A dileucine motif is involved in plasma membrane expression and endocytosis of rat sodium taurocholate cotransporting polypeptide (Ntcp)

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Stross C, Kluge S, Weissenberger K, Winands E, Häussinger D, Kubitz R. A dileucine motif is involved in plasma membrane expression and endocytosis of rat sodium taurocholate cotransporting polypeptide (Ntcp). Am J Physiol Gastrointest Liver Physiol 305: G722–G730, 2013.—The sodium taurocholate cotransporting polypeptide (Ntcp) is the major uptake transporter for bile salts into liver parenchymal cells, and PKC-mediated endocytosis was shown to regulate the number of Ntcp molecules at the plasma membrane. In this study, mechanisms of Ntcp internalization were analyzed by flow cytometry, immunofluorescence, and Western blot analyses in HepG2 cells. PKC activation induced endocytosis of Ntcp from the plasma membrane by ~30%. Endocytosis of Ntcp was clathrin dependent and was followed by lysosomal degradation. A dileucine motif located in the third intracellular loop of Ntcp was essential for endocytosis but also for processing and plasma membrane targeting, suggesting a dual function of this motif for intracellular trafficking of Ntcp. Mutation of two of five potential phosphorylation sites surrounding the dileucine motif (Thr225 and Ser226) inhibited PKC-mediated endocytosis. In conclusion, we could identify a motif, which is critical for Ntcp plasma membrane localization. Endocytic retrieval protects hepatocytes from elevated bile salt concentrations and is of special interest, because NTCP has been identified as a receptor for the hepatitis B and D virus.

Endocytosis; internalization motif; protein kinase; transporter

THE BILE SALT TRANSPORTER Ntcp/NTCP (rodent and human orthologs, respectively, of the sodium taurocholate cotransporting polypeptide) is exclusively located at the sinusoidal membrane of hepatocytes. It is responsible for the transport of bile acids including taurocholate, di- and trihydroxy bile acids (12, 21), and steroid sulfates as well as drugs conjugated to bile acids (1, 21). Ntcp [together with members of the organic anion transporting polypeptide (OATP) family] is essential for the enterohepatic circulation of bile acids. It mediates the recycling of more than 90% of bile acids from portal blood (7).

Human NTCP is a polypeptide with an apparent mass of 38 kDa, which is further increased by glycosylation in the mature, plasma membrane-bound form. Computer analysis predicted a topology of seven transmembrane domains with an extracellular NH₂– and an intracellular COOH-terminus (11).

High intracellular bile salt concentrations are detrimental to hepatocytes. For that reason, Ntcp expression is tightly regulated at the transcriptional level by negative feedback mechanisms. High bile acid levels induce suppression of NTCP expression via the nuclear factors FXR and Shp (2). In pathological conditions such as primary biliary cirrhosis (35, 36), cholestatic alcoholic hepatitis (36), and progressive familial intrahepatic cholestasis (16), NTCP is strongly downregulated. During inflammation, the cytokines IL-1β and TNF-α suppress transcription of the NTCP gene (8, 32).

In regard to the long half-lives of other hepatic transporter proteins it can be assumed that transcriptional downregulation of NTCP/Ntcp is a slow process (17). In contrast, regulation at the posttranscriptional level serves for rapid adjustments of bile salt uptake. On a short-term scale, transport capacity of rat Ntcp can be increased by insertion into the sinusoidal membrane (9). Exocytosis of Ntcp from an intracellular pool is stimulated via cAMP and involves dephosphorylation of a serine residue in the third intracellular loop (3). Recently, we could show internalization of Ntcp as an additional mechanism of rapid regulation of bile salt uptake (23, 30). This may protect hepatocytes against high levels of intracellular bile acids.

Internalized membrane proteins can be recycled from early endosomes or a late recycling compartment back to the plasma membrane, be sequestered in endosomes, or be transported to late endosomes and lysosomes for degradation (29). In this study, we investigated mechanisms for endocytosis and subsequent processing of Ntcp. Recently, human NTCP has been identified as the receptor for hepatitis B and D viruses (HBV/HDV) (34); therefore endocytosis of NTCP may be relevant for cellular entry of HBV/HDV.

MATERIALS AND METHODS

Antibodies and reagents. The monoclonal anti-GFP antibody (JL-8) was obtained from Clontech (Heidelberg, Germany), the monoclonal anti-FLAG M2 antibody and the monoclonal anti-Na⁺-K⁺-ATPase (M7-PB-E9) antibody from Sigma Aldrich (Deisenhofen, Germany), the monoclonal GADPH antibody from Millipore (Schwalbach, Germany), the polyclonal anti-PK-Cα antibody (sc-208) from Santa Cruz Biotechnology (Santa Cruz, CA), the polyclonal anti-Lamp1 antibody from Abcam (Cambridge, UK), the monoclonal anti-PDI (protein disulfide-isomerase) antibody (RL77) from Thermofinn Scientific (Waltham, MA), and the monoclonal anti-GM130 (Golgi matrix protein of 130 kDa) antibody (35GM130) from BD Biosciences (San Jose, CA). The Fab fragments of an anti-mouse IgG antibody coupled to the fluorescent dye Pacific Blue and Hoechst 34580 were purchased from Invitrogen (Karlsruhe, Germany). Accutase and bafloilocin A1 were purchased from Millipore (Schwalbach, Germany). The horseradish peroxidase (POD)-conjugated secondary anti-mouse antibody was obtained from Bio-Rad (Munich, Germany). Secondary anti-rabbit antibodies conjugated with Cy3 were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), antimouse antibody conjugated to Alexa Fluor 546 from Invitrogen. Chlorpromazine hydrochloride (CPZ) was obtained from Alexis Biotech.

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Cloning of FLAG-tagged Ntcp-EYFP and mutagenesis. FLAG-tagged Ntcp was subcloned from a Ntcp-EGFP construct with a modified FLAG tag of the sequence DYKDE with the primer pair 5'-ATGGACTACAAGGACGATGACGATAAGATGGAGGTGC-3' coding for a start codon, followed by the classic FLAG tag (DYKDDDK) and 5'-TGGATCCCGATTGTCCCATGTACCAGAATT-CAGG-3'. The resulting PCR products were cloned into the pcR2.1 TOPO vector (Invitrogen). After propagation in Escherichia coli the insert were excised with BamHI and ligated into the pEYFP-N1 vector (Clontech). The accuracy of the resulting plasmids was confirmed by sequencing. Point mutations were introduced with the Stratagene (La Jolla, CA) QuickChange MultiSite-Directed Mutagenesis Kit. The primers for the respective mutations are given in Table 1. The accuracy of the resulting plasmids was confirmed by sequencing.

Cell culture and transfection. Untransfected and stably transfected FLAG-Ntcp-EGFP 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 previously (20). Stable cell lines were maintained in medium supplemented with 0.35 mg/ml Geneticin (Biochrom, Berlin, Germany) as selection agent. Three independent clones from two cloning strategies (30) with regular expression of FLAG-Ntcp-EGFP at the plasma membrane were used. Notably, for transient expression FLAG-Ntcp-EYFP (enhanced yellow fluorescent protein) was transfected into HepG2 cells by using FuGene HD (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s guidelines.

Cell lysis and Western blot analysis. Cells were lysed in Triton containing lysis buffer [1% Triton X-100, 10 mM Tris, pH 7.5, 150 mM NaCl] and protease inhibitors (Complete, Roche Diagnostics). Equal protein amounts of the samples were separated by SDS polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. Ntcp was detected with the anti-GFP antibody and secondary POD-coupled antibody by use of enhanced chemiluminescence (Perkin Elmer, Waltham, MA).

Immunoprecipitation studies. HepG2 cells were transiently transfected with FLAG-Ntcp-EYFP or FLAG-Ntcp-LLAA2-EYFP, respectively, and incubated for 48 h. For inhibition of clathrin-mediated endocytosis, cells were incubated with CPZ for 24 h, starting 24 h posttransfection. For immunoprecipitation of GFP-tagged proteins, cells were lysed in lysis buffer provided with the μMACS Epitope Tag Protein Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Immunoprecipitation was performed according to the manufacturer’s guidelines with 1 mg of protein per sample. Proteins were separated by SDS polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose membranes, and detected by appropriate primary antibodies and secondary POD-coupled antibodies and by the use of enhanced chemiluminescence (Perkin Elmer).

Immunofluorescence, confocal laser scanning microscopy, and densitometric analysis. Immunostaining and confocal laser scanning microscopy (LSM 510, Zeiss, Oberkochen, Germany) were performed as described recently (16). Staining was performed on cells grown on glass coverslips, which were fixed and permeabilized with methanol (100%, 4°C, 1 min). The Lamp1 (1:100), Na⁺/K⁺-ATPase (1:500), GM130 (1:100), or PDI (1:1,500) antibodies were applied for 2 h, and the appropriate secondary antibody conjugated to Cy3 or Alexa Fluor 546 at a dilution of 1:500 and the nuclear dye Hoechst at a dilution of 1:20,000 were applied for 1 h.

Flow cytometry. HepG2 cells stably expressing FLAG-Ntcp-EGFP were cultured in 12-well culture plates until subconfluence. They were treated with the PKC activator phorbol 12-myristate 13-acetate (PMA; dissolved in 0.1% DMSO) or with 0.1% DMSO (control) for the indicated time at 37°C. The proteasome inhibitor MG132, the lysosomal inhibitor bafilomycin A1, and the endocytosis inhibitor CPZ were applied 30 min before PMA as indicated. For cell degradation assays, cells were preincubated with cycloheximide to prevent PMA-induced de novo synthesis of FLAG-Ntcp-EGFP from the transgene. After stimulation, cells were washed with ice-cold phosphate-buffered saline (PBS) and incubated with Accutase at 37°C for 4 min. Cells were transferred into 1.5-ml tubes, centrifuged for 30 s at 4,500 g, resuspended in PBS, and filtered through 70-μm gauge to enrich single cells.

The anti-Flag M2 antibody was labeled with the anti-mouse IgG-Fab fragment coupled to Pacific Blue according to manufacturer’s guidelines at a ratio of 1:3 (antibody: Fab fragment). Cells were then incubated with the labeled antibody at a dilution of 1:500 for 45 min at 4°C. Cell size, granularity, and fluorescence intensities were measured by a FACSCanto Flow Cytometer (Becton Dickinson, Heidelberg, Germany) with excitation at 488 and 405 nm, respectively. EGFP fluorescence was measured at 530 ± 30 nm, Pacific Blue fluorescence at 450 ± 50 nm.

Wild-type and transiently transfected HepG2 cells were analyzed 2 days after transfection. Transfected cells were gated by comparison of EYFP fluorescence with untransfected wild-type HepG2 cells.

Statistics. For the stably transfected cells data were obtained from 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). Fluorescence intensities of EGFP and Pacific Blue were displayed as cumulative histograms (sigmoid curves) for easier comparison of different conditions.

For the transient transfection experiments measurements of 10,000 single events in the gated population of YFP-positive cells were analyzed with the Diva Software (Becton Dickinson) for comparison of mean values. Data were obtained from at least three independent experiments.

For the Pacific Blue fluorescence detected, mean values of measurements from unlabeled cells (labeling mix without FLAG-antibody) were subtracted from mean values of stained cells. Changes in Ntcp surface and total expression were 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 statistical significant.

RESULTS

PKC-mediated degradation of Ntcp. Recently, we demonstrated PKC-dependent endocytosis of Ntcp upon stimulation

| Table 1. Sequences of primers, which were used for Ntcp mutagenesis |
|---------------------------------|---------------------------------|
| LLAA1                           | 5'- GCCCTGGGCGATGATGCCCGCCTTTATAGCTATACGAAAGGC-3' |
| LLAA2                           | 5'- GCTTATGAGAACGAGGCGGCGTTTTGACTG2-3' |
| S213A                           | 5'- CAGGGGCGATGATGCCCGCCTTTATAGCTATACGAAAGGC-3' |
| T219A                           | 5'- GCTTATGAGAACGAGGCGGCGTTTTGACTG2-3' |
| T225A                           | 5'- CAGGGGCGATGATGCCCGCCTTTATAGCTATACGAAAGGC-3' |
| S226A                           | 5'- GCTTATGAGAACGAGGCGGCGTTTTGACTG2-3' |
| S227A                           | 5'- CAGGGGCGATGATGCCCGCCTTTATAGCTATACGAAAGGC-3' |
| T225A/S226A                     | 5'- CAGGGGCGATGATGCCCGCCTTTATAGCTATACGAAAGGC-3' |

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with the phorbol ester PMA (30). Here, the fate of internalized transporter was studied in HepG2 cells expressing FLAG-Ntcp-EGFP.

Ntcp at the plasma membrane was stained by the extracellularly located FLAG tag in unpermeabilized cells and analyzed by flow cytometry. Overall Ntcp expression was quantified by EGFP fluorescence. The transgene promoter is induced by PMA; therefore cells were preincubated with the protein synthesis inhibitor cycloheximide to prevent de novo Ntcp expression. PMA induces significant endocytosis within 30 min, reaching a maximum after 2 h. This is represented by a shift of the cumulative histograms to lower FLAG-dependent fluorescence in the overlay presentation (Fig. 1A). Endocytosis was followed by a reduction of total Ntcp (measured by EGFP fluorescence) between 1 and 2 h of PMA treatment (Fig. 1, A and B). Together, PKC-dependent internalization and degradation induced a loss of ~30% of Ntcp. Under control conditions (DMSO) no such effects on Ntcp redistribution or expression were visible (Fig. 1B).

In Western blot analyses FLAG-Ntcp-EYFP was detected at ~86 kDa, representing the complex-glycosylated fully processed and plasma-membrane-bound transporter. After 2 h of PMA treatment, expression of complex-glycosylated Ntcp is diminished, indicating the loss of mature transporter (Fig. 1C).

Ntcp is degraded in lysosomes. To analyze the underlying pathway of degradation, FLAG-Ntcp-EGFP cells were preincubated with the proteasome inhibitor MG132 or the lysosomal inhibitor bafilomycin A1, respectively, prior to 6-h stimulation with PMA. As shown by flow cytometric analyses of EGFP expression, downregulation of Ntcp is partly reversed by pretreatment with MG132 and fully reversed by pretreatment with bafilomycin A1. Neither inhibitor influences steady-state expression of Ntcp (Fig. 2A). Furthermore, colocalization of Ntcp with the lysosomal marker protein Lamp-1 (lysosomal-associated membrane protein 1) after 4 h of PMA stimulation can be visualized by confocal laser scanning microscopy (Fig. 2B). These results suggest that upon PKC activation internalization of Ntcp is followed by lysosomal degradation.

Clathrin-dependent endocytosis of Ntcp. Membrane proteins can be internalized via a clathrin- or via a caveolin-dependent pathway. Filipin, an inhibitor of caveolin-mediated endocytosis (26), could not inhibit PMA-induced Ntcp endocytosis (data not shown). However, preincubation with CPZ, an inhibitor of clathrin-mediated internalization, partly reversed PMA-stimulated internalization as shown by flow cytometry and immunoﬂuorescence (Fig. 3, A and B). This suggests Ntcp endocytosis via a clathrin-dependent pathway.

Ntcp plasma membrane targeting and endocytosis is mediated via a dileucine motif. Clathrin-dependent cargo proteins usually contain short sequence motifs in their intracellular parts necessary for their recognition. Three possible motifs have
been described: so-called dileucine motifs, tetrapeptides with the sequence YXX\(\alpha\) (\(\alpha\) represents a hydrophobic amino acid), or NPXY (25). The putative intracellular sequences of Ntcp contain two potential dileucine motifs: the first, L136L137, in the second intracellular loop and the second, L222L223, in the third intracellular loop (Fig. 4). Mutations of both leucine residues to alanine in these two motifs (LLAA1 and LLAA2, respectively) were introduced into a FLAG-Ntcp-EYFP construct and were transiently overexpressed in HepG2 cells. Mutation of the second dileucine motif (LLAA2) resulted in strongly diminished plasma membrane expression (represented by the FLAG-dependent fluorescence) whereas total Ntcp expression (represented by EYFP-dependent fluorescence) is only slightly reduced as shown by flow cytometry (Fig. 5A). In line with this, microscopy revealed an intracellular predominance of the LLAA2 mutant, whereas Ntcp wild-type and the LLAA1 mutant are expressed at the plasma membrane (Fig. 5B). At high expression levels, the LLAA2 mutant accumulated in large cytoplasmic granules, which colocalized with marker proteins of the Golgi apparatus and in part of the endoplasmic reticulum (Fig. 6, C and F). In addition, immunoprecipitation of FLAG-Ntcp LLAA2-EYFP showed an accumulation of core-glycosylated immature Ntcp. At the same time, expression of the complex-glycosylated transporter, which represents Ntcp at the plasma membrane, was diminished (Fig. 7A). This ratio of complex- and core-glycosylated Ntcp remains un-
changed by inhibition of clathrin-mediated endocytosis by CPZ (Fig. 7B), indicating disturbed plasma membrane targeting of the mutant rather than a recycling defect. Upon PMA stimulation FLAG-Ntcp LLAA1-EYFP was slightly less internalized than the wild-type construct, whereas endocytosis of FLAG-Ntcp LLAA2-EYFP was abrogated (Fig. 5A). These results suggest a dual function of the dileucine motif L222L223, involving plasma membrane targeting on the one hand and endocytosis of Ntcp on the other hand.

PKC-α interacts with Ntcp. We have shown recently that PMA-induced endocytosis of Ntcp is in part mediated by PKC-α (30). Therefore, direct interaction of PKC-α with Ntcp was investigated. Indeed, endogenous PKC-α was found to coprecipitate with FLAG-Ntcp-EYFP in transiently or stably transfected cells. This interaction is independent of the dileucine motif (Fig. 7A) and is not influenced by PMA stimulation (data not shown).

Serine and threonine residues are part of the internalization motif. It has been described that dileucine motif-mediated internalization can be regulated by phosphorylation of surrounding serine residues (25). Since PKC-α is a serine/threonine kinase, the potential role of such residues in vicinity to the dileucine motif was investigated. Mutations to alanine were introduced into a FLAG-Ntcp-EYFP construct at five positions in the third intracellular loop (S213A, T219A, T225A, S226A, and S227A; and T225A/S226A double mutation) and transiently expressed in HepG2 cells. Flow cytometric analysis of PMA-stimulated internalization showed significantly reduced endocytosis of T225A and S226A mutants compared with the wild type Ntcp. Mutation at the other positions did not affect Ntcp internalization (data not shown). These results suggest an involvement of T225 and S226 in PKC-mediated Ntcp endocytosis.

DISCUSSION

NTCP is the major uptake transporter for bile salts into liver parenchymal cells. It is of particular importance because it influences bile salt concentrations in blood and hepatocytes, thereby affecting bile salt-dependent signaling pathways in hepatic and extrahepatic tissues. Recently, we described endocytosis of rat Ntcp (30) as well as human NTCP (unpublished data) as a rapid mechanism of regulating the plasma membrane availability of this transporter in stably transfected HepG2...
cells. This might be a physiologically relevant mechanism, because several in vivo situations with inconsistent reductions in NTCP/Ntcp protein and mRNA were described. For example, in bile duct-ligated rats, Ntcp protein was downregulated by more than 90%, whereas Ntcp mRNA was only reduced by ~60% (10). Similarly, in humans with progressive familial intrahepatic cholestasis (16) or in mdr2 knockout mice (19), NTCP/Ntcp protein but not mRNA was shown to be downregulated. This might be explained by increased transporter endocytosis and subsequent degradation.

Indeed, in stably transfected HepG2 cells we can show that total Ntcp expression begins to decrease 90 min after PMA-induced endocytosis, leading to a loss of ~30% of protein after 4 h. Because of lower sensitivity compared with flow cytometric measurements, no Ntcp is detectable any more 4 h after PMA stimulation in Western blot analyses. These data demonstrate for the first time degradation of Ntcp as a mechanism of posttranscriptional downregulation. Lysosomal degradation is the predominant pathway for Ntcp removal since the lysosomal inhibitor bafilomycin A1 completely prevents PKC-dependent loss of Ntcp. We further addressed the question, whether the internalization of Ntcp is mediated by clathrin- or caveolin-dependent processes. Inhibition of caveolin-mediated endocytosis by filipin does not block PMA-induced Ntcp downregulation (data not shown). In contrast, preincubation with CPZ prevents Ntcp endocytosis. CPZ causes the clathrin lattice to assemble on endosomal membranes and at the same time prevents...
the assembly of coated pits at the plasma membrane (33). Therefore, our data indicate an involvement of clathrin-coated pits in Ntcp endocytosis.

PKC-mediated endocytosis has been described for several other transporters like OATP2B1 (18), the ileal apical sodium-dependent bile acid transporter ASBT (28), the bile salt export pump BSEP, and the canalicular transporter MRP2 (multidrug resistance-associated protein 2) (6), establishing this pathway as a general mechanism of rapidly regulating transporter availability at the plasma membrane.

Incorporation of membrane proteins in clathrin-coated vesicles depends on the presence of signals that have been defined on the basis of protein sequence manipulation (5). Two major classes of motifs have been described referred to as “tyrosine based” and “dileucine based” according to the identity of their most critical residues (4). Ntcp contains two potential dileucine motifs: the first, leucine 136-leucine 137 (L136L137), in the second intracellular loop and the second, leucine 222-leucine 223 (L222L223), in the third intracellular loop. Dileucine motifs interact with the heterotetrameric adaptor protein complexes AP-1, -2, or -3, which are part of clathrin coats and are involved in internalization, lysosomal targeting, and basolateral sorting. Mutation of the dileucine motif L222L223 abrogates PKC-mediated endocytosis of Ntcp. In some transmembrane proteins with such motifs, a phosphoacceptor serine NH2-terminal to the dileucine sequence adds to the strength of the

Fig. 7. Wild-type Ntcp and the dileucine mutant associate with PKC-α. A: untransfected cells (Ø) and cells transfected with FLAG-Ntcp-EYFP and FLAG-Ntcp LLAA2-EYFP, respectively, were subjected to immunoprecipitation of Ntcp via the EYFP tag. Mutation of the dileucine motif L222L223 leads to an accumulation of immature core-glycosylated Ntcp and a reduction of mature complex-glycosylated Ntcp compared with wild-type in immunoprecipitation studies. Both wild-type and mutant Ntcp coprecipitate PKC-α. B: inhibition of clathrin-mediated endocytosis did not change the ratio of core- and complex-glycosylated Ntcp of wild-type or mutated Ntcp. Therefore, the occurrence of the low-molecular-weight form of mutated Ntcp is independent of endocytosis. Differences in the total quantity of precipitated protein per lane reflect variations in transfection efficiency.

![Fig. 7](image.png)

Fig. 8. Serine and threonine residues involved in Ntcp endocytosis. Mutation of T225 and S226, but not S213, T219, and S227, to alanine reduces PMA (50 nM; 1 h)-induced internalization of Ntcp as shown by flow cytometry. Mean values ± SE of at least 3 independent experiments are given, calculated as described in MATERIALS AND METHODS. Controls were set to 100%. *Statistical significance of the differences in internalization.
signals. Since PKC-α is a serine/threonine kinase that might regulate internalization of Ntcp by phosphorylation, all serine and threonine residues in the third intracellular loop surrounding the dileucine motif were mutated to alanine. Interestingly, none of the amino-terminally located residues (in front of the dileucine) influences PMA-induced Ntcp endocytosis, but mutation of T225 and S226 COOH-terminal to the dileucine motif significantly diminishes internalization. It has been suggested that Ntcp is constitutively phosphorylated and that dephosphorylation of S226 is involved in cAMP-mediated Ntcp translocation to the plasma membrane (3, 4). Vice versa, phosphorylation of this residue might stimulate endocytosis. Although we found an interaction of the transporter with PKC-α, we were not able to show an increase in Ntcp phosphorylation in response to PMA stimulation in immunoblot analyses with phosphospecific antibodies. This might be a matter of detection limits. Alternatively, PKC-α might phosphorylate not Ntcp but proteins of the endocytic machinery, thereby stimulating internalization. In this case, mutation of T225 and S226 might have an inhibitory effect due to interfering with adaptor binding to the dileucine motif.

Interestingly, mutation of L222 and L223 not only abolishes Ntcp endocytosis but additionally results in a 60% reduced plasma membrane expression whereas overall Ntcp expression is only slightly reduced. Ntcp/NTCP is exclusively localized at the basolateral membrane of hepatocytes but the mechanisms underlying its sorting have not been fully elucidated. In a study by Sun et al. (31), deletion of the 56-amino acid COOH-terminal tail of Ntcp resulted in only a partial loss of basolateral sorting fidelity in MDCK cells. Therefore, it was speculated that the COOH-terminal tail of Ntcp was not predominant for basolateral targeting and that additional sorting signals may be present. In the polarized hepatoma cell line HepG2 we find a strong contribution of the dileucine motif L222L223 to Ntcp plasma membrane localization. In many cases, basolateral sorting signals are colinear with internalization signals (13). The amino acids in their vicinity are supposed to define whether they function as determinants for endocytosis, basolateral targeting, or both (22). In Western blot analyses we can show an accumulation of the core-glycosylated immature form and a reduction of the mature complex-glycosylated form of the Ntcp dileucine mutant compared with wild-type Ntcp. However, this altered distribution is not caused by deglycosylation following endocytosis and defective recycling of the mutant because inhibition of clathrin-mediated endocytosis had no effect on the ratio of the complex- and the core-glycosylated form, i.e., did not increase the amount of complex-glycosylated Ntcp. In addition, colocalization analysis with a Golgi marker protein showed a distinct accumulation of the dileucine mutant in the maturation pathway. Taken together, these results suggest a defect in targeting of the dileucine mutant from the Golgi to the plasma membrane.

The sequence surrounding the dileucine motif is highly similar in rat Ntcp and human NTCP (MTPHLLATSSL vs. MTPHLLATSSL). Therefore, it is not surprising that human NTCP is downregulated in the same way in response to PMA stimulation (data not shown). Several polymorphisms of NTCP are described (15, 24, 27), including a variant of this motif (14). In this variant, an isoleucine is replaced by a threonine (p.I223T). It was detected in populations with different ethnic backgrounds and leads to reduced taurocholate uptake, reduced plasma membrane expression, and intracellular retention (14), as would be proposed for defective membrane targeting. Regarding our studies on rat Ntcp, we expect a similar function of this leucine-isoleucine (LI) motif as internalization and basolateral sorting signal. In view of the recently described function of NTCP as a HBV/HDV receptor (34) a reduction of membrane targeting and endocytosis of NTCP would be advantageous in terms of infectability. This may explain the relatively high allele frequency of the variant p.I223T, which has been found to be 5.5% in an African-American population.

In conclusion, we propose PKC-mediated internalization of Ntcp and subsequent lysosomal degradation as a rapid mechanism of regulating transporter availability and half-life. Analyzing the molecular details of this process, we could identify a motif located in the third intracellular loop of Ntcp/NTCP mediating transporter endocytosis and plasma membrane sorting. Since downregulation of Ntcp seems favorable for the protection of hepatocytes from toxic bile salt concentrations, it will be interesting to identify further physiological or pharmacological stimuli influencing these processes.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


