CDCA, TUDC, GUDC, and UDCA (1 μM) according to Eq. 7 in the text. The PS_{Ntcp} of each bile salt was divided by that of TC. Data represent means ± SE.
REFERENCES


