Protein kinase C induces endocytosis of the sodium taurocholate cotransporting polypeptide

Claudia Stross,* Angelika Helmer,* Katrin Weissenberger, Boris Görg, Verena Keitel, Dieter Häussinger, and Ralf Kubitz

Department of Gastroenterology, Hepatology and Infectiology, Heinrich-Heine University, Düsseldorf, Germany

Submitted 13 April 2010; accepted in final form 4 June 2010

TRANSPORTER PROTEINS ARE REGULATED at multiple levels such as transcription, translation, protein maturation, covalent modification, and degradation as well as vesicular insertion and retrieval (11).

Endo- and exocytosis of transporters have been increasingly recognized as a mode of rapid adaptation to a changing environment. For example, the glucose transporter GLUT4 is exocytosed in response to insulin, thereby regulating glucose uptake into muscle and adipose tissue cells (3, 7, 46). Similarly, anisoosmolarity stimulates the insertion of the water channel aquaporin 2 into the apical membrane of kidney cells for acute adjustments of water homeostasis (16, 41, 47). Likewise, the canalicular multidrug resistance-association protein Mrp2 (19, 24, 43) and the bile salt export pump Bsep (28, 42) are regulated by endocytic processes in hepatocytes.

The liver is challenged by varying substrate delivery through the portal vein, necessitating adaptable transport mechanisms in liver cells. In particular, bile salt concentrations may vary considerably after food intake in response to gallbladder contraction and bile salt recycling within the enterohepatic circulation (40). The sodium taurocholate cotransporting polypeptide (Ntcp) and Bsep are part of this recycling pathway. Ntcp takes up bile salts into hepatocytes utilizing the sodium gradient generated by the Na⁺-K⁺-ATPase (45). In fact, it is the major uptake system for conjugated bile salts from blood into hepatocytes. Ntcp is one determinant of intracellular bile salt concentration in hepatocytes, thereby influencing several bile salt-dependent processes, including gene transcription (37), hepaticcellular apoptosis (9, 12, 38, 39), and bile flow (34). In cholestatic liver diseases, Ntcp is downregulated at the levels of mRNA and protein (8, 14). On a short-term scale transport capacity of Ntcp can be increased by insertion into the sinusoidal membrane (6). Exocytosis of Ntcp is stimulated via cAMP (6) and involves dephosphorylation of Ntcp at serine and threonine sites (33). By contrast, endocytosis of Ntcp seems favorable for the protection of hepatocytes from toxic bile salt concentrations (8, 29); however, this mechanism has not been described in detail.

In this study, we identified protein kinase C (PKC) activation as a distinct signal pathway, which rapidly downregulates the surface expression of Ntcp. Internalization is quantified by a recently established method, which utilizes flow cytometry (20). This technique may serve as a screening tool for the identification of additional “translocators” of Ntcp.

MATERIALS AND METHODS

Antibodies and reagents. The anti-rat Ntcp antibody (K4) (45) was a gift from Dr. B. Stieger and Dr. P. Meier (Universitätsspital Zürich). The anti-FLAG M2 antibody and the Na⁺-K⁺-ATPase antibody (c-subunit) were obtained from Sigma-Aldrich (Deisenhofen, Germany). The Fab fragments of an anti-mouse IgG antibody coupled to the tandem fluorescent dye AlexaFluor 647-R-PE or Pacific blue, respectively, were purchased from Invitrogen (Karlsruhe, Germany). Accutase was purchased from Millipore (Schwalbach, Germany). 4,6-Diamidino-2-phenylindole (DAPI) was obtained from Invitrogen.

Cloning of FLAG-tagged Ntcp-EGFP and mCherry-tagged PKCa. Two different FLAG tags were cloned to the NH₂ terminus of rat Ntcp using partially overlapping oligonucleotides. The first primer pair was 5'-ATG GAC TAC AAC GAC GAT GAC GAT AAC AGG-3' and 5'-CTT ATC GTC ATC GTC TTT GCA GTC CAT CCT-3' and coded for a start codon, followed by the classic FLAG tag (DYKDDDK). The second pair was 5'-CCG GAC TAC AAC GAC GAT GAC GAT AAC AGG-3' and 5'-CTT ATC GTC ATC GTC TTT GCA GTC CAT GCG GCC CT-3', which codes for a FLAG tag with an improved start codon (Kozak sequence). The free ends of the annealed products were complementary to the restriction sites of the endonuclease Var911. The products were cloned into the Ntcp-enhanced green fluorescent protein (EGFP) plasmid (26) (Fig. 1A) after digestion with Var911 in frame to the 5'-site of Ntcp. Human PKCα (22) was subcloned from the pEFYP-N1 into the mCherry vector (Clontech Laboratories, Mountain View, CA) by restriction with NheI and...
that were fixed with paraformaldehyde (4%) or that were fixed and 

Depending on experimental design, staining was performed on cells 

Oberkochen, Germany) were performed as described recently (14). 

ing of Ntcp and confocal laser scanning microscopy (LSM 510, Zeiss, 

Germany) according to the manufacturer’s instructions. 

PKC /H9251 

PKC/H11005 

PKC inhibitors bisindolylmaleimide I (BIM I) or Gö6976 (Calbio-

chem, Bad Sodem, Germany) were applied 30 min before phorbol-

12-myristate-13-acetate (PMA). For cell death assays, 175 μM tau-

rolithocholate sulfate (TLCS, Sigma Aldrich) was applied for 6 h 

following PMA treatment. Thereafter, cells were washed with ice-

cold phosphate buffered saline (PBS) and incubated either with 0.05% 

trypsin/0.02% EDTA or with Accutase at 37°C. Cells were transferred 

into 1.5 ml tubes, centrifuged for 30 s at 4,500 g, resuspended in PBS, 

and filtered through 70 μm gauze to enrich single cells. 

The anti-Flag M2 antibody was labeled with the anti-mouse IgG-Fab fragment coupled to AlexaFluor 647-PE or to Pacific blue, 

respectively, according to manufacturer’s guidelines at a ratio of 1:3 

(antibody:Fab fragment). Cells were then incubated with the labeled 

antibody at dilution of 1:2,500 and 1:500, respectively, for 45 min 

at 4°C. For determination of the cell death rate, cells were incubated 

with the nucleic acid stain DAPI (1 μg/ml) for 5 min at room 

temperature. 

Cell size, granularity, and fluorescence intensities were measured by a FACScalibur or a FACSCan Flow Cytometer (Becton Dick-

inson, Heidelberg, Germany) with excitation at 488 and 405 nm, 

respectively. EGFP fluorescence was measured at 530 ± 30 nm, 

Pacific blue and DAPI fluorescence at 450 ± 50 nm, and the 

fluorescence of AlexaFluor 647-PE was acquired above 670 nm. 

The emission light of EGFP is zero at wavelengths > 670 nm and the 

emission light of AlexaFluor 647-PE is zero at wavelengths < 550 

nm; therefore, cross talk between the emitted fluorescence of the two 

fluorochromes was avoided. Likewise, no cross talk was observed 

between EGFP and Pacific blue fluorescence when used with the 

FACScanto II cytometer. 

Statistics. Data were obtained from at least three independent cell 

preparations. Results were reproduced with three different clones. 

Primary data consisted of nongated measurements of at least 10,000 

single events, which were further analyzed by the FCS-Express 

software (DeNovo Software, Los Angeles, CA). Cells were gated 

for their characteristic forward scatter (cell size) and sideward scatter 

(granularity) except in cell death analyses. Fluorescence intensities of 

EGFP, Pacific blue, and AlexaFluor 647-PE were displayed as 

cumulative histograms (sigmoid curves) for easier comparison of 

different conditions. 

The geometric mean values of measurements from unlabeled cells 

(labeling mix without FLAG-antibody) were subtracted from mean 

values of stained cells. Changes in Ntcp surface expression were 

Fig. 1. Topology and expression of FLAG-Ntcp-EGFP in HepG2 cells. 

A: putative topology of FLAG-Ntcp-EGFP with 7 transmembrane domains, an 
extracellular FLAG tag (●), and the intracellular enhanced green fluorescent 
protein EGFP. B: FLAG-Ntcp-EGFP in stably transfected HepG2 cells is 

predominantly localized at the plasma membrane. Bar = 20 μm. C: HepG2 
cells were trypsinized for 0, 7, 30, or 60 min before cell lysis. In subsequent 
Western blot analysis with the anti-Ntcp antibody, the amount of FLAG-Ntcp-

EGFP remains unchanged during the first 30 min of trypsination. 

KpnI (NEB, Frankfurt am Main, Germany). The accuracy of the 

resulting plasmids was confirmed by sequencing. 

Generation of stably expressing FLAG-Ntcp-EGFP cells and 

transfection. HepG2 cells were cultured in Dulbecco’s modified 

Eagle’s medium Nutrimix F12 (DMEM-F12; Invitrogen) containing 

10% fetal calf serum (PAA, Coelbe, Germany), as described recently 

(21). FLAG-Ntcp-EGFP was transfected into HepG2 cells by using 

Lipofectamine 2000 (Invitrogen) according to the manufacturer’s 
guidelines. Stable cell lines were established with 0.5% of geneticin as 

selection agent. Three independent clones from the two cloning 

strategies (see above for details) with regular expression of FLAG-

Ntcp-EGFP at the plasma membrane (Fig. 1B) were used for further 

experiments. PKCs fused to mCherry was transfected into these 

clones by using Atractene transfection reagent (Qiagen, Hilden, 

Germany) according to the manufacturer’s instructions. 

Immunofluorescence analyses and transport assays. Immunostain-
ing of Ntcp and confocal laser scanning microscopy (LSM 510, Zeiss, 

Oberkochen, Germany) were performed as described recently (14). 

Depending on experimental design, staining was performed on cells 

that were fixed with paraformaldehyde (4%) or that were fixed and 

permeabilized with methanol (100%, 4°C, 1 min). Uptake of tauro-

cholate in the first 5 min (linear range) was measured as described 

previously (23) and was performed in triplicates for each condition 

and cell preparation. The uptake values were analyzed for Michaelis-

Menten kinetics by utilizing nonlinear regression data analysis from a 

computerized model (GraphPad, PRISM, San Diego, CA). Details for 

live cell imaging are given in the supplementary materials. Total 

internal reflection fluorescence (TIRF) microscopy (TIRFM) was 

performed on a Zeiss AxioObserver.Z1 with an oil immersion objective 

(Plan Fluar ×100, 1.45 numerical aperture) combined with a 

TIRFM module. The TIRF laser module was equipped with a 100 mW 

Lasos77 series Argon-Laser (Lasos Laser, Ebersberg, Germany). 

Fluorescence was collected by using a TIRFM filter set consisting of 

483–493 nm for excitation and 500–550 nm for emission. Image 

acquisition was performed with a Sony ICX-285 DCC camera (array 

size: 1,366 × 1,044). Epifluorescence pictures were acquired by use 
of a HXP 120 lamp (Zeiss). Evanescent field calibration was 

performed by using the TIRF-angle adjustment kit (Zeiss) and 

fluorescent latex beads (100 nm diameter) according to the manufacturer’s 

protocol. Evanescent wave penetration depth was calculated to ~150 nm 

by use of the ImageJ plugin “Calc TIRF” (written by Sebastian Rhode, 

Institute for Biophysics, SDT Group, University of Linz). 

Flow cytometry. HepG2 cells stably expressing FLAG-Ntcp-EGFP 

and wild-type HepG2 cells were cultured in 12-well culture plates 

until subconfluence. They were treated with PKC activators (dissolved 
in 0.1% DMSO) or with 0.1% DMSO (control) for 1 h at 37°C. The 

PKC inhibitors bisindolylmaleimide I (BIM I) or Gö6976 (Calbio-

chem, Bad Sodem, Germany) were applied 30 min before phorbol-

12-myristate-13-acetate (PMA). For cell death assays, 175 μM tau-

rolithocholate sulfate (TLCS, Sigma Aldrich) was applied for 6 h 

following PMA treatment. Thereafter, cells were washed with ice-
cold phosphate buffered saline (PBS) and incubated either with 0.05% 
trypsin/0.02% EDTA or with Accutase at 37°C. Cells were transferred 

into 1.5 ml tubes, centrifuged for 30 s at 4,500 g, resuspended in PBS, 

and filtered through 70 μm gauze to enrich single cells.
displayed as percent changes in relation to the untreated control (=100%). Values were analyzed by the one-sided Student’s *t*-test for unpaired samples with a *P* value <0.05 considered to be statistically significant.

**RESULTS**

**Stable expression of FLAG-Ntcp-EGFP in HepG2 cells.** To examine endocytosis of Ntcp, HepG2 cells were transfected with a plasmid coding for Ntcp with a FLAG tag at the NH$_2$ terminus and the enhanced green fluorescent protein EGFP at the COOH terminus (Fig. 1A). Significant amounts of Ntcp were targeted to the plasma membrane as shown by confocal laser scanning microscopy (Fig. 1B). Some green fluorescence was found in intracellular vesicular structures at various distances from the plasma membrane, representing the localization of Ntcp within intracellular compartments.

The expression of the FLAG-Ntcp-EGFP protein was confirmed by Western blotting. An 80-kDa band was detected, which corresponds to the expected molecular mass of the complex-glycosylated Ntcp-fusion protein (Fig. 1C), as described earlier (25). Whereas there was no significant taurocholate uptake in nontransfected HepG2 cells, taurocholate uptake by transfected cells was inhibited by treatment with 10 nM PMA (Fig. 2). Reversal of Ntcp internalization by PMA was blocked by pretreatment with 1 μM bisindolylmaleimide I (BIM) for 20 min (Fig. 3).

**Fig. 2.** Reduction in sodium taurocholate co-transporting protein (Ntcp) surface expression. Plasma membrane-bound Ntcp (green) is reduced upon stimulation with PMA as shown by total internal reflection fluorescence microscopy (TIRF), which detected EGFP fluorescence within a distance of 150 nm. In parallel, Ntcp vesicles (red) appear intracellular, as shown by standard epifluorescence.

**Fig. 3.** Activation of protein kinase C induces retrieval of Ntcp as shown by fluorescence microscopy. Activation of PKCs by PMA (50 nM; 1 h) or thymeleatoxin (TTX; 100 nM; 1 h) induced a decrease of Ntcp fluorescence at the cell membrane and concomitant formation of intracellular vesicles containing Ntcp. Preincubation with the PKC inhibitor bisindolylmaleimide I (BIM; 1 μM) for 20 min inhibits PMA, and TTX induced internalization. Bars = 20 μm.
transport in Ntcp expressing HepG2 cells was saturable, with an apparent $K_m$ value of 37.9 $\mu$M (Supplemental Fig. S1), which is slightly higher than the $K_m$ value of untagged Ntcp (10, 25). It is concluded that both tags (FLAG and EGFP) allowed normal expression, localization, and function of Ntcp in HepG2 cells.

**Topology of the FLAG-Ntcp-EGFP.** In nonpermeabilized HepG2 cells, FLAG-Ntcp-EGFP was not detectable by the anti-EGFP antibody (Supplemental Fig. S2A), whereas colocalization of green fluorescence and the red fluorescence from the cyanin3-labeled anti-EGFP antibody was observed in permeabilized cells (Supplemental Fig. S2B). Therefore, the COOH terminus of Ntcp localizes intracellularly. In contrast, the anti-FLAG antibody M2 detected FLAG-Ntcp-EGFP at the plasma membrane of nonpermeabilized cells, but not in intracellular compartments, verifying the extracellular position of the NH$_2$ terminus of Ntcp (Supplemental Fig. S2C). The anti-FLAG antibody only detected intracellular vesicles after permeabilization of cell membranes (Supplemental Fig. S2D).

Activators of protein kinase C induce a retrieval of FLAG-Ntcp-EGFP. Immunofluorescence studies showed that treatment of FLAG-Ntcp-EGFP-HepG2 cells with the PKC activator PMA for up to 1 h induced the internalization of this transporter and the formation of vesicular structures (Fig. 3 and Supplemental Movie S1). Along with vesicle formation there was a reduction of Ntcp in the plasma membrane as shown by total internal reflection fluorescence microscopy (TIRFM, Fig. 2). Likewise, the appearance of Ntcp in vesicles was observed after treatment with thymeleatoxin (TTX), an activator of the classical, Ca$^{2+}$-dependent conventional PKCs (cPKCs: PKC$\alpha$, PKC$\beta$I, PKC$\beta$II, and PKC$\gamma$) (Fig. 3).

PMA-induced retrieval of Ntcp could be blocked by pretreatment with BIM I, an inhibitor of classical and novel PKC (Fig. 3 and Supplemental Movie S2). These data strongly support the model that PKC activation mediates the retrieval of Ntcp from the plasma membrane in HepG2 cells.

Fig. 4. Selective Ntcp internalization in response to PKC activation. A–C: cotransfected mCherry-tagged PKC$\alpha$ translocates to the plasma membrane within 5 min of PMA stimulation and stays there during Ntcp internalization. Bar = 10 $\mu$m. D and E: PMA stimulation for 1 h induces internalization of Ntcp but not of the Na$^+/K^+$-ATPase costained in red. Bar = 10 $\mu$m.
suggest the involvement of cPKCs in Ntcp internalization. Stimulation with PMA induced a rapid membrane translocation of cotransfected PKCα fused to mCherry, indicating activation of this cPKC isoform (Fig. 4, A–C). Since there is no general colocalization of PKCs and Ntcp in vesicles, the kinase may be important for vesicle formation but not for further stabilization or trafficking.

In contrast to Ntcp, the Na\(^{+}\)-K\(^{+}\)-ATPase, another integral protein of the plasma membrane, remained at the cell membrane after PMA treatment (Fig. 4, D and E), thereby demonstrating that the internalization process induced by PMA discriminates between different membrane proteins.

Analysis of Ntcp endocytosis by flow cytometry. To analyze PMA-induced endocytosis of Ntcp in more detail, a flow cytometry technique was developed making use of the extracellular FLAG tag to determine cell surface expression of Ntcp. This method allows the comparison of membrane bound and total Ntcp and a quantification of transporter internalization.

For flow cytometry HepG2 cells were detached from culture plates by trypsin or Accutase. Complete separation of cells was achieved after 7 min of incubation with trypsin or 4 min of incubation with Accutase. As shown by Western blot analysis, no significant breakdown of Ntcp was observed during the time period used for detachment (Fig. 1C).

After detachment, cells were stained with the AlexaFluor 647-R-PE-labeled FLAG antibody. At a dilution of 1:2,500 only FLAG-Ntcp-EGFP but not untransfected cells were significantly labeled by the anti-FLAG/647-R-PE complex (Fig. 5A). Neither the 647-R-PE fluorescence nor green autofluorescence of HepG2 cells influenced the EGFP fluorescence of Ntcp transfected cells (Fig. 5B).

The extracellular FLAG tag was used for cell surface labeling of Ntcp in nonpermeabilized cells to quantify membrane localization of Ntcp. PMA and TTX induced a significant reduction in surface expression of Ntcp as shown by a reduction of FLAG-dependent fluorescence by 36.2 ± 6.9 and 25.5 ± 3.9%, respectively (Fig. 6A). In contrast, total Ntcp (=EGFP-dependent fluorescence), forward scatter, and side-ward scatter remained unchanged (Fig. 6A). In line with data from fluorescence microscopy, preincubation with BIM I and the cPKC-specific inhibitor Gö6976 inhibited PMA-induced Ntcp internalization (Figs. 6, B and C) without changes of EGFP fluorescence.

Along with the reduction in FLAG-associated fluorescence, taurocholate uptake was decreased to 75 ± 4% (n = 3) in FLAG-Ntcp-EGFP expressing HepG2 cells after 1 h of PMA treatment compared with controls, suggesting that the decrease in surface expression of Ntcp is associated with a reduced transport capacity.

PKC-mediated protection against cytotoxic bile salt concentrations. Bile salts such as TLCS are known to induce cell death in hepatocytes. To analyze the effects of Ntcp endocytosis on bile salt toxicity, FLAG-Ntcp-EGFP cells were incubated with TLCS (175 μM, 6 h) with or without PMA pretreatment (50 nM, 1 h). The percentage of dead cells was determined by nuclear DAPI incorporation and flow cytometry. TLCS treatment increased cell death rate ~1.9-fold compared with control. Downregulation of Ntcp by a 1-h pretreatment with PMA lowered this rate to 1.1-fold. This protective effect was shown to be PKC dependent because it could be prevented by the inhibitor BIM I (Fig. 7A). Furthermore, significant changes in cell morphology were observed in almost all TLCS-treated cells whereas cell morphology was preserved in PMA-pretreated cells (Fig. 7B). Wild-type HepG2 cells, which do not express Ntcp endogenously, were resistant to TLCS-induced cell death (Fig. 7A). Incubation with TLCS (100 μM) at the same time reduced \(^{[3]H}\)taurocholate uptake by ~64% (data not shown). Taken together, these findings suggest that TLCS is transported via Ntcp and that PKC stimulated endocytosis of Ntcp protects cells from effects of toxic bile salt concentrations.

DISCUSSION

Exo- and endocytosis can adjust the abundance of transporter molecules, thereby switching substrate fluxes on and off (30, 44). This study demonstrates that PKC activation induces endocytosis of Ntcp, the major uptake transporter for bile salts into liver parenchymal cells. Ntcp is of particular importance because it influences bile salt concentrations in blood and hepatocytes (27) and thereby alters bile salt-dependent signaling pathways in hepatic and extrahepatic tissues (13). Although insertion of Ntcp into the sinusoidal membrane in response to cAMP formation and downstream signaling pathways has been studied in detail (31, 32, 48, 49), regulated endocytosis of Ntcp on a short-term scale has not yet been described. The existence of such a mechanism is likely, because in humans with pro-
gressive familial intrahepatic cholestasis (14) or in mdr2 knockout mice (18) NTCP/Ntcp protein (human/rodent ortholog) but not mRNA was shown to be downregulated, suggesting a posttranscriptional mechanism of regulation. Furthermore, in bile duct-ligated rats, Ntcp protein was downregulated by more than 90%, whereas Ntcp mRNA was reduced by only ~60% (8). Under all these conditions, decrease of NTCP/Ntcp protein may be due to reduced delivery of this transporter through the secretory pathway or due to increased transporter endocytosis and subsequent degradation.

Our immunofluorescence data reveal a protein kinase C-dependent internalization of the sinusoidal bile salt transporter...
Ntcp in stably transfected HepG2 cells. Endocytosis was demonstrated by laser scanning, live cell, and TIRF microscopy; however, all these methods have the disadvantage of limited sensitivity and/or lack of quantitative measurements. We therefore developed a method based on flow cytometry, which overcomes some of the limitations of the microscopic and other techniques used for the analysis of subcellular transporter distribution [e.g., separation of membranes by differential centrifugation (5, 50) or sucrose gradients (4, 17)]. By the combination of the localization-dependent labeling with flow cytometry, transporter distribution in each cell of a large cell population can be analyzed. This allows statistical evaluation and quantification and results in a higher sensitivity for measuring endocytosis compared with fluorescence microscopy. Especially, vesicles in close vicinity to the plasma membrane or very small vesicles may not be detected by fluorescence microscopy, which is not a limitation in flow cytometry where the reduction in surface labeling of transporter proteins is measured.

As shown here, in FLAG-Ntcp-EGFP-expressing HepG2 cells forward scatter, side scatter, and EGFP fluorescence were almost identical under different conditions whereas FLAG-dependent fluorescence displayed significant changes. These data underline the robustness of the method (Fig. 6).

The fraction of Ntcp, which was endocytosed from the plasma membrane in response to PKC activation, was ~25–
PKC-INDUCED ENDOCYTOSIS OF NTCP

36%, as quantified by flow cytometry. This was in good agreement with uptake studies, which showed a reduction of taurocholate uptake of 25%. The correlation between endocytosis and reduced uptake suggests a direct link between activity and localization of Ntcp. It is known that cAMP activates Ntcp by PP2B-dependent dephosphorylation and exocytosis (2, 33). Consequently, phosphorylation of Ntcp within the plasma membrane may be the initial event to turn off Ntcp, an issue that needs to be investigated.

Ntcp is known to be downregulated in cholestatic liver diseases on a transcriptional level. Transcriptional downregulation is probably a slow process regarding other hepatic transporter half-times of several days (15). In contrast, Ntcp internalization is a fast mechanism as shown here and may therefore serve for rapid adjustments of bile salt uptake. Reduced bile salt uptake into hepatocytes is proposed to protect cells from toxic bile salt concentrations (8). Indeed, our data suggest that rapid endocytosis of Ntcp protects cells from harmful effects of TLCS. Other sulfated steroids such as progesterone metabolites are NTCP substrates and might be involved in the development of cholestatic disorders like intrahepatic cholestasis of pregnancy (1). Endocytosis of NTCP should lead to a reduced hepatocellular uptake of progesterone derivatives as well and therefore ameliorate their potential cholestatic effects. It will be interesting to analyze the potential effects of nonbile acid NTCP substrates on transporter internalization. Such a mechanism might play a role in conditions with elevated serum bile acids without obvious liver damage like asymptomatic hypercholanemia of pregnancy (35).

In conclusion, short-term downregulation of Ntcp by endocytosis may be an important mechanism to protect hepatocytes from toxic effects of bile salts (36). The method presented here may be used to further identify physiological regulators of Ntcp membrane expression and to discover “translocators,” as yet unknown pharmacological stimuli of endo- and exocytosis.

ACKNOWLEDGMENTS
Expert technical assistance by Elisabeth Winands and assistance by Thomas Kühlkamp is gratefully acknowledged.

GRANTS
This study was supported by the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 575 “Experimentelle Hepatologie.”

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES