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VIRUS-CELL INTERACTIONS

NTCP Oligomerization Occurs Downstream of the NTCP-EGFR Interaction during Hepatitis B Virus Internalization

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ABSTRACT Sodium taurocholate cotransporting polypeptide (NTCP) is a receptor that is essential for hepatitis B virus (HBV) entry into the host cell. A number of HBV entry inhibitors targeting NTCP have been reported to date; these inhibitors have facilitated a mechanistic analysis of the viral entry process. However, the mechanism of HBV internalization into host cells after interaction of virus with NTCP remains largely unknown. Recently, we reported that troglitazone, a thiazolidinedione derivative, specifically inhibits both HBV internalization and NTCP oligomerization, resulting in inhibition of HBV infection. Here, using troglitazone as a chemical probe to investigate entry process, the contribution of NTCP oligomerization to HBV internalization was evaluated. Using surface plasmon resonance and transporter kinetics, we found that troglitazone directly interacts with NTCP and noncompetitively interferes with NTCP-mediated bile acid uptake, suggesting that troglitazone allosterically binds to NTCP, rather than to the bile acid-binding pocket. Additionally, alanine scanning mutagenesis showed that a mutation at phenylalanine 274 of NTCP (F274A) caused a loss of HBV susceptibility and disrupted both the oligomerization of NTCP and HBV internalization without affecting viral attachment to the cell surface. An inhibitor of the interaction between NTCP and epidermal growth factor receptor (EGFR), another host cofactor essential for HBV internalization, impeded NTCP oligomerization. Meanwhile, coimmunoprecipitation analysis revealed that neither troglitazone nor the F274A mutation in NTCP affects the NTCP-EGFR interaction. These findings suggest that NTCP oligomerization is initiated downstream of the NTCP-EGFR interaction and then triggers HBV internalization. This study provides significant insight into the HBV entry mechanisms.

IMPORTANCE Hepatitis B virus (HBV) infection is mediated by a specific interaction with sodium taurocholate cotransporting polypeptide (NTCP), a viral entry receptor. Although the virus-receptor interactions are believed to trigger viral internalization into host cells, the exact molecular mechanisms of HBV internalization are not understood. In this study, we revealed the mode of action whereby troglitazone, a specific inhibitor of HBV internalization, impedes NTCP oligomerization and identified NTCP phenylalanine 274 as a residue essential for this oligomerization. We further analyzed the association between NTCP oligomerization and HBV internalization, a process that is mediated by epidermal growth factor receptor (EGFR), another essential host cofactor for HBV internalization. Our study provides critical information on the mechanism of HBV entry and suggests that oligomerization of the viral receptor serves as an attractive target for drug discovery.

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Hepatitis B virus (HBV), an enveloped DNA virus, specifically infects hepatocytes, and the resulting chronic infection is a major risk factor for the development to liver cirrhosis and hepatocellular carcinoma (1). Sodium taurocholate cotransporting polypeptide/solute carrier family 10A1 (NTCP/SLC10A1), which is specifically expressed in the liver and known as a transporter for bile acid uptake into hepatocytes, was identified as an HBV entry receptor in 2012 (2). This discovery has greatly accelerated mechanistic studies, especially in the field of HBV entry, and has facilitated the development of anti-HBV agents (3, 4).

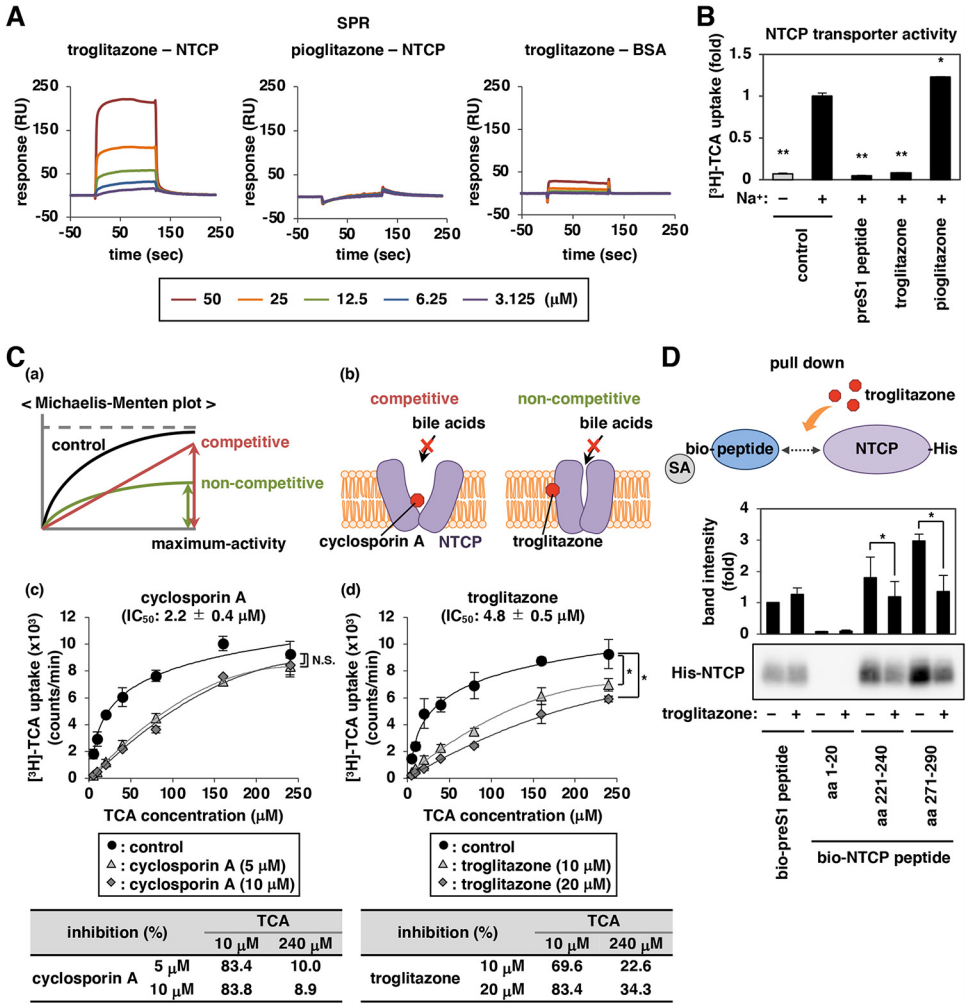
Viral entry is a critical process for the initiation, spread, and maintenance of infection and is an attractive target for the development of antiviral agents (5, 6). HBV infects host hepatocytes through a multistep process. Initially, HBV attaches to the cell surface through binding to heparan sulfate proteoglycans in a nonspecific and low-affinity manner (7). Subsequently, cell surface virions generate a specific and high-affinity interaction between the preS1 region of HBV large surface protein (LHBs) and its viral receptor, NTCP (2). The preS1-NTCP interaction is believed to trigger internalization of the virus into the cell via endocytic machinery. However, the molecular mechanism regulating the internalization of HBV remains poorly understood.

Recently, we reported that troglitazone specifically inhibits HBV internalization while also disrupting the oligomerization of NTCP (8). This finding suggests the possibility that NTCP oligomerization plays a critical role in the HBV internalization pathway and serves as a novel target for the development of anti-HBV agents. Therefore, in this study, we investigated the contribution of NTCP oligomerization to HBV internalization by analyzing the molecular mechanism whereby troglitazone inhibits NTCP oligomerization. We also identified an NTCP residue that is responsible for oligomerization of the protein. In addition, we clarified the functional relevance between NTCP oligomerization and epidermal growth factor receptor (EGFR)-mediated endocytosis, which we previously reported as a pivotal trigger of HBV internalization (9, 10). Thus, our study provides novel information on the mechanism of viral entry, deepening our understanding of the HBV life cycle.

RESULTS

Troglitazone directly interacts with NTCP to inhibit NTCP oligomerization. We previously reported that troglitazone inhibits HBV infection by interrupting viral internalization and interferes with NTCP oligomerization (8). These findings raised the question of how troglitazone regulates NTCP oligomerization. To investigate whether troglitazone interacts with NTCP, we used surface plasmon resonance (SPR), making use of sensor chip-immobilized recombinant His-tagged NTCP (His-NTCP) or bovine serum albumin (BSA) as a negative control. Pioglitazone was also used as a negative control, given previous work showing that pioglitazone has no effect on HBV infection and NTCP oligomerization (8). As shown in Fig. 1A, the injection of troglitazone, but not that of pioglitazone, across recombinant NTCP produced signals in an apparently dose-dependent manner (Fig. 1A, left and middle). These troglitazone-dependent signals were not evident with the BSA-immobilized chip (Fig. 1A, right).

Most compounds known to bind with NTCP, including cyclosporin A, irbesartan, ezetimibe, and ritonavir, reportedly inhibit its transporter activity (11–14). Therefore, we next assessed whether troglitazone affected NTCP-mediated bile acid uptake. NTCP transporter activity was evaluated by incubating HepG2-hNTCP-C4 cells, a HepG2-derived cell line engineered to overexpress human NTCP (15), with [^{3}H]taurocholic acid (TCA) in either a sodium-free or a sodium-containing buffer in the presence or absence of compounds. As shown in Fig. 1B, preS1 peptide (used as a positive control), a lipopeptide consisting of myristoylated amino acids (aa) 2 to 48 of the preS1 region,



inhibition (%)		TCA 10 μM	TCA 240 μM
cyclosporin A	5 μM	83.4	10.0
	10 μM	83.8	8.9

inhibition (%)		TCA 10 μM	TCA 240 μM
troglitazone	10 μM	69.6	22.6
	20 μM	83.4	34.3

FIG 1 Troglitazone directly interacts with NTCP to inhibit NTCP oligomerization. (A) Surface plasmon resonance (SPR) analysis of the interaction between compounds (troglitazone or pioglitazone) and recombinant His-tagged NTCP (His-NTCP) or BSA. Various concentrations (50, 25, 12.5, 6.25, and 3.125 μM) of compounds were injected from 0 to 120 s onto a sensor chip on which the indicated protein had been immobilized, followed by application of compound-free buffer. The SPR responses are indicated in resonance units (RU). (B) NTCP-dependent bile acid uptake was measured by quantifying [^{3}H]taurocholic acid (TCA) as a substrate in HepG2-hNTCP-C4 cells in the presence or absence of preS1 peptide (100 nM), troglitazone (25 μM), or pioglitazone (25 μM) in either sodium-free (gray) or sodium-containing (black) buffer. (C) (a and b) Michaelis-Menten plot in which reduced maximum activity indicates a noncompetitive mode of inhibition (green), while little or no reduction in the maximum activity indicates competitive inhibition of the target protein (red). Kinetics of NTCP transporter activity were analyzed following treatment with or without cyclosporin A (c) or troglitazone (d) at either 0 (black), 5 (light gray), or 10 (dark gray) μM and various concentrations of TCA. (D) Interactions between recombinant full-length His-NTCP and the fragment peptides derived from NTCP were examined by pulldown assay. Biotinylated (bio) peptides of preS1 (aa 2 to 48) or NTCP fragments (20-aa lengths corresponding to the indicated regions) immobilized on streptavidin (SA)-agarose beads were incubated with His-NTCP in the presence or absence of troglitazone. Coimmunoprecipitated His-NTCP was detected with anti-His antibody by immunoblotting. Quantitative densitometry is shown in the upper graph. Data are means and standard deviations (SD). Statistical significance was determined using a two-tailed nonpaired Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

decreased the sodium-dependent TCA uptake (16). Treatment with troglitazone, but not pioglitazone, showed an apparent reduction of TCA uptake.

It is difficult to clearly define the structural requirement of NTCP for compound interaction, since the crystal structure of NTCP has not yet been solved. Therefore, we next investigated the mode of action for the troglitazone-NTCP interaction by using transporter kinetics analysis to determine if troglitazone targets the bile acid-binding pocket of NTCP. Competitive inhibition would indicate that the compound targets the bile acid-binding pocket of NTCP, while noncompetitive inhibition would suggest an

allosteric interaction with NTCP distinct from the bile acid-binding pocket (Fig. 1C, panels a and b). As shown in Fig. 1C, panel c, cyclosporin A potently inhibited the TCA uptake at a low TCA concentration (10 μM) but did not significantly reduce the maximum transporter activity, indicating its competitive inhibition of NTCP transporter activity (17). In contrast, the TCA uptake in the presence of troglitazone was significantly reduced even at the highest TCA concentration tested (240 μM), suggesting a noncompetitive manner of inhibition (Fig. 1C, panel d).

Furthermore, we performed an *in vitro* pulldown assay to assess the contribution of the interaction of troglitazone with NTCP to the inhibition of NTCP oligomerization. In this assay, we tested whether troglitazone affects the binding interaction between His-NTCP and two biotinylated NTCP fragment peptides. The two peptides consisted of aa 221 to 240 and aa 271 to 290 of NTCP; these regions were previously identified as regions responsible for NTCP oligomerization (8). His-NTCP was incubated with these biotinylated NTCP fragment peptides (or with biotinylated preS1 peptide) that had been immobilized on streptavidin-agarose beads. The coprecipitated His-NTCP then was detected by immunoblotting with anti-His antibody. As shown in Fig. 1D, treatment with troglitazone prevented the NTCP-NTCP peptide interaction without affecting the NTCP-preS1 peptide interaction. These data suggested that troglitazone directly interacts with NTCP at a site that is distinct from the bile acid-binding pocket and that troglitazone binding interferes with NTCP oligomerization.

Identification of regions responsible for NTCP oligomerization. We next used the alanine scanning approach to identify amino acids important for the function of two NTCP regions (aa 221 to 240 and aa 271 to 290) previously identified as regions responsible for oligomerization (8). We used the alanine-variant peptides in which three adjacent amino acids in NTCP peptides (aa 221 to 240 and aa 271 to 290) were replaced sequentially with an alanine triplet (Fig. 2A, left). The effect of these mutant NTCP peptides on HBV infection, compared with that of wild-type peptides, was evaluated by measuring the levels of HBs antigens in the culture supernatant. Replacing any three amino acids in the aa 221–240 region did not result in an apparent reduction in inhibition of HBV infection ability compared to the wild-type peptide (aa 221 to 240) (Fig. 2A, panel a). In contrast, the inhibition of HBV infection by the wild-type NTCP peptide (aa 271 to 290) was markedly attenuated by the introduction of mutations at any of the three regions (aa 274 to 276, aa 283 to 285, and aa 289 to 290) (Fig. 2A, panel b). This result suggested that these three regions (aa 274 to 276, aa 283 to 285, and aa 289 to 290) are essential for the NTCP peptide (aa 271 to 290) to block HBV infection. Consistent with this inference, we found that these three mutant peptides (274–276A, 283–285A, and 289–290A) decreased the interaction with His-NTCP in pulldown assays compared with the wild-type peptide (aa 271 to 290) (Fig. 2B).

F274 is required for NTCP oligomerization and HBV preS1 internalization. To identify amino acids essential for NTCP oligomerization, we generated eight NTCP constructs encoding proteins possessing single mutations within individual elements (aa 274 to 276, aa 283 to 285, and aa 289 to 290) (Fig. 3A). As a first step, we examined the effect of these single mutations on HBV attachment using a fluorescently labeled myristoylated preS1 peptide (preS1 probe). This preS1 region (aa 2 to 48) of the large HBs antigen is essential for its binding to NTCP (18). HepG2 cells transfected with an expression plasmid encoding wild-type or mutant NTCP were incubated with the preS1 probe. As shown in Fig. 3B, single mutations were not associated with clear reductions in the attachment of the preS1 probe compared to the wild-type. This result suggested that these mutant NTCPs retain the ability to bind HBV. As a next step, we determined the effect of each of the single mutations in NTCP protein on HBV infection. Notably, only the alanine mutation at the position 274 of NTCP (NTCP-F274A) significantly decreased HBV infection (Fig. 3C), suggesting that the phenylalanine at position 274 is required for the process of HBV infection after viral attachment. We further investigated the influence of NTCP-F274A on preS1-mediated HBV internalization (8, 9, 14). HepG2 cells expressing wild-type NTCP (NTCP-WT) or NTCP-F274A were incubated with the preS1 probe at 37°C. After 8 h of incubation, the preS1 probe was observed to

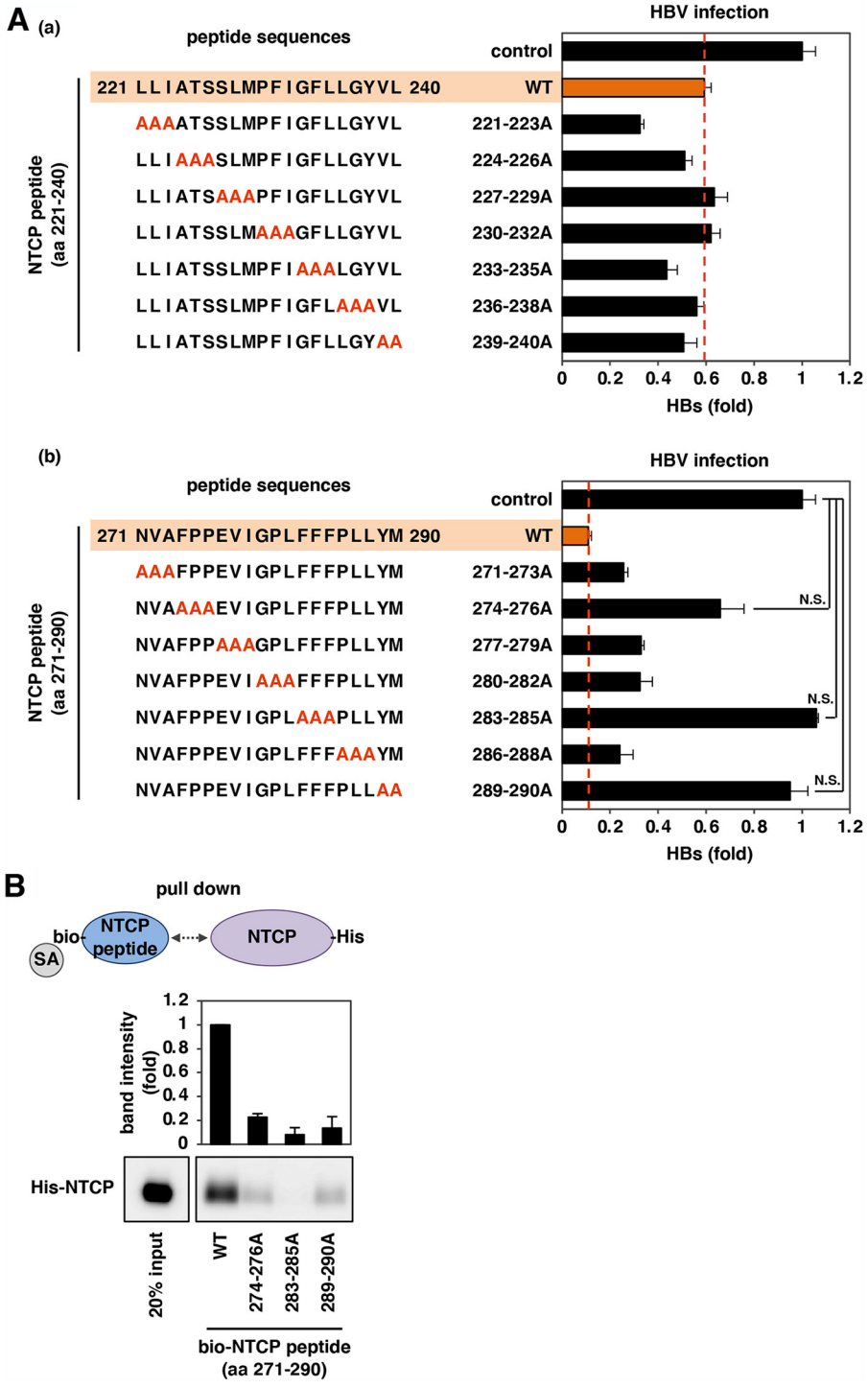


FIG 2 Identification of regions responsible for NTCP oligomerization. (A) HBV inhibition activity of alanine-variant NTCP peptides (20-aa lengths, with the mutated regions indicated in red) was evaluated by HBV infection assay. HepG2-hNTCP-C4 cells treated with or without NTCP peptides were inoculated with HBV for 16 h. HBV infection was determined by HBs antigen level in the culture supernatant at 12 days postinoculation. (a) Inhibition activity of NTCP peptide (aa 221 to 240) (red dotted line) was compared with those of seven mutant peptides (221–223A, 224–226A, 227–229A, 230–232A, 233–235A, 236–238A, and 239–240A). (b) Inhibition activity of NTCP peptide (aa 271 to 290) (red dotted line) was compared with those of seven mutant peptides (271–273A, 274–276A, 277–279A, 280–282A, 283–285A, 286–288A, and 289–290A). (B) Interactions between recombinant full-length His-NTCP and the NTCP peptides were examined by pulldown assay, as described in the Fig. 1D legend. Quantitative densitometry is shown in the upper graph. Data are means and SD. Statistical significance was determined using a two-tailed non-paired Student's *t*-test.

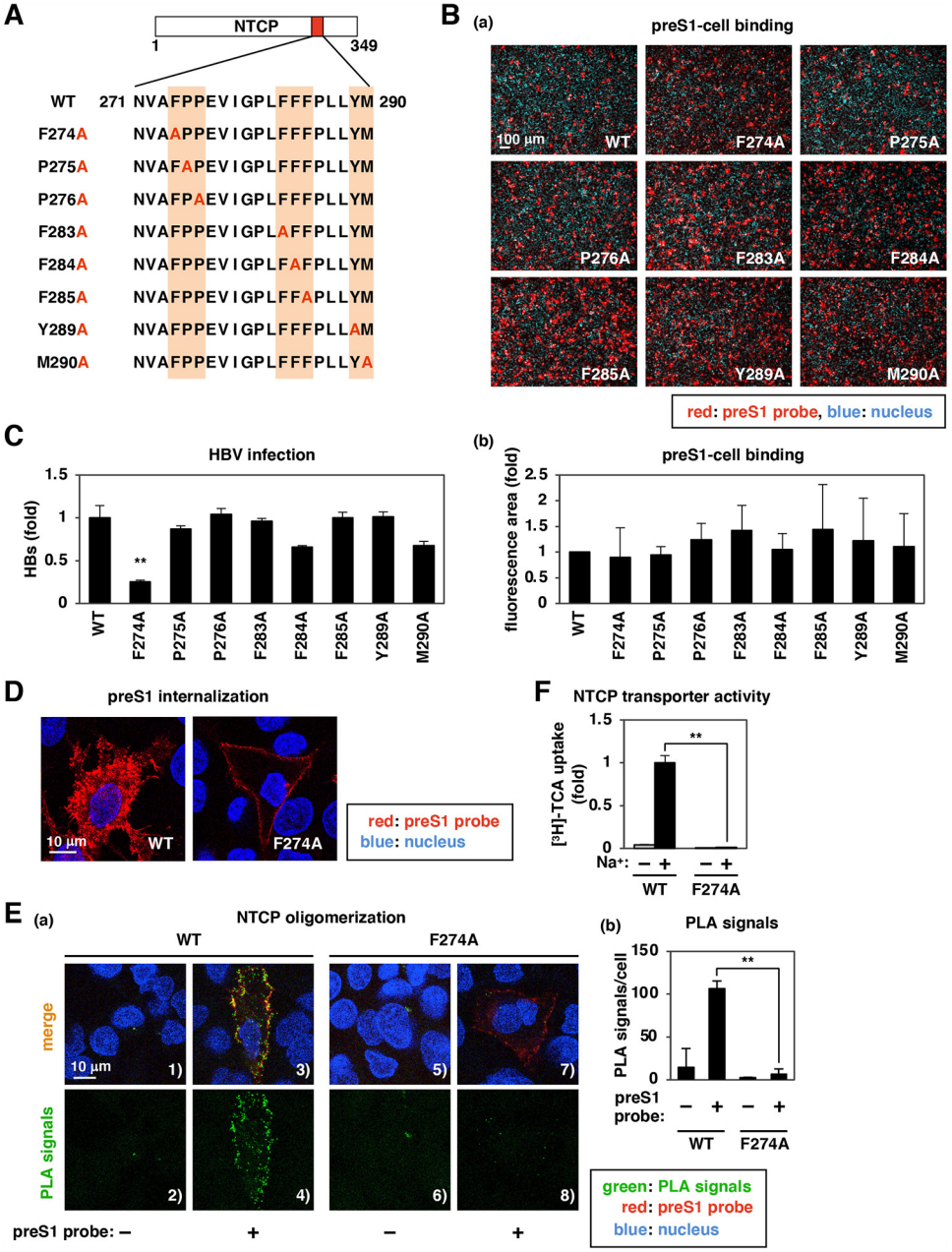


FIG 3 NTCP residue F274 is essential for both NTCP oligomerization and HBV infection. (A) Schematic representation of the amino acid sequences of NTCPs possessing single mutations (in red) within each element (aa 274 to 276, aa 283 to 285, and aa 289 to 290) identified as regions important for NTCP oligomerization in Fig. 2. (B) HBV preS1 attachment to host cells was examined in HepG2 cells transfected with expression plasmids encoding the wild-type or mutant NTCP. The cells were exposed for 30 min at 37°C to TAMRA-labeled preS1 peptide (preS1 probe) (red) and then were stained for the nucleus (blue) by DAPI (a). PreS1 fluorescence intensities were quantified using Dynamic Cell Count (Keyence) (b). (C) HepG2 cells overexpressing the wild-type or mutant NTCP were infected with HBV, and the supernatant HBs antigen was quantified at 12 days postinoculation. (D) HepG2 cells overexpressing the wild-type or mutant NTCP (F274A) were exposed for 8 h at 37°C to preS1 probe (red) and then were stained for the nucleus (blue) by DAPI. Merged patterns are shown in lower panels. (E) Proximity ligation assay (PLA) to evaluate NTCP oligomerization. HepG2 cells overexpressing both myc-NTCP (WT or F274A) and HA-NTCP (WT or F274A) were treated for 30 min at 4°C with or without preS1 probe (red), and then the PLA signal (green) produced by the proximity of anti-myc and anti-HA antibodies was detected with Duolink PLA. The nucleus was also stained with DAPI (blue). (a) The lower panels show the PLA signal (green) only, and the upper panels show the merged images of green (PLA signal), red (preS1 probe), and blue (nucleus) signals. (b) PLA signals in the experiment reported in panel a were quantified using Dynamic Cell Count. (F) NTCP transporter activity was measured by using [^{3}H]taurocholic acid as a substrate in HepG2 cells overexpressing the wild-type or mutant NTCP (F274A) in either sodium-free or sodium-containing buffer, as described in Materials and Methods. Intracellular ^{3}H counts taken up into the cells are shown. Data are means and SD. Statistical significance was determined using a two-tailed nonpaired Student's *t* test (**, $P < 0.01$).

form punctate structures within the cells expressing NTCP-WT (Fig. 3D). In contrast, most of the preS1 probe remained on the cell surface in cells expressing NTCP-F274A. We then examined whether the effect seen in NTCP-F274A was accompanied by and correlated with impaired NTCP oligomerization. NTCP oligomerization was evaluated by employing the proximity ligation assay (PLA), which can detect protein-protein interactions within cells. HepG2 cells transiently expressing both myc-tagged (myc-NTCP-WT or -F274A) and HA-tagged (HA-NTCP-WT or -F274A) NTCP were incubated for 30 min at 4°C with or without preS1 probe and then assessed for PLA signals produced by myc-HA proximity. Upon incubation with preS1 peptide, the observed level of PLA fluorescence was markedly increased at the cell membrane expressing NTCP-WT (Fig. 3E, panel a, images 2 and 4), as previously reported (8), but not NTCP-F274A (Fig. 3E, panel a, images 6 and 8). This mutagenesis analysis revealed that phenylalanine 274 of NTCP is critical for NTCP oligomerization and HBV internalization and implied a strong association between these two events. In addition, as shown in Fig. 3F, NTCP-F274A was defective in bile acid transporting activity, raising the possibility that NTCP oligomerization is also associated with its bile acid uptake.

Functional association between NTCP oligomerization and EGFR-mediated HBV internalization. We further validated the association of NTCP oligomerization with EGFR-mediated HBV internalization into cells. In a previous paper, we reported that the peptide aa 131 to 150 of NTCP (a region that includes G144 and G148, responsible for the interaction with EGFR) impedes the NTCP-EGFR interaction and HBV internalization (9, 10). We also demonstrated that inactivation of EGFR by gefitinib, an EGFR autophosphorylation inhibitor, interferes with HBV internalization. In the present study, we sought to use these inhibitors as tools. Interestingly, treatment with troglitazone (positive control) and NTCP peptide (aa 131 to 150) clearly inhibited NTCP oligomerization (Fig. 4A, panel a, image 2 versus 4 or 2 versus 6), while gefitinib had no inhibitory effect (Fig. 4A, panel a, image 2 versus 8). These observations led us to hypothesize that NTCP oligomerization occurs downstream of the NTCP-EGFR interaction. Consistent with these results and our hypothesis, NTCP-G144A/G148A (full-length NTCP harboring G144A and G148A mutations), previously shown to lose the ability to bind to EGFR (9), did not undergo NTCP oligomerization (Fig. 4B). Moreover, a coimmunoprecipitation assay showed that the NTCP-EGFR interaction is not impaired under conditions in which NTCP oligomerization is impeded by troglitazone treatment (Fig. 4C, panel a) or by expression of NTCP-F274A (Fig. 4C, panel b). These results supported the hypothesis that NTCP oligomerization occurs at a step following the NTCP-EGFR interaction (Fig. 5).

DISCUSSION

In a previous report, we showed that troglitazone specifically interferes with HBV internalization into host cells and the oligomerization of NTCP (8). The results of the present study suggested that troglitazone inhibits HBV internalization activity via a direct interaction with NTCP. A number of HBV entry inhibitors have been reported to directly interact with NTCP, including a lipopeptide (Myrcludex B), cyclosporins, vanitaracin A, NPD8716, rapamycin, and WL4 (2, 17, 19–22), and these compounds were found to inhibit the attachment of HBV to the host cell surface. Two primary regions within the NTCP protein, aa 84 to 87 and aa 157 to 165, have been reported to mediate HBV entry (16, 23). NTCP with mutations in aa 157 to 165 is deficient in the attachment of preS1. Point mutations of the NTCP residues involved in either bile acid binding (N262, Q293/L294, and S267) or sodium binding (Q68, S105/N106, E257, and Q261) also have been reported to suppress preS1 binding and thus HBV infection, indicating that the regions of importance for bile acid transport overlap those required for viral attachment (24). Consistent with these findings, most of the above HBV attachment inhibitors also have the ability to inhibit NTCP-mediated bile acid uptake. Among these inhibitors, cyclosporin A directly competes with bile acid for overlapping binding sites on NTCP (17). However, troglitazone interfered with NTCP-mediated bile acid uptake in the present study, even though the compound does not affect the preS1 binding to

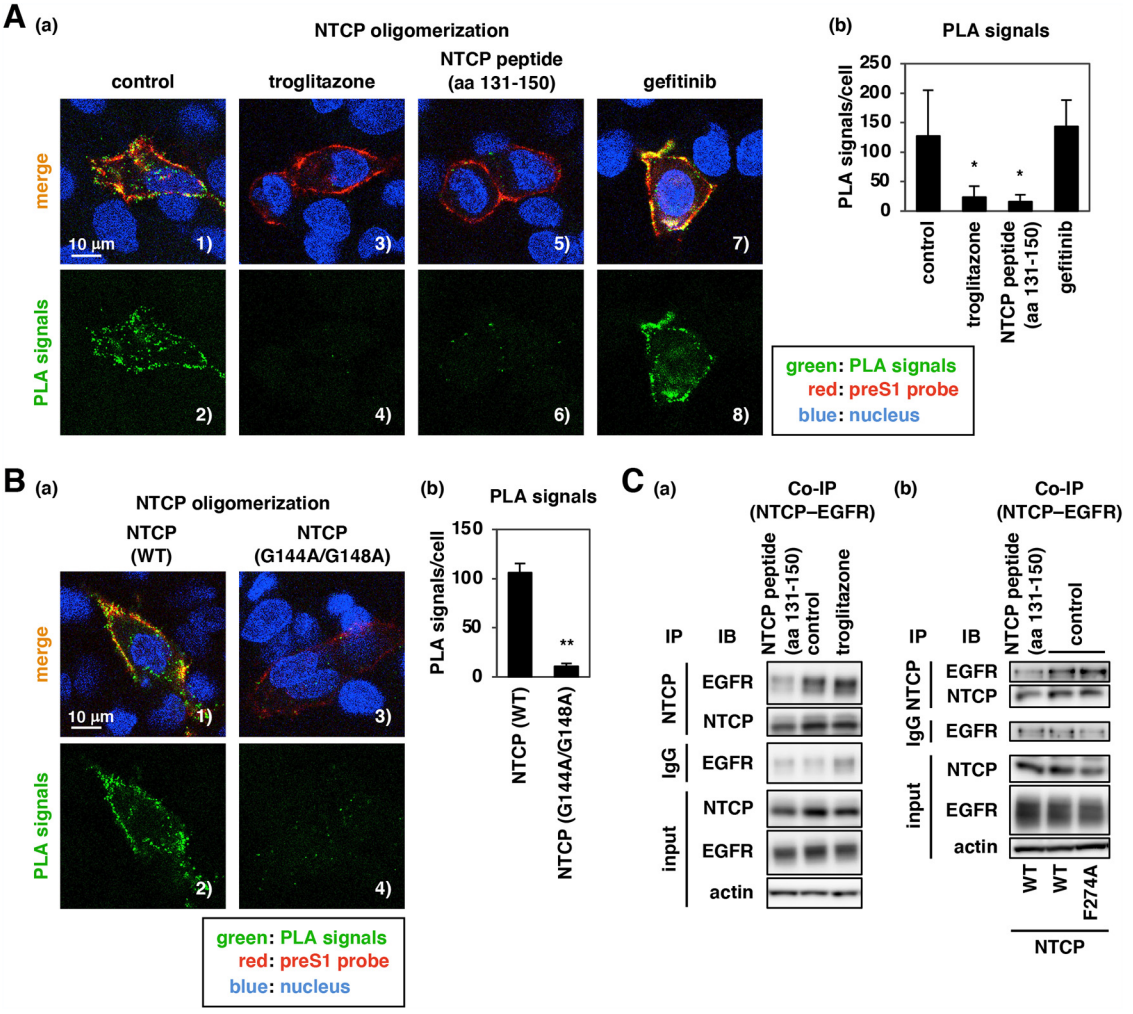
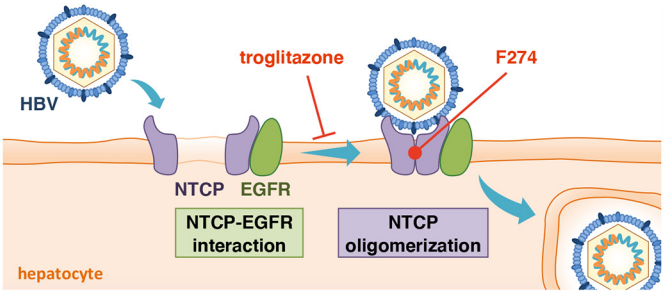


FIG 4 Functional relevance of the association between NTCP oligomerization and EGFR-mediated HBV internalization. (A) Effect of NTCP peptide (aa 131 to 150) and gefitinib on NTCP oligomerization was evaluated by PLA, as described in the Fig. 3E legend. The PLA signal was detected in HepG2 cells overexpressing both myc-NTCP and HA-NTCP following treatment with or without the indicated compounds for 30 min at 4°C in the presence of preS1 probe. (a) The lower panels show the PLA signal (green) only, and the upper panels are the merged images of green (PLA signal), red (preS1 probe), and blue (nucleus). (b) PLA signals in the experiment reported in panel a were quantified using Dynamic Cell Count (Keyence). (B) Effect of mutations in NTCP (G144A/G148A) on NTCP oligomerization was evaluated by PLA, as described in the Fig. 3E legend. The PLA signal was detected in HepG2 cells overexpressing both myc-NTCP (WT or G144A/G148A) and HA-NTCP (WT) following treatment with preS1 probe. (a) The lower panels show the PLA signal (green) only, and the upper panels are the merged images of green (PLA signal), red (preS1 probe), and blue (nucleus). (b) PLA signals in the experiment reported in panel a were quantified using Dynamic Cell Count. (C) 293T cells overexpressing HA-EGFR and myc-NTCP (WT [a and b] or F274A [b]) were treated with or without NTCP peptide (aa 131 to 150) or troglitazone and then harvested for immunoprecipitation with anti-myc antibody (or normal mouse IgG as a negative control). The precipitates or total cell lysates were assessed for NTCP, EGFR, and actin by immunoblotting. Data are means and SD. Statistical significance was determined using a two-tailed nonpaired Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

the cells. Furthermore, our results suggested that troglitazone exhibits noncompetitive inhibition with bile acid uptake and thus directly interacts with NTCP at a site distinct from the preS1 binding region and the bile acid-binding pocket. We propose that direct binding of troglitazone to NTCP induces conformational changes in NTCP, resulting in impairment of bile acid uptake by NTCP. These observations also suggested that NTCP oligomerization is impeded by the interaction of troglitazone with the transmembrane domain of the NTCP protein or by the conformational change induced by troglitazone binding.

Alanine scanning mutagenesis analysis revealed that F274 of NTCP is required for both NTCP oligomerization and HBV internalization (Fig. 3 and 5). Given that the crystal structure of the NTCP protein has not been solved to date, it is difficult to predict



	HBV preS1 attachment	NTCP-EGFR interaction	NTCP oligomerization	HBV preS1 internalization
troglitazone	○	○	×	×
NTCP mutant (F274A)	○	○	×	×
NTCP peptide (aa 131-150)	○	×	×	×
NTCP mutant (G144A/G148A)	○	×	×	×

○: no inhibition, ×: inhibition

FIG 5 Proposed model of HBV internalization involving NTCP oligomerization and EGFR. Upon HBV attachment, NTCP first interacts with EGFR, followed by NTCP oligomerization. Troglitazone impedes the NTCP oligomerization, and the phenylalanine at position 274 of NTCP is essential for the oligomerization. Formation of the complex consisting of HBV virion, EGFR, and NTCP oligomer, followed by EGFR autophosphorylation, allows its internalization via endocytosis. Results from Fig. 1 to 4 are summarized at the bottom.

which NTCP residues will be proximal to F274 in the folded protein. Although the physiological significance of NTCP oligomerization remains poorly defined, the present study indicates that NTCP oligomerization is significant for trafficking of this protein. Additionally, the current work demonstrates the role of NTCP oligomerization in HBV internalization.

Our recent finding that EGFR triggers the internalization of HBV through EGFR's direct interaction with NTCP (9) raised the question of which events of HBV internalization occur first: NTCP oligomerization or the NTCP-EGFR interaction. Although further analysis will be needed to clarify how NTCP oligomerization mediates the internalization of HBV, our study assumed that HBV internalization is promoted by increasing the local concentration of NTCP via NTCP's interaction with EGFR. We also previously reported that the EGFR endocytosis machinery involves the phosphorylation of EGFR and subsequent recruitment of adaptor molecules such as AP2A1 and EPS15 (10). Therefore, NTCP oligomerization may contribute to the recruitment of these adaptor molecules. In the present study, we used fluorescence-tagged preS1 to visualize the internalization of the HBV envelope (14). Future analysis of HBV internalization using labeled HBV particles also will be important, although the labeling of HBV particles is currently difficult and represents a technical challenge. However, our observations provide significant information and tools to further investigate the HBV entry process.

Clinically, sustained undetectable serum levels of HBs antigens and of HBV DNA are the primary goals of treatment and have been defined as a "functional cure." Recently, it was reported that Myrcludex B led to the functional cure of HBV in some patients with chronic hepatitis B and D when combined with pegylated interferon alpha (25), suggesting the potential merit of entry inhibitors for treatment, as well as for the prevention of infection. Therefore, NTCP oligomerization involved in HBV entry is expected to be an attractive novel target for the development of antivirals.

MATERIALS AND METHODS

Reagents. Troglitazone was purchased from AdooQ BioScience. Pioglitazone and gefitinib were purchased from Sigma-Aldrich. Cyclosporin A was purchased from Abcam. [^{3}H]taurocholic acid was purchased from American Radiolabeled Chemicals, Inc. PreS1 peptides consisting of aa 2 to 48 of the HBV preS1 region, myristoylated at the amino terminus and with or without carboxy-terminal conjugation to

TABLE 1 PCR primers used for plasmid construction

Name	Sequence
NTCP	F1 5′-TAGTAGCTCGAGATGGAGGCCCACAACGCGTC-3′
	R1 5′-TTATTAGAATTCGACTAAGCGTAATCTGGAACATCGTATGGG-3′
F274A	F2 5′-GTGGCCGCCCCACCTGAAGTCATTGGACCACTT-3′
	R2 5′-GACTTCAGGTGGGGCGGCCACATTGAGGATGGTGG-3′
P275A	F 5′-GTGGCCTTTGCCCCTGAAGTCATTGGACCACTT-3′
	R 5′-GACTTCAGGGGCAAAGGCCACATTGAGGATGGTGG-3′
P276A	F 5′-GTGGCCTTTCCAGCCGAAGTCATTGGACCACTT-3′
	R 5′-GACTTCGGCTGGAAAGGCCACATTGAGGATGGTGG-3′
F283A	F 5′-CCACTTGCCTTCTTTCCCCTCCTCTACATGATTTTCC-3′
	R 5′-GAGGGGAAAGAAGGCAAGTGGTCCAATGACTTCAGGTGG-3′
F284A	F 5′-CCACTTTTCGCCTTTCCCCTCCTCTACATGATTTTCC-3′
	R 5′-GAGGGGAAAGGCGAAAAGTGGTCCAATGACTTCAGGTGG-3′
F285A	F 5′-CCACTTTTCTTCGCCCCCCTCCTCTACATGATTTTCC-3′
	R 5′-GAGGGGGGCGAAGAAAAGTGGTCCAATGACTTCAGGTGG-3′
Y289A	F 5′-CTCCTCGCCATGATTTTCCAGCTTGGAGAAGGGC-3′
	R 5′-GAAAATCATGGCGAGGAGGGGAAAGAAGAAAAGTGG-3′
M290A	F 5′-CTCCTCTACGCCATTTTCCAGCTTGGAGAAGGGC-3′
	R 5′-GAAAATGGCGTAGAGGAGGGGAAAGAAGAAAAGTGG-3′

6-carboxytetramethylrhodamine (TAMRA), were synthesized by Scrum, Inc. The NTCP peptide fragment library was synthesized by Sigma-Aldrich and Scrum, Inc.

Plasmid construction. The expression plasmids pEF4-NTCP-myc-His, pCS-EF-NTCP-HA, pEF4-NTCP-G144A,G148A-myc-His, and pCS-EF-EGFR-HA were constructed as described previously (8, 9, 17). For the construction of pCS-EF-NTCP-HA carrying a point mutation (Fig. 3A), DNA fragments were amplified by PCR using pCS-EF-NTCP-HA as a template and two separate primer pairs, the first consisting of F1 and R2 and the second consisting of F2 and R1. Primer sequences are shown in Table 1. The resulting products were further amplified by overlap extension PCR with F1 and R1 and then inserted between XhoI and EcoRI sites of pCS-EF-NTCP-HA to generate pCS-EF-NTCP-F274A-HA. Other NTCP mutants were prepared by the same method using the indicated primer sets (Table 1).

Cell culture. HepG2-hNTCP-C4, Hep38.7-Tet, and HepG2 cells were cultured at 37°C and 5% CO_2 in growth medium consisting of Dulbecco's modified Eagle medium (DMEM)/F-12 plus GlutaMAX (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (pH 7.4), and 5 μg/ml insulin (15, 26). 293T cells were cultured at 37°C and 5% CO_2 in growth medium consisting of DMEM (high glucose) (Wako) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (pH 7.4), 100 μM nonessential amino acids, and 1 mM sodium pyruvate (27).

SPR analysis. The binding affinity of compounds for recombinant His-tagged NTCP (His-NTCP) (17) or for bovine serum albumin (BSA) (used as a negative control) was evaluated by SPR analysis using a Biacore X100 (GE Healthcare) instrument as described previously (20). Compounds diluted to the indicated concentrations with running buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, 0.05% Tween 20, and 5% dimethyl sulfoxide [DMSO]) were injected for 120 s over His-NTCP or BSA that had been immobilized on a CM5 sensor chips. The resulting data were analyzed using Biacore X100 evaluation software (GE Healthcare, v. 2.0.1).

NTCP transporter assay. HepG2-hNTCP-C4 cells were incubated with [^{3}H]taurocholic acid (TCA) in uptake buffer containing 136 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.1 mM KH_2PO_4, 1.8 mM $CaCl_2$, 10 mM D-glucose, and 10 mM HEPES (pH 7.4) at 37°C for 15 min to allow TCA uptake into the cells in the presence of compounds. After washing out of free [^{3}H]TCA, cells were lysed and intracellular radioactivity was measured (19).

NTCP transporter kinetics analysis. HepG2-hNTCP-C4 cells were treated with various concentrations of TCA (5, 10, 20, 40, 80, 160, and 240 μM) in uptake buffer in the presence or absence of compounds, and the intracellular ^{3}H count for quantifying TCA uptake was measured. Concentration-dependent [^{3}H]TCA uptake is shown in a Michaelis-Menten plot (17).

Pulldown assay. His-NTCP was incubated at 4°C for 4 h with either biotinylated NTCP peptide fragments (corresponding to 20-aa lengths of the NTCP sequence) or preS1 peptide (as a positive control). Biotinylated peptides then were immunoprecipitated with streptavidin beads, and the resulting precipitates were dissolved in sample buffer. Coprecipitated His-NTCP was detected by immunoblot analysis with anti-His antibody (8, 28).

HBV preparation and infection. HBV derived from the culture supernatant of Hep38.7-Tet cells was prepared as described previously (12). HBV was inoculated at 12,000 (Fig. 2A and 3C) genome equivalents (GEq) per cell in the presence of 4% polyethylene glycol 8000 (PEG 8000) (Sigma-Aldrich; P2139) at 37°C for 16 h, as described previously (29).

Detection of HBs antigens. HBs antigens were quantified by enzyme-linked immunosorbent assay (ELISA) using anti-HBs antibody (LifeSpan BioSciences) essentially as described previously (22).

PreS1 binding assay. PreS1-cell surface attachment was evaluated by incubating the cells at 37°C for 30 min in the presence of TAMRA-labeled peptide spanning aa 2 to 48 of the myristoylated preS1 region (preS1 probe), as described previously (19).

PreS1 internalization assay. HepG2-hNTCP-C4 cells were exposed to preS1 probe at 37°C for 8 h to allow the attachment and internalization of the preS1 probe. After the washing out of free preS1 probe and fixing of the cells, the nuclei were stained with 200 ng/ml 4′,6-diamidino-2-phenylindole (DAPI). PreS1 internalization was observed by using confocal microscopy (8).

PLA. NTCP oligomerization was evaluated using PLA (Sigma-Aldrich) in cells transfected with expression plasmids for both myc-tagged NTCP and hemagglutinin (HA)-tagged NTCP and treated with preS1 probe. The PLA signal produced using anti-myc (Santa Cruz Biotechnology; A-14) and anti-HA (Abcam; HA-7) antibodies was detected according to the manufacturer's protocol (8). PLA signals in the experiment were quantified using Dynamic Cell Count (Keyence).

Coimmunoprecipitation analysis. 293T cells were lysed in a buffer containing 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% NP-40, and complete protease inhibitor, followed by immunoprecipitation with anti-myc (Santa Cruz Biotechnology; 9E10) and anti-HA (Abcam; HA-7) antibodies or mouse normal IgG as a negative control, essentially as described previously (30).

Statistics. Statistical significance was determined using a two-tailed nonpaired Student's *t* test. *P* values of <0.05 were considered significant.

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